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Low frequency sonophoresis mediated transdermal and intradermal delivery of ketoprofen

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ABSTRACT

The objective of this study was to test low frequency sonophoresis at 20 kHz for delivery of ketoprofen into and across the skin. Permeation studies were carried out *in vitro* on excised hairless rat skin over a period of 24 h using Franz diffusion cells after which, skin samples were subjected to skin extraction to quantify the amount of drug present in skin. Parameters like ultrasound application time, duty cycle coupling medium and distance of ultrasound horn from skin were optimized. Transepidermal water loss (TEWL) was measured to indicate the extent of barrier disruption following sonophoresis. Confocal microscopy was used to visualize dye penetration through sonophoresis treated skin. Application of ultrasound significantly enhanced permeation of ketoprofen from 74.87 ± 5.27 μg/cm^2 for passive delivery to 491.37 ± 48.78 μg/cm^2 for sonophoresis. Drug levels in skin layers increased from 34.69 ± 7.25 μg following passive permeation to 212.62 ± 45.69 μg following sonophoresis. TEWL increased from 31.6 ± 0.02 (passive) to 69.5 ± 12.60 (sonophoresis) indicating disruption of barrier properties. Confocal microscopy images depicted enhanced dye penetration through sonophoresis treated skin confirming barrier disruption. Low frequency sonophoresis with optimized ultrasound parameters can be effectively used to actively enhance transdermal and topical delivery of ketoprofen.

1. Introduction

Ketoprofen is a non steroidal anti-inflammatory drug predominantly used in treatment of rheumatoid arthritis and osteoarthritis (Cagnie et al., 2003; Maestrelli et al., 2005). It is also used to relieve minor aches and menstrual pain (Cho and Choi, 1998). Ketoprofen has analgesic, antipyretic and anti-inflammatory properties (Goosen et al., 1998). It acts as an anti-inflammatory agent by reversible inhibition of cyclooxygenase 1 and 2 enzymes leading to reduced formation of prostaglandin precursors (Paolino et al., 2002). Ketoprofen is commercially available as oral dosage forms having a maximum dose of 300 mg/day (Cho and Choi, 1998). However, oral administration of ketoprofen is associated with gastrointestinal side effects such as abdominal pain, ulceration and irritation of gastric mucosa (Maestrelli et al., 2006). In addition, ketoprofen also has a short elimination half life making it necessary to administer the drug 3–4 times a day (Rhee et al., 2001; Cheng et al., 2007). Transdermal delivery of ketoprofen is a potential alternative route of administration having several advantages over oral delivery. This route of administration would avoid gastrointestinal side effects (Beetge et al., 2000). It is also beneficial for drugs with a short half life and can help maintain consistent plasma drug levels for longer durations of time (Alvarez-Roman et al., 2003). However, delivery through skin is limited by its barrier properties. Hence, it is necessary to employ enhancement techniques to assist transdermal delivery of ketoprofen.

Several techniques have been employed to improve transdermal delivery of ketoprofen. Different transdermal formulations such as liposomes (Maestrelli et al., 2005), ointments, creams (Gürol et al., 1996), gels (Goosen et al., 1998; Beetge et al., 2000), patches (Shinkai et al., 2008, 2011) incorporating various permeation enhancers have been studied. Physical enhancement techniques (Banga, 2011) such as microneedles treatment (So et al., 2009), iontophoresis (Panus et al., 1997; Tashiro et al., 2000) and therapeutic frequency sonophoresis (1 MHz) (Sharma et al., 1999; Cagnie et al., 2003) have also been investigated for transdermal delivery of ketoprofen.

Sonophoresis or phonophoresis implies application of ultrasound energy to drive molecules into and across skin (Polat et al., 2011b; Mutoh et al., 2003). Depending on the frequency of ultrasound used, it can be further classified into low frequency ultrasound (20–100 kHz) and therapeutic frequency ultrasound (1–3 MHz). The exact mechanism behind enhancement of transdermal delivery by sonophoresis is not yet known. However, acoustic

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cavitation (formation and oscillation of microbubbles in the coupling medium) is thought to play an important role in ultrasound assisted delivery (Tang et al., 2002; Ueda et al., 2009). Collapse of these microbubbles on the surface of skin (stratum corneum) leads to skin permeabilization.

Ketoprofen is being used extensively in physical therapy for management of musculoskeletal pain. Therapeutic frequency ultrasound is being used as an enhancement technique to assist delivery of ketoprofen through skin (Cagnie et al., 2003). Recent literature suggests that low frequency sonophoresis would be a more effective technique in enhancing transdermal delivery of small hydrophilic molecules (Sarheed and Abdul Rasool, 2011) as well as macromolecules (Mitragotri et al., 1995, 1996; Polat et al., 2010a,b). However, sonophoretic enhancement of transdermal permeation is highly variable from drug to drug (Mitragotri et al., 1997). Hence, in this study, low frequency sonophoresis (20 kHz) was investigated as a possible enhancement approach for transdermal delivery of ketoprofen and optimization of ultrasound parameters was carried out for delivery of this moderately lipophilic molecule. This is the first study reporting transdermal delivery of ketoprofen using low frequency sonophoresis.

Transdermal delivery of ketoprofen was studied across hairless rat skin, *in vitro* using static vertical Franz diffusion cells. Permeation studies were done over a period of 24 h and drug levels obtained in the receptor compartment were quantified. Drug levels in the skin were also quantified using skin extraction assay. The effect of using different coupling media for sonophoresis, the minimum effective ultrasound application time, the effect of duty cycle and the effect of distance of ultrasound horn from skin was studied. Confocal microscopy was used to visualize enhancement of intradermal delivery following sonophoresis.

2. Materials and methods

2.1. Materials

Ketoprofen was obtained from Sigma–Aldrich (St. Louis, MO, USA). Phosphate buffered saline, ethanol, acetonitrile, methanol, propylene glycol, potassium phosphate monobasic (KH_2PO_4) and ortho-phosphoric acid were purchased from Fisher Scientific (NJ, USA). Calcein was obtained from Holles Laboratories Inc., Cohasset, MA, USA. 3M Transpore tape for tape stripping was obtained from 3M (St. Paul, MN, USA). Centrifuge tubes for skin extraction studies were obtained from MedSupply Partners (Atlanta, GA). De-ionized water was used to prepare all solutions required in this study and for HPLC analysis.

Male CD Hairless rats were obtained from Charles River Laboratories (Wilmington, MA, USA). The rats were quarantined and housed in Mercer University animal facility. All animal procedures were carried out as per the protocol approved by Institutional Animal Care and Use Committee (IACUC) at Mercer University.

2.2. Skin isolation and preparation

Hairless rats were euthanized using carbon dioxide asphyxiation and abdominal skin was isolated for *in vitro* studies. Freshly isolated full thickness skin was used for each experiment. After isolation, the subcutaneous fat adhering to the skin was carefully removed and the skin was cut into pieces of appropriate size for mounting on the receptor compartment of vertical Franz diffusion cells.

2.3. Sonophoresis equipment and application

Sonophoresis was carried out using a sonicator operating at a frequency of 20 kHz (VCX 500, Sonics and Materials, Newtown, CT, USA). Briefly, a piece of skin was placed under the sonicator horn such that the epidermal surface of the skin was facing the horn. The distance between the skin and horn was either 0.3 cm or 0.6 cm depending on the experiment. A donor chamber (cylindrical shaped chamber made of glass, open from both ends) of the vertical Franz diffusion cells was then placed over the skin. Coupling medium for sonophoresis consisted of either water or 1% Sodium lauryl sulfate (SLS) and was contained in the donor chamber. The sonicator horn was then placed in the coupling medium and ultrasound was activated for a predetermined time. After ultrasound pretreatment, skin was dabbed with kimwipes and mounted Franz diffusion cells for permeation studies. Fig. 1 depicts a schematic diagram for application of sonophoresis.

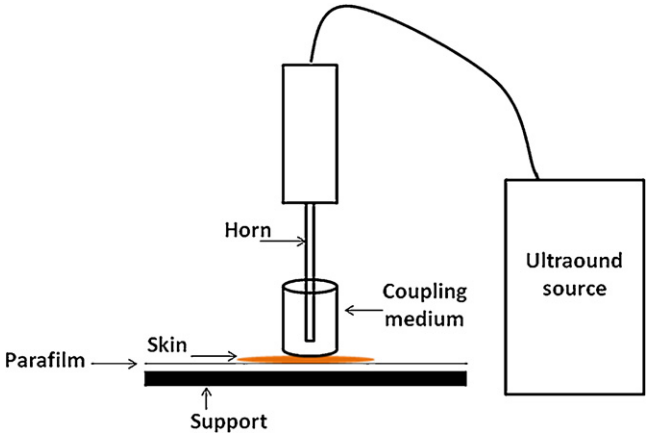

Fig. 1. Schematic diagram for sonophoresis setup.

2.4. Determination of ultrasound intensity

Intensity of ultrasound was determined using a calorimetric method. Water (50 g) was placed in an insulated beaker and ultrasound was activated for predetermined time periods at various duty cycles. The temperature of water was measured using a thermometer before and after ultrasound activation. The intensity of ultrasound was calculated according to the following formula:

$$I = \left(\frac{m_{\text{water}} \times Cp_{\text{water}}}{A}\right)\frac{dT}{dt}$$

where I is the intensity of ultrasound (W/cm^2), m is the mass of water (50 g), Cp is the specific heat capacity of water (4.18 J/g °C), A is the surface area of the sonicator horn and dT/dt is the rate of change of temperature of water.

2.5. Permeation studies

In vitro permeation studies were carried out using vertical Franz diffusion cells. Full thickness hairless rat skin was used for permeation studies. Hairless rat skin has been used as an *in vitro* skin model for active transdermal transport studies (Ueda et al., 1995; Boucaud et al., 2001; Mutoh et al., 2003; Morimoto et al., 2005; Kumar and Lin, 2009; Xu et al., 2011). The Franz cells were cleaned and their receptor compartments were filled with receptor medium. The receptor medium comprised of 5 ml of PBS (1×): ethanol (50:50%, v/v). The skin samples which were pretreated with ultrasound were then mounted on Franz cells such that the epidermal surface of skin now faced the donor compartment of the cells. Similar procedure was followed for passive delivery studies except that the skin samples were not subjected to ultrasound pretreatment. After skin mounting, the donor compartments were placed on the skin surface and the Franz cell assembly was held

together with the help of clamps. The effective area of diffusion with this assembly was 0.64 cm^2. The donor formulation consisted of saturated solution of ketoprofen in 50% propylene glycol containing 3.5 mg/ml drug. Donor formulation (300 μl) was placed in the donor compartments. Receptor medium samples (0.5 ml) were withdrawn from the receptor compartments at predetermined time points over a period of 24 h. The withdrawn volume of receptor medium was replenished at each time point by fresh receptor medium. All studies were carried out with $n \geq 3$. The samples obtained were analyzed by a stability indicating HPLC assay.

2.6. Skin extraction

Skin extraction procedure was carried out to determine drug levels in skin. Skin samples were removed from Franz diffusion cells at the end of permeation experiments. The skin surface was thoroughly cleaned by dabbing it three times with Q-tips soaked in receptor medium. Skin was then tape stripped two times using 3M Transpore tapes to ensure that the formulation is completely removed from the surface. Kimwipes were used to dry the skin surface. After the cleaning procedure, skin samples were minced manually using a pair of scissors and added to centrifuge tubes. Extraction solvent (methanol:water – 50:50%, v/v, 4 ml) was added to minced skin and extraction was carried out by overnight shaking on roller shaker (New Brunswick Scientific Co. Inc., NJ, USA). The tubes were then centrifuged to separate skin from the solvent and the supernatant extract was filtered and analyzed by a stability indicating HPLC assay.

2.7. Recovery studies

Recovery studies were carried out *in vitro* in hairless rat skin to determine the efficiency of skin extraction. Standard solutions of ketoprofen were prepared (100 μg/ml, 200 μg/ml, 500 μg/ml and 1000 μg/ml) and 50 μl of each solution (containing 5, 10, 25 and 50 μg drug respectively) was injected in hairless rat skin samples in triplicate. The skin pieces were allowed to equilibrate with drug for a period of 2 h and were then subjected to the extraction procedure mentioned above. The extraction efficiency was determined using a standard curve and average recovery for the extraction method was 73.19 ± 2.86%. This factor was taken into consideration for quantification of drug in underlying skin samples. The amount of drug calculated from HPLC analysis was divided by a factor of 73.19/100 = 0.7319 to determine the actual amount of drug in skin samples.

2.8. Optimization of sonophoresis parameters for transdermal and topical delivery of ketoprofen

Ultrasound parameters such as application time, coupling medium, duty cycle and distance of horn from skin were optimized. In order to determine the minimum effective application time, sonophoresis was carried out for 0.5 min, 1 min and 2 min at 100% duty cycle with 1% SLS as coupling medium. The effect of using different coupling media during sonophoresis was investigated. Sonophoresis (2 min at 100% duty cycle) was carried out using either water or 1% SLS as coupling medium. The effect of using pulsed ultrasound at 50% duty cycle (5 s on, 5 s off) was also determined. Finally, the effect of distance of ultrasound horn from skin was determined by placing the horn at a distance of 0.3 cm and 0.6 cm from skin.

2.9. Transepidermal water loss measurements

Transepidermal water loss (TEWL) can be used to predict the barrier properties of skin (Shimada et al., 2008). If the skin barrier is compromised, TEWL values are elevated (Fluhr et al., 2006). TEWL measurements were done *in vitro* on Franz diffusion cells using a closed chamber evaporimeter (Vapometer, Delfin Technologies Ltd., Kuopio, Finland) Skin pieces were subjected to ultrasound treatment for 1–2 min. Immediately after treatment, skin was mounted on the receptor compartment of Franz cells containing water as the receptor medium. Skin was allowed to equilibrate for a period of 30 s and TEWL measurements were then performed over the next 10 s. Intact (dry) skin as well as hydrated skin (hydrated for 1–2 min respectively and wiped dry) were used as controls.

2.10. Visualization of dye permeation through sonophoresis treated skin

Confocal microscopy (Zeiss LSM 510 META, Goettingen, Germany) was used to visualize permeation of calcein through sonophoresis treated skin. Skin piece was subjected to ultrasound treatment (2 min, 100% duty cycle, 1% SLS as coupling medium, 0.3 cm horn distance) and mounted on vertical Franz diffusion cells. Calcein (Holles Laboratories Inc., Cohasset, MA, USA) was added to the donor compartment. After 24 h, skin was removed from Franz diffusion cells, cleaned with Q-tips soaked in water and dried with kimwipes. A small piece of skin was cut out from the diffusion area (0.64 cm^2) and embedded in OCT medium. After freezing at −20 °C, vertical sections (10 μm) were cut using AO HistoSTAT Microtome, Buffalo, NY. These sections were observed under confocal microscope.

2.11. Quantitative analysis

Ketoprofen was quantified using high performance liquid chromatography. A stability indicating assay modified from literature was used for analysis of ketoprofen (Bempong and Bhattacharyya, 2005). Alliance system (Waters Corp, Milford, MA, USA) was used with a photodiode array detector (Waters 2996) operating at 233 nm. The column used was Waters YMC ODS AQ C18; 250 mm × 4.6 mm. Isocratic elution was carried out at a flow rate of 1 ml/min using phosphate buffer pH 3.5:acetonitrile:water (8:49:43%, v/v) as the mobile phase. The column temperature was maintained at 35 °C. Injection volume used was 20 μl. The total run time was 9 min and the retention time of ketoprofen was 5.3 min. The limit of detection was 0.1 μg/ml and the limit of quantification was 0.5 μg/ml. The range of the standards was 0.5–50 μg/ml. The assay was sensitive in the range tested. The assay procedure was validated with respect to intra and inter-day variability and precision. The relative standard deviation values were 0.2–3%, 0.2–2.3% and 0.17% respectively.

2.12. Statistical analysis

ANOVA (analysis of variance) and Student's *t*-test were used for statistical analysis. Tukey's analysis was used to determine honestly significant difference.

3. Results and discussion

Transdermal delivery of ketoprofen is a potential alternative to oral delivery since the former helps avoid gastrointestinal side effects. Topical gel formulations of ketoprofen are available in the market for treatment of rheumatoid arthritis and osteoarthritis. However, transdermal delivery of ketoprofen has been limited by the barrier properties of skin. Several formulations such as poultices (Sheu et al., 2002), liposomes containing ketoprofen–cyclodextrin complexes (Maestrelli et al., 2006) or lecithin microemulsions have been tested to enhance delivery. Microemulsions (o/w) made up

of soyabean lecithin have been shown to be beneficial in enhancing transdermal delivery of ketoprofen across human cadaver skin over conventional formulations such as gels or o/w and w/o creams (Paolino et al., 2002). Also incorporation of 10% ethanol in carbopol gels has been shown to enhance the partition coefficient of ketoprofen across full thickness porcine ear skin (Ceschel et al., 2002). Active enhancement techniques such as iontophoresis, microneedles treatment and therapeutic frequency sonophoresis have also been investigated to enhance transdermal delivery of ketoprofen. Transdermal delivery of ketoprofen could be achieved in vivo in human subjects with cathodal iontophoresis at a current density of 0.28 mA/cm^2 for 40 min (Panus et al., 1997). Similarly insertion of microneedles array coated with ketoprofen gel in male Sprague-Dawley rats for 10 min could achieve 1.86 fold and 2.86 fold increase in AUC and C_{max} in comparison with application of ketoprofen gel alone (So et al., 2009). Sonophoresis is an alternative technique which can be used to effectively enhance transdermal permeation of drugs. This technique has been commonly used to assist delivery of topically applied anesthetics, counter irritants and anti-inflammatory agents (Maruani et al., 2010a). Additionally sonophoresis of histamine is being investigated as a positive control for allergy testing (Maruani et al., 2010b). Therapeutic frequency sonophoresis (1 MHz, 1.5 W/cm^2) for 5 min in both pulsed and continuous mode has been shown to be beneficial for transdermal delivery of ketoprofen. Delivery with sonophoresis resulted in higher accumulation of ketoprofen in synovial tissue, which is the major site of inflammation in arthritis conditions (Cagnie et al., 2003). Acoustic cavitation has been identified as an important mechanism of action for sonophoresis mediated enhancement in transdermal delivery. Since cavitation is inversely proportional to the frequency of ultrasound, low frequency sonophoresis (20–100 kHz) has been more effective in enhancing transdermal delivery of small hydrophilic molecules as well as macromolecules. Hence, in this study we have employed low frequency sonophoresis as an enhancement technique assisting transdermal delivery of ketoprofen. Using this enhancement technique, we have investigated the possibility of achieving significant permeabilization of skin using lower application times.

Sonophoresis was carried out at a constant frequency of 20 kHz and intensity of 6.9 W/cm^2. Ultrasound parameters tested for optimized drug delivery included ultrasound application time, coupling media used, duty cycle and distance of the ultrasound horn from skin.

3.1. Determination of minimum effective application time

It has been suggested that enhancement in skin permeability caused by sonophoresis depends on ultrasound parameters such as frequency, intensity and duration of ultrasound application. Since the instrument used in this study works at a constant frequency and intensity, it would be important to determine the optimum duration of ultrasound application to significantly enhance transdermal and topical delivery of ketoprofen. Mitragotri et al. (2000a) have shown that there exists a threshold dose of ultrasound energy below which sonophoresis would not cause a significant increase in skin conductivity. However, if the applied energy exceeds the threshold dose, skin conductivity and hence permeability would increase with increase in ultrasound energy. The total ultrasound energy applied during sonophoresis can be calculated as energy = intensity × application time × duty cycle. Increase in application time would thus, increase the total ultrasound energy delivered. The minimum effective application time would indicate a condition at which the threshold dose of ultrasound energy is delivered causing a significant enhancement in permeation of ketoprofen. Different ultrasound application times, ranging from 60 s to 4 h (Singer et al., 1998; Terahara et al., 2002; Weimann and Wu, 2002; Alvarez-Roman et al., 2003; Merino et al., 2003; Mutoh et al., 2003; Lee et al., 2004), have been used for enhancement of transdermal delivery. Permeation and skin extraction studies were performed to determine the minimum effective application time to enhance transdermal and topical delivery of ketoprofen, respectively. Application times of 0.5 min and 1 min did not significantly enhance transdermal or topical delivery of ketoprofen over passive permeation. However 2 min of ultrasound application (20 kHz, 6.9 W/cm^2, 100% duty cycle) resulted in significantly higher transdermal delivery (Fig. 2A, $p < 0.05$). Permeation of ketoprofen increased from 74.87 ± 5.27 μg/cm^2 (passive delivery) to 491.37 ± 48.78 μg/cm^2 after 2 min of ultrasound pretreatment. Similar results were observed for skin extraction studies where the amount of drug accumulating in skin increased significantly (Fig. 2B, $p < 0.05$) from 34.69 ± 7.25 μg following passive permeation to 212.62 ± 45.69 μg. An application time of 5 min is typically used in physical therapy studies for transdermal delivery of ketoprofen using therapeutic frequency sonophoresis. Low frequency sonophoresis can permit reduction in the application time to 2 min with a significant enhancement in intradermal and transdermal delivery of this drug.

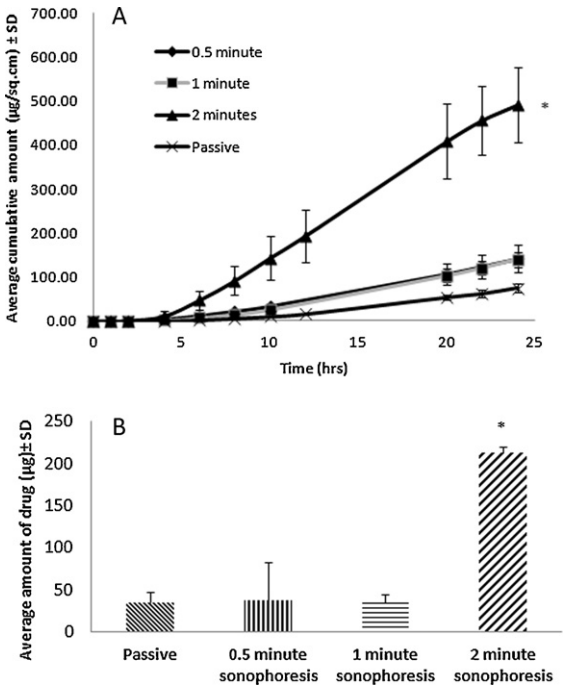

Fig. 2. Effect of sonophoresis application time on transdermal and intradermal delivery of ketoprofen (100% duty cycle, 1% SLS coupling medium, 0.3 cm distance between ultrasound horn and skin). (A) Permeation profile depicting transdermal delivery, (B) skin accumulation depicting intradermal delivery. * indicates significant difference from other groups ($p < 0.05$).

3.2. Effect of 1% sodium lauryl sulfate in coupling medium

It is known that addition of a surfactant such as sodium lauryl sulfate (SLS) to the coupling medium during sonophoresis can enhance drug delivery several fold over using sonophoresis alone. In a study by Mitragotri et al. (2000b) treatment of porcine skin with 1% SLS alone, ultrasound alone (20 kHz, 10 W/cm^2) and combination treatment resulted in an increase in mannitol permeability of 3 fold, 8 fold and 200 fold respectively over passive permeation demonstrating synergistic effect of sonophoresis-SLS treatment. The mechanism of this synergistic enhancement has been studied using microscopy as well as using an aqueous pore pathway model. Paliwal et al. (2006) have demonstrated increased occurrence of

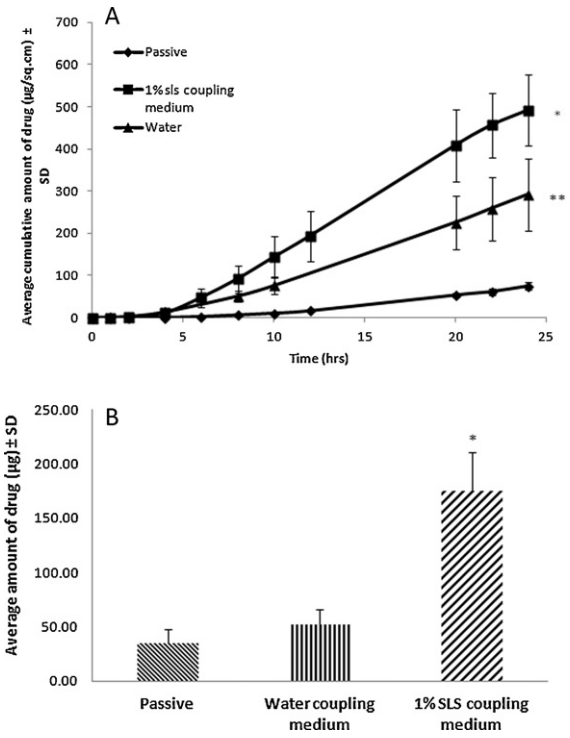

Fig. 3. Effect of using different coupling media on transdermal and intradermal delivery of ketoprofen (2 min application time, 100% duty cycle, 0.3 cm distance between ultrasound horn and skin). (A) Permeation profile depicting transdermal delivery, (B) skin accumulation depicting intradermal delivery. * indicates significant difference from other groups ($p < 0.05$). ** indicates significant difference from passive permeation ($p < 0.05$).

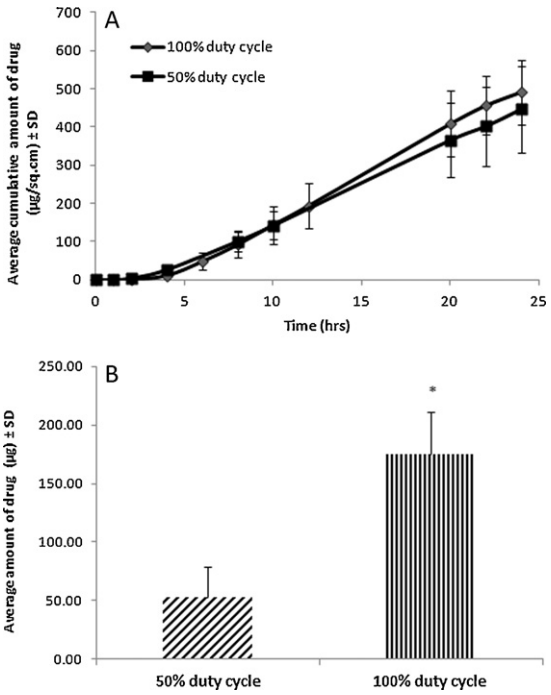

Fig. 4. Effect of duty cycle on transdermal and intradermal delivery of ketoprofen (2 min application time, 1% SLS coupling medium, 0.3 cm distance between ultrasound horn and skin). (A) Permeation profile depicting transdermal delivery, (B) skin accumulation depicting intradermal delivery. * indicates significant difference from other groups ($p < 0.05$).

lacunar regions (imperfections in stratum corneum) after ultrasound treatment using transmission electron microscopy. Addition of 1% SLS resulted in an increase in dimensions of lacunar regions thereby increasing uptake of quantum dots in skin. It is also claimed that SLS may be responsible for increasing pore radii in the non-LTR regions of sonophoresis treated skin (Polat et al., 2011a). The aqueous pore pathway model suggests that sonophoresis-SLS treatment leads to more consistent skin perturbation in comparison to sonophoresis treatment alone. The combination treatment also results in more direct pathways being created in skin based on porosity to tortuosity ratio. Also, the treatment times required to achieve the same level of skin electrical resistivity were 5–12 times longer with sonophoresis alone compared to combination treatment (Polat et al., 2010b). The synergistic effect of using SLS with sonophoresis has been typically tested using hydrophilic molecules. In this study we tested the effect of using 1% SLS as the coupling medium on transdermal permeation and intradermal accumulation of a moderately lipophilic compound, ketoprofen. Sonophoresis with 1% SLS enhanced transdermal delivery of ketoprofen significantly over sonophoresis with water as the coupling medium (Fig. 3A). Also, the accumulation of drug in skin significantly increased when sonophoresis was carried out with 1% SLS as the coupling medium (Fig. 3B). Sonophoresis-SLS treatment resulted in 6.5 fold enhancement in permeation and 5 fold enhancement in skin accumulation over passive delivery. In comparison, sonophoresis treatment alone resulted in 3.8 fold and 1.4 fold enhancement in permeation and skin accumulation over passive delivery respectively. Sonophoresis-SLS treatment assisted enhancements in delivery of a moderately lipophilic molecule, ketoprofen were modest in comparison to the orders-of-magnitude enhancements observed for hydrophilic molecules. This may be due to short durations of ultrasound treatment used in this study protocol. However, addition of 1% SLS in the coupling medium significantly enhanced ketoprofen delivery over using sonophoresis treatment alone and this treatment regimen can be used to assist low frequency sonophoresis mediated delivery of moderately lipophilic molecules.

3.3. Effect of duty cycle

The effect of pulsed application of ultrasound (50% duty cycle: 5 s on 5 s off) on transdermal permeation and intradermal accumulation of ketoprofen was investigated in comparison to continuous application (100% duty cycle). Pulsed ultrasound is known to avoid heating in the coupling medium and hence can be a more patient compliant sonophoresis regimen. In order to study the effect of duty cycle, the total time of ultrasound exposure was kept constant at 2 min, The pulsed treatment was carried out for a total time of 4 min, with ultrasound being active for 2 min, in pulses of 5 s. The pulsed ultrasound treatment was equally effective as the continuous treatment in enhancing transdermal delivery of ketoprofen over passive permeation. No significant difference was observed in permeation of ketoprofen after continuous and pulsed ultrasound treatments (Fig. 4A, $p > 0.05$). Similar results have been obtained by Mitragotri et al. (2000a) where enhancements in skin conductivity values were found to be independent of duty cycle used. However, skin extraction studies revealed that intradermal accumulation of ketoprofen was enhanced only with the continuous ultrasound treatment. A possible reason for this may be faster increase in temperature of the coupling medium (data not shown) seen with continuous ultrasound treatment as compared to pulsed treatment (Merino et al., 2003) Another explanation that seems to support this phenomenon would be that pulsed ultrasound was used with a surfactant (SLS) in the coupling medium. When pulsed ultrasound is applied in presence of a surfactant, some of the cavitation bubbles have a tendency to dissolve back in solution (Polat et al., 2011b). This would result in lower cavitation which may possibly lower permeability of deeper

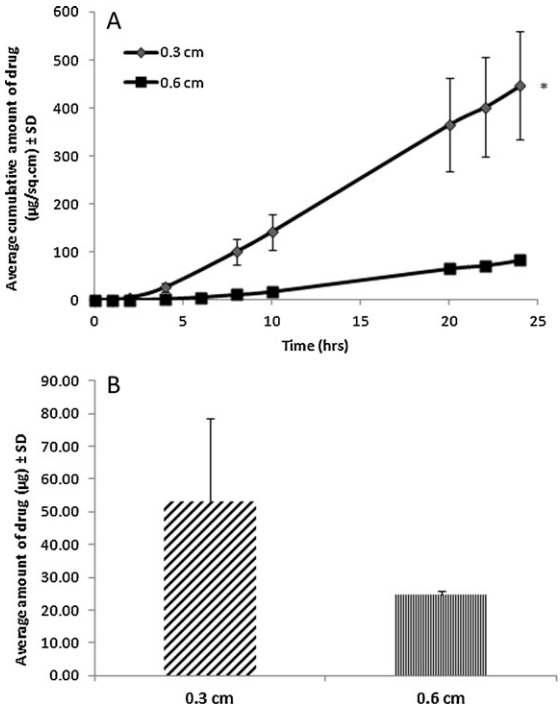

Fig. 5. Effect of distance of ultrasound horn from skin surface on transdermal and intradermal delivery of ketoprofen (2 min application time, 1% SLS coupling medium, 50% duty cycle). (A) Permeation profile depicting transdermal delivery, (B) skin accumulation depicting intradermal delivery. * indicates significant difference from other groups ($p < 0.05$).

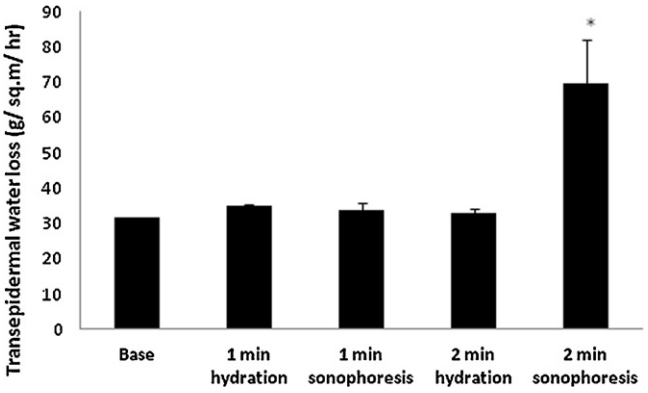

Fig. 6. Transepidermal water loss measurements following sonophoresis. Base value is the measurement on intact skin. * indicates significant difference from other groups ($p < 0.05$).

layers of skin reducing the amount of drug available to form a depot in skin layers.

3.4. Effect of distance between ultrasound horn and skin

The effect of distance between ultrasound horn and skin on sonophoresis assisted delivery of ketoprofen was investigated. Distances between horn and skin previously investigated range from zero tip displacement (horn in contact with skin) to 4 cm, with 0.3 cm to 1 cm distances typically used for low frequency sonophoresis (Polat et al., 2011b). Permeation of ketoprofen (50% duty cycle, 1% SLS coupling medium) was inversely proportional to the distance between ultrasound horn and skin surface. Permeation decreased significantly when the distance between ultrasound horn and skin was increased from 0.3 cm to 0.6 cm (Fig. 5A, $p < 0.05$). Increasing distance between ultrasound horn and skin is known to reduce cavitation effects hence decreasing permeability (Terahara et al., 2002). Since continuous ultrasound was not used in this case, no significant difference was observed in skin accumulation of ketoprofen with increasing distance between ultrasound horn and skin (Fig. 5B).

3.5. Transepidermal water loss measurements

Transepidermal water loss (TEWL) can be used as an indicator of skin barrier function. The intact skin barrier (stratum corneum) maintains homeostasis by preventing loss of water from the body. Disruption of the barrier, caused by physical or chemical perturbation or due to a disease condition would increase loss of water across skin elevating the TEWL values (Kalia et al., 2000; Shimada et al., 2008). TEWL can thus be used to estimate the extent of skin barrier disruption. In this study, TEWL values were used to predict the minimum effective application time required to enhance skin permeability using low frequency sonophoresis, Application times of 1 min and 2 min were studied. TEWL increased significantly from a base value of 31.6 ± 0.12 g/m^2 h to 69.5 ± 12.6 g/m^2 h following sonophoresis for 2 min (100% duty cycle, water as

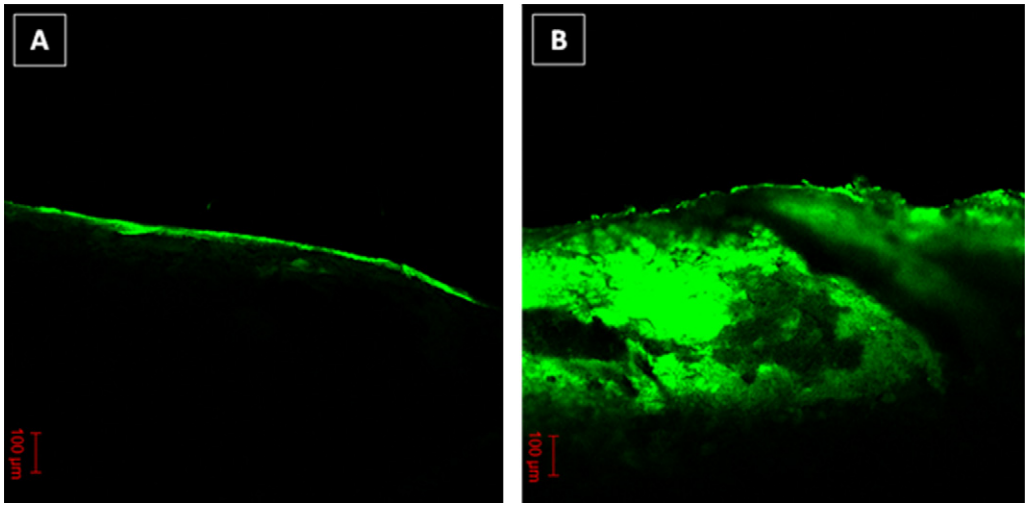

Fig. 7. Confocal microscopy images of vertical sections of hairless rat skin depicting intradermal delivery of calcein. (A) Following passive permeation, (B) Following 2 min sonophoresis treatment (100% duty cycle, 1% SLS coupling medium, 0.3 cm distance between ultrasound horn and skin).

coupling medium). Lower sonophoresis application time of 1 min did not result in significant increase in TEWL from base value. This data (Fig. 6) supports the results from the permeation study (Fig. 2A) confirming that 2 min is the minimum effective application time required to enhance transdermal delivery using low frequency sonophoresis. Since hydration of skin samples during sonophoresis can lead to overestimation of TEWL values, control experiments were performed where TEWL was measured after 1 and 2 min of hydration. Hydration of skin did not increase TEWL in comparison to base value confirming that hydration caused by the coupling medium did not contribute to the elevated TEWL values after treatment with sonophoresis.

3.6. Visualization of dye permeation through sonophoresis treated skin

Confocal microscopy was used to visualize dye accumulation in skin treated with optimized conditions of sonophoresis (2 min, 100% duty cycle, 1% SLS coupling medium). Vertical sections of skin observed under confocal microscope indicate that sonophoresis enhanced permeation of the dye calcein into skin. In comparison to passive delivery of calcein (Fig. 7A) higher accumulation of the dye was observed after sonophoresis treatment (Fig. 7B). This data supports our findings that continuous application of ultrasound results in increased accumulation of molecules in skin layers. Thus, in addition to enhancing permeation of drugs through skin, sonophoresis under optimized conditions can potentially increase intradermal accumulation of molecules (Morimoto et al., 2005).

4. Conclusions

Low frequency sonophoresis with optimized parameters (2 min application, 100% duty cycle, 0.3 cm distance between horn and skin, 1% SLS as coupling medium) could enhance transdermal and topical delivery of ketoprofen in comparison to passive delivery. Permeation, skin extraction and TEWL values indicated 2 min as the minimum effective application time for sonophoretic enhancement in transdermal and topical delivery. Permeation of ketoprofen was not affected by duty cycle. However increasing distance between ultrasound horn and skin decreased permeation. Sonophoresis assisted enhancement in topical delivery could be visualized by dye permeation studies. Overall, low frequency sonophoresis was an effective active enhancement technique enhancing transdermal and topical delivery of ketoprofen.

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