Fluvastatin as a Micropore Lifetime Enhancer for Sustained Delivery Across Microneedle-Treated Skin

PRIYANKA GHOSH,[1,2] NICOLE K. BROGDEN,[1] AUDRA L. STINCHCOMB[1,2,3]

[1]Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536-0082
[2]Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Maryland 21201
[3]AllTranz, Inc., Lexington, Kentucky 40505

ABSTRACT: Microneedles (MNs), a physical skin permeation enhancement technique, facilitate drug delivery across the skin, thus enhancing the number of drugs that can be delivered transdermally in therapeutically relevant concentrations. The micropores created in the skin by MNs reseal because of normal healing processes of the skin, thus limiting the duration of the drug delivery window. Pore lifetime enhancement strategies can increase the effectiveness of MNs as a drug delivery mechanism by prolonging the delivery window. Fluvastatin (FLU), a HMGCoA reductase inhibitor, was used in this study to enhance the pore lifetime by inhibiting the synthesis of cholesterol, a major component of the stratum corneum lipids. The study showed that using FLU as a pretreatment it is possible to enhance the pore lifetime of MN-treated skin and thus allow for sustained drug delivery. The skin recovered within a 30–45-min time period following the removal of occlusion, and there was no significant irritation observed due to the treatment compared to the control sites. Thus, it can be concluded that localized skin treatment with FLU can be used to extend micropore lifetime and deliver drugs for up to 7 days across MN-treated skin. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:652–660, 2014
Keywords: fluvastatin; microneedles; pore lifetime; transepidermal water loss; pharmacokinetics; transdermal drug delivery; controlled release/delivery; formulation vehicle; spectroscopy

INTRODUCTION

Transdermal drug delivery allows the delivery of drugs across the skin. For drug delivery purposes, the skin is best described as a three-layer model. From top down, these layers are stratum corneum (SC), the epidermis, and the dermis. Among these, the SC is the major rate-limiting step for the delivery of most drugs across the skin, because of its rigid structure and high lipophilicity.[1] A very small subset of molecules can effectively cross the SC barrier in therapeutically relevant amounts.[2] The structure of the SC is composed of dead keratinized remains of once rapidly dividing epidermal cells bound tightly by a lipid matrix.[2] The intercellular domain of the SC is composed of three different lipid molecules, namely, cholesterol, free fatty acids, and ceramide in equimolar ratio.[3,4] There is a plethora of literature on the proposed mechanisms of recovery after SC disruption using different methods of evaluation, such as tape stripping, acetone treatment, surfactants, etc. Formation and release of lamellar body contents is a major process in regeneration of the SC barrier following disruption or normal wear and tear. There is an initial burst (0–30 min) release of preformed lamellar body contents followed by the upregulation of lamellar body synthesis, which includes both lipid precursors and hydrolytic enzymes.[5,6] Following release at the interface of the SC and epidermis, the lamellar body contents undergo extracellular processing to form comparatively nonpolar SC lipids

from their polar precursors. Thus, a host of enzymes and biochemical pathways are involved in proper functioning of the SC barrier. The modulation of a number of the precursors and the enzymes involved lead to malformation of the SC barrier following insult.[3,5] A constant molar ratio of the lipids is one of the most important parameters in barrier formation and a decrease in synthesis of any of the three lipids or their precursors leads to a delay because of malformation of lamellar bodies.[7]

Microneedles (MNs) are an alternative technique used to permeabilize the SC barrier and increase the number of drugs that can be delivered transdermally. It is a physical enhancement technique.[8] There are a number of different types of MNs and application techniques. Solid MNs are used to permeabilize the skin followed by the application of drug over the treated area, or drug is coated onto the MN itself. Polymer MNs are used to load drug into the polymer for delivery. Hollow MNs are used in conjunction with an infusion pump to facilitate the delivery of hormones and vaccines over short periods of time.[8–10] The effectiveness of MNs as a drug delivery vehicle has been established in the literature over the last decade.[11–15] The MN delivery system is very useful for short-term delivery, over a few hours.[16,17] However, the efficacy of the technique is limited for chronic therapy because of normal healing processes of the skin, which lead to resealing of the micropores anywhere between a 48–72-h timeframe under occlusion.[18–21] Thus, drug can only be delivered across MN-treated skin for a maximum of 3 days under occlusion.[15,22] Lifetime of the micropore when exposed to air is much shorter and ranges from 15 min to a few hours depending on the MN geometry, animal model, and detection method used for evaluation.[18–20] The short time frame of micropore resealing can be explained in terms of enhanced transepidermal water loss (TEWL) in the absence of occlusion. TEWL is the

most important signal for barrier recovery and lamellar body secretion following insult to the skin.[23] Decreased TEWL under occlusion slows down the recovery processes of the skin.

Biochemical enhancers/lipid biosynthesis inhibitors prevent the synthesis of the essential lipids required for lamellar body synthesis and thus the proper formation of the SC. Local concentrations of specific inhibitors of the three lipid synthesis pathways, namely, cholesterol, fatty acids, and ceramides, can be used to alter the molar ratio and thus delay barrier recovery.[4] The inhibitors can be used either as a pretreatment or in the formulation. Some of the inhibitors that can be used for the above mechanism are 5-(tetradecycloxy)-2-furancarboxylic acid (TOFA) for fatty acid synthesis, fluvastatin (FLU) for cholesterol synthesis, and β-chloroalanine (BCA) for ceramide synthesis.[4]

The goal of the current study was to evaluate the effectiveness of FLU, an HMGCoA reductase (an important enzyme of the cholesterol synthesis pathway) inhibitor, as a pore lifetime enhancement agent for sustained drug delivery across MN-treated skin. Solid stainless steel MNs were used to permeabilize the skin. This technique is also known as the "poke (press) and patch" approach and is advantageous for sustained delivery because the MN is only used to permeabilize the skin and does not remain in contact with skin thereafter.[8] The current efforts were directed toward enhancing the drug delivery window to 7 days, the ideal transdermal patch wear time, by using a biochemical enhancement technique in addition to MN.

The model compound for the drug delivery project was naltrexone (NTX). It is a μ-opioid receptor antagonist used for alcohol and opioid addiction treatment. The currently approved dosage forms include an oral and an extended-release intramuscular injection.[24,25] The oral dosage form has issues with variable bioavailability and compliance in its treatment population, because of daily dosing and side effects.[24,26] The extended-release intramuscular injection is difficult to remove if there is a need for emergency opiate treatment, and also leads to serious injection site reactions.[25] Thus, NTX is a suitable candidate for transdermal patch development and an active delivery system in the form of MNs was used in this project, as the drug cannot be delivered in therapeutic concentrations via passive transdermal delivery.[15] It has been previously shown that by using MNs, NTX can be delivered at therapeutic levels for 2–3 days in humans.[15]

EXPERIMENTAL SECTION

Materials

Naltrexone HCl was purchased from Mallinckrodt (St. Louis, Missouri), and FLU sodium was purchased from Cayman chemical (Ann Arbor, Michigan). Propylene glycol (PG) and ethanol (200 proof) were purchased from Sigma (St. Louis, Missouri). Acetic acid, ammonium acetate, and benzyl alcohol were obtained from Fisher Scientific (Fair Lawn, New Jersey). 1-Octanesulfonate, sodium salt was obtained from Regis Technologies, Inc (Morton Grove, Illinois). Trifluoroacetic acid (TFA), triethylamine (TEA), methanol, ethyl acetate, and acetonitrile (ACN) were obtained from EMD chemicals (Gibbstown, New Jersey). Natrosol® (hydroxyethlycellulose) was obtained from Ashland (Wilmington, Delaware). Ethanol (70%) was obtained from Ricca chemical (Arlington, Texas). Sterile water for injection was obtained from Hospira (Lake Forest, Illi-

nois) and water was purified using a NANOpure DIamond™, Barnstead water filtration system for all *in vitro* experiments.

High-Pressure Liquid Chromatography Methods

Naltrexone and FLU from *in vitro* studies were quantified using high-pressure liquid chromatography (HPLC). The HPLC system consisted of a Waters 717 plus auto-sampler, a Waters 600 quaternary pump, and a Waters 2487 dual-wavelength absorbance detector with Waters Empower™ software (Milford,MA). A Perkin Elmer Brownlee™ Spheri 5 VL C18 column (5 μm, 220×4.6 mm^2) and a C18 guard column (15×3.2 mm^2) were used with the UV detector set at a wavelength of 280 nm for NTX and 305 nm for FLU. The mobile phase consisted of 65:35 (v/v) ACN: (0.1% TFA with 0.065% 1-octane sulfonic acid sodium salt, adjusted to pH 3.0 with TEA aqueous phase). Samples were run at a flow rate of 1.5 mL/min. The injection volume used was 100 μL for all samples.

In Vitro Experiments

The *in vitro* studies were carried out to look at the flux across MN-treated skin and the skin concentrations of NTX and FLU in the skin. Full thickness Yucatan miniature pig skin was used for all *in vitro* experiments. All pig tissue harvesting experiments were carried out under IACUC approved protocols at the University of Kentucky. Fresh skin was cleaned to remove the excess subcutaneous fat, dermatomed and stored at $-20°$C. Skin was thawed and cut into small square pieces on the day of the diffusion experiment. The thickness was measured for each individual piece of skin and the average thickness of all treatment groups was maintained between 1.4 and 1.8 mm. Skin was next treated with a 5 MN in-plane array, 10 times in one direction and 10 times in a mutually perpendicular direction to generate a total of 100 nonoverlapping MN insertions within the active treatment area of 0.95 cm^2. Each MN (triangular in shape) was 750 μm long, 200 μm wide (at the base) and 75 μm thick (thickness of metal used) and the inter needle spacing was 1.35 μm. Interindividual variability for MN application was not evaluated during the current study, and all treatments were carried out by the same investigator. The pressure associated with MN application was kept consistent throughout the studies. Five treatment groups were used based on the four different vehicles used for FLU and a control for NTX only; all studies were performed in triplicate. Saturated NTX gel was prepared by mixing 90 mg/mL of NTX HCl (saturated solution) with PG (10%). A 3% hydroxyethylcellulose (HEC) gel of the NTX solution was used for the *in vitro* studies. The four different FLU treatments were FLU in 200 proof ethanol, acetone, 7:3 PG–ethanol, and 1:2:1 PG–ethanol–water. All the vehicles for FLU were based on previous studies looking at the recovery of the skin or commonly used drug deposition methods.[4,27] The concentration of FLU was 1.5% for all the formulations and 40 μL of the formulation was applied to each cell. The receiver solution was water alkalified to pH 7.4 containing 20% ethanol at 37°C. The temperature of the diffusion cell skin surface was maintained at 32°C. Samples were collected every 6 h for 48 h. All samples were analyzed using HPLC. The steady state flux of NTX was calculated using the steady-state portion of the cumulative amount permeated versus time plot. The skin concentrations of both drugs were also determined at the end of the study by extracting the drug overnight into 10 mL of ACN,

and injecting the extracted sample onto the HPLC column after appropriate dilutions.

Pharmacokinetic Studies

Pharmacokinetic (PK) studies were carried out in hairless guinea pigs (HGP) using FLU and NTX to evaluate the effectiveness of FLU as a pore lifetime enhancement technique. All animal studies were approved by the IACUC at University of Kentucky and University of Maryland. All animals used had jugular vein catheters. Catheters were either implanted by laboratory staff or animals were purchased with catheters from Charles River. Three treatment groups were used for the PK studies. Treatment group: 300 µL of NTX gel + 40 µL of FLU in ethanol (200 proof); vehicle control group: 300 µL of NTX gel + 40 µL of ethanol (200 proof); MN control group: 200 µL of NTX gel. For PK studies, preparation of the NTX gel was similar to the *in vitro* studies except 1% benzyl alcohol was added as an antibacterial agent. All sites were treated twice with a 50 MN array to generate a total of 100 nonoverlapping MN pores and there were two treatment sites per animal. The array was rotated 45° following the first insertion to ensure nonoverlapping pores and was held in place for 15–20 s for each application. (The consistency of pore formation can be verified using PK analysis of the data, TEWL and data from staining studies). FLU was deposited using the ethanol solution in the active treatment group; only ethanol was used in the vehicle control group followed by NTX gel for all three groups. All sites were occluded using patches[28] for 7 days (treatment and vehicle control groups) or 4 days (MN control group) following treatment. Two hundred microliter blood samples were withdrawn from the catheter at regular intervals into collection tubes precoated with 500 IU/mL heparin. The blood samples were immediately centrifuged at 10,000g for 3 min; plasma was separated and stored at −80°C until analysis. All *in vivo* studies were carried out at least in triplicate.

Liquid chromatography/mass spectrometry (LC/MS–MS) Analysis of Plasma Samples

All plasma samples were extracted using a previously validated method.[29] Five hundred microliter of 1:1 ACN–ethyl acetate was added to 100 µL of plasma for protein precipitation. The mixture was vortexed for 15 s and centrifuged for 20 min at 12,000 g. The supernatant was removed carefully without disturbing the pellets and dried under nitrogen in a glass test tube. The extract was suspended in 100 µL ACN, vortexed, sonicated for 10 min, and transferred to HPLC vials with low-volume inserts for injection. For plasma standards, the 100-µL of the blank plasma was spiked with 10 µL of ACN standards and extracted following the same method as above.

The LC/MS–MS system consisted of HPLC Waters Alliance 2695 Separations Module, Waters Micromass® Quattro MicroTM API Tandem Mass Spectrometer, and Masslynx Chromatography software with Waters Quanlynx (V. 4.1) analysis software. A Waters Atlantis Silica HILIC column (5 µ, 150 × 2.1 mm^2) and guard column (10 × 2.1 mm^2) were used for LC separation. The mobile phase composition was methanol with 0.1% acetic acid–20 mM ammonium acetate (95:5), the flow rate was 0.5 mL/min, and positive mode atmospheric pressure chemical ionization was used for detection of NTX (APCI$^+$). Multiple reaction monitoring was carried out with the following parent to daughter ion transitions for NTX m/z 341.8→323.8. The

corona voltage was 3.5 µA, cone voltage 25 V, extractor 2 V, RF lens 0.3 V, source temp 130°C, and APCI probe temperature 575°C. Nitrogen gas was used as a nebulization and drying gas at flow rates of 50 and 350 L/h, respectively. Injection volume was 40 µL.

PK Analysis

The plasma concentration versus time data obtained using the MS were modeled by fitting data to a noncompartmental model with extravascular output (WinNonlin Professional; version 4.0; Pharsight Corporation, Mountain View, California) to obtain PK parameters like area under the curve (AUC), maximum concentration (C_{max}), and time to maximum concentration (T_{max}).

Reversibility/Recovery of Pores

Recovery of the skin following the removal of treatment is a concern for studies involving local topical inhibitors. The reversibility/recovery of the pores following removal of occlusion was studied using TEWL in a HGP model. Five different sites were chosen on the dorsal region of an animal. The treatment sites were site 1: MN + NTX gel + FLU (200 proof ethanol), site 2: MN + NTX gel + 200 proof ethanol, site 3: MN + placebo gel, site 4: no MN treatment + placebo gel, and site 5: occlusion only. All sites were marked and cleaned on the day of the treatment. Sites 1, 2, and 3 were treated twice, with 50 MN arrays (620 µm), (the two treatments being mutually perpendicular to each other to give a total of 100 nonoverlapping insertions) and TEWL readings were obtained before and immediately following treatment at all sites using a TEWL evaporimeter (cyberDERM, Media, Pennsylvania). The concentration and amount of NTX gel and FLU were consistent with the PK studies. A placebo gel was used for site 3 and site 4. The placebo gel was similar in composition to the NTX gel with 10% PG, 1% benzyl alcohol, and 3% HEC as the gelling agent. Forty microliter of the 1.5% FLU in ethanol or 200 proof ethanol was applied to the treated skin at site 1 and 2, respectively, and 300 µL of NTX–placebo gel was applied to sites 1–4. All sites were occluded using a transdermal patch[29] secured in place with Bioclusive™ tape. The occlusive patch was removed after 7 days and TEWL measurements were obtained for 30–45 min at all sites or until values returned to baseline (pretreatment values). All experiments were conducted at least in triplicate.

Skin Irritation

Local skin irritation can be one of the major drawbacks of topical/transdermal delivery, so irritation studies were conducted to look at the effect of local FLU treatment on the skin in HGP. The studies were conducted using the same treatment groups as the recovery studies. Chroma Meter (CR-400; Konica, Minolta, Japan) readings were obtained, in triplicate, at each site before and immediately after the treatment. The data were used to record changes in the color of the skin from baseline based on three different color axes, the red-green axis (a^*), the black-white axis (L^*), and the yellow-blue axis (b^*). The sites were then occluded for 7 days and readings were obtained after removal of occlusion. The degree of redness of the skin or the change in erythema of the skin was measured by the change in the red-green axis (Δa^*) values. Skin irritation studies were conducted in triplicate.

Staining/Pore Visualization Studies

Pore visualization studies were carried out to look at the pores on the skin following treatment with FLU. Two sites were used on the same animal for these studies. Site 1: MN + NTX gel + FLU (200 proof ethanol) and site 2: MN–NTX gel + 200 proof ethanol. The amount and concentration of gels were consistent with other studies. The skin was occluded for 7 days following treatment. Gentian violet was used to stain the skin after removal of the occlusive patch. The dye stains viable epidermis and not *SC*, so a grid can be clearly visualized for MN treatment/presence of micropores in the skin, whereas no staining can be observed on skin with intact *SC*.

Statistical Analysis

Data for all experiments are reported as mean ± SD. Statistical analysis of data was carried out with Students' *t*-test and one way ANOVA with post hoc Tukey's pairwise tests, if required, using GraphPad Prism® software, version 5.04 software. $p < 0.05$ was considered to be statistically significant.

RESULTS

Analytical Methods

Naltrexone and FLU were quantified in ACN for skin samples and receiver samples from *in vitro* diffusion studies using HPLC. The retention times were 2.2 and 3.2 min for NTX and FLU, respectively. All retention times are reported ±0.1 min. All standard curves were linear in the range of 100–10,000 ng/mL, $r^2 > 0.99$.

Plasma samples were analyzed for NTX concentration using a LC/MS–MS assay. The standard curves of NTX in ACN and plasma were linear in the range of 1–75 ng/mL, $r^2 \geq 0.98$.

Diffusion Studies

The *in vitro* diffusion studies were carried out to optimize the formulations for *in vivo* studies. All formulations for FLU were chosen based on previous publications on skin recovery following FLU treatment. The NTX was formulated in 10% PG containing formulation because concentrations higher than 10% of PG have been shown to decrease flux across MN-treated skin significantly.[30] The *in vitro* results (Fig. 1) indicated that there was no significant difference in flux across MN-treated skin among the five different treatment groups ($p > 0.05$). The skin concentration data indicated that there was no significant difference in the concentration of NTX or FLU in the skin irrespective of the method of FLU deposition ($p > 0.05$). Ethanol (200 proof) was chosen as the vehicle for FLU deposition from these studies for all *in vivo* studies.

PK Studies

The PK studies were carried out to evaluate the effect of FLU treatment on micropore closure *in vivo* in a HGP model. The three treatment groups were evaluated either for 168 h (7 days) for the treatment and vehicle control groups, or for 96 h (4 days) for MN control. The results (Fig. 2) indicated that detectable levels of NTX were present in the plasma for 72 h for all study groups. The last detectable plasma concentration for the vehicle control group was 144 h (6 days) and for the treatment group was 168 h (7 days). Data were included for PK analysis from studies where quantifiable plasma levels of NTX were
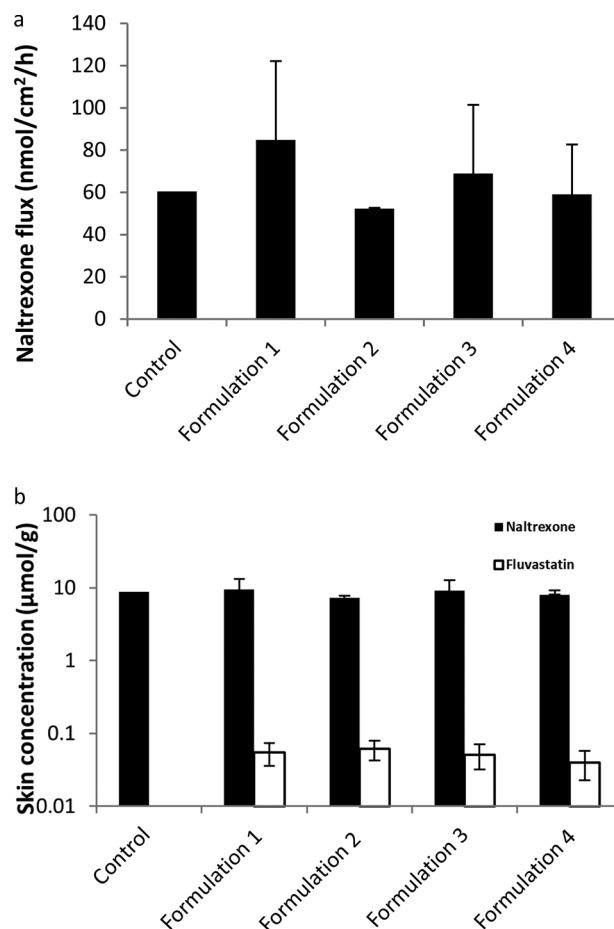
Figure 1. *In vitro* formulation optimization for application of FLU along with NTX gel. Flux of NTX across MN-treated skin (top panel), skin concentration of NTX, and FLU across MN-treated skin (bottom panel). FLU in 200 proof ethanol (formulation 1), acetone (formulation 2), PG–ethanol = 7:3 (formulation 3), and PG–ethanol–water = 1:2:1 (formulation 4). $n \geq 3$ for all studies except control NTX. $p > 0.05$ for flux and skin concentration data among all groups.

obtained for more than 96 h for the treatment and vehicle control groups. The WinNonlin parameters (Table 1) indicated that the AUC for plasma concentration versus time plots were not significantly different from each other for the treatment group and the vehicle control group ($p > 0.05$). However, the time of last detectable plasma concentration (T_{last}) for both the treatment group (158.8 ± 21.6 h) and the vehicle control group (130.7 ± 23.0 h) were significantly higher than the MN control group (67.3 ± 8.0 h) ($p < 0.05$). There was no lag time observed with MN-enhanced delivery in any of the three groups. There was no significant difference in maximum drug concentration (C_{max}) among the three groups ($p > 0.05$). The average plasma concentration was 41.6 ± 2.9 ng/mL. T_{max} or time to maximum plasma concentration was reached between 15 min and 4 h for all studies.

Recovery Studies

The recovery of the skin, that is, regeneration of the barrier function of the SC was studied using TEWL. The studies were carried out to look at the effect of the local concentration of FLU on the recovery/healing of the skin upon removal of occlusion.

Table 1. WinNonlin Parameters from PK Studies

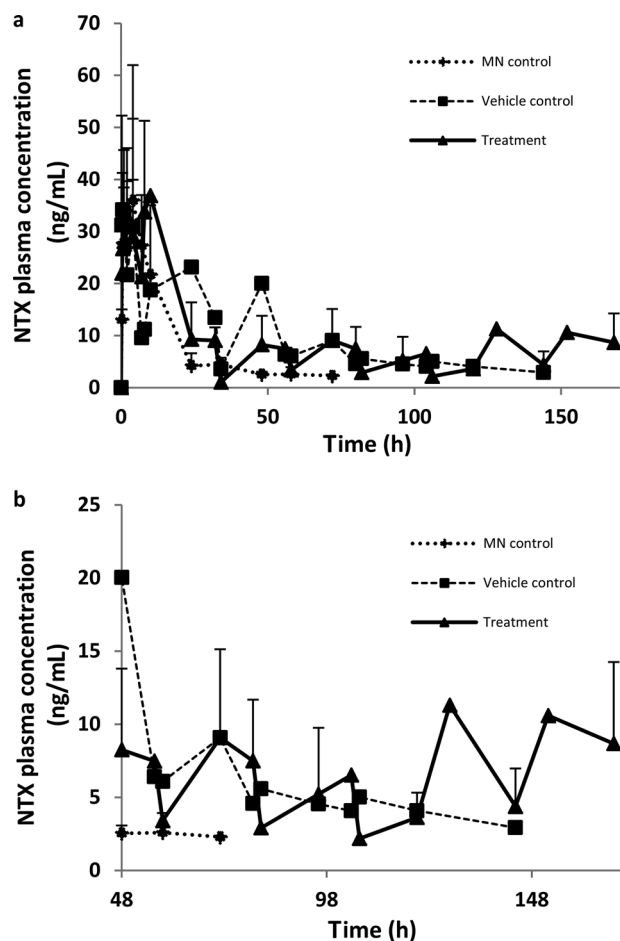|	Treatment Group	Vehicle Control	MN Control
C_{max} (ng/mL)	40.5 ± 14.7	44.9 ± 18.9	39.4 ± 11.5
T_{max} (h)	3.6 ± 4.0	1.7 ± 2.0	2.8 ± 2.0
AUC (ng h/mL) (time for AUC calculation, h)	1493.2 ± 800.9 (158.8 ± 21.6)	1596.2 ± 1096.3 (130.7 ± 23.0)	603.3 ± 199.1 (67.3 ± 8.0)
AUC_{last}/T_{last} (ng/mL)	9.3 ± 4.5	11.9 ± 6.7	9.3 ± 4.3
N	5	3	3

Figure 2. Naltrexone plasma concentrations in HGP following application of MN and FLU, MN and ethanol vehicle control, or MN only. Full profile (top panel) and later time points (bottom panel). $n \geq 3$ for all studies. The data indicated that there was no statistical difference in AUC between the FLU-treated group and vehicle control group. T_{last} was significantly higher for the treatment and vehicle control groups compared with the MN-only control.
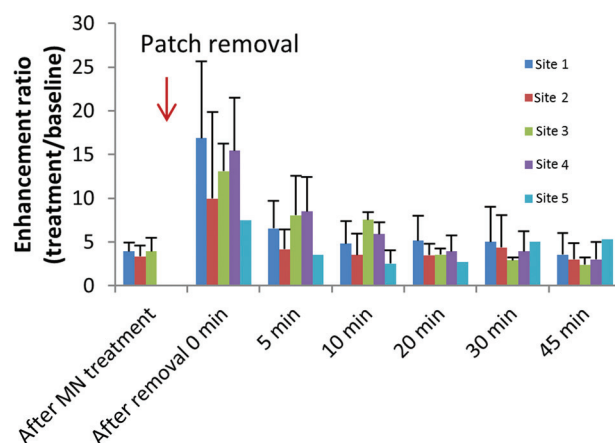

Figure 3. Recovery of skin following 7-day treatment with FLU using TEWL. Measurements were obtained before, immediately following treatment, and after removal of patches at 7 days. Site 1: MN + NTX gel + FLU (200 proof ethanol), site 2: MN + NTX gel + 200 proof ethanol, site 3: MN + placebo gel, site 4: no MN treatment + placebo gel, and site 5: occlusion only, $n = 3$. Data are presented as a ratio of TEWL enhancement over baseline. $p > 0.05$ among all sites.
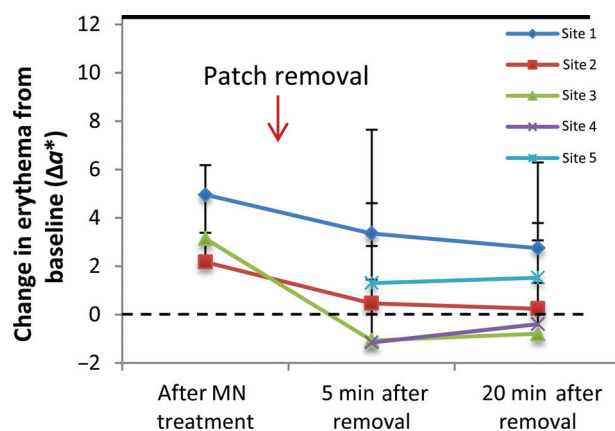

Figure 4. Change in erythema of the skin following 7-day treatment with FLU using a Chroma Meter. Measurements were obtained before, immediately following treatment, and following patch removal at 7 days. Site 1: MN + NTX gel + FLU (200 proof ethanol), site 2: MN + NTX gel + 200 proof ethanol, site 3: MN + placebo gel, site 4: no MN treatment + placebo gel, and site 5: occlusion only, $n = 3$, $p > 0.05$ among all sites. Positive control for skin irritation is from ref 31 (black solid line), baseline (black dashed line).

The recovery data (Fig. 3) are represented as a ratio of the posttreatment values to the pretreatment intact skin/baseline values. Values with an enhancement ratio of less than 1 were not used for the calculation as values below baseline indicate complete reversal of treatment. The recovery studies indicated that there were no significant differences among the five treatment groups used for the recovery studies ($p > 0.05$).

Irritation Studies

The irritation studies were carried out to look at the effect of local FLU treatment on the skin at 7 days. The data (Fig. 4)

indicated that there were no significant differences in skin irritation among the treatment groups immediately following treatment or following removal of the patches at 7 days following treatment ($p > 0.05$). The established positive control treatment for irritation studies in HGP is 0.5% sodium lauryl
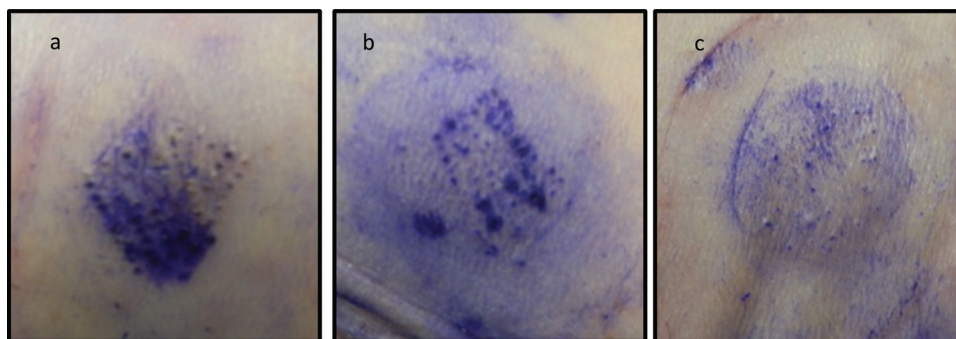
Figure 5. Gentian violet staining in HGP at 7 days following treatment with 1.5% FLU in 200 proof ethanol (a) and (b) or 200 proof ethanol only (c). The images show that pores can be visualized at 7 days following FLU treatment in HGP, whereas staining of pores cannot be visualized in the ethanol control.

sulfate solution and the corresponding $\Delta a*$ value is 12.3.[31] All the experimental values were significantly lower ($p < 0.05$).

Staining Studies

The staining studies (Fig. 5) indicated that pores were present in the FLU-treated group at the end of the 7 day patch application period compared with the absence of pores in the ethanol-treated vehicle control group. In some of the studies, staining was observed in the region surrounding the micropores for the treatment group. FLU is a known inhibitor of the cholesterol synthesis pathway; therefore, it can influence the normal turnover of the intact skin in addition to the healing of the micropores. This process can lead to staining of the regions around the micropores. Minimal staining was also observed in the ethanol control group; however, the grid because of MN application was not observed in this group at 7 days. The staining in this group could be attributed to the use of ethanol followed by 7 days of occlusion.

DISCUSSION

Microneedle-enhanced transdermal delivery has been established as a safe, effective, and pain-free method for drug delivery over the last decade. However, the resealing of the micropores is a rate-limiting step in effective delivery of drugs across MN-treated skin for more than 48 h. Pore lifetime enhancement methods have been explored in the literature to enhance the drug delivery window. One such method is topical application of Solaraze® (3% diclofenac sodium, 2.5% hyaluronic acid), a nonsteroidal anti-inflammatory drug. The hypothesis was that inhibition of the cyclooxygenase pathway would lead to decreased local subclinical inflammation following MN treatment, thus enhancing the drug delivery window. The results from impedance spectroscopy, TEWL, and PK studies in animal models and humans indicated that daily/alternate day application of diclofenac allowed drug delivery for a period of 7 days compared to 2–3 days in the absence of a pore lifetime enhancement agent in formulation, following MN treatment.[18,22,32,33] However, the studies required frequent application of diclofenac, which is not ideal for a 7-day transdermal patch system and formulating high concentrations of diclofenac at the skin surface pH of around 5 was difficult because of the physicochemical properties of the molecule. A codrug approach with naltrexone–diclofenac codrugs is currently under investigation to solve the formulation issues.[34,35]

The current study was designed to evaluate the role of FLU, a lipid biosynthesis inhibitor, as a pore lifetime enhancement agent. Expression levels of mRNA of most enzymes of the lipid biosynthesis pathway are upregulated following insults to the SC barrier.[5] Inhibitors of these enzymes have been shown to delay barrier recovery following acetone or surfactant treatment.[4] Since the exact nature of barrier insult following MN treatment is not well established, the goal of the current study was to evaluate the effect of FLU as a one-time topical application on micropore healing and drug delivery using PK and TEWL for evaluation. It was believed that frequent reapplication or formulation issues would not arise with the FLU treatment as compared with diclofenac, as only very small concentrations of potent lipid biosynthesis inhibitors were required for previously published studies. The hypothesis was that downregulation of cholesterol synthesis would lead to delay in barrier recovery and enhance the drug delivery window. FLU (MW 411.47 Da, log P 4.6, and pKa 4.27) was chosen over other lipid biosynthesis inhibitors because it is a well-known statin drug, and the dosage, metabolism, PK, and toxicity profile are well established in humans.[24,36] The lowest therapeutically relevant oral daily dose is 20 mg and the oral bioavaibility is 9%–50%.[24] Therefore, even if the bioavaibility is 100% from the topical formulation, the maximum delivered dose would be 1.2 mg over 7 days, which is significantly below the therapeutic dose. In comparison, TOFA and BCA are investigational drugs with very limited data being available on dosage and toxicity.

Optimization of formulation for *in vivo* studies was required to maximize FLU skin concentration and NTX flux. Four different methods were chosen for FLU deposition. The results indicated that there was no significant difference in NTX flux or skin concentration of FLU using any of the deposition methods. Ethanol (200 proof) was chosen for *in vivo* studies because it is a frequently used topical excipient and most of the 40 μL ethanol used was allowed to evaporate before application of the drug (NTX) gel. Acetone is similar in action to ethanol; however, it caused irritation in the MN-treated animal models (data not shown) and thus was not used for *in vivo* studies. The other two formulations tested contained either 70% or 25% PG. PG has been shown to interact with the underlying dermis and microchannels in MN-treated skin.[29] The high viscosity of PG-rich formulations significantly decreased flux across MN-treated skin, as compared with an aqueous formulation.[30] The formulations also contained large amounts of ethanol, a known permeation enhancer, and evaporation of the ethanol

from these was not as efficient, because of the presence of PG. Solubility of FLU was not an issue with any of the formulations. Therefore, 40 μL of 1.5% FLU in 200 proof ethanol was chosen as the vehicle for FLU deposition for all *in vivo* studies. Ethanol is a well-known chemical enhancer used in the topical/transdermal industry. It acts by replacing the water molecules in the lipid polar head groups as well as the protein regions of the *SC*, thus enhancing free volume and permeability of molecules across the skin.[37] Therefore, a control was used in PK, recovery, and irritation studies to evaluate the effect of ethanol alone on micropore lifetime in addition to the treatment group.

Pharmacokinetic studies are the most direct measure of drug delivery and micropore closure. Three different groups were evaluated to study micropore lifetime enhancement: FLU in ethanol and MN, ethanol only and MN, and MN only in HGP. A non-MN-treated control was not evaluated for animal studies as it is known that permeation of ionized species such as NTX HCl is limited across the intact *SC*. The PK data indicated that for the MN only control, plasma concentration dropped below detectable levels within the 48–72-h time frame. These data are in agreement with previously published PK data where the drug delivery window following one time application of MN has been shown to be between 48–72 h.[15,18] The treatment group showed detectable NTX levels for the entire length of the study, as compared with the vehicle control group, which showed detectable levels only until 144 h. The AUC values for the treatment group and the vehicle control group were not significantly different from each other indicating that ethanol treatment alone also enhanced the drug delivery following MN treatment. As mentioned before, ethanol is a known permeation enhancer used in passive transdermal delivery. However, the role of ethanol has never been evaluated in MN-enhanced delivery. Since an intact skin control with ethanol treatment was not evaluated in the current study, it is difficult to assess the exact mechanism via which the drug delivery window was enhanced. A direct comparison is difficult because evaluations of NTX plasma concentrations resulting from delivery of NTX HCl across intact skin are limited by the LLOQ of the LC/MS–MS assay. The C_{max} and T_{max} values indicated that treatment of all animals were consistent across the treatment groups. Drug was quantified in plasma within 15 min of treatment indicating that there is no significant lag time associated with the patch system. Such profiles are commonly observed for MN treatment and immediate delivery in addition to sustained release is one of the advantages of such a delivery system. The shape of the plasma concentration time curve is consistent with reported profiles of MN-enhanced delivery, where a peak is observed immediately following patch application followed by a decline in the plasma concentration because of resealing of the micropores. Such data can be modeled using a three-compartment model for drug permeation across skin, PK of the drug in the body, and healing of the micropores.[29] Rate of micropore resealing in the presence of an active pore lifetime enhancement agent can then be estimated if permeation values are known from *in vitro* studies and PK estimates are obtained from *in vivo* studies. Thus, from the PK studies it can be concluded that FLU in an ethanol vehicle can be used to enhance the drug delivery window following MN treatment for up to 7 days. Ethanol treatment alone also had some enhancement effect on the micropore lifetime; however, the exact mechanism of ethanol action is not known.

Recovery of the skin, that is, regeneration of barrier function and irritation of skin following local application of biochemical inhibitors and permeation enhancers are important aspects during the development of transdermal delivery systems for chemical enhancement techniques.[38,39] Therefore, recovery of the skin following removal of treatment at 7 days was monitored using TEWL. Five sites were compared for the recovery and irritation studies. In addition to the treatment group and vehicle control, site 3 was used to evaluate the role of the NTX gel, site 4 was used to evaluate the effect of MN treatment, and site 5 was only occlusion used to control for TEWL enhancement at all sites because of occlusion alone. There was no significant difference among the sites following the removal of treatment or at any of the later time points indicating that recovery of the skin was not influenced by the presence of FLU or ethanol. Recovery of the skin was expected, as removal of occlusion leads to increased TEWL across disrupted *SC*, which in turn acts as one of the major signals for lamellar body release and skin healing.[23] Irritation data also indicated that there was no significant difference among the sites (Δa^* values), and all sites were much lower compared with the positive control in HGP, the most sensitive model for skin irritation for transdermal and topical studies.

The staining studies were conducted to visualize the micropores at the end of the 7-day period. The studies were used as a visualization technique for the presence of micropores for correlation with PK data. The studies indicated that micropores could be clearly visualized in FLU-treated groups; however, the visibility in the ethanol control group was minimal at 7 days. The minimal staining could be because of the effect of ethanol under occlusion at the study site. NTX concentration in the plasma of the ethanol-treated group was not detectable at 7 days, indicating that micropores had resealed by that time. A time-dependent study for the ethanol-treated group with time points on days 5 and 6 in addition to day 7 would help with better correlation of pore visualization to the PK data. Additionally, the healing of the micropores is a gradual process; therefore, although the presence of minimal staining could indicate the presence of partial impairment of the *SC* barrier, the viability of the pores can only be assessed via PK studies. The FLU-treated group showed additional staining in the regions between the micropores. Cholesterol is required for the normal turnover process of the SC in addition to healing. Thus, it might be possible that local application of the inhibitors decreased cholesterol synthesis in the entire treated region leading to malformation of the barrier. However, this should not be of additional concern for FLU treatment because both recovery and irritation studies indicated that there was no significant effect of the treatment following removal among the groups.

Thus, overall, the FLU in ethanol treatment was effective in enhancing the drug delivery window of NTX for up to 7 days, but the exact role of ethanol and FLU could not be evaluated in this study. Further, pretreatment with MN and FLU before application of the NTX gel is a three-step process that is not practical outside a clinic setting and could have also led to variablity in the AUC values. Therefore, further optimization of formulation is required for incorporation of NTX and FLU into a single formulation. The other inhibitors of the lipid biosynthetic pathway in addition to FLU could be evaluated separately or in combination to understand their role in micropore lifetime enhancement. Synergistic effects of enhancement strategies and inhibitors have been reported previously.[40]

CONCLUSIONS

The current study was conducted to evaluate the role of FLU as a pore lifetime enhancement agent. The data indicated that FLU pretreatment allowed delivery of significant levels of NTX for 7 days, whereas treatment with the ethanol vehicle allowed delivery up to 6 days. Recovery of the skin following the removal of treatment and irritation issues because of the local application of FLU was not significant with the current drug delivery system. Staining studies further confirmed the presence of micropores on the skin at 7 days for the FLU treatment. Thus, in conclusion, FLU in ethanol was effective in enhancing micropore lifetime; however, the exact mechanism of enhancement could not be clearly evaluated from the current studies, and further evaluation is needed.

ACKNOWLEDGMENTS

NOTES

The content of the present work is largely based on data included in Priyanka Ghosh's Ph.D. dissertation, Formulation optimization for pore lifetime enhancement and sustained drug delivery across microneedle treated skin, University of Kentucky, 2013

REFERENCES

1. Flynn GL. 1996. Cutaneous and transdermal delivery: Processes and systems of delivery. 3rd ed. Dekker, New York: Modern Pharmaceutics.

2. Paudel KS, Milewski M, Swadley CL, Brogden NK, Ghosh P, Stinchcomb AL. 2010. Challenges and opportunities in dermal/transdermal delivery. Ther Deliv 1:109–131.

3. Elias PM, Tsai J, Menon GK, Holleran WM, Feingold KR. 2002. The potential of metabolic interventions to enhance transdermal drug delivery. J Investig Dermatol Symp Proc 7:79–85.

4. Tsai JC, Guy RH, Thornfeldt CR, Gao WN, Feingold KR, Elias PM. 1996. Metabolic approaches to enhance transdermal drug delivery. 1. Effect of lipid synthesis inhibitors. J Pharm Sci 85:643–648.

5. Harris IR, Farrell AM, Grunfeld C, Holleran WM, Elias PM, Feingold KR. 1997. Permeability barrier disruption coordinately regulates mRNA levels for key enzymes of cholesterol, fatty acid, and ceramide synthesis in the epidermis. J Investig Dermatol 109:783–787.

6. Menon GK, Feingold KR, Elias PM. 1992. Lamellar body secretory response to barrier disruption. J Investig Dermatol 98:279–289.

7. Grubauer G, Feingold KR, Harris RM, Elias PM. 1989. Lipid content and lipid type as determinants of the epidermal permeability barrier. J Lipid Res 30:89–96.

8. Prausnitz MR. 2004. Microneedles for transdermal drug delivery. Adv Drug Deliv Rev 56:581–587.

9. Prausnitz MR, Mitragotri S, Langer R. 2004. Current status and future potential of transdermal drug delivery. Nat Rev Drug Discov 3:115–124.

10. Prausnitz MR, Langer R. 2008. Transdermal drug delivery. Nat Biotechnol 26:1261–1268.

11. Davis SP, Martanto W, Allen MG, Prausnitz MR. 2005. Hollow metal microneedles for insulin delivery to diabetic rats. IEEE Trans Biomed Eng 52:909–915.

12. Gill HS, Prausnitz MR. 2007. Coated microneedles for transdermal delivery. J Control Release 117:227–237.

13. Henry S, McAllister DV, Allen MG, Prausnitz MR. 1998. Microfabricated microneedles: A novel approach to transdermal drug delivery. J Pharm Sci 87:922–925.

14. Martanto W, Moore J, Kashlan O, Kamath R, Wang P, O'Neal J, Prausnitz M. 2006. Microinfusion using hollow microneedles. Pharm Res 23:104–113.

15. Wermeling DP, Banks SL, Hudson DA, Gill HS, Gupta J, Prausnitz MR, Stinchcomb AL. 2008. Microneedles permit transdermal delivery of a skin-impermeant medication to humans. Proc Natl Acad Sci USA 105:2058–2063.

16. Daddona P, Matriano J, Mandema J, Maa YF. 2011. Parathyroid hormone (1–34)-coated microneedle patch system: Clinical pharmacokinetics and pharmacodynamics for treatment of osteoporosis. Pharm Res 28:159–165.

17. Zhang Y, Brown K, Siebenaler K, Determan A, Dohmeier D, Hansen K. 2012. Development of lidocaine-coated microneedle product for rapid, safe, and prolonged local analgesic action. Pharm Res 29:170–177.

18. Banks S, Paudel K, Brogden N, Loftin C, Stinchcomb A. 2011. Diclofenac enables prolonged delivery of naltrexone through microneedle-treated skin. Pharm Res 28:1211–1219.

19. Gupta J, Gill HS, Andrews SN, Prausnitz MR. 2011. Kinetics of skin resealing after insertion of microneedles in human subjects. J Control Release 154:148–155.

20. Kalluri H, Banga A. 2011. Formation and closure of microchannels in skin following microporation. Pharm Res 28:82–94.

21. Banks SL, Pinninti RR, Gill HS, Paudel KS, Crooks PA, Brogden NK, Prausnitz MR, Stinchcomb AL. 2010. Transdermal delivery of naltrexol and skin permeability lifetime after microneedle treatment in hairless guinea pigs. J Pharm Sci 99:3072–3080.

22. Brogden NK. 2012. Clinical evaluation of novel methods for extending microneedle pore lifetime. PhD. Thesis University of Kentucky.

23. Grubauer G, Elias PM, Feingold KR. 1989. Transepidermal water loss: The signal for recovery of barrier structure and function. J Lipid Res 30:323–333.

24. Micromedex2.0. Accessed July 1, 2013, at: http://www.micromedexsolutions.com/micromedex2/librarian.

25. Vivitrol. Alkermes. Accessed July 1, 2013, at: http://www.vivitrol.com/.

26. Revia. Accessed July 1, 2013, at: http://www.rxlist.com/revia-drug.htm.

27. Mao-Qiang M, Feingold KR, Thornfeldt CR, Elias PM. 1996. Optimization of physiological lipid mixtures for barrier repair. J Investig Dermatol 106:1096–1101.

28. Paudel KS, Nalluri BN, Hammell DC, Valiveti S, Kiptoo P, Hamad MO, Crooks PA, Stinchcomb AL. 2005. Transdermal delivery of naltrexone and its active metabolite 6-beta-naltrexol in human skin in vitro and guinea pigs in vivo. J Pharm Sci 94:1965–1975.

29. Milewski M. 2011. Microneedle-assisted transdermal delivery of naltrexone species: In vitro permeation and in vivo pharmacokinetic studies. PhD. Thesis. Lexington, Kentucky: University of Kentucky.

30. Milewski M, Stinchcomb A. 2011. Vehicle composition influence on the microneedle-enhanced transdermal flux of naltrexone hydrochloride. Pharm Res 28:124–134.

31. Andersen F, Hedegaard K, Petersen TK, Bindslev-Jensen C, Fullerton A, Andersen KE. 2006. The hairless guinea-pig as a model for treatment of cumulative irritation in humans. Skin Res Technol 12:60–67.

32. Brogden NK, Milewski M, Ghosh P, Hardi L, Crofford LJ, Stinchcomb AL. 2012. Diclofenac delays micropore closure following microneedle treatment in human subjects. J Control Release 163:220–229.

33. Brogden N, Banks S, Crofford L, Stinchcomb A. 2013. Diclofenac enables unprecedented week-long microneedle-enhanced delivery of a skin impermeable medication in humans. Pharm Res 30:1947–1955.

34. Ghosh P, Pinninti RR, Hammell DC, Paudel KS, Stinchcomb AL. 2013. Development of a codrug approach for sustained drug delivery across microneedle-treated skin. J Pharm Sci 102:1458–1467.

35. Ghosh P. 2013. Formulation optimization for pore lifetime enhancement and sustained drug delivery across microneedle treated skin. PhD. Thesis. Lexington, Kentucky: University of Kentucky.

36. American Chemical Society. Accessed July 1, 2013. www.scifinder.cas.org.

37. Kurihara-Bergstrom T, Knutson K, DeNoble LJ, Goates CY. 1990. Percutaneous absorption enhancement of an ionic molecule by ethanol–water systems in human skin. Pharm Res 7:762–766.

38. Barry BW. 1987. Mode of action of penetration enhancers in human skin. J Control Release 6:85–97.

39. Thong HY, Zhai H, Maibach HI. 2007. Percutaneous penetration enhancers: An overview. Skin Pharmacol Physiol 20:272–282.

40. Arora A, Kisak E, Karande P, Newsam J, Mitragotri S. 2010. Multicomponent chemical enhancer formulations for transdermal drug delivery: More is not always better. J Control Release 144:175–180.