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Effect of Solvent Interaction & Soluble Microneedles on Skin Permeability to Drug Molecules

Abhijeet Maurya

University of Mississippi

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EFFECT OF SOLVENT INTERACTION & SOLUBLE MICRONEEDLES ON
SKIN PERMEABILITY TO DRUG MOLECULES

A Dissertation presented for the Doctor of Philosophy Degree
The University of Mississippi

Abhijeet Maurya
December 2018
ABSTRACT

Skin forms a formidable barrier protecting the human body from external environmental rigors and excessive loss of water; maintaining equilibrium. The barrier properties of the skin can be attributed to its unique macromolecular organization and morphology. As a route for drug administration, skin presents a large surface area and can be used for both systemic and localized targeted drug delivery applications offering several advantages over conventional drug therapy; avoidance of first pass metabolism, patient compliance, sustained or controlled delivery for an extended period, to name a few. However, the organized structure of the skin, since intended to prevent entry of adverse chemicals, poses a formidable challenge to molecular transport. From a drug delivery perspective, skin is different from GIT in anatomy and functionality, the former being more permeable to drug molecules. Through various peer reviewed research on the drug transport kinetics through skin, it has been realized that the primary barrier to cutaneous drug transport resides in the Stratum Corneum (SC), the uppermost layer of the skin. The 15-20 μm thick lipophilic, torturous morphology of the SC resembles a brick and mortar structure and imposes a limitation on percutaneous drug transport with only a few molecules having the prerequisite physicochemical characteristics to permeate the intact SC. Thus, drug penetration and subsequent diffusion across the SC is a passive process leading to constraints on the amount of drug that is deliverable to achieve the desired therapeutic effect. To increase the number of candidates for cutaneous delivery and to attain appropriate dose levels requires application of certain enhancement strategies. These approaches employ different mechanisms; (i) an external
driving force by iontophoresis (ii) reversible modulation of the SC barrier function by chemical penetration enhancers (iii) creating “easy access” transport channels by microneedles. Nevertheless, a thorough understanding of the molecular transport process across the skin is requisite before formulation strategies could be employed to deliver drugs across the skin in a therapeutically pertinent time-frame. The research presented in this dissertation addresses the knowledge gap that pertains to percutaneous drug absorption by investigating the transport of drug molecules into the skin after a short-term exposure (5 minutes) to aqueous and ethanolic drug solution. Further, the research demonstrates the effect of chemical & physical enhancement approaches: chemical penetration enhancers and microneedles on skin permeability to drug molecules.
ACKNOWLEDGEMENTS

The path towards completing this dissertation has been intense and torturous, a path that has tested my limits of critical thinking, intellect and patience. This thesis represents not only my work at Faser 104, it is a lesson learned in resolution, discipline and sincerity, traits that I have tried to learn from various people I have worked with at the Department of Pharmaceutics and Drug Delivery at The University of Mississippi who I wish to acknowledge.

The completion of this thesis is thanks in large part to my advisor, professor Dr. S. Narasimha Murthy. Without his direction and moral support this thesis would have been far-fetched. My sincere gratitude to him for being tolerant with me at times when I was not at my best. As an unparalleled example of dedication and hard work, Dr. Murthy has educated me over the years on the importance of having curiosity and to be able to do the best you can, with what you have. I thank my thesis committee, Dr. Michael Repka, Dr. Seongbong Jo and Dr. N. P. Dhammika Nanayakkara for agreeing to review my work and giving their valuable time in correcting this thesis.

I would like to acknowledge the help that I received from the faculty at The Institute of Drug Delivery and Biomedical Research, Bangalore, India. Their work on the in vivo study with Iron microneedles made it possible for me to publish the work in the Journal of Pharmaceutical Sciences. I have received a lot of logistical support from Ms. Deborah King that has made this journey far less stressful. I thank her for the time and efforts.
To my parents, for all the calls that I did not make, for all the time that I was not home, I am sorry. At the end, my journey towards being intellectually superior was not worth for the sacrifices you have made.
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\[ a \ p = 0.0374 \]
\[ b \ p = 0.0136 \]
\[ c \ p = 0.0212 \]
\[ d \ p = 0.2612 \]
CHAPTER 1

Unveiling the mechanism of drug penetration into Stratum Corneum during the short duration exposure of topical formulation

1. Abstract

The physicochemical and formulation factors influencing the penetration of drug molecules from topical products into the skin and the mechanisms of drug permeation are well investigated and reported in the literature. However, the mechanism of absorption of drugs during short term exposure is not given enough importance. In this project, the extent of absorption of drug molecules into the skin from aqueous and ethanolic solutions following a 5 min application period was investigated. The experiments demonstrated measurable magnitude of absorption into the skin for all the molecules tested despite the duration of exposure being a few min. Among the two solvents used, absorption was greater from aqueous drug solution than ethanolic solution. The results suggest that an alternative penetration pathway; herein referred to as the convective transport pathway, is likely responsible for the rapid and significant uptake of drug molecules during initial few minutes of exposure. Additionally, absorption through the convective transport pathways is a function of the physico-chemical nature of the formulation vehicle rather than the API.
2. Introduction

Skin, as the largest organ in the body presents a large surface area and serves to perform dual function; acting as a barrier to the entry of harmful chemicals and toxins in the environment and preventing excessive loss of water from the body, thereby maintaining homeostasis. Improved understanding of the structure and composition of the skin has led to the realization of its selective permeability and forms the basis for the development of various formulation strategies for local and systemic afflictions\(^1,2\). Several predictive modelling approaches have been suggested in this regard as a tool to assess permeability of a compound\(^3-5\). This is of relevance towards development of better drug delivery and formulation approaches and most importantly, for risk assessment of industrial & warfare toxins. The predictive modeling approaches involve regression equations that are constructed using data generated from \textit{in vitro} steady state permeation experiments\(^6\). While these equations and the steady state experiments provide an accurate estimate of chemical’s rate of absorption, it does not take into consideration the fraction of the applied dose entering the stratum corneum at an early phase of transport process. It also does not provide a rational representation of the real-life situation where the contact of a chemical with the skin is much shorter unlike the lengthy conventional steady-state experiments. There have been few reported instances of absorption of drug molecules upon brief application periods of the compounds on skin. Maibach et al., studied the effect of application time and concentration of methyl nicotinate (MN) from an aqueous solution as an erythematic response upon application to human subjects. The authors observed rapid transport of MN upon exposure times ranging from 15s to 1200s as evidenced by radial erythematous vasodilatory response\(^7\). Similar observations were published by Guy et al., for the vasodilatory effect of hexyl nicotinate, measured using Laser Doppler
Velocimetry. For an application duration of only 15 seconds, the authors observed considerable pharmacodynamic activity; the magnitude of the response was determined by the type of carrier vehicle employed. These reports present interesting illustrations for the penetration of a chemical molecule after short application periods to the skin and the effect of vehicle on the rate and extent of absorption. While these studies present data generated from a controlled experimental setting, several case studies concerning instances of occupational exposure by incidental transfer of drug molecules have been published. Some of these reports include manifestation of hirsutism (hair growth) in female partners of men undergoing topical testosterone therapy and respiratory depression/death caused by accidental transfer of fentanyl transdermal patch. These reports suggest that the notion of absorption of a chemical after a short exposure period is generally accepted but rarely confirmed and there is lack of profound experimental data that can provide comprehensive insights into the mechanism leading to the short-term penetration of molecules into the skin. In this study, we have investigated the mechanism leading to the penetration of molecules into the skin following a brief exposure to drug product. In vitro experiments were performed across full thickness porcine skin on a series of test molecules for an exposure duration of 5 minutes. Simultaneously, the effect of vehicle on short-term absorption was studied by comparing the extent of penetration obtained from aqueous and ethanolic vehicles. The penetration depth profile of the test molecules into the SC was obtained through sequential tape-stripping procedure. An exposure duration of 5 minutes was a conscious selection to have a duration short enough to test the premise of a pre-steady state penetration and from an analytical perspective; to have a quantifiable measure of the penetrating solute.
3. Materials and Methods

3.1 Materials

Diclofenac sodium, caffeine & nicotine was sourced from Sigma Aldrich Inc. (St. Louis, Missouri). Fentanyl was procured from Noramco Inc. (Athens, GA). For tape stripping experiments, Transpore™ tape (# 1527-1) was obtained from 3M Science (St. Paul, MN). The porcine skin was obtained from a local abattoir. Buffers & solutions for experiments was prepared in deionized water. All other chemicals and reagents used were of analytical grade.

3.2 Epidermis preparation

The hairs from the porcine skin was shaved off using electric razor and all the adhering subcutaneous fat and exogenous tissues were removed carefully. Skin was cut into small rectangular pieces and placed in an Iso-Pouch™ (Topical products Testing LLC, University, MS). The Iso-Pouch™ maintains an isothermal environment across the entire surface area of the enclosed skin tissue and prevents loss of stratum corneum lipids. The Iso-Pouch™ was then immersed in a water bath maintained at 60ºC for 2 minutes following which, the epidermis was carefully teased off from the dermis. The peeled epidermis was mounted on to glass slides and stored at 4ºC. The stored epidermis was used within 3 days.

3.3 In vitro Study

3.3.1 Short-term penetration

The study was carried out using Vertical Franz diffusion cells having a 1.5 cm² diffusion area. The cells were mounted with freshly prepared porcine full thickness and the receiver
compartment was filled with pH 7.4 PBS buffer. Prior to the experiments, integrity of the skin was checked by measuring the electrical resistance at a frequency of 10 Hz and low voltage of 100 mV. The skin showing a 10 kΩ/cm² resistance value or greater was exclusively used for the experiments. The skin was wiped with Kimwipes® to remove the buffer before initiating the in vitro experiment. Saturated concentrations of the drug was prepared by adding excess amount of drug in water or ethanol. The solution was vortexed for 12 hours and centrifuged. The supernatant was collected as the saturated concentrated solution for further in vitro experiments. The study was conducted by placing the concentrated drug solution in aqueous or ethanolic medium in the donor chamber and allowing it to remain in contact with the skin for only 5 minutes. The donor drug solution was removed following the exposure time and the diffusion set-up was dismantled. This followed an immediate washing of the skin with water to remove any adhering drug solution. The skin was immediately secured on a glass plate and swabbed with Kimwipes® to remove residual drug solution. The rim of the donor chamber formed a demarcation of the diffusion area on the skin. The amount of drug absorbed into the stratum corneum after the exposure period was determined by tape-stripping. Adhesive tape (3M), cut to the size that covers the entire diffusion area was gently pressed on the skin and peeled off using forceps. The procedure was repeated 20 times (20 tape-strips) to ensure complete removal of the stratum corneum. The tape strips were placed into vials and extracted for the adhering drug by placing them in an extraction medium comprising of methanol & water in a 1:1 ratio. The samples were analyzed using HPLC and LC MS/MS12-14.

3.3.2 Long-term permeation
Long-term permeation experiments were carried out using saturated aqueous and ethanolic solutions of caffeine and salicylic acid across freshly excised and intact epidermis. Franz diffusion cell was used and PBS of pH 7.4 formed the receiver medium and was continuously stirred at 600 rpm. The epidermis was clamped between the donor and receiver chamber and the integrity was checked by measuring the resistance of the epidermis across an electrical circuit. The permeation study was initiated by replacing the PBS in the donor chamber with the test drug solution. The study was conducted for 6 hours and at pre-determined time points samples were collected and replaced with the blank receiver medium. The samples were analyzed for the amount of drug permeated across the epidermis using HPLC.

3.4 In vivo Study in human volunteers

The in-vivo studies on human volunteers was performed at the Institute for Drug Delivery and Biomedical Research, Bangalore (IDBR) (Protocol # VIPS/2014/14). The study was carried out on 6 subjects. The volar forearm of each subject was wiped off with water soaked Kimwipes®. A 2 cm$^2$ area was circled with a marker on the left and right volar forearms on the flattest plane possible to outline the area of application. An adhesive backing membrane tape was cut out to form a chamber having an area of 1.5 cm$^2$. The chamber was fixed to the pre-determined (circled) area of application on the forearm. The subjects were asked to extend their forearms over the work bench with the volar forearm facing upwards. A 0.5 ml of aqueous or alcoholic solution of caffeine (5 mg/ml) was dosed into chambers fixed on the arm. The solutions remain unoccluded and was allowed to remain in contact for 3 min. After 3 min of exposure the solution was removed, and the chamber was discarded. The exposed area (1.5 cm$^2$) was wiped off with kimwipes to remove any adhering drug solution and then tape-stripped 20 times. The tape strips were placed in the
extracting medium (50:50; methanol: water) and vortexed for 12 hours to extract the adhering drug. The amount of drug in the stratum corneum was subsequently quantified by HPLC.

4. Results & Discussion

Skin is a multi-layered tissue comprising of the stratum corneum (SC), viable epidermis and dermis. The transport of a drug molecule from its initial application on the skin surface to its intended site of action involves a series of partitioning and diffusion processes across these regions and each layer of this composite presents a barrier to the passage of an applied chemical molecule. However, from a cutaneous drug delivery perspective, SC forms the primary incomprehensible barrier, a function which can largely be ascribed to its brick and mortar morphology. The description can be appropriated to the arrangement of the protein keratin cells, the corneocytes constituting the brick which is suspended in an extracellular lipid matrix which forms the mortar. The SC barrier is a 15 -20 µm thick dynamic biphasic medium which presents different routes for the penetration of drug molecules; however, the lipidic route is widely recognized as the principal transport medium and a major contributor to the overall drug transport. The lipids in the SC comprising of ceramides, cholesterol and non-essential fatty acids assumes a multilamellar coherent bilayer morphology resulting in a tortuous, convoluted pathway for the transport of drug molecules. It is because of this unique composition and absolute morphology, that absorption across the stratum corneum is essentially a slow process relative to passive absorption cross other biological barriers\textsuperscript{15-17}. Thus, based on this heterogeneity of the SC it is only appropriate to measure the potency of chemical through long-term \textit{in vitro} steady state permeation experiments. Such studies involve drawing a time dependent cumulative concentration profile of the permeating molecule across an excised skin tissue. The flux of the molecule is calculated as the slope of the
linear portion of the graph indicating a period of steady state of the chemical into the skin tissue. The steady state flux is widely expressed by Fick’s first law diffusion principle which states that the solute flux is a product of permeability coefficient and the concentration gradient. Various models estimating permeability coefficient (Kp) have been established to quantify percutaneous absorption such as the widely accepted Potts & Guys regression equation which gives permeability estimates based on partition coefficient and molecular size of the permeating species. While the Potts and Guys equation predicts Kp values for a range of molecular weight (< 750) and logP values (-3 to +6), these predictions are made assuming long term steady state permeability conditions. It also effectively assumes that the solute transport occurs exclusively only through the lipidic pathway and the magnitude of the predictions are based on the physico-chemical properties of the solute. However, for certain chemicals, steady state may never be achieved or could take a longer time. Thus, it is important to understand the proceedings leading up to the steady-state conditions especially in the event of an accidental or occupational exposure where the timespan could be much shorter than these conventional long-term permeation experiments. The period preceding the steady-state, characterized as part of the pre-steady state phase is an initial absorption phase that accounts for the solute required to load the SC and is often under-represented to contribute to the overall permeability status of the compound with only a few reports addressing the significance of this short-term absorption period. In this direction, different modelling techniques have been proposed that predicts mass absorbed during the short-term pre-steady state. The first of these modelling techniques was proposed by the US-EPA and Bunge et al. Various other simulation approaches have been adopted for risk assessment from brief exposure of organic chemicals. While these modelling techniques are important from a regulatory perspective, a thorough experimental investigation of the penetration post application of a chemical compound
and before a steady state is achieved can yield greater insight into the mechanisms governing the transport process. This current study was designed to understand the drug transport into the stratum corneum when a permeant is exposed to skin for a short duration.

<table>
<thead>
<tr>
<th>Drug Molecule</th>
<th>Physico-chemical properties</th>
<th>Molecular weight (g/mol)</th>
<th>Melting Point</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>logP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caffeine</td>
<td>-0.07</td>
<td>194.19</td>
<td>238ºC</td>
</tr>
<tr>
<td>Diclofenac Sodium</td>
<td>0.7</td>
<td>318.129</td>
<td>288-290 ºC</td>
</tr>
<tr>
<td>Nicotine</td>
<td>1.17</td>
<td>162.23</td>
<td>-79ºC</td>
</tr>
<tr>
<td>Salicylic Acid</td>
<td>2.26</td>
<td>138.12</td>
<td>158.6ºC</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>4.05</td>
<td>336.47</td>
<td>83-84ºC</td>
</tr>
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Table 1.1: Physico-chemical properties of the test molecules employed for short-term exposure study

**Short term drug penetration studies**

Figure 1.1 presents SC concentration-depth profile of caffeine (logP: -0.07), diclofenac sodium (log P: 0.7) and salicylic acid (logP: 2.26) from saturated aqueous and alcoholic solution obtained through sequential tape stripping after a 5-minute contact interval. The penetration profile of the test molecules is presented as a function of the position of the solute in the SC to provide an objective analysis of the depth and distribution of the solute in the stratum corneum. Significant amounts of caffeine penetrated into the SC in the short-term study. Similarly, a significant amount of drug penetrated into even deeper layers of stratum corneum in case of salicylic acid and
diclofenac as well. Considering the difference in physico-chemical properties of caffeine, diclofenac sodium and salicylic acid (Table 1), steady state calculations of permeation rates cannot account for the substantial absorption observed into the skin during first few minutes of exposure (5 min in this case). This data clearly suggests the presence of a pathway which is independent of solute properties. This alternate pathway of drug absorption is apparently relatively rapidly accessible and does not appear to depend on the properties of the solute.

Two solvent systems were chosen in this experiment to understand the role of vehicle on the absorption of drugs into the stratum corneum. From figure 1, it is evident that the amount of caffeine penetrating the SC was higher for aqueous solution than for the ethanolic solution. A similar phenomenon was observed with diclofenac sodium and salicylic acid solutions as well (figure 1.1). Ethanol is known to be an excellent drug penetration enhancer for the drugs considered in this study. On the other hand, water, as compared to ethanol, does not form a good solvent system for salicylic acid (100% ionization, limited solubility). Despite of these counteracting factors, the absorption of drug molecules into the SC was significantly higher (p value less than 0.005) from aqueous solution as compared to alcoholic solution that were saturated with the drug. The reason for saturating the drug in respective solvents was to eliminate the influence of thermodynamic potential on the drug penetration from different solvents.
Aqueous Solution Alcoholic Solution

Amount of caffeine penetrated (µg/mg of stratum corneum)

Number of Tape Strips

A

B

Amount of diclofenac sodium penetrated (µg/mg of stratum corneum)

Number of Tape Strips

Aqueous solution Alcoholic Solution

1

2 to 5

6 to 10

11 to 15

16 to 20
Figure 1.1: *In vitro* Stratum Corneum concentration-depth profile of [A] caffeine, [B] diclofenac sodium and [C] salicylic acid for short-term exposure study from saturated concentration drug solutions. N=6

Further, to understand upto what extent the thermodynamic potential of drugs in aqueous and alcoholic solution influences their short-term penetration into the stratum corneum, skin penetration studies were performed at a concentration of 5 mg/ml (below the saturation concentration). The studies involved aqueous and alcoholic solutions of caffeine or diclofenac sodium at 5 mg/mL concentration. At this concentration, the thermodynamic activity of caffeine in aqueous and alcoholic solution is 0.28 & 0.63 respectively (caffeine saturation solubility in water (pH 6.8) and alcohol is 18 mg/mL and 9 mg/mL respectively). Similarly, in case of diclofenac sodium, the thermodynamic activity in aqueous and alcoholic solvent is 0.25 and 0.59 (diclofenac sodium saturation solubility in water (pH 6.8) and alcohol is 20 mg/mL and 8.5 mg/mL.
respectively). The results from figure 1.2 indicate that though the thermodynamic potential of caffeine and diclofenac sodium was lesser in aqueous solution (than alcoholic solution), the amount of drug penetrated into the different layers of stratum corneum was relatively greater when compared to that obtained from alcoholic drug solution; a trend similar to the short-term penetration studies from saturated drug solution (figure 1.1). The results for both these experiments indicate that thermodynamic activity of the drug in the vehicle and the extend of drug penetration during short-term penetration is not positively correlated; a deviation from conventional drug absorption principle.
Figure 1.2: *In vitro* Stratum Corneum concentration-depth profile of [A] caffeine and [B] diclofenac sodium for short-term exposure study from 5 mg/mL concentration drug solution. N=6.
The results provide a mechanistic understanding of the principles governing the transport during short-term contact or the unsteady state period. The fact that any amount of solute absorbed in the SC could elicit a local response and can act as a reservoir to diffuse through to reach the systemic circulation in the later stages reflects the pragmatic significance of this study especially for drug molecules with a lower toxic threshold potential. In this context, this study was extended to investigate the short-contact penetrability of fentanyl and nicotine from aqueous and ethanolic drug solution in vitro across full thickness porcine skin. A 1 mg/mL concentrated drug solution of fentanyl and nicotine was exposed to the skin for a 5-minute contact duration and the amount penetrating the skin was determined. The concentration-depth penetration profile obtained were in correspondence with the previous experiments demonstrating significant absorption into the SC from both the solvents with greater penetration obtained from aqueous drug solution than from alcoholic (Figure 1.3). It is reported that the mean plasma concentration of fentanyl in accidental deaths involving abuse is only 26.4 ng/mL.25 Thus, the extent of penetration observed for fentanyl and nicotine into the skin after a short exposure of only 5 minutes indicates that any accidental or occupational exposure to such chemicals could lead to a precarious situation if left unchecked.

**In vivo studies in human subjects**

To further corroborate the results of the in vitro experiments, short-term penetration studies were performed in vivo in healthy human volunteers. Caffeine at a concentration of 5mg/mL in alcohol and water was selected as model formulations to perform studies in human subjects. The results of the in vivo studies is in agreement with the in vitro studies (figure 1.3). First, the drug penetrated even into the deeper stratum corneum layers in significant levels within a short duration of exposure. Second, although less prominent (due to small sample size), results are in agreement
with the fact the solvent properties influence the extent of penetration of drug into the skin during the early stages of absorption. The results prompt us to speculate the presence of a solvent penetration pathway in the skin. The mechanism of transport of drug dissolved by the way of penetration of vehicle is termed as convective transport pathway in this manuscript. The convective transport of drugs into the stratum corneum appear to depend on the physicochemical properties of the solvent rather than the solute. A rational interpretation for the greater penetration of water compared to that of ethanol can be based upon the contention that the water has a lesser molecular weight (18.02 g/mol) and molecular volume (18.02 mL) than ethanol (46.07 g/mol & 59.3 mL respectively)\textsuperscript{26}.

The outcome of the above discussed experiments is in agreement with the reported evidences on short term drug absorption\textsuperscript{7,9,27}. Smith and coworkers investigated the absorption mechanism of dichloroethylsulfide (mustard gas) into the skin. Application of mustard gas on skin of human subjects moistened with water and ethanol resulted in rapid absorption (< 5 minutes) from both the solvents studied. However, absorption was greater into skin saturated with water\textsuperscript{27}. The fact that mustard gas in only sparingly soluble in water (0.07\%) and yet facilitates greater penetration into skin than ethanol in which it is readily soluble prompted the authors to speculate that water facilitates the rapid transport of mustard gas into the skin through a capillary action and surface adsorption. Additionally, the data presented in this research paper follows the theory proposed by Scheuplein in his report on the diffusional process across intact epidermis, suggesting the anatomical presence of organized short-circuit pathways which can transpire a rapid influx of solute\textsuperscript{17}. 
Figure 1.3: In-vivo Stratum Corneum concentration-depth profile of Caffeine for short-term exposure study in human subjects from 5 mg/mL concentrated drug solution. The penetration data from tape-strips 1 to 5 has been intentionally omitted to offset any accidental impurities during the tape-strip experiment. N = 6

In vitro Permeability studies

As mentioned earlier, ethanol has been reported to act as a permeability enhancer for several drugs including caffeine and salicylic acid\textsuperscript{28,29}. The property of ethanol as a penetration enhancer was not evident in short term exposure studies performed in the current project. To provide validity to the in vitro studies performed in the current study, long term permeation studies were performed using aqueous and alcoholic solution of caffeine.
Figure 1.4 demonstrates the long-term permeation profile of caffeine and salicylic acid from aqueous and ethanolic solvent medium across epidermis. It can be observed from the results that the permeation kinetics of the long-term study contrasts with the short-term penetration experiments where absorption from aqueous solution was significantly superior to that from the ethanolic solvent for both the test molecules. In case of long-term permeation experiments, the cumulative amount of caffeine permeated across the epidermis from ethanolic and aqueous solvent was $22.89 \pm 4.11 \mu g/cm^2$ & $11.89 \pm 3.67 \mu g/cm^2$ respectively. A similar trend was observed for salicylic acid where the cumulative amount permeated was $32.34 \pm 8.17$ & $15.45 \pm 4.74 \mu g/cm^2$ from ethanolic and aqueous solution respectively. This paradox in absorption behavior of the test molecules during the short-term experiment and the cumulative permeation profile obtained following the long-term experiments could be attributed to the fact that during the early stages of an exposure event, rate of absorption into the SC is rapid by virtue of the convective transport pathways which provides a direct route of entry for the drug solution. After this rapid influx period, the permeation of drug molecules through the skin becomes a passive process as the drug has to diffuse through the convoluted lipidic regions of the SC. This permeation process is a function of the physico-chemical properties of the drug molecules and its interaction with the lipids in the SC. The increased permeability from the ethanolic solvent of the drug molecule is the result of the permeation enhancing ability of ethanol acting primarily by extracting the lipids of the bilayer medium thus reducing the resistance of the continuous pathway for the passage of drug molecules. Permeation enhancement by solvent action is a gradual process and transpires only at the later stages of permeation and is thus not evident during the short-term contact of ethanolic drug solution.
Figure 1.4: *In vitro* long-term permeation profile of [A] caffeine and [B] salicylic acid across epidermis from aqueous and ethanolic drug solution. N= 6
Significance of short-term dermal drug transport

In the absence of any anatomical structural evidences the existence and location of convective transport pathways still remains hypothetical. Nevertheless, the results of the short-term drug penetration studies clearly provide a reasonable functional evidence to believe that the formulation vehicle exploits the convective transport pathways to transport drug into the stratum corneum. Depending on its pharmacological nature, the solute absorbed in the SC during short term exposure could either elicit a local response and/or diffuse eventually into the circulation eliciting a systemic effect. Particularly, in case of agents with lower toxic threshold potential, the extent of drug penetration into skin is of pragmatic significance. In this context, this study was extended to investigate the short-contact penetrability of fentanyl and nicotine from aqueous and ethanolic drug solution in vitro across full thickness porcine skin. 1 mg/mL concentrated drug solution of fentanyl and nicotine was exposed to the skin for a 5-minute contact duration and the amount penetrating the skin was determined. The concentration-depth penetration profile obtained were in correspondence with the previous experiments demonstrating significant absorption into the SC from both the solvents with greater penetration obtained from aqueous drug solution than from alcoholic (figure 1.5). It is reported that the mean plasma concentration of fentanyl in accidental deaths involving abuse is only 26.4 ng/mL. Thus, the extent of penetration observed for fentanyl and nicotine into the skin after a short exposure of only 5 minutes indicates that any accidental or occupational exposure to such chemicals could lead to a precarious situation if left unchecked.
**Figure 1.5:** *In vitro* Stratum Corneum concentration-depth profile of [A] fentanyl and [B] nicotine for short-term exposure study from 1mg/mL concentrated drug solution. N= 6
5. Conclusion

Observations from the extent of absorption for the compounds tested for the short-term penetration study and from published peer-reviewed experimental evidences, it can be inferred that absorption of molecules following short application times is instantaneous and is likely by the virtue of convective transport pathways in the SC. The drug transport via convective transport pathways is predominantly determined by the physico-chemical properties of the solvent and is independent of the physico-chemical nature of the solute. The short-term exposure studies are significant in assessing the risk associated with using dermal/transdermal products containing potent medicaments. It also is important to investigate the extent of penetration of chemicals from the occupational safety perspective.
CHAPTER 2

Pretreatment with Skin Permeability Enhancers: Importance of Duration and Composition on the Delivery of Diclofenac Sodium

1. Abstract

The use of chemical penetration enhancers (CPEs) is one of the most common approaches to improve the dermal and transdermal delivery of drugs. However, often, incorporation of CPEs in the formulation poses compatibility and stability challenges. Moreover, incorporation of enhancers in the formulation leads to prolonged exposure to skin increasing the concern of causing skin reactions. This study was undertaken to assess whether pretreatment with CPEs is a rational approach to enhance the permeation of diclofenac sodium. In vitro experiments were performed across porcine epidermis pretreated with propylene glycol or oleic acid or their combinations for 0.5, 2, and 4 h, respectively. Pretreatment with combination of oleic acid in propylene glycol was found to enhance the permeation of diclofenac sodium significantly only at 10% and 20% (v/v) level, and only when the pretreatment duration was 0.5 h. Longer durations of pretreatment and higher concentration of oleic acid in propylene glycol did not enhance the permeation of diclofenac sodium. In vivo dermatokinetic studies were carried out on Sprague–Dawley rats. A twofold increase in AUC and C_{max} was observed in case of rats pretreated with enhancers over the group
that was pretreated with buffer. In conclusion, this study showed that composition of the enhancers and duration of pretreatment are crucial in determining the efficacy of CPEs.

2. Introduction

The delivery of drugs through skin is an established alternative to other drug delivery systems. The popularity of transdermal drug delivery systems (TDDS) can be attributed to the advantages that it holds over other drug delivery systems. Noninvasiveness, patient compliance, potential for controlled/sustained delivery are few among those many advantages that TDDS has to offer over conventional forms of drug delivery systems. However, skin is less permeable to high molecular weight and polar drugs. Therefore, the number of potential drugs that can be administered transdermally are categorically very small, which emphasizes the need for developing techniques that can improve the permeability of skin. The poor permeability properties of the skin is attributed to the stratum corneum (SC), the “dead” outermost layer of the epidermis. Various approaches have been investigated to breach the barrier property of the SC to enhance the permeation of drugs. Generally, these approaches are divided into physical, biochemical, and chemical methods. Iontophoresis, microneedles, prodrugs, and barrier perturbation with chemical penetration enhancers (CPEs) are the techniques that are employed either singly or in combination to improve drug delivery across the epidermis.

Stratum corneum is an arrangement of corneocytes embedded in a lipid cast. This pattern gives SC the property to be confined to the external environment. CPEs have the ability to reversibly modulate the SC barrier and thereby improve uptake of permeants. Earlier studies on the mechanism of the action of CPEs has suggested that most enhancers act primarily on the lipidic
regions of the SC, thereby promoting easy permeation of drug molecules. Additionally, protein components in the corneocytes contribute to the overall barrier property of the SC. Some enhancers are also known to interact with the protein components, thus assisting better permeation. The enhancers that can simultaneously act on the lipid and the protein regions is likely to be more effective.\textsuperscript{34,35} Generally, CPEs are incorporated along with the transdermal formulation.\textsuperscript{36} However, incorporation of CPEs often poses formulation problems such as immiscibility, incompatibility, and interactions. The other alternative method to promote the percutaneous absorption of drugs is to pretreat the skin with those CPEs that perturb the SC barrier. The primary purpose of the present paper is to rationalize that pretreatment of epidermis is a potential and pragmatic approach to enhance permeation of drugs across the epidermis. The effect of pretreating the skin with different concentrations of oleic acid in propylene glycol on percutaneous absorption of diclofenac sodium, a widely used nonsteroidal anti-inflammatory drug, was investigated in this project. Permeation studies were carried out with aqueous solution of diclofenac sodium; however, to substantiate the pretreatment approach for achieving enhancement, permeation was also performed with HPMC (hydroxypropyl methylcellulose) gel system incorporated with 1% diclofenac sodium and a commercial diclofenac sodium Voltaren\textsuperscript{®} gel formulation. Further, to assess the feasibility of this approach, dermatokinetic profile of the drug was evaluated by cutaneous microdialysis in rats.

3. **Materials and Methods**

3.1 Materials
Diclofenac sodium, oleic acid, isopropyl myristate, propylene glycol, 1-phenyl piperazine, and phosphate-buffered saline (PBS; pH 7.4) were purchased from Sigma–Aldrich Inc. (St. Louis, Missouri). Ag/AgCl wire was purchased from Alfa Aesar (Ward Hill, Massachusetts). All other chemicals and reagents used were of analytical grade. All solutions were prepared in deionized water. Diclofenac sodium gel (1%) was prepared by dissolving HPMC (Methocel E4M premium) powder in one-fifth of the required total amount of water as hot water with continuous agitation until a uniform dispersion is obtained. Drug was dissolved in remainder of the water and was added to the polymer solution with continuous stirring. The mixture was left overnight for effecting complete hydration of the polymer.

3.2 Epidermis preparation

Porcine whole skin from the abdominal region was obtained from a local abattoir. The hair from the skin was shaved off using an electric razor and all the adhering subcutaneous fat and exogenous tissues were removed carefully. Skin was cut into small pieces and wrapped into aluminum foil and then was immersed in water maintained at 60ºC for 2 minutes following which the epidermis was carefully teased off from the dermis. The peeled epidermis was mounted onto glass slides and stored at 4ºC. The stored epidermis was used within 3 days.

3.3 In vitro Study

3.3.1 Experimental setup for permeation studies

In vitro permeation studies were performed across porcine epidermis with Franz diffusion cells. Prior to use, the epidermis was thawed at room temperature for 1 h. The epidermal membrane was carefully mounted on the Franz diffusion cell (SC facing the donor side) having a receiver
volume capacity of 5 mL and was fastened with a rigid clamp. The donor and receiver compartments were filled with PBS (pH 7.4). The integrity of epidermis was checked before starting the experiment by measuring the resistance at a frequency of 10 Hz and low voltage of 100 mV. The epidermis having resistance value greater than 20 KΩ/cm² only was used for permeation studies. During permeation studies, the receiver compartment buffer was stirred throughout the experiment to maintain sink conditions. This setup was maintained at 37°C by a water circulator.

3.3.2 Pretreatment of Epidermis

After measuring the initial electrical resistance, the donor compartment was replaced with 0.5 mL of enhancer solution following which the donor chamber was sealed off with a parafilm. The CPEs used for pretreatment were oleic acid or propylene glycol or their combinations. The enhancer solution was kept in contact with the epidermis for 0.5, 2, and 4 h. Following pretreatment, the enhancer solution was discarded, and the epidermis carefully washed with methanol and wiped off with cotton swabs to remove any adhering enhancer solution. Permeation studies were carried out across the epidermis that was pretreated for 0.5, 2, and 4 h, respectively, with oleic acid or propylene glycol in their neat form or their combinations. Epidermis pretreated with PBS for 0.5, 2, and 4 h was used as a control.

3.3.3 In vitro permeation studies

Permeation was carried out by placing a saturated solution of diclofenac sodium or 1% diclofenac sodium HPMC gel or a 1% Voltaren® gel formulation in the donor chamber for 24 h,
and samples were withdrawn at different time points from the receiver compartment and analyzed using HPLC.\textsuperscript{37}

3.3.4 Extraction of diclofenac sodium from epidermis

At the end of permeation studies, any adhering formulation to the epidermis was removed by washing it with methanol and water. The active diffusion area (0.64 cm\(^2\)) of the epidermis was cut off using a biopsy punch and weighed. The epidermis was then homogenized in methanol using a tissue homogenizer (Tissue miser; Fischer Scientific, Pittsburgh, PA). This solution was kept on a Labquake\textsuperscript{TM} shaker for 24 h for effecting complete extraction of diclofenac sodium. Thereafter, the solution was centrifuged for 15 minutes at 1027 g. The supernatant was collected and was directly injected into HPLC to measure the content of diclofenac sodium in the epidermis. The validity of this procedure was established by spiking known amounts of diclofenac sodium in blank homogenates of epidermis–methanol solution followed by an extraction procedure similar to above. The percentage recovery was found to be greater than 98%.

3.4 \textit{In vivo study}

3.4.1 Dermal Microdialysis in Rats

Microdialysis performed in Sprague–Dawley rats (200–250 g) was an adaptation of a previously reported procedure.\textsuperscript{38} The animal studies were approved by the Institutional Animal Care and Use Committee (IUCAC) at the University of Mississippi (Protocol # 11-016). The hairs from the abdominal region of the skin were shaved off with clippers 1 day prior to the study. On the day of experiment, rats were anaesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) by an intraperitoneal injection. The dorsal region of the skin was punctured with a 20-gauge needle.
A linear microdialysis probe having a 5-mm membrane length with a molecular weight cut off of 30 kDa was inserted through the needle. Thereafter, the needle was removed leaving the dialysis window implanted in the dermal region. The probe was then equilibrated with isotonic PBS (perfusate pH 7.4) for 1 h. During the entire length of the study, PBS (perfusate pH 7.4) was continuously perfused through the probes at a flow rate of 2 µL/min. A cylindrical chamber with an area of 1.77 cm² was glued to the rat skin at the site of probe implantation, and pretreatment of the skin was effected by placing the enhancer solution in this chamber. After 0.5 h, the enhancer solution was removed from the surface, wiped off with cotton swabs and washed with methanol. This was followed by application of 1% diclofenac sodium HPMC gel (1 g) at the pretreated skin area and performing microdialysis for 8 h. At this point, the gel was removed from the application site and microdialysis was further continued for another 4 h. A similar procedure was followed when gel was applied topically to the skin pretreated with PBS (control) for 0.5 h and the drug sampled by microdialysis. The probe recovery was determined \textit{in vivo} by retro-dialysis method where a known drug concentration solution in PBS was perfused through the probe and dialysate collected every hour for 2 h and analyzed by HPLC. The loss of the drug from the perfusate to the extracellular fluid represents the percentage recovery that is calculated using the recovery formula\textsuperscript{38,39}.

\[
\text{(\% Recovery)} = 100 - \left( \frac{\text{Concentration of dialysate}}{\text{Concentration of perfusate}} \times 100 \right)
\]
3.5 Statistical Analysis

Statistical analysis of the data was concluded by using a GraphPad® Insta-5 software. The level of significance between parameters was determined by applying unpaired t-test/one-way analysis of variance. A p value of less than 0.05 was considered as statistically relevant.

4 Results and Discussion

4.1 In vitro permeation studies

i. Diclofenac Sodium solution

In this project, diclofenac sodium was used as a model candidate to study the permeability enhancement of epidermis following pretreatment with enhancers. Diclofenac Sodium has a log P value of approximately 1 and has been reported to be not highly permeable because of moderate lipophilicity. It was evident in the present study, as the permeation flux of diclofenac sodium across PBS treated epidermis was only 1.28 ± 0.24 µg/cm$^2$/h, which was in agreement with the earlier reports.$^{40,41}$ Diclofenac is an analgesic drug available in the form of ointments and gels for the treatment of regional inflammation and pain. It is also available in the form of transdermal patches for systemic delivery. Penetration of therapeutically effective amount of diclofenac is crucial in determining the efficacy of treatment. Chemical enhancers could be used as one of the potential ways of enhancing the dermal and transdermal drug delivery of diclofenac. Isopropyl myristate, nonionic surfactants, hydrogenated soya phospholipids, n-octanol, and decanol, cyclic monoterpenes are certain chemicals that have been reported in the past to have enhanced the permeation of diclofenac sodium across the SC.$^{42-47}$ In some cases, the combination of CPEs showed a synergistic effect and is thus more effective than when each of them was used.
Propylene glycol is widely used as one of the ingredients in various topical formulations. It is used as a vehicle for lipophilic drugs and as a co-solvent for inherent enhancers such as azones, fatty acids, and fatty alcohols. A section of literature reports on the role of propylene glycol as a penetration enhancer suggesting that its action is based on skin. Barry and coworkers reported that propylene glycol could also act as a penetration enhancer under suitable conditions. Oleic acid is a long-chain monounsaturated fatty acid with a cis configuration and has been found to improve the transdermal delivery of various hydrophilic and lipophilic drugs. Extensive literature on the mechanism of enhancer activity of oleic acid proposes that it modifies the nature of lipid domains of the SC. It is reported that oleic acid incorporates a cis double bond in the lipid bilayers and is thus kinked. Few other reports on the mechanism of action of oleic acid indicate the formation of separate phase within the bilayer lipids and induction of a discrete lipid domain within SC lipid bilayers. Generally, oleic acid and propylene glycol are directly incorporated into the topical or transdermal products. During the development of topical products, incorporation of enhancers might lead to immiscibility, interaction, and incompatibilities with other ingredients present in the formulation. Moreover, incorporation of enhancers in the formulation leads to prolonged exposure to skin increasing the concern of causing skin reactions. In a study performed by Tanojo et al. to assess the safety of long-term exposure to oleic acid and propylene glycol, using laser Doppler velocimetry as a tool, the authors found that prolonged (3-24 h) occlusive application of these enhancers lead to significant skin irritation and inflammation. Often, short-term exposure to enhancers might be sufficient to bring about safe and reversible enhancement in the skin permeability and prolonged exposure might not prove advantageous or rather have negative impact on the skin. Therefore, the present study sought the option of using the enhancers as pretreating agents. The epidermis was pretreated for 0.5, 2, or 4
h with an objective of enhancing the transepidermal permeation of diclofenac sodium. Following pretreatment, donor phase was discarded, and the epidermis was gently washed with methanol. The contact time between the epidermis and methanol was less than 1 minute. Sloan et al. have clearly demonstrated that methanol has no disruptive effects on the skin barrier. Even in the present study, in case of control (pretreated with PBS and washed with methanol), no significant alteration of epidermal barrier was observed that agrees with Sloan’s findings.58 Pretreatment with neat oleic acid or propylene glycol alone (at 100% levels) for 0.5, 2, and 4 h resulted in an average flux of 0.63 ± 0.35 and 0.30 ± 0.11 µg/cm²/h, which was low when compared with the flux of diclofenac sodium across PBS-pretreated epidermis (1.28 ± 0.24 µg/cm²/h). Apparently, oleic acid and propylene glycol in their neat forms were found to be permeation retardants rather than enhancers when used as pretreating agents (Figure 2.1). However, when used in combination, significant enhancement was observed only at 10% and 20% oleic acid levels in propylene glycol and only when the pretreatment duration was 0.5 h (Figure 2.2). Apparently, 0.5-h pretreatment appears to be “just sufficient time” to bring about the possible changes in the epidermal barrier and any increase in the pretreatment duration would not help in enhancing the permeability to the drug further. Moreover, pretreatment for more than required duration could even decrease the epidermal permeability for some unknown reason (Figure 2.2). In agreement with the permeation data, the amount of diclofenac sodium retained in the epidermis was also significantly higher in epidermis pretreated for 0.5 h duration with 10% or 20% oleic acid in propylene glycol (Figure 2.3). The retention of diclofenac sodium in the epidermis relates well with the flux values across the epidermis. The above data clearly suggest that optimizing the duration of pretreatment and the composition of enhancer are crucial to achieve optimal drug delivery.
Figure 2.1. Transepidermal permeation flux of diclofenac across the porcine epidermis pretreated with PBS, oleic acid, and propylene glycol for 0.5, 2, and 4 h. The data points represented in the graph are an average of $n = 12 \pm \text{S.D.}$.

Flux across PBS pretreated epidermis was $1.28 \pm 0.24 \text{ (µg/cm}^2/\text{h)}$. 
Figure 2.2. Transepidermal permeation flux of diclofenac sodium across the porcine epidermis pretreated for 0.5, 2, and 4 h pretreatment duration with different concentration of oleic acid in propylene glycol solution. The data points represented in the graph are an average of $n = 12 \pm S.D.$
Figure 2.3. Amount of drug retained in the epidermis pretreated with different concentration of oleic acid in propylene glycol for 0.5, 2, and 4 h. The data points represented in the graph are an average of $n = 12 \pm$ S.D.

ii. Diclofenac Sodium gel

It is customary to carry out permeation studies with aqueous drug solutions; however, gel systems are appropriate formulation choice considering its relevance in clinical application. Often the observations elucidated using aqueous solutions would not translate absolutely when gel systems are used because of the differences in nature and microenvironment between the two
systems. Therefore, it is important to investigate whether the phenomenon observed in case of solutions holds good in case of semisolids as well. In this study, diclofenac sodium HPMC gel (1%) was subjected to permeation studies across epidermis pretreated with PBS (control) and epidermis pretreated with 10% oleic acid in propylene glycol for 0.5 h. The difference in permeation between the enhancer pretreated and PBS-pretreated epidermis was evident with the former showing a fourfold enhancement in flux \((0.422 \pm 0.086 \, \mu g/cm^2/h)\) compared with that across the later \((0.095 \pm 0.04 \, \mu g/cm^2/h)\) (Figure 2.4).

\[\text{Figure 2.4. In vitro permeation profile of diclofenac sodium across enhancer pretreated (10% oleic acid in propylene glycol) (□) versus PBS pretreated epidermis (●) (duration of pretreatment 0.5 h) following application of 1% diclofenac sodium in HPMC gel. The data points represented in the graph are an average of } n = 3 \pm \text{ S.D.}\]
To evaluate further the applicability of pretreatment approach, permeation studies were carried out using Voltaren® gel across pretreated and PBS-treated epidermis. Permeation profile thus obtained showed a threefold enhancement in flux values for enhancer pretreated epidermis (3.79 ± 1.00 µg/cm²/h) than with the PBS-pretreated epidermis (1.69 ± 0.31 µg/cm²/h) (Figure 2.5). The results thus obtained clearly suggest that pretreatment with 10% oleic acid in propylene glycol for 0.5 h holds promise for achieving enhanced drug delivery into and across skin that could be of clinical significance.

**Figure 2.5.** *In vitro* permeation profile of diclofenac sodium across enhancer pretreated (10% oleic acid in propylene glycol) (□) versus PBS pretreated epidermis (●) (duration of pretreatment 0.5 h) following application of Voltaren® gel formulation. The data points represented in the graph are an average of $n = 3 ±$ S.D.
4.2 Dermatokinetics of diclofenac sodium in rats

During the process of development, often some of the novel drug delivery approaches do not escalate to subsequent steps. For example, many in vitro observations do not translate in vivo because of the differences between excised tissue and tissue in the animal model.\textsuperscript{59} Similarly, disparity between preclinical and clinical studies is very common because of anatomical and physiological differences between the animal model and humans. Therefore, to provide additional validity to the in vitro observations, in this study, preclinical evaluation of the pretreatment approach was performed in Sprague–Dawley rat model. Dermatokinetic studies were performed by sampling drug form the dermal extracellular fluid using a minimally invasive technique, microdialysis. Microdialysis allows continuous sampling of unbound drug in the extracellular fluid of a local tissue. In cutaneous microdialysis, a probe consisting of a semipermeable dialysis membrane is implanted in the dermal region of the skin. When the probe is perfused with an isotonic solution, a concentration gradient is created that allows for diffusion of drug from the dermal extracellular fluid into the perfusing fluid across the semipermeable membrane. Cutaneous microdialysis for the assessment of enhancer activity following topical application of drugs has been previously reported.\textsuperscript{60} This technique has also been successfully employed to study pharmacokinetics of drugs in rat dermis following topical application.\textsuperscript{61}

In this study, 1% diclofenac sodium gel was applied topically, and the drug was sampled from the dermal region following pretreatment with either enhancer or PBS for 0.5 h. Dermatokinetic parameters obtained from dermal concentration–time profile of diclofenac sodium is shown in Table 2.1. The dermal extracellular fluid (ECF)-time profile presented in Figure 2.6 indicates the kinetics of only the unbound drug concentration and it does not refer to the total
amount of drug obtained from the topical formulation. Particularly in case of diclofenac sodium, the unbound drug sampled was relatively less because of its high protein binding ability. The in vivo recovery of diclofenac sodium obtained from the retro-dialysis method was 35%.

Generally, to assess one mode of drug delivery in comparison to other in terms of bioavailability, it has been suggested to consider the differences in the AUCs and $C_{\text{max}}$ values. In this study, there was about twofold increase in $\text{AUC}_{(0-t)}$ and $C_{\text{max}}$ in the enhancer pretreated rats over the PBS-pretreated control group. However, the enhancement factor observed in vitro across the porcine epidermis was fourfold that turned into only twofold, in vivo in rat model. This disparity could be attributed to the differences in the structure and lipid composition in the SC between rat skin and porcine skin. The dermal extracellular fluid concentration–time profile of drug represented in Figure 2.6 shows the clearance profile of drug from the dermal region after the formulation was removed from the skin surface at the 8th hour. A drastic drop in the dermal drug concentration could be noted from 8th to 9th hour following an exponential disposition. The rate constant calculated using log10 for concentration values starting 8th to 12th hour shows that there was no significant difference between the control and pretreated groups (0.70 ± 0.23 h$^{-1}$ vs. 0.79 ± 0.18 h$^{-1}$), indicating that pretreatment did not lead to an enhanced reservoir formation (as in porcine epidermis, in vitro) in the skin at levels significant to cause any differences in the kinetics of dermal clearance of diclofenac sodium.
### Table 2.1. Dermatokinetic Parameters of Diclofenac Sodium in Rats pretreated with PBS and Enhancer (10% oleic acid in propylene glycol). 1% diclofenac sodium in HPMC gel was applied after pretreating the skin for 0.5 h.

<table>
<thead>
<tr>
<th>Dermatokinetic parameters</th>
<th>PBS Pretreated Skin</th>
<th>Enhancer Pretreated Skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC$_{(0-t)}$ (ng.h/mL)</td>
<td>1242.28 ± 13.94</td>
<td>25085.54 ± 48.85</td>
</tr>
<tr>
<td>C$_{\text{max}}$ (ng/mL)</td>
<td>176.54 ± 57.64</td>
<td>394.09 ± 49.85</td>
</tr>
<tr>
<td>T$_{\text{max}}$ (h)</td>
<td>6.50 ± 2.12</td>
<td>8</td>
</tr>
</tbody>
</table>
Figure 2.6. Diclofenac sodium concentration in the dermal extracellular fluid of rats pretreated with PBS (●) and 10% oleic acid in propylene glycol enhancer (□) for 0.5 h. Data points represent an average of \( n = 3 \pm S.D. \)

5 Conclusion

The studies suggest that chemicals that lack the skin permeability enhancing ability in neat form could turn into effective enhancers in certain compositions. There is need for a thorough screening of enhancers in different compositions to figure out the appropriate composition of enhancers. The myth that the duration of pretreatment and extent of increase in skin permeability
is positively correlated may not be true in all cases. As in this present study, it may be necessary to optimize the duration of pretreatment to exploit the CPEs for dermal/transdermal drug delivery.
CHAPTER 3

Rapidly Dissolving Microneedle Patches for Transdermal Iron Replenishment Therapy

1. Abstract

The prevalence of Iron Deficiency Anemia (IDA) is predominant in women and children especially in developing countries. The disorder affects cognitive functions and physical activity. While oral iron supplementation and parenteral therapy remains the preferred choice of treatment, gastric side effects and risk of iron overload decreases adherence to therapy. Transdermal route is an established approach which circumvents the side-effects associated with conventional therapy. In this project, an attempt was made to investigate the use of rapidly dissolving microneedles loaded with Ferric pyrophosphate (FPP) as a potential therapeutic approach for management of IDA. Microneedle array patches were made using the micro molding technique and tested in vitro using rat skin to check the duration required for dissolution/disappearance of needles. The ability of FPP loaded microneedles to replenish iron was investigated in anemic rats. Rats were fed iron deficient diet for 5 weeks to induce IDA following which microneedle treatment was initiated. Recovery of rats from anemic state was monitored by measuring hematological and biochemical parameters. Results from in vivo study displayed significant improvements in hemoglobin and serum iron levels after two-week treatment with FPP loaded microneedles. The study effectively demonstrated the potential of microneedle mediate iron replenishment for treatment of IDA.
2. Introduction

Iron is a principal component required for the synthesis of oxygen transporters hemoglobin and myoglobin thorough Erythropoiesis. Generally, Iron deficiency anemia (IDA) is a condition which develops when the dietary iron consumption & iron stores are not able to meet the body’s iron requirement. The requirement for iron outweighs the intake especially affecting adolescent girls in their menstrual cycle that endure depleted iron stores due to menstrual iron loses. Pregnant women are at a greater risk because a major proportion of the iron stores are utilized in the development of fetus and placenta. Iron malabsorption due to gastrectomy and bypass gastric surgery is one of the major causative factors for IDA. Several drugs can induce IDA by reducing iron absorption from GIT or by increasing blood loss. IDA can have severe clinical manifestation and is often underdiagnosed. In adults, common symptoms of IDA include retardation of physical performance, productivity and functional efficacy. Children acquiring IDA suffer from physiological as well psychological implications & cognitive impairment. In pregnant women IDA is associated with preterm labor, maternal mortality and fetal death. A major neurological ramification of IDA is the restless leg syndrome which is an index of reduced iron levels in the brain. Intervention strategies for treatment of IDA is well established with oral iron supplementation being the first line of therapy. Several extended and immediate release formulations of iron (ferrous and ferric salts) are available in the market for treating IDA. Since duodenum is the predominant absorption site for iron, only a fraction of oral dose (10 to 15%) is absorbed from the GIT. To be able to cope up with the excessive iron demands these iron medications are usually prescribed to be taken at frequent intervals and having longer dosage regimens often producing gastric complications such as nausea, heartburn, pain, constipation and
diarrhea which can be attributed to higher iron doses. Parenteral iron therapy is an alternative therapeutic strategy which is preferred in severe cases of IDA. Although parenteral iron therapy is more effective than oral, it is invasive and is often associated with safety issues such as oxidative stress due to iron overload, anaphylactic reactions and infection. Additionally, parenteral route presents economical barriers as it needs to be performed under medical supervision. Transdermal delivery of iron is a practical approach and has been successfully explored in the past for the delivery of ferric pyrophosphate (FPP). Transdermal delivery of iron across skin with FPP was explored using different passive and active enhancement strategies including iontophoresis, chemical penetration enhancers and microporation. While these exploratory strategies present an interesting prospect for the delivery of iron across the skin (and possibly a short-term approach) an iron fortification technology that works for both acute and long-term management of IDA would present a better undertaking. Microneedles are minimally invasive miniature drug delivery devices that upon application on the skin creates reversible transient pores briefly circumventing the stratum corneum barrier. Soluble microneedles are manufactured using biodegradable polymers and an incorporated drug payload. Upon insertion, these soluble microneedles dissolve in the interstitial fluid of the skin releasing the embedded drug molecule thereby providing a direct route of drug delivery, an approach similar to bolus dermal injection, however devoid of any pain & bioburden unlike the invasive and anaphylactic attributes of an injection. In this direction, Modepalli et al. investigated the feasibility of this approach and established the in vivo dermal kinetics and safety profile of FPP delivered from soluble microneedles. The present work is an extension of this study and explores the prospects of using hyaluronic acid microneedles loaded with FPP for transdermal iron replenishment. Soluble FPP was used as the iron source because of its aqueous solubility and proven stability and safety profile. Microneedles were made using
hyaluronic acid as the rapidly dissolving biodegradable polymer and the fabrication process followed a previously reported protocol\textsuperscript{73}. IDA was induced in Sprague dawley rats and subsequently treated with microneedles loaded with FPP. Hematological and biochemical parameters were measured to confirm the recovery of rats from an iron deficient anemic states to the normal healthy states.

3. Materials and Methods

3.1 Materials

Iron pyrophosphate (Ferric pyrophosphate) soluble crystals as a source of iron was obtained from Sigma-Aldrich (St. Louis, MO). Hyaluronic acid obtained from Bloomage Freda Biopharm USA Inc. (Parsippany, NJ) was used as a casting material for microneedles. Ferrover\textsuperscript{®} iron reagent was purchased from HACH (Loveland, CO). Serum Iron kit was obtained from Cliniqa Corporation (San Macros, CA). All other chemicals and reagents used for the study were procured from Fischer Scientific (Fairway, NJ) and were of analytical standards.

3.2 Preparation of rapidly dissolving microneedle patches loaded with FPP

Microneedles were fabricated by mold casting method using Hyaluronic acid (HA) as the casting material. Briefly, FPP was mixed with distilled water to make a 250 mg/mL concentrated solution. HA (molecular weight of approximately 10 kDs) was then added to this aqueous FPP solution to make a 50% w/w solution of HA. The HA-FPP viscous blend was then poured over PDMS micromolds and centrifuged at 4150 X g for 5 minutes to depress the solution into mold cavities. Excess solution outside the cavities was pipetted out and replaced with blank solution (without FPP) of high molecular weight HA (30-40 kDa) to serve as the needle base. The
micromold was kept overnight in a desiccator to facilitate drying following which the microneedles were detached from the molds. Final microneedles were observed under an optical microscope before *in vitro* and *in vivo* studies to check for any surface imperfections. Microneedles patches with absolute needle morphology were exclusively used for the study.

3.3 Microscopic evaluation of FPP loaded microneedle patch

Microneedles were evaluated for their morphological characteristics by Scanning Electron microscopy (JSM-5600, JEOL Ltd., Tokyo, Japan). The patches were fixed on aluminum stabs by using glued carbon tapes. The samples were then sputter coated with gold (Hummer 6.2 sputter coater, Anatech USA, Union City, California) and subsequently subjected to electron microscopic evaluation of their morphology and topography.

3.4 *In vitro* Study

3.4.1 Quantification of total iron content in soluble microneedles

Total iron content loaded in the microneedles was determined by performing *in vitro* dissolution studies. A single microneedle array was enclosed in a hermetically sealed chamber with only the needle shaft exposed to the dissolution medium. Dissolution study was initiated by mounting the microneedle-chamber in a vertical Franz diffusion cell (0.64 cm², 5mL volume), the needle shafts facing the receiver compartment and the setup secured using clamps. The receptor fluid consisted of pH 5.0 PBS. The experiment was conducted for 5 minutes after which the setup was dismantled, and the receiver compartment collected in its entirety and analyzed for total iron content using Ferrover® iron reagent.
3.4.2 Skin deposition Study

Skin deposition experiment was conducted *in vitro* on excised rat skin. A single microneedle patch was applied on the rat skin and held in its place for 5 minutes. Thereafter, the microneedle patch was detached, and the skin washed with water to discard residual peripheral iron. Iron content in the skin was determined by digesting the skin with 1 N sodium hydroxide and analyzing for iron using Ferrover® Iron reagent.

3.5 *In vivo* Study

*In vivo* study was performed on Male Sprague-Dawley rats (Charles River, Hollister, CA), weighing 250–275 g. Approval for *in vivo* animal experiments was obtained from the Institutional Animal Care and Use Committee (IACUC) of The University of Mississippi (Approval No # 10-013). The animals were housed in the animal care facility and allowed access to standard rat diet and water ad libitum for one week. The rats were tested for their basal hematological and biochemical parameters by using a Sysmex-XP100 model Hematology analyzer. Briefly, blood was withdrawn using the retro-orbital bleeding technique and collected into micro centrifuge tubes. For hematological measurements, 0.5 ml of the blood was collected into heparin coated micro tubes and analyzed for hemoglobin (Hb), Red blood Cell (RBC) count and Hematocrit (HCT). Serum iron concentration as a biochemical parameter was measured by collecting 1 mL blood sample in a centrifuge tube and allowing it to clot at room temperature. After 15 minutes, clots were removed by centrifuging at 3000 rpm for 20 mins. Serum was separated as the upper clean layer and analyzed for Serum iron concentration (µg/mL) using a Serum Iron Assay Kit.

3.5.1 Induction of Iron Deficiency Anemia (IDA)
Rats were induced with IDA following a previously reported diet protocol. A standard reference diet was prepared as per the compositional guidelines of the American Institute of Nutrition (AIN-76) having all the ingredients in required quantities present except for the iron concentration\textsuperscript{74}. The iron concentration was kept at a stringent 2 to 6 ppm compared to the 34.25 ppm suggested in the guidelines. Rats were kept on this custom-made iron deficient diet throughout the duration of the experiment. Hematological and biochemical parameters were evaluated every week to check the extent of Iron deficiency. The induction of IDA in rats was acknowledged by measuring the hematological and biochemical parameters after 5 weeks of being kept on Iron deficient diet.

3.5.2 Application of microneedles loaded with FPP to Anemic rats

FPP loaded microneedles were applied to the skin following induction of anemia to the rats. Prior to the administration of microneedles patches, the intended site of application was determined (dorsal region) and the spot was shaved off to have a uniform area for microneedle administration. The patches were then applied to the application site and secured using an adhesive bandage for 5 minutes. The fate of microneedles post application to the rat skin was studied using bright field microscopy (Olympus Trinocular microscope BX53). The recovery of rats from an anemic state was monitored every week by measuring the hematological and biochemical parameters. The course of the treatment was stopped upon the complete revival of the rats from the anemic state to the healthy state which was confirmed by the recovery of the hematological and biochemical parameters to the basal values.
3.6 Statistical Analysis

GraphPad® Instat Software was used for statistical analysis. The level of significance between parameters obtained at healthy and anemic states and between anemic states and post microneedle treatment of the rat was determined by applying an unpaired t-test. A P-value less than 0.05 was considered as statistically significant.

4 Results and Discussion

4.1 Morpho-metrics of FPP-Soluble Microneedles

Since FPP is poorly permeable across the skin owing to its unfavorable physiochemical properties, (log P: -1.4, Molecular weight: 745.22 g/mol) it is difficult to deliver therapeutic amounts of FPP using passive transdermal delivery approach especially in case of severe iron deficiency conditions. Previous studies on transdermal delivery of FPP have reported significant enhancement in the delivery of iron across the skin using a combination of active & passive enhancement techniques. The reported in vitro study was successfully extrapolated to in vivo experiments. HA has an established safety profile due to its wide use in dermatological products. Previous studies using HA as the matrix material for microneedles have demonstrated microneedles with good mechanical strength. For the present study, soluble FPP loaded HA microneedles were fabricated using PDMS micro-mold (master mold) by a micro molding process. The microneedles were evaluated for their morphological attributes using SEM (Figure 3.1). A single microneedle patch constituted a 0.5 cm² area over a 10 x 10 array platform, all needles spaced at 464.31 ± 12.822 µm distance apart from each other and measured 467.59 ± 15.23 µm in
height & $183.29 \pm 18.68 \, \mu m$ in base width (aspect ratio = 2.5) with a tip diameter of $6.48 \pm 1.22 \, \mu m$.

**Figure 3.1:** Scanning electron microscopic images of microneedles loaded with FPP at different magnification. (a) 40X (b) 100X (c) 250X. Microneedles were made using a reverse transcription molding process. Each patch comprises of 100 needles arranged in a 10 X 10 array.

4.2 *In vitro* dissolution and skin deposition of FPP from soluble microneedles

Two different molecular weights of HA were used for forming the microneedle patch which serves dual purpose. Low molecular weight HA forms the needle shafts which provides...
immediate release of FPP within minutes upon insertion in the skin. High molecular weight HA forms a base with remarkable mechanical support. The results from *in vitro* dissolution study shows that the total FPP contained in a single microneedle patch was 196.65±30.76 µg. The microneedles were subjected to skin deposition experiments in excised rat skin to observe disappearance of microneedles in the skin within a short span of time. Figure 3.2A and 3.2B represents the optical microscopic images of microneedles before and after insertion into the skin. The amount of iron recovered from skin after 5 minutes application of the microneedle patch was 130.54±18.64 µg which is about 66% of the total load in the microneedles. The difference in the amount loaded to the amount delivered into skin could be attributed to the partial penetration of the microneedles into the skin owing to its morphology.

![Figure 3.2](image)

**Figure 3.2**: Optical microscopic images of the FPP-loaded microneedle patch [A]. Two-step fabrication process yields an array with FPP localized only in the shaft. The picture represents needles before application. [B] The picture represents needles after in vitro application to the excised rat skin.
4.3 *In vivo* evaluation of Microneedles loaded with FPP in anemic rat model

IDA is acquired due to reduced iron levels in the body, therefore diagnostic investigations of the condition should provide substantial evidence of anemia as well as of low iron stores. Several laboratory tests are available for the diagnosis of IDA. According to the World Health Organization (WHO), anemia is defined as a condition arising from the reduction of red blood cells or when the blood hemoglobin concentration is 2 counts below the normal mean population levels\(^77\). Since low blood hemoglobin concentration and RBC count are the characteristic manifestation of anemia, these hematological parameters are widely considered to be the most appropriate indicators of anemia. Additional screening test for anemia includes measuring percentage Hematocrit which is a measure of the space the red blood cells takes up in the whole blood. Serum iron levels reflects a section of all the iron that circulates in the body as transferrin bound iron and hence is a critical biochemical parameter of the iron status. Thus, an exact interpretation of IDA involves measuring for the low levels of hematological and biochemical parameters which are the clinical indexes of IDA\(^78,79\). In the present study, rats were induced with iron deficiency anemia following a 5-week regimen of an iron deficient diet (2 – 6 ppm iron concentration). A significant reduction in the levels of both hemoglobin & serum iron concentration was observed from their basal values at the healthy states which confirmed the induction of IDA. Table 3.1 shows the mean values of the measured hematological and biochemical parameters in the healthy and the subsequent iron deficient anemic state of the rats.
Table 3.1: Observed Mean Hematological and Biochemical Parameters Obtained at Healthy and Anemia States of the Rats. Substantial reduction in all the measured parameters confirmed the induction of anemia after a 5-week regimen of iron deficient diet. Significant difference in all the parameters between healthy and anemic states was observed except for the Serum iron Concentration. Data points represent an average of n = 4 ± S.D.

\[ \begin{array}{|l|c|c|}
\hline
\text{Parameters evaluated} & \text{Healthy Rats} & \text{Anemic Rats} \\
\hline
\text{Hemoglobin (g/dl)} & 17.35±2.79 & 13.575±0.53^a \\
\hline
\text{RBC (x 10^{12}/L)} & 9.79±1.02 & 8.015±0.135^b \\
\hline
\text{Hematocrit (%)} & 54.4±7.16 & 43.15±1.21^c \\
\hline
\text{Serum Iron Concentration (%)} & 123.27±48.06 & 84.95±38.85^d \\
\hline
\end{array} \]

\[ ^a p = 0.0374 \quad ^b p = 0.0136 \quad ^c p = 0.0212 \quad ^d p = 0.2612 \]

The daily recommended oral dose for the treatment of iron deficiency anemia in adults is in the range of 150 to 200 mg of elemental iron\textsuperscript{66,80}. Since it is estimated that only 10% to 15% of the total iron taken orally undergoes absorption, a 325 mg of ferrous sulphate tablet containing 65 mg of elemental iron prescribed 3 times a day will provide 195 mg of elemental iron of which approximately 20 mg of iron is absorbed \textsuperscript{81-84}. Thus, anemic rats weighing 150-200 grams would require around 50 µg of elemental iron daily to revive iron stores. In the present study, a single FPP loaded microneedle patch was able to deliver approximately 130 µg of FPP across excised rat skin (corresponding to 15 µg of elemental iron). Thus, to achieve the target iron levels and accounting for the higher demands of iron in rats, a dosage regimen comprising of 4 microneedles patches per day per rat was determined to be an appropriate course for the treatment. An added
therapeutic objective was to achieve rapid improvements in the anemic states of the rat to prevent death due to extreme anemia. An application duration of 5 minutes ensured maximum dissolution of the needles which was confirmed by bright field microscopic images (Figure 3.3).

Figure 3.3: Bright field microscopic images of microneedles loaded with FPP (A) before and (B) after in vivo application of rats

An effective treatment of iron deficiency anemia was accomplished by a 1.5 g/dl recovery in the hemoglobin levels within 1 week of treatment with FPP loaded microneedles followed by a steady improvement in RBC count and percentage hematocrit levels. The microneedle treatment was continued for the second week. Figure 3.4 shows a gradual increase in the hematological and biochemical parameters to the target levels over a two-week treatment period with microneedles. It is widely accepted that an iron replacement therapy is deemed appropriate when the hemoglobin levels in the body is improved by 2 g/dl. Our findings demonstrate that the mean hemoglobin levels of 13.575±0.53 g/dl at the anemic states of the rats increased to 15.35±0.66 g/dl after a 2-week treatment with microneedles (p=0.0057). Target levels were attained for RBC count and hematocrit which suggests that enough iron was delivered from the microneedles to reinstate
regular erythropoiesis. A more significant recovery of iron was attained in the serum with serum iron concentration increasing from $84.95\pm38.85 \, \mu g/mL$ at the anemic state to $163.12\pm5.15 \, \mu g/mL$ post the treatment duration ($p=0.0072$). The microneedle treatment was stopped after two-weeks following the successful recovery of anemic rats.

**Figure 3.4:** Target recovery levels to achieve homeostasis and gradual revival of the blood count and iron stores in anemic rats over a 2-week treatment period with FPP-loaded microneedles (FPP-MN). The difference in all the measured parameters between microneedle-treated rats and anemic rats was significant with $p$ value less than 0.05. Data points represent an average of $n = 4 \pm$ standard deviation.
5 Conclusion

Regardless of its widespread occurrence and well-recognized etiology, management of IDA is often accompanied with undesirable side effects and insufficient response to treatment. The present work addresses this unmet medical need and demonstrates an early stage proof of concept effort to understand the efficacy of microneedle mediated approach to replenish iron stores in iron deficient rats. Soluble microneedles loaded with therapeutic doses of FPP was able to deliver enough elemental iron across the skin to correct induced iron deficiency anemia in rats. Considerable replenishment of the iron stores was achieved as evidenced from the serum iron levels. In humans, however, this approach to deliver iron could be very well suited to correct latent iron deficiency (LID) or mild anemic conditions especially in infants and paediatric population. Iron deficiency during maternal stages is often translated into late preterm and term infants as LID which manifests abnormal neural development (due to negative iron status in the brain). The regular dose of iron to regulate LID is about 1-3 mg of elemental iron per day. Thus, to achieve the target iron levels, a 10-15 cm$^2$ patch would be sufficient in patients with LID to moderate iron deficiency. In severe cases of Iron deficiency such as Iron Deficiency Anemia where more Iron needs to be delivered to suffice the demands, alternative formulation strategies could be employed such as increasing drug loading in the microneedles or by reducing microneedle aspect ratio to incorporate more needles/cm$^2$ of patch area. This mode of therapy circumvents the traditional iron restoration approaches of oral and parental administration by providing a more practical patient-specific approach. Depending upon the severity of anemia and the level of iron in the body, dose individualization is possible. More importantly, microneedle mediated iron restoration does not cause any undesirable GI effects or localized infections as repeated application does not encourage
any unwanted microbial contamination. Thus, microneedle mediated treatment of IDA has favorable regulatory prospects and demands further clinical attention.
CHAPTER 4

Evaluation of soluble fentanyl microneedles for Anti-Nociceptive activity

1. Abstract

The use of opioids for treating acute and chronic pain condition is a common clinical practice. However, the analgesic activity is mediated through the central pathway, which although effective, leads to various adverse effects such as dependence, abuse and respiratory depression. Fentanyl is an opioid analgesic that is available as injection and transdermal patch for pain management. Further, systemic exposure of fentanyl often leads to serious central side effects and has major abuse potential requiring stringent regulatory controls and label warnings on disposal. This research project evaluates the regional antinociceptive efficacy of fentanyl delivered from soluble microneedles. The microneedle patches were formulated with low drug loading and tested for their antinociceptive activity in rats by measuring the paw withdrawal latencies, post application of the patches, to the plantar surface of the hind paw when exposed to a thermal stimulus. The results indicate that regional delivery of fentanyl mediated through soluble microneedles provides an effective anti-nociceptive activity. The onset of analgesic activity was faster with microneedle patch (0.5 hour) when compared to the adhesive dermal patch (6 hours).
This study thus demonstrates the effectiveness of microneedle mediated pain management for immediate pain relief.

2. Introduction

The International Association for the Study of Pain (IASP) describes pain as an aggressive sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage\textsuperscript{86}. The definition suggests that pain can also be perceived in the absence of tissue damage. It also identifies pain to be instinctive as perception of pain differs in each individual depending on their emotional and sensory capacity and experiences with injuries. Pain can be classified in many ways; however, the most common types of pain is the nociceptive pain (NP). NP arises when a nociceptive stimulus or a noxious stimulus is detected that leads to the activation of the nociceptors\textsuperscript{87,88}. These are the sensory receptors of the peripheral somatosensory nervous system expressed throughout the body, including the visceral tissues that respond to mechanical, thermal and chemical stimulus. Activated nociceptors initiate a neural process of encoding the stimulus leading to a signal transduction from the peripheral to the central nervous system. Depending on the site of origin, NP can be categorized into three types, namely cutaneous, somatic and visceral\textsuperscript{89}. While cutaneous and somatic NP is constant and typically well localized in a small region, visceral pain originates from internal organs, is episodic and not confined to one region. NP is characteristically acute and temporary, however, depending upon the conditions it can become chronic\textsuperscript{89,90}. The current research focuses on developing a new drug delivery approach for the management of cutaneous nociceptive pain (CNP). Common attributes of a CNP includes sharp stinging pain that is localized to the site of injury or inflammation. Treatment approaches for CNP requires understanding of the stimulus that leads to nociceptor activation and mostly rely on
administration of nonsteroidal anti-inflammatory drugs (NSAIDs). Since CNP responds well to opioids, it is commonly prescribed in chronic pain conditions especially when conventional pain medications do not provide adequate pain relief. The therapeutic objective of these treatment modalities is to provide adequate pain relief by maintaining stable drug concentrations at site of action. However, because of unwanted systemic exposure, both NSAID’s and opioids have been reported to exhibit side effects which includes stomach pain, ulcers, bleeding, physical dependence and addiction. Cutaneous drug administration in the form of a creams, spray lotions or a patch directly at the peripheral site of origin of the pain can convey drug concentrations at lower doses with minimal plasma exposure and without compromising the therapeutic potential. This approach reduces adverse effects that are encountered with various conventional formulations. However, the barrier properties of the skin limits absorption of drugs after local application. Drug permeation across the skin is a slow process and often requires multiple application regimen to attain therapeutically effective concentrations required for pain relief. Additionally, when immediate pain relief is desired, conventional formulations cannot deliver the drug fast enough to provide a quick onset of action. In the event when rapid drug concentrations are required locally into the skin, breaching the skin barrier is an effective technique. To address this unmet need, the present work explores the prospect of using rapidly dissolving microneedles loaded with fentanyl for inducing immediate anti-nociception. Microneedles are minimally invasive miniature drug delivery devices that can be fabricated with biodegradable polymeric materials and an active payload. When applied, these microneedles channel the skin and releases the payload. Depending upon the molecular weight of the polymer used for fabrication, onset of microneedle dissolution in the skin can be controlled, thus providing a window for achieving rapid drug concentrations into the skin. Fentanyl is a pure opioid receptor agonist that has been widely used for chronic cancer.
pain as well as postoperative pain management. Fentanyl and its analogues in the form of infusions and transdermal patches provide prolonged analgesic activity by maintaining sustained plasma levels\textsuperscript{95,100}. However, activation of central opioid systems leads to several undesired side effects such as addiction, nausea and respiratory depression. Since opioid receptors are widely expressed on the peripheral nervous system, locally applied fentanyl is effective in treating acute pain conditions\textsuperscript{101}. The proposed project tests this hypothesis by evaluating the antinociceptive activity of fentanyl in a rat model by using the hot plate method. The paw withdrawal latency of the hind-paw treated with fentanyl microneedles was compared with the non-treated control paw and the efficacy of this treatment approach for anti-nociceptive activity was evaluated.

3. Materials and Methods

3.1 Materials

Fentanyl was procured from Noramco Inc. (Athens, GA). Hyaluronic acid formed the skeleton for microneedles and was purchased from Bloomage Freda Biopharm USA Inc. (Parsippany, NJ). For the transdermal patch, Duro-Tak\textsuperscript{TM} 87-2677 pressure sensitive adhesive was obtained as a gift sample from Henkel Corporation (Bridgewater, NJ). Super-Polyfoil obtained from Sigma–Aldrich Inc. (St. Louis, Missouri) was used as a backing membrane. Scotchpak\textsuperscript{TM} obtained from 3M was used as a release liner. All other chemicals and reagents used for the study were of analytical grade. Fresh porcine skin was used for all the \emph{in vitro} skin deposition experiments and was obtained from the local abattoir.

3.2 Preparation of fentanyl microneedles
Microneedles were fabricated by mold casting method using Hyaluronic acid (HA) as the casting material. Briefly, 50% w/w solution of HA (molecular weight of approximately 10 kDs) in distilled water was mixed with 15 mg/mL of fentanyl. The solution was poured over PDMS micro molds and centrifuged at 4150 X g for 5 minutes to depress the solution into mold cavities. Excess solution outside the cavities was pipetted out and replaced with blank solution (without fentanyl) of high molecular weight HA (30-40 kDa) to serve as the needle base. The micro mold was kept overnight in a desiccator to facilitate drying following which the microneedles were detached from the molds. Final microneedles were examined by optical microscope and needles with complete morphology were used for further in vitro and in vivo experiments.

3.3 Scanning Electron Microscopy (SEM)

The fabricated microneedles were subjected to Scanning Electron Microscopy (JSM-5600, JEOL Ltd., Tokyo, Japan). Previously reported procedure was followed\textsuperscript{102}. Briefly, microneedles were fixed on aluminum stabs using glued carbon tapes. The samples were then sputter coated with gold (Hummer 6.2 sputter coater, Anatech USA, Union City, California) and observed by a Scanning Electron Microscope to derive its morphology and topography.

3.4 Microneedle-skin insertion imaging

Insertion characteristics of the microneedle patch was determined in vitro on rat skin using microneedle fabricated with CM-Dil fluorescent red dye. Briefly, dye loaded microneedle patch was applied onto the rat skin for 2 minutes and removed immediately. Thereafter, the skin was gently wiped with Kim-wipes\textsuperscript{®} and placed upon glass slide. The skin was observed using a Nikon
eclipse Ti2 fluorescent microscope with Tetramethyl rhodamine filter and emission wavelength of 555 nm.

3.5 Preparation of fentanyl dermal patch

Fentanyl patches were prepared by solvent evaporation casting technique. Fentanyl was dissolved in ethyl acetate and ethanol in a 1:1 ratio. Duro-Tak 87-2677 was selected as the adhesive polymer based on the Henkel’s drug in polymer solubility calculator. An appropriate amount of the adhesive (per gram of the dried weight of the polymer) was added to the drug solution and agitated at room temperature for 1 hr and later stirred overnight to ensure proper mixing. The homogenous drug-polymeric solution was then coated onto a silicon backing membrane. To remove solvents, the patches were first maintained at room temperature for 15 minutes followed by drying in an oven at 40°C for 12 hours. The dried patches were capped with a release liner and stored at room temperature until used for further studies. The test patches were evaluated microscopically and patches without any crystals and air-bubbles were exclusively used for in vitro and in vivo studies\textsuperscript{103}. The dermal patches were prepared with 3 different fentanyl loading doses of 0.25, 0.5 and 1% to determine the appropriate strength required for inducing analgesia. Each patch was 10 cm\textsuperscript{2} in area.

3.6 In vitro Study

3.6.1 In- vitro drug release from fentanyl dermal patch

The in vitro drug release characteristics of the patch was evaluated for 24 h at 32°C using Franz diffusion cells having an active diffusion area of 0.64 cm\textsuperscript{2} and 5 mL receiver volume capacity. The formulated patches of Fentanyl were punched out to a 1 cm\textsuperscript{2} area for the release
study. Subsequently, the release liner was removed, and the patch was applied on the diffusion cell with the adhesive side facing the receiver compartment. The receiver compartment consisted of de-aerated pH 6.8 phosphate buffered saline which was continuously stirred at 600 rpm throughout the experiment. The solubility of the drug in the receiver medium at 32°C was 0.75 mg/mL. This along with the replacement of sample aliquots with fresh buffer maintained the sink conditions for the duration of release experiment\textsuperscript{104}. At predetermined time points 0.5 mL of the receptor solution was sampled and replaced with blank receiver medium solution. Samples were analyzed using a previously reported HPLC method\textsuperscript{105}.

3.6.2 \textit{In-vitro} skin penetration study

The \textit{in-vitro} skin penetration study was conducted across freshly excised Sprague-Dawley rat skin. The abdominal skin area of the animal was shaved off with electrical clippers and excised using surgical scissors. The subcutaneous fat was removed, and the skin cleaned with PBS. The prepared skin was then mounted on a Franz-diffusion cell (similar setup as used for \textit{in vitro} release testing) and secured between the receiver and donor chamber using metal clamps. The donor and receiver compartment was filled with PBS (pH 6.8) and the integrity of skin was checked by measuring the resistance at a frequency of 10 Hz and low voltage of 100 mV. The skin with a resistance value greater than 10 KΩ/cm\textsuperscript{2} was exclusively used for the studies\textsuperscript{70}. For the microneedle patch, skin penetration experiment was carried out by applying the patch on the rat skin for a 0.5-hour duration. Later, the skin was removed from the set-up and washed first with methanol-soaked Q-tips\textsuperscript{®} and then with water to remove any superficially unabsorbed fentanyl. The skin was dissolved in 2 mL 1 N sodium hydroxide solution and extracted for fentanyl by a solvent-solvent extraction procedure using acetonitrile (2 mL) as the organic immiscible solvent.
The extraction procedure was standardized by using known fentanyl standards and calculating percent fentanyl recovery from the organic phase. Samples were injected into HPLC for quantitative analysis. A similar procedure was followed for skin penetration study from the fentanyl dermal patch. Application time for the patch was varied until relevant levels of fentanyl required for eliciting an analgesic response was achieved.

*In vivo study*

3.6.3 Anti-nociceptive activity

Male Sprague-Dawley rats (Charles River, Hollister, CA), weighing 250–275 g were housed in groups of three in the animal care facility at the University of Mississippi, Oxford, Mississippi under a 12 h light/dark cycle and were allowed access to standard rat chow and water ad libitum. All animals were tested during the 12 h light cycle (7 a.m.–7 p.m.) on successive days. Experiments followed American Association of Laboratory Animal Care guidelines, were approved by the Institutional Animal Care and Use Committee (IACUC) of UM (Approval No.16-016 dated 02/12/2016) and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of pain. The animals were randomly grouped into two groups of six animals each. Group I was treated with fentanyl microneedles and group II with fentanyl dermal patch.

**Hot Plate Analgesia:** The animals were allowed several days of acclimatization for handling. Two days prior to experimentation they were allowed to acclimatize for baseline thermal latency. On the day of the experiment, the patches were applied on the plantar surface of the hind limbs and secured using an adhesive bandage. The patches were removed (After 0.5 h for the
group I and periodically after 0.5, 2, 4 & 6 h for group II) and the animals were subjected to hot plate analgesia testing using a hot plate analgesia meter (IITC Life Science Inc., Woodland Hill, CA). Hot plate analgesia tests were carried out at 48.5°C. Each test was recorded using a hand-held video camera. Video footages were analyzed to determine the paw withdrawal latency \((\text{PWL})\), the time it took for the rat to lift and start licking its paw\(^{107}\). A cut-off period of 45 s was given to avoid any potential thermal injury to the rat’s paw. A minimum of three replicates was run for each rat. The maximum possible analgesia was calculated following a reported method as follows\(^{108}\):

\[
\text{MPA} = \frac{\text{Reaction time for treatment} - \text{reaction time for control}}{\text{45 seconds react}} \times 100
\]

Statistical Analysis

The mean and standard deviation was calculated for all \textit{in vitro} and \textit{in vivo} experiments. The level of significance between pre-treatment latency response and the post-treatment latency responses was determined by applying student t-test. A p-value less than 0.05 was considered as statistically significant.

4 Results and Discussion

4.1 Characterization of fentanyl microneedle patch

Hyaluronic acid (HA) is a biocompatible and bio erodible polysaccharide that has been used for biomedical applications and has been a common ingredient of various dermatological preparations\(^{109,110}\). Its application as a casting material for microneedles have resulted in formation of nonflexible microneedle arrays that have the necessary mechanical strength to penetrate through
the stratum corneum, dissolving within minutes in the interstitial fluid to release the contained drug payload\textsuperscript{111,112}. Aqueous blend of HA has the appropriate viscoelastic property to efficiently seep inside the mold cavities on centrifugation and upon drying facilitates easy detachment of microneedles from the mold. For the present study, fentanyl microneedles was fabricated using a previously reported micromolding technique\textsuperscript{73}. Low molecular weight HA (< 10k Daltons) formed the needle shaft whereas the base support was fabricated from a high molecular weight HA (< 40k Daltons). Scanning electron microscopic images of the fabricated microneedles are shown in Figure 4.1. The microneedle patch comprised of 100 needles organized in an array of 10 x 10 on a 0.25 cm\textsuperscript{2} base plate, each needle at 464.14 ± 15.5 µm distance apart from each other and measured 523.33 ± 0.55 µm in height with a tip diameter of 5.52 ± 1.85 µm.

**Figure 4.1:** Scanning electron microscopic images of microneedle patch loaded with fentanyl at [A] 35X and [B] 50X magnification. Each patch comprises of 10 x 10 array of 100 microneedles and 0.25 cm\textsuperscript{2} area

4.2 Skin penetration & dissolution kinetics of fentanyl microneedle patch
Rapidly dissolving microneedles provides an excellent platform for delivering drug across the skin in a minimally invasive manner. In this study, HA microneedles was mold casted by a transcription process. This fabrication method renders the drug to be encapsulated solely in the needle shafts while maintaining the needle base drug free. The microneedles were subjected to in vitro skin penetration studies in excised rat skin. Upon insertion, the microneedles dissolved quickly in the intestinal fluid of the skin to release the encapsulated drug in its entirety. Bright field microscopic images (Olympus Trinocular microscope BX53) before and after application of microneedles to the rat skin confirms dissolution of microneedles after 0.5 h of application (Figure 4.2). The intended fentanyl drug loading in a single microneedle patch of 0.25 cm² area was 5 µg. However, the amount of fentanyl retained in the skin after microneedle application was 4.38 ± 0.55 µg. Fluorescent microcopy was used to observe the micro conduits created by the microneedles application in vitro on rat skin. Figure 4.3 shows the perforations created by the microneedles upon insertion onto the skin and confirms the formation of micro conduits by the microneedles.
Figure 4.2: Bright field microscopic images of microneedles loaded with fentanyl before [A] and after [B] to the skin for 5 minutes

Figure 4.3: Fluorescence perforation into the skin [B] following pretreatment with microneedles loaded with CM-Dil lipophilic fluorescent dye [A]

4.3 In vitro study

4.3.1 In vitro release kinetics from fentanyl dermal patch

The fentanyl dermal patches were subjected to in vitro drug release testing (IVRT) in a Franz diffusion cell. Figure 4.4 represents the cumulative amount of drug released in sink medium from the patches with different drug loading. The release profile of all patches tested followed the square root of time linear kinetics. Based on the IVRT results, the adhesive patch with 1% drug loading and 1 cm² area was selected for further in vitro and in vivo experiments. This was also a
conscious selection to choose the patch with a drug loading that can deliver enough drug across the skin to elicit local anti-nociceptive effect without systemic exposure.

![Graph showing in vitro fentanyl release kinetics from three drug-in-adhesive patches with different drug loading.](image)

**Figure 4.4:** *In vitro* fentanyl release kinetics from three drug-in-adhesive patches with different drug loading. (△) 0.25%, (○) 0.5%, (□) 1%. The data points represented in the graph are an average of n=3 ± S.D

4.3.2 Skin penetration study from fentanyl dermal patch

This experiment was performed to determine the application time required for the fentanyl dermal patch to achieve fentanyl levels in the skin analogous to that obtained from the microneedle patch (after 0.5-hour application time). *In vitro* skin penetration testing for the selected fentanyl dermal patch (1% drug loading, 1 cm$^2$ area) was performed by applying the patch on excised rat skin mounted on a Franz diffusion cell. At different time points, the patch was removed, and
amount of fentanyl absorbed into the skin from the patch was quantified. The results indicate that after 6 hours of patch application time the amount of fentanyl extracted from the skin was 4.07 ± 0.90 µg (Figure 4.5). Thus 6 hours application time was required to achieve fentanyl levels across the skin from the adhesive patch that are comparable to that attained from the 0.5- hour application time of the microneedles patch.

![Figure 4.5: In vitro skin deposition study from fentanyl dermal patch with 1% drug loading. The data points represented in the graph are an average of n=4 ± S.D.](image)

4.4 In vivo study

4.4.1 Anti-nociceptive activity
a. Fentanyl microneedle patch

The hind paw withdrawal latency in response to an external thermal stimulus was measured using hot plate analgesiometer. The baseline withdrawal latency of the hind paw was recorded to be 13.94 ± 1.76 seconds. After application of the microneedles patch for 0.5 hours, the mean paw withdrawal response time significantly (p < 0.0001) increased to 36.72 ± 2.09 seconds. The results clearly demonstrates the feasibility of the fentanyl microneedle patch to induce an analgesic/anti-nociceptive response.

b. Fentanyl dermal patch

Fentanyl dermal patch was used as a reference standard to compare the relative onset of anti-nociceptive activity obtained from the fentanyl microneedle patches. Rats were checked for their response to the thermal stimulus at 0.5, 2, 4, & 6 hours post application of the fentanyl dermal patch. The paw withdrawal responses thus obtained showed no increase in the reaction time/PWL between the control left paw and the test right paw at 0.5 and 2 h after patch application. The PWL subsequently increased at 4 h and at 6 h, the analgesic activity of fentanyl from dermal patch was the maximum (figure 6). Fentanyl dermal patch applied on the right hind paw produced a potent anti-nociceptive effect with an increase in paw withdrawal latency to 32.72 ± 8.02 seconds at 6 h (relative to 13.33 ± 2.38 seconds observed for the control left paw). The MPA values as represented in figure 6 indicates that the anti-nociceptive response obtained from the application of fentanyl dermal patch is comparable to the fentanyl microneedle group (P<0.0001), however the onset of anti-nociceptive response is much faster for the microneedle group (0.5 hours) than that obtained from the fentanyl dermal adhesive patch (6 hours). This can be attributed to the fact that upon
insertion, the microneedles bypass the rigid stratum corneum barrier of the skin and release the entire encapsulated drug payload, however, for the fentanyl dermal patch, the drug undergoes a long lag in the torturous lipidic pathway of the stratum corneum before it can exhibit any anti-nociceptive response. Additionally, duration of analgesic effect from dermal and the microneedle patch was checked post removal of the patch from the application side (microneedle patch removed at 0.5 h; dermal patch removed at 6 h). The MPA values gradually decreased suggesting that the analgesic activity was sustained for almost 2 hours post application of the fentanyl dermal or microneedle patch. The rats were also checked for any systemic exposure of fentanyl post application of the microneedle and dermal patch by withdrawing blood by tail-vein sampling method and analyzing for fentanyl. The blood was collected in a heparin coated centrifuge tube and centrifuged at 1200 rpm for 5 minutes to separate plasma. 50 µL of plasma was collected and mixed with acetonitrile to precipitate proteins. The sample was centrifuged and 10 µL of the supernatant solution was injected into HPLC for analysis of fentanyl. Detectable levels of fentanyl was not observed in rat plasma after application of microneedle and drug-in-adhesive patch suggesting that drug loading in both these formulations was sufficient enough to achieve localized anti-nociceptive activity, however, not enough to elicit plasma exposure.
Figure 4.6: Maximum possible analgesia (MPA) (%) in response to the thermal stimulus after application of Fentanyl microneedle vs Fentanyl dermal patch to the plantar surface of the hind paw in a rat model (hot plate analgesia). The MPA was 0.5 hr for Fentanyl microneedle patch vs 6 hours for Fentanyl dermal patch. The paw withdrawal latency was measured immediately following removal of the test patches (microneedle or dermal). The control in the experiment is the untreated paw. The data points represented in the graph are an average of n=6 ± S.D

4.5 Fentanyl for topical Anti-nociceptive activity

The primary afferents from the periphery transmits sensory information (such as the perception of pain) to the several regions of the brain through the dorsal horn of the spinal cord. Thus, the dorsal horn acts as a relay site where peripheral nerve impulses are modulated before they are conveyed centrally to induce perception and response. This neurological mechanism of
encrypting and processing a noxious stimulus is referred to as nociception\textsuperscript{113}. Thus, dorsal horn was an essential target for the development of analgesics and because it was traditionally accepted that anti nociception takes place exclusively in the central nervous system, formulations were designed to achieve stable plasma drug concentrations for inducing analgesia. In this direction, opioids have been one of the most widely used analgesics for the treatment of acute and chronic pain conditions\textsuperscript{114}. Three different types of opioid receptors have been recognized, the Mu(\(\mu\)), Delta(\(\delta\)) & Kappa (\(\kappa\)) that are anatomically distributed throughout the central nervous system in the spinal cord, brainstem and Thalamus\textsuperscript{95}. Activation of the opioid receptors by external agonist such Morphine and fentanyl induces analgesic activity and anti-nociception, however often with other elicit effects such as respiratory depression, euphoria, sedation, decreased GI mobility and physical dependence\textsuperscript{115,116}. Reports from several research groups have suggested presence of opioids receptors on the peripheral terminals of thinly myelinated and unmyelinated cutaneous sensory fibers which are upregulated during conditions of inflammation\textsuperscript{117-119}. Study conducted on the antinociceptive behavior of opioids by Stein et al. revealed that peripheral application of opioids that are agonists to mu, delta and kappa receptors actuates analgesia in experimentally induced inflammation through a receptor-substrate action further confirming the functional activity of opioids at these cutaneous sites\textsuperscript{101}. Local application of exogenous opioid receptor agonist at peripheral site is an attractive therapeutic approach for pain management and also provides for the opioids to be used at a lower dose thereby avoiding central side effects. Since most of clinically applied opioids function through mu receptor activation, fentanyl has been an ideal opioid agonist for inducing analgesic activity as it predominantly interacts with the mu-receptors\textsuperscript{120,121}. It is available in the form of transdermal patches, buccal tablets and injections for the clinical management of acute and chronic pain. Compared to other opioids in its class, fentanyl
shows rapid onset of action and is 80 to 100 time more potent than morphine. Since most of the opioids receptors expressed peripherally are mu-subtypes and owing to its lipophilic nature, fentanyl presents an ideal case for inducing anti nociception at sub systemic doses upon local application. A previous study on the anti-nociceptive potential of fentanyl conducted by Kaiser et al demonstrated that fentanyl showed a localized, potent and prolonged anti-nociceptive effect when injected at very lower doses (0.5 µg to 1 µg) into the rat brachial plexus sheath, thus confirming the validity of the approach. However, acute pain management goals require immediate pain relief at doses that are devoid of central side effects with a non-invasive treatment approach. The present work addresses these challenges by developing a rapidly dissolving fentanyl microneedle patch which upon insertion in the skin releases the encapsulated fentanyl within minutes at the epidermal-dermal junction of the skin, providing a rapid antinociceptive effect.

5 Conclusion

The Mu opioid receptor present on the peripheral somatic sites can be exploited to produce anti-nociceptive effect upon local application of exogenous opioids. This is especially convenient for treating acute pain situations which requires immediate relief from pain. Current therapeutic practices for treating acute pain are invasive, induce several side-effects and may require co-administration with other NSAID’s or anesthetics. This research presents a unique approach for management of local cutaneous nociceptive pain by using rapidly dissolving fentanyl microneedle patch. The developed microneedle patches loaded with systemically inert doses of fentanyl provides for an excellent therapeutic strategy that is devoid of any central side effects such as respiratory depression, nausea, addiction and sedation. Additionally, fentanyl microneedles patch
exhibits quick onset of action compared to conventional dermal patch. However, this mode of opioid delivery needs further clinical investigations.
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EDUCATION

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SKILLS

Pre-Formulation  Thermogravimetric Analysis (TGA), Digital Scanning Calorimetry (DSC), Dynamic Vapor Sorption (DVS), LOD
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JOURNAL PUBLICATIONS


PROJECTED PUBLICATIONS

1. A. Maurya, SN Murthy. Unveiling the mechanism of drug penetration into Stratum Corneum during the short duration exposure of topical formulation. *Journal: TBD*

BOOK CHAPTERS

SELECT PRESENTATION/CONFERENCE PROCEEDINGS


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SCIENTIFIC AND PROFESSIONAL OUTREACH

Co-Moderator
- Dermatopharmaceutics Focus Group (DFG), AAPS, San Diego, 2014
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- Dermatopharmaceutics Focus Group Travel ship award for AAPS annual meeting, San Diego. Paramagnetic Medium to enhance the Magnetophoretic Transdermal Delivery of Drugs

2013
- Initiative of RhoChi, Honors Society
2012

- Graduate student council research grant, The University of Mississippi
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