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Improve the Permeation and Deep Tissue Penetration of Topical NSAIDs with Prodrugs

By

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A dissertation submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy in the Department of Biomedical and Pharmaceutical Sciences

Idaho State University

Fall 2014

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This work is dedicated to my beloved Mom (Florence Lobo),

who believed in me to do this.

ACKNOWLEDGEMENTS

First of all I would like to thank the Department of Biomedical and Pharmaceutical Sciences, for deeming me worthy to conduct good and honest research and offering me an admit at this prestigious university. I am also grateful to the department for providing me with all the facilities I needed to conduct my research and make this work come true.

I am also grateful to Dr. James C.K. Lai and Dr. James Bigelow for all the support they have provided me, and all the encouragement they have provided me through the years. I am very grateful to Dr. Byron Bennett for all the help and direction he provided especially in the chemical synthesis portion of my research work, and especially thankful to him for taking time out to personally coach me through the chemistry sections for my comprehensive exam. I am very thankful to Dr. Mike Thomas for providing the Financial Support and guidance for seeing me through my tenure here. I am obliged to Dr. Karl De Jesus for training me on the NMR. I would also like to thank Dr. Shawn Bearden and Dr. Jean Pfau for all the help they provided me when I was over at the Biology building micro slicing tissue samples or trying to work the optical microscope. I would like to thank Barbara Taylor for all her support and for everything she does for us students.

I also want to acknowledge my family and especially my Mom for believing in me, and making this decision to pursue a Ph.D. easier for me. She is a real sport and is completely responsible for where I am today.

And last but not the least, I would like to thank Dr. Guang Yan for giving me the opportunity to pursue my Ph.D. here in the US. Through the years, I thank him for teaching me all that he knows and being a great supervisor. I will always be indebted to him for all his guidance and wisdom he has given me.

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DISSERTATION ABSTRACT

In the recent years, research in Transdermal and Topical drug delivery has been mainly emphasizing on improving the penetration of molecular entities through the skin and their localization at the site of application. The penetration enhancement techniques that depend on compromising the stratum corneum of the skin have been extensively studied and are well established and incorporate techniques such as use of penetrants, use of microneedles, tape stripping etc. Our approach to answer this problem of skin penetration is to attempt to alter the core physicochemical properties of the drug itself by forming ester prodrugs of it. We have attempted to study the change in the physicochemical properties of the drug brought about by esterification, and to evaluate how these changes in physicochemical properties affect the skin permeability properties. Apart from the permeability, we have also attempted to answer the problem of localization and deep tissue penetration of topically administered drugs. Systemic appearance of topically applied drug have been the main concern to deep tissue delivery of drugs, and with the limited research in this particular area, the protein binding property of drugs have been labelled to be the main contributor. A clear understanding of this property and how it affects deep tissue penetration would be useful in gaining knowledge in this inadequately explored area. Our attempt in exploring this issue is to prepare appropriate ester prodrug of the chemical entity and use iontophoresis as a delivery enhancement technique and use animal models to evaluate how these two techniques contribute to localization and deep tissue penetration of the drug.

CHAPTER 1 Introduction and Literature Review

Topical delivery of drug entities have been gaining grounds in drug delivery research mainly because of its ease of administration and the lack of adverse effects associated with the route of delivery. Drugs such as NSAIDs and corticosteroids which mainly prescribed for long term ailments such as arthritis and other musculoskeletal pain disorders are turning out to be good candidates to be reformulated into topical dosage forms. A reason for this is the widely studied and identified gastrointestinal and metabolic adverse effects associated with these drugs(Dequeker 1999). As an alternative towards therapy, selective COX-2 inhibitors have been proposed as an effective treatment to these musculoskeletal disorders. However, since the introductions of the first COX-2 inhibitor based medication in the market (Celebrex® – Celecoxib), almost all of the other specific COX-2 inhibitors have been withdrawn from the market owing to their involvement in cardiovascular complications(Cannon CP 2012). As of December 2011, only Celebrex remains as the only COX-2 inhibitor based drug in the market today. As per the study by Cannon et al, not only was it found for COX-2 inhibitors, but systemic appearance of traditional NSAIDs (except Naproxen) are also associated with increased vascular complications and myocardial infarctions. Hence, there is an urgent need to reconsider chronic therapy of ailments such as arthritis and other musculoskeletal disorders especially when they are prescribed to high risk patients over the age of 65.

Research today to alleviate this issue has mainly concentrated on direct local delivery of the medicament to joints or muscles where the pain related complications reside. And the most viable route of administration to achieve this is through the Topical route. There are

many advantages to delivering medications for pain therapy through this route(Jorge LL 2010), some of which include

- Reduce Gastric disturbances
- Avoid first pass hepatic metabolism
- Reduce variations in Serum concentrations
- Reduce systemic appearance of drug and hence reduce adverse reactions

Despite the obvious advantages, topical drug delivery systems have not received the appropriate attention as should be expected owing to the perceived advantages. There are various factors that make topical drug delivery a more challenging alternative to the oral route. Some of these factors that have caught the eye of the formulation scientist today are –

1.1Permeation through the Stratum Corneum

The Stratum Corneum is the outer most layer of the skin and provides the vital barrier function to the body. The Stratum Corneum has an average thickness of around 10µm, but varies widely throughout the body, ranging from 400-600µm on the palms soles to just 2-3µm on the eyelids. The entities that provide the layer with its formidable barrier properties are called the corneocytes. These are basically highly keratinized, dead cells that are present as a 10 – 25 layer thick barrier that makes up the Stratum Corneum(Wiechers 1989). These corneocytes are embedded in a continuous, lipid – enriched intercellular matrix that consists of multilamellar structures. It is these tightly cemented, lipid depleted keratinocytes amongst the highly lipidic matrix, held together by tightly bound structures known as corneodesmosomes, that makes the Stratum Corneum

highly lipidic and potentially impermeable to most of the extraneous molecules exposed to the body.

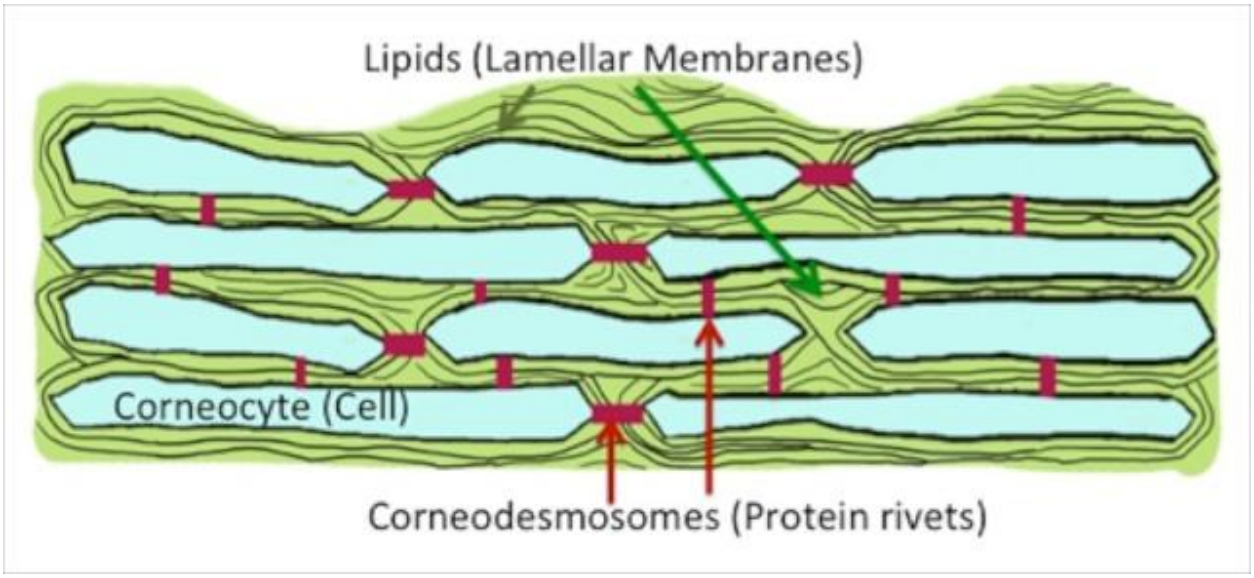

Figure 1 Structure of Stratum Corneum*(Elias 2013)*

1.1.1 Pathways for drug penetration

The permeation of drugs through the skin can follow one of the two routes mentioned (Flynn and Stewart 1988)

- Passage through the epidermis itself –

It is today generally believed that the transepidermal pathway is the primary pathway to drug penetrations for a majority of the drugs. This is where the hydrophobic nature of the Stratum Corneum is the biggest challenge. It is believed that the movement of drug along the lipidic matrix around the corneocytes is the predominant pathway for drug transfer (G. Flynn 1985).

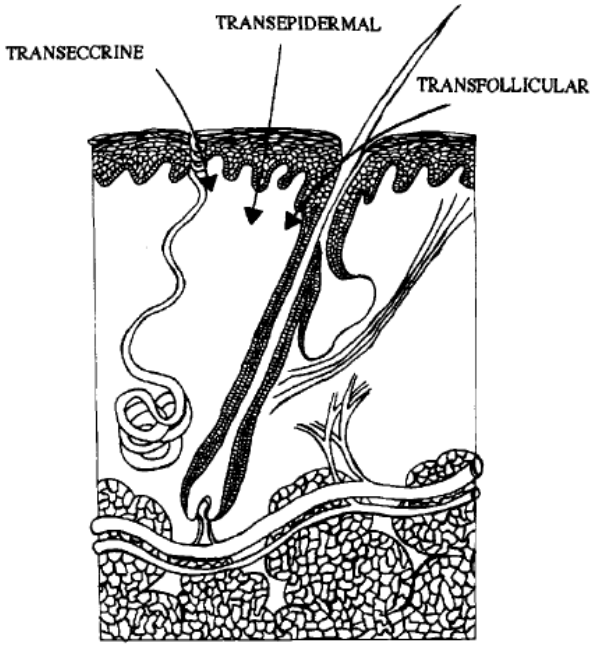

Figure 2 Possible routes for percutaneous absorption *(G. Flynn 1985)*

After exiting the Stratum Corneum, the drug then enters into the wet cell mass of the epidermis, which is a layer that that forms an irregular, papillate interface with the dermis. The dermis is predominantly a mesh of collagen fibers which is the primary structural element of the skin. Wide spaces between the collagen fibers consists of watery gel called the ground substance. Because the epidermis and dermis lack the basic physicochemical distinction, both these layers are considered to be a single field of diffusion. This difference in properties in the Stratum Corneum and the lower viable epidermis and the dermis make the diffusion of drug molecules difficult through the skin. Polar entities would find it a challenge making its way through stratum Corneum, however the lipophilic molecules that make it through his layer, would find passing through the viable epidermis and the dermis difficult.

- Diffusion through shunts – Hair follicles and eccrine glands

This appendageal route is only a secondary route for the transport of drugs through the skin. The pilosebaceous and eccrine gland are ubiquitous throughout the body excepting a few regions. The presence of eccrine glands are frequent and numerous (400 glands/cm^2), however their opening are very tiny and collective contribute to a very minute fraction of the total skin surface area. Hence, contribution to these routes towards diffusion of drugs is very little. And to add, these cavities are very active and due to the retroactive flow of its contents, do not contribute much to drug diffusion. But the eccrine glands are beneficial in the absorption of highly polar molecules and ionized entities that appear on the skin.

The pilosebaceous glands are less frequent than eccrine glands (50-100 glands/cm^2), but cover more surface area owing to the larger openings. Their ducts are filled with lipoidal medium called sebum, and lipoidal drugs usually dissolve in them and pass through the shaft to the base of hair follicle. This is a favorable route for the absorption of particulate agents such as nanoparticles.

1.1.2 Ideal physicochemical properties for percutaneous absorption

As we have seen earlier, the major barriers to drug penetration is the stratum Corneum, which is a tightly bound compact lipidic layer, making out the outermost layer of the skin. The fate of a drug molecule as a topical agent depends on how easily it can pass through his layer.

The properties of a drug that would influence the fate of the flux of a drug through the skin could be perceived in the steady state flux equation

$$\frac{d_m}{d_t} = \frac{DC_0\,K}{h} \quad \textit{Equation 1}$$

Where D is the diffusion coefficient, C_0 is the donor drug concentration, K is the partition coefficient and h is the thickness of the membrane

There have been various properties of drugs that have been studied and explored as contributors to skin penetration, and have been important database in the formulation of drugs as topicals.

Molecular weight –As per a study done by a team in Netherlands (Bos JD 2000), an extensive research on chemical entities such as contact allergens, cyclic immunosuppressants belonging to the class of cyclosporine, widely approved drugs for transdermal or topical delivery gave the conclusion for 500 Daltons to be the ceiling molecular weight for penetration through the skin. The paper also further goes on to talk about this rule to be applicable provided the formulation aspects are not taken into account, but there are not many examples of larger MW compounds that are used topically with formulation penetrants either. It does however show tacrolimus and ascomycin (822 and 811 Daltons resp), as these are absorbed through the skin despite being larger molecules. In another study, the diffusivity of drug molecules were collected and analyzed, and a review of permeation data of around 90 compounds with molecular weight ranging from 18 to 750 Daltons suggested that the skin permeation was found to decrease exponentially with increasing molecular weight(Potts RO 1992).

Log P – Also known as the partition coefficient of a molecule, the Log P value is found to be the major determinant for passage of drug molecule through a biological membrane. A study to examine the exposure and toxicity potential of ampiphilic compounds found the molecules with a positive Log P value to have more potential to cross the skin as compared to the ones with negative Log P values (Korinth G 2012). Another study that

evaluated data of widely used NSAIDs and their flux data across Human epidermis found that there is a parabolic relationship between the flux value and Log P and actually an optimum Log P of 2.5 and 3 is viable(Singh P, Skin permeability and local tissue concentrations of nonsteroidal anti-inflammatory drugs after topical application 1994).

Melting point–A study conducted involving cyclizine alkyl analogues and their fluxes through human epidermal membranes concluded to indicate that water solubility and melting points are determinant for how much of the drug passed through the membrane(Monene LM 2005). It was reported that the compounds with higher aqueous solubility and lower melting points were found to show higher fluxes through the epidermal membranes.

1.1.3 Methods to increase drug permeation through the skin

There have been various efforts to facilitate the flux of drugs molecules across the skin, and most of these have focused to increase the penetrations of the molecules through this outermost layer, the Stratum Corneum. These efforts could be broadly divided based on what approach has been adopted(Eric Wane Smith 1995);

Altering the physicochemical properties of the drug itself -There are various ways by which the intrinsic properties of a chemical entity can be reversibly changed to offer better transient properties to effect permeation. The most widely investigated approach is the study of esters as prodrugs. Many studies have experimented with correlating various properties of the prepared esters especially Log P, and studying the effect of change in this property to the permeation of the prodrug (Bansal AK 2001). Esters have also been prepared to alter and balance both the solubility and Log P properties of an NSAID to get an optimum balance between the two properties to maximize flux through skin (Rautio J

2000). Another set of prodrugs that also have been studied extensively are amides. These chemical entities provides synthesis chemists better flexibility in balancing the solubility and Log P values and these are found to be much more stable enzymatically than ester prodrugs. Again with amides, the Log P value is found to be the predominant factor and studies with a series of amide prodrugs have shown the more lipid soluble one to have the highest flux (Majumdar S 2007). Studies have also been done on amides to retard the deep penetration of amide prodrugs as they were to be used as sunscreens, and concluded to show that the amides with a Log P > 5 showed enhanced concentration only in the upper layers of the epidermis and failed to penetrate deeper (Yan YD 2011).

Ion Pairs are another way to improve the skin permeation properties of drugs especially those that are highly ionized in nature. The molecular size of the counter ion and lipophilicity of the ion pair expressed as a Log P parameters in octanol/water system were found to be the major determinants in predicting the skin flux (Megwa SA 2000).

Saturation/Supersaturation degrees of a drug in a medium has also been explored and shown to affect permeation of the drug through a biological membrane. A relationship between the flux of a drug moiety and its thermodynamic activity in the medium has been derived (Higuchi 1960).

$$\frac{dM}{dT} = \frac{aD}{\gamma h} \quad \textit{Equation 2}$$

Where dM/dT is the flux rate of the drug, α is the thermodynamic activity in the medium and γ is the activity coefficient in the membrane, h is the membrane thickness and D is the diffusion coefficient. Supersaturated solutions of Oestradiol in PG/water systems in presence of appropriate antinucleating agents were shown to have up to 19 folds

improved flux through human skin as compared to the unsaturated dispersions of the drug (Megrab 1995).

External factors to improve drug permeation - chemical or Physical methods–These techniques principally rely on modifying the structure of the Stratum Corneum for effecting drug permeation.

Chemical Enhancers

In literature, various chemical enhancers have been studied and stated to have improved the skin permeation of chemicals such as alcohols, essential oils, DMSO, terpenes, surfactants and azones. The principle involved in the mode of actions of these chemical penetrants can be broadly classified into one of the three concepts based on the Lipid-protein Partitioning Theory (Barry 1991)–

- Altering the intercellular lipid
- Altering the Intracellular protein domains of the Stratum Corneum
- Or act as a co-enhancer to increase the partitioning of the drug into the skin

The most widely thought of and safest chemical enhancer would be water. It acts as an enhancer irrespective of the properties of the chemical moiety. The mode of action of water is by getting incorporated and increasing the water content of the Stratum Corneum, which otherwise is a standard value of 15 – 20%. This hydration is proposed to swell and open up the structure of the Stratum Corneum, thus allowing the permeation of drugs(Warner RR 2003). As a result, use of transdermal patches or oil based ointments

have been proposed and studied as an occlusive medium to improve the permeation of drug entities through the skin (Ladenheim D 1996).

Other enhancers that act by lipid disruption or fluidization include DMSO, terpenes, oleic acid, and alcohols. These compounds are known to incorporate themselves into the lipid bilayers of the intercellular and cellular matrix, thus forming microcavities and increasing the free volume fraction of the biological membrane. Compounds such as DMSO and alcohols disperse themselves uniformly throughout the lipid membrane, whereas oleic acid and terpenes pool themselves at the lipid domains and form micropores for the passage of polar molecules. These compounds have a profound effect on the passage of polar molecules (Reddy LH 2001), but some studies have also shown them to increase the flux of lipophilic drugs(Scott RC 1987).

Co-solvents are a third category of chemical enhancers that do not particularly disrupt the structure of the Stratum Corneum, but aid in the partitioning and solubilization of the drug in the Stratum Corneum. Some of the popular co-solvents studied for topical drug delivery are ethanol, propylene glycol, N-methyl pyrrolidone etc. A study showed propylene glycol acting as a cosolvent and further improving the flux profile of metronidazole in the presence of azone as a penetrant (Wotton, et al. 1985).

Physical Enhancers

The most widely studied physical techniques for increasing percutaneous absorption can be further based on if they are disruptive wave techniques or if they are transient Stratum Corneum removal or bypass techniques. The former could be techniques such as Iontophoresis or Sonophoresis and the latter includes techniques such as using

microneedles, SC removal techniques such as tape stripping and laser ablation, or jet propelled nanoparticles (Benson 2005). In this particular chapter, we would be discussing about iontophoresis in detail, as it's the principal enhancement technique used for this project.

Iontophoresis

Iontophoresis is basically the use of a constant electromotive force to facilitate the movement of charged molecule across a biological membrane. The technique not only relies on the movement of the extraneous ions in the electric field, but also on the electrical potential gradient across the skin to alter the permeability of the skin by inducing rearrangements in the assembly of the lipids, proteins or water molecules that make up the structure of the skin. Even through the electric current is shown to cause changes in the structural features of the Stratum Corneum, the transappendageal route along with special transient high admittance zones formed during the iontophoresis are shown to have high densities of the movement of polar drugs (Grimnes 1984).

For drug delivery, a solutions of the drug is usually contained in a solution or a gel having one of the electrodes and a second electrode which is called the return electrode attached to another part of the body.

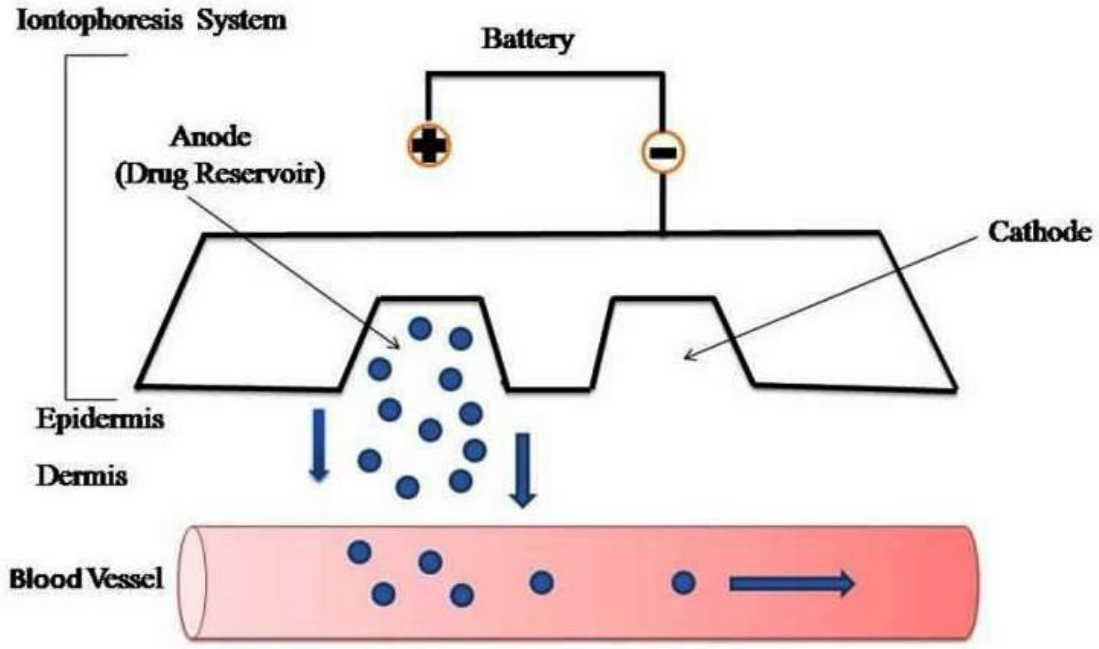

Figure 3 Schematic diagram of iontophoresis for drug delivery to skin*(Panzade 2012)*

When an electric current is applied to a concentrated drug solution, the ions move under the influence of both the concentration gradient across the membrane and of the electric field; which could be explained by the Nernst Plank Equation(Singh J 1996)

$$J = -D\left(\frac{dc}{dx}\right) - \frac{DCZeF}{RT}\frac{dE}{dx} \textit{Equation 3}$$

Where D is the diffusion coefficient of solute across the membrane, C is the concentration of ions with valence Z, F is the Faraday constant, R is the Gas constant and T is the absolute temperature,

For a drug molecule, there are various factors that would influence its delivery efficiency which will be discussed later, but the efficiency can be elaborated on by simplifying the above equation into

$$Ed = fi\,\frac{F}{Mw}$$ *Equation 4*

Where fi is the plot of the steady state flux vs the current density. Hence the drug delivery efficiency can be described as the mol of drug ion that cross the skin for each mol of electrons flowing through the external circuit.

The amount of drug passing through the membrane in the presence of the external electric field can be indicated as

$$Md = \frac{Ed.Id.A.Mw}{Zd.F}$$ *Equation 5*

Where Ed is the current efficiency for drug transport, Id is the current density in mA/cm^2, A is the electrode area in cm^2, Mw is the molecular weight in g/mol and Zd is the valence of the drug ion.

The efficiency of the iontophoresis process to that of the drug transport by passive diffusion can be indicated as the Enhancement factor and can be calculated as

$$E = \frac{J\,iont}{J\,pass}$$ *Equation 6*

Where J_{iont} and J_{pass} are the fluxes of the drug in iontophoresis and passive conditions respectively.

Factors influencing iontophoretic movement of a drug

There are various factors that affect the movement efficiency of a drug molecule in the presence of an electric field. Many of these factors are represented in the above equations and can be elaborated into

Molecular size – As of now, researchers have come out with various correlations of molecular weight and drug movement across a biological membrane through iontophoresis, and in all, the amount of drug diffusing through iontophoresis is inversely related to the molecular weight. These correlations however cannot be applied throughout different classes of compounds and depend on other properties of drugs too.

A study of both divalent and monovalent inorganic ions showed the former to show lesser flux through skin, but for the monovalent ions, a correlation with the molecular weight was obtained (Phipps JB 1989).

$$10_{log\ iontophoretic\ flux} = 1.236 - 0.0032\ M \qquad \textit{Equation 7}$$

Where M is the molecular weight.

On a study with n-alkanols of varying chain length, it was reported that the iontophoretic enhancement values decresed linearly with increasing chainlengths (Terzo SD 1989).

Competing ions- The drug concerned has to compete with other ions in the donor solution with similar charge for the transfer of charge. These ions travel in the same direction of the drug and are also called as Co-ions. The flux of the drug therefore would be a fraction of the charge transfer by the drug to the total charge transfer by all the ions present.

Equation 8

$$J_d = \frac{t_d I}{Z_d F}$$

$$J_e = \frac{t_e I}{Z_e F`}$$

Where J_d and J_e are the fluxes of drug and competing ion, t_d and t_e are the transport numbers for the drug and competing ion, Z_d and Z_e are the valence of drug and ion and F is the Faradays constant

Counter ions – These ions migrate in the opposite direction to the drug movement, and if skin iontophoresis is studied, then the counter ion usually belongs in the skin which is usually Sodium ions. However, when performing *in vitro* studies, it is seen that when potassium ions are present, the flux of the drug is usually reduced as opposed to sodium ions (Terzo SD 1989).

pH–Not only is the pH important to keep the drug in concern in its ionized state(R. M. Siddiqui O 1985), but pH also is known to affect the ionization state of the protein in the skin, thus affecting drug movement through the membrane. The skin had an intrinsic negative charge and maintains the integrity at neutral and lower pHs, however at higher pHs, the charge is lost, thus reducing movement of solvent and drugs. The effect of pH is more profound in the delivery of macro proteins such as insulin (S. Y. Siddiqui O 1987).

Apart from increasing the permeability of drugs through the skin, another aspect that is a challenge to topical drug delivery is localization and deep tissue penetration.

1.2 Localization and deep tissue penetration at the site of application

Topical skin delivery is different from transdermal in that a local effect is desired in the former, i.e. the drug is not intended to be taken up by the systemic circulation once it goes through the skin. On observing the vasculature of the skin, it can be seen that epidermis does not have a blood supply. Only the deepest layer of the Stratum Basale receives direct nutrition from the blood supply. The dermis is rich in fine capillaries and larger blood vessels and these lymphatic vessels and move on into the deeper subcutaneous tissue.

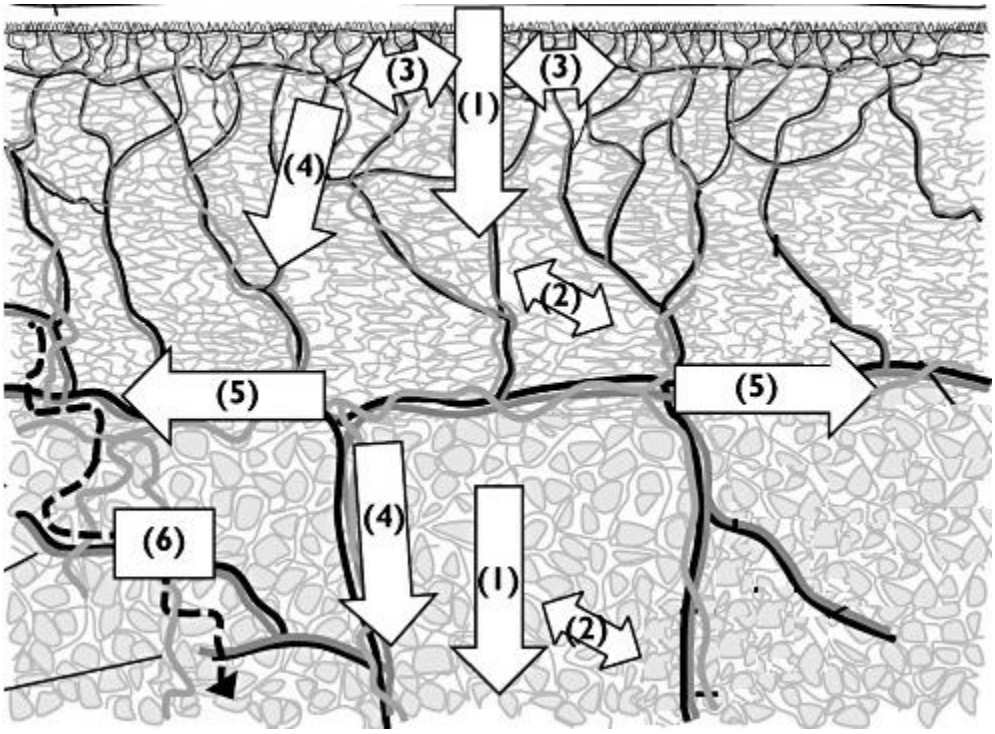

Figure 4 Fate of a drug delivered percutaneously after it passes through the upper layers of the skin (1) Diffusion in the skin, (2) Binding to collagen and tissue proteins, (3) High capillary permeability, (4) Binding to blood proteins and high axial convection via blood

vessels and transport by lymphatics, (5) Radial clearance via vasculature and (6) Interstitial flow parallel and perpendicular to capillaries*(Dancik Y 2012)*

There have been various studies reported who have evaluated the systemic absorption of drug from the site of local delivery and the factors responsible for it. An elaborate study by a team in Australia has studied the distribution of classes of drugs belonging to different physicochemical groups in anaesthetized and sacrificed rats. Based on the study and various other studies that will be discussed, the distribution fate of a drug can be laid on upon a few important factors.

Fraction protein bound vs Unbound

The fraction unbound of the drug is said to be vital for the deep tissue penetration of drugs. A study working on 10 model drugs has reported that the clearance of the drugs from the upper skin to the lower muscle tissue is directly correlated with the fraction unbound percentage of the drug i.e. the larger the unbound fraction, more would be the drug washed out from the upper skin tissue to the lower muscle tissue (Higaki K 2002). However there were still doubts that were raised in the study as to the correlation of the unbound fraction and the systemic absorption of the drug as this was also found to be higher. In yet another study, the fraction unbound was shown to be the major determinant in the tissue layers upto the lower fascia, but did not show to be a major determinant in the lower superficial or deep muscle layers. (Singh P 1996). Another study involving salicylic acid and lidocaine showed the contribution of tissue concentration to deep tissue penetration can only be justified upto a depth of 3-4 mm for a drug like salicylic acid which is highly protein bound, and any drug appearing below this level is predominantly due to systemic circulation (Singh P 1993). The same was not observed for Lidocaine,

where tissue concentrations of the drug were found to be much higher after iontophoresis, and presence of drug in systemic circulation was very low.

Blood circulation

A study using a perfusate system running through a rat hindlimb perfusate model showed that with increasing flow rates of the perfusate, the clearance of the drug into the perfusate increased and the drug appearance in the lower tissue decreased (Cross SE, Effect of perfusion flow rate on the tissue uptake of solutes after dermal application using the rat isolated perfused hindlimb preparation. 1994)(Cross SE 1996). It was also observed that when the albumin in the perfusate was replaced by dextran, a similar fate for the drugs was not observed as the drugs did not perfuse in to the vessels. These studies don't only emphasize the effect of blood perfusion rate on the deep tissue penetration fate, but also corroborate the significance of protein binding of the drug on drug clearance from the site of application. In all the above studies, it is seen that widely used and popular NSAIDs are ideal candidate for study of deep tissue penetration as most of these drugs show a high protein bound state in the physiology and are huge compromised in their ability to show local retention and deep tissue penetration when applied topically.

General Hypothesis for Dissertation

In the literature survey done above, it is evident that Topical delivery systems have a lot of advantage over oral and parenteral routes of administration. It is also apparent that plain application of drugts topically is not completely achieving what is desired out of the route because the skin forms a formidable barrier against penetrations of xenobiotic molecules through, and also does not allow for deep tissue penetration. Working on NSAIDs seems to be a rational choice because of the widely known adverse effects that are associated with the oral administration route. I addition, these drug are taken to treat diseases such as arthritis and muscle spasms that are amenable to topical therapy due to their proximity to the skin surface. Hence, we will start with two model drugs, diclofenac and ketoprofen, and prepare appropriate prodrugs of these NSAIDs. We also intend to prepared prodrugs for ketoprofen to be amenable to iontophoresis delivery and here the emphasis will not only to evaluate the effect of the prodrugs, but also the enhancement effect of the iontophoretic method on permeation and deep tissue penetration. . These prodrugs will be evaluated extensively for all their physicochemical properties and further go on to measure their permeation rate as opposed to the parent. Appropriate models will have to be developed and optimized *in vitro* and *in vivo,* not only to measure the permeation efficiency of the prodrug thr4ough biological membranes, but efficient models will also have to be optimized to evaluate the localization and deep tissue penetration of the prodrugs versus the parent. Following preparation of the prodrugs and evaluating their efficiency, we also do plan to prepare optimized formulations for these prodrugs to be delivered in, possible Transdermal patches for the first Diclofenac prodrugs.

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CHAPTER 2 Evaluation of Diclofenac Prodrugs for Enhancing Transdermal Delivery

This project was supported partly by the INBRE program, NIH Grant Nos P20 RR016454 (National Center for Research Resources) and P20 GM103408 (National Institute of General Medical Sciences)

Abstract

The purpose of this study was to evaluate the approach of using diclofenac acid (DA) prodrugs for enhancing transdermal delivery. Methanol diclofenac ester (MD), ethylene glycol diclofenac ester (ED), glycerol diclofenac ester (GD), and 1,3-propylene glycol diclofenac ester (PD) were synthesized and evaluated for their physicochemical properties such as solubilities, octanol/water partition coefficients, stratum corneum/water partition coefficients, hydrolysis rates, and bioconversion rates. *In vitro* fluxes across human epidermal membrane (HEM) in Franz diffusion cell were determined on DA, MD, ED, GD, and PD saturated aqueous solutions. The formation of GD and ED led to the prodrugs with higher aqueous solubilities and lower partition coefficients than the parent drug. Prodrugs with improved aqueous solubility showed better fluxes across HEM in aqueous solution than that of the parent drug, with GD showing the highest aqueous solubility and also the highest flux. Overall, diclofenac prodrugs with improved hydrophilicity than the parent drug could be utilized for enhancing transdermal diclofenac delivery.

Introduction

Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) with strong analgesic and anti-inflammatory effect. It has been used for pain relief in conditions such as osteoarthritis, usually in oral dosage forms. However, long-term use of oral NSAIDs like diclofenac can cause stomach problems including ulcers and bleeding (McCarthy, 1998), and they may also increase the risk of heart attacks and strokes (Friedewald et al., 2008). On the other hand, topical NSAIDs may show less side effects commonly observed in the oral products due to less systemic exposure (Kienzler et al., 2010). FDA approved the first topical NSAID prescription product, Voltaren (diclofenac sodium) gel for osteoarthritis in 2007, and more recently approved a diclofenac topical solution (Pennsaid) for osteoarthritis. In addition, FDA also approved a diclofenac plaster patch (Flector Patch) for acute pain due to minor strains and sprains. Clinical studies have demonstrated that topical diclofenac products show better pain relief than the placebo counterparts (Barthel et al., 2009; Galer, 2011).

It has been postulated that the efficacy of topical diclofenac is due to direct penetration of the drug to affected joint tissue (Kienzler et al., 2010). One study showed that application of diclofenac gel on one of the knees in patients with osteoarthritis in both knees, the drug synovial fluid concentrations in both knees were not significantly different (Radermacher et al., 1991). This result indicates that diclofenac present in synovial fluid was not from direct penetration but from redistribution by systemic blood circulation. On the other hand, oral diclofenac showed significantly higher plasma concentration, synovial membrane tissue concentration and synovial fluid concentration, and also demonstrated better therapeutic efficacy than those by topical applications (Miyatake et al., 2009).

Additionally, topical diclofenac gel was only modestly effective against osteoarthritis pain in comparison to the placebo, since the placebo group also had substantial improvements in pain scores and function scores, possibly from the manual massage on the affected joint during application (Lin et al., 2004). Those results suggest that current topical diclofenac products might need improvement in diclofenac delivery to the local tissues for better efficacy. One possible approach for enhancing topical delivery to the local tissues would be to increase the diclofenac drug permeation rate across the skin.

Prodrug approach has been widely investigated for enhancing transdermal delivery (Rautio et al., 1998; Valiveti et al., 2005). By modifying the chemical structure of the parent drug, the formed prodrug may show different physicochemical properties including solubility, lipophilicity, and partition coefficient, which may render the prodrug more favorable for skin penetration. After entering into the body, the prodrug will convert back to its parent drug to exhibit the pharmacological effect. Diclofenac is a weak acid with an octanol/water partition coefficient of 4.9 from the Drug Bank website report, which is above the optimal partition coefficient (usually between 1 and 3) for transdermal drug delivery (Guy and Hadgraft, 2003). Presumably, converting diclofenac to some appropriate prodrugs within the optimal partition coefficient range could achieve higher transdermal penetration. Diclofenac prodrugs have been widely explored, but mostly for the purposes of reducing gastric irritation after oral administration or improving anti-inflammatory efficacy (Abo-Ghalia et al., 1999; Halen et al., 2009; Ribeiro et al., 2007), with the exception of two studies on diclofenac prodrugs for enhancing transdermal delivery (Bonina et al., 2001; Jilani et al., 2003). In those two studies, diclofenac prodrugs were formed by converting diclofenac to diclofenac esters (formed ester with ethylene glycol in

one study, and formed esters with different chain length of polyoxyethylene glycols in the other study), and better transdermal delivery was observed. In the present study, we were to investigate a wider range of diclofenac prodrugs (forming diclofenac ester prodrugs with methanol, ethylene glycol, glycerol, and 1,3-propylen glycol), with the purpose of better understanding diclofenac prodrugs for enhancing transdermal diclofenac delivery.

Experimental

Material

Diclofenac sodium salt (USP grade) was obtained from Gallipot (St Paul, Minnesota). Methanol, propylene glycol, 1,3-propylene glycol, ethylene glycol, glycerol, N,N-Dicyclohexylcarbodiimide (DCC) and 4-pyrrolidonopyridine were obtained from Sigma (St. Louis, MO). Ethyl acetate, and hexane were obtained from BDH chemicals. Silica gel, ultra pure, 40 to 60 µm was obtained from ACROS organics (New Jersey, USA). Dimethyl Sulfoxide – d6 was obtained from Cambridge Isotopes Laboratories (Andover, MA). Isopropyl Myristate (IPM) and 1-Octanol was obtained from Spectrum Chemicals (New Brunswick, NJ). 0.5% trypsin solution was obtained from Atlanta Biologicals (Lawrenceville, GA). Human cadaver skin was obtained from New York Fire Fighter Skin Bank. Human epidermal membrane (HEM), the epidermis layer of the skin, was prepared by heat stripping method as reported before (Peck et al., 1995).

Converting Diclofenac Sodium to Diclofenac Acid (DA)

Diclofenac sodium (10 grams) was dispersed into 200 ml purified water, and then 6.8 ml of hydrochloride acid (6N) was gradually added to the mixture under continuous stirring. After all the acid was added, the mixture was sonicated in a sonication bath for 0.5 hour. After sonication, 50 ml of ethyl acetate was added to the beaker to dissolve the solid. The ethyl acetate layer was separated in a separation funnel, and washed with purified water twice. Then the ethyl acetate solution was mixed with anhydrous zinc sulfate to eliminate the water content, and filtered through No.1 filter paper. Then ethyl acetate was evaporated and diclofenac acid was obtained.

Synthesis of Diclofenac Ester Prodrugs

Diclofenac ester prodrugs were synthesized following the method for Ketoraolac ester prodrug synthesis method (Doh et al., 2003) with some modifications. Specifically, Diclofenac acid (0.00304 mol) was dissolved in 19 ml of acetone, then 24 mg of 4-pyrrolidinopyridine was added and dissolved. After that, 0.00916 mole of alcohol (methanol, ethylene glycol, glycerol, or 1,3-propylene glycol, depending on the prodrug to be synthesized) was added to the solution and stirred. To the solution, 777 mg of DCC was added. This reaction mixture was kept on continuous stirring for 18 hours at room temperature. Then the reaction mixture was centrifuged at 1000 rpm for 10 minutes. Supernatant was collected, and the remaining solid was washed with additional 20 ml acetone under vortexing for 5 mins, and then centrifuged again at 1000 rpm for 10 minutes to collect the supernatant. Both supernatants were combined and evaporated in a vacuum oven at room temperature to obtain the crude ester product. The crude product was then dissolved in 1 ml of ethyl acetate and purified through flash chromatography. 1H NMR spectra were obtained in DMSO-d6 at 300 MHz, JEOL ECX300 instrument.

1) Diclofenac Acid (DA): ^{1}H NMR (300 MHz DMSO-d6) δ 12.68 (1H, s), 7.50 (2H, d, 8.25), 7.22 – 7.13 (3H, m), 7.03(1H, t, 7.77), 6.82(1H, t, 7.32), 6.25(1H, d, 7.35), 3.67(1H, s)
2) Methanol Diclofenac Ester (MD): MD was purified by flash silica gel column chromatography eluting with 90% Hexane and 10% ethyl acetate and obtained with about 90% yield. The final product is a white crystal solid. ^{1}H NMR (300 MHz DMSO-d6) δ 7.51 (2H, d), 7.21-7.14 (2H, m), 7.07-7.00(2H, m), 6.81(1H, t), 6.21(1H, d), 3.78(2H, s), 3.62(3H, s)

3) Ethylene Glycol Diclofenac Ester (ED): ED was purified by flash silica gel column chromatography eluting with 60% Hexane and 40% ethyl acetate and obtained with about 80% yield. The final product is a white waxy semisolid. ^{1}H NMR (300Mz, DMSO-d6) δ 7.51 (2H, d), 7.20-7.15(2H, m), 7.06-7.00(2H, m), 6.82(1H, t), 6.22(1H, d), 4.82(1H, t), 4.07(2H, t), 3.78(2H, s), 3.59-3.54(2H, m)

4) Glycerol Diclofenac Ester (GD): GD was purified by flash silica gel column chromatography eluting with 20% Hexane and 80% ethyl acetate and obtained with about 75% yield. The final product is a transparent viscous liquid. ^{1}H NMR (300 MHz, DMSO-D6) δ 7.54(2H, d), 7.24-7.18(2H, m), 7.08-7.06(2H, m), 6.85(1H, t), 6.25(1H, d), 4.94(1H, d), 4.66(1H, t), 4.12(1H, dd), 3.99(1H, dd), 3.82(2H, s), 3.72(1H, m), 3.37-3.34(2H, m)

5) 1,3 Propylene Glycol Diclofenac Ester (PD): PD was purified by flash silica gel column chromatography eluting with 80% Hexane and 20% ethyl acetate and obtained with about 80% yield. The final product is a pale yellow solid. ^{1}H NMR (300 MHz, DMSO-D6) δ 7.50(2H, d), 7.20-7.15(2H, m), 7.05-7.00(2H, m), 6.81(1H, t), 6.22(1H, d), 4.50(1H, t), 4.10(2H, t), 3.76(2H, s), 3.42-3.38(2H, m), 1.70(2H, p).

Solubility Determination

The solubilities of the diclofenac prodrugs in deionized water or diclofenac acid in 0.02 M phosphate buffer at pH 2.5 (0.02 M NaHPO4 solution adjusted to pH 2.5 with 6N HCl) were determined by adding excessive amount of drug or prodrugs into 10 ml of the solvent and equilibrating at room temperature under stirring condition. At 3, 6, 12, and 24 hours, the solution was centrifuged at 3000 rpm for 10 min, 0.5 ml of supernatant was taken and diluted with equal volume of water immediately and then analyzed with HPLC.

The solubilities of the prodrugs and diclofenac acid in 5% (v/v) propylene glycol in water or phosphate buffer solution at pH 2.5 at room temperature were determined with the method above. In addition, the solubilities of the prodrugs and diclofenac acid in hexane and in isopropylmyristate (IPM) was also determined in similar way, except the samples in IPM was diluted with DMSO, and samples in hexane were first evaporated to dry and then re-dissolved with DMSO.

Partition Coefficient Determination

To determine the partition coefficient in octanol/water system, the diclofenac prodrugs or diclofenac acid were first dissolved in octanol, and then 10 times the volume of purified water (or 0.02 M phosphate buffer at pH 2.5 for DA) was added and equilibrated on a shaking platform at room temperature for 24 hours. The octanol phase and water phase were separated by centrifugation, and the drug concentrations in the aqueous phase and the octanol phase after equilibrium were measured by HPLC method. The partition coefficient was calculated by equation: $\text{Log}P_{O/W} = \text{Log}\ (C_{octanol} / C_{water})$.

To determine the partition coefficient of diclofenac acid and its prodrugs in stratum corneum / water system, the stratum corneum was first isolated with a previous method (He et al., 2003) with some modifications. HEM was placed with the viable epidermis side down on a filter paper in a petridish. The filter paper was wetted with phosphate buffer saline (PBS) solution at pH7.4 and then 0.5% trypsin was added to get a final concentration of 0.25% with the liquid level match the top of the HEM. The petridish was incubated at 37°C for 24 hrs. After the treatment, the HEM was removed carefully and put into deionized water. After the epidermis layer fell off, the stratum corneum was taken and rinsed three times in deionized water, and then air dried. Diclofenac acid or the prodrugs

were dissolved in 5% propylene glycol solution in water (or 0.02 M phosphate buffer at pH 2.5 for DA), and the dried stratum corneum was weighed and put into the drug solutions. The solutions were equilibrated under shaking condition with an orbital shaker for 24 hrs. Then the stratum corneum was removed from the solution and the any excess solution was blotted away carefully, and weighed immediately. The stratum corneum was then extracted with 5 ml methanol for 24 hrs and analyzed with HPLC for the amount of drug taken by the stratum corneum. In addition, the drug concentration of aqueous solution after equilibrium was also measured. The drug concentration in stratum corneum is determined based on the stratum corneum dry weight and also the amount of drug taken by it. The partition coefficient was calculated by equation: $LogK_{SC/W} = Log (C_{Stratum\ Corneum}/C_{Water})$.

Degradation Rate Determination

Diclofenac prodrugs of appropriate concentration in PBS solution (pH 7.4) with addition of 30% DMSO were prepared. 30% of DMSO in PBS at pH 7.4 was used for the purpose of increasing the solubility of prodrugs in the solution. The initial concentrations of the prodrugs in the solutions were similar. The solutions were placed in a water bath shaker at 37 ^{o}C. Samples were taken from the solution at 0 min, 10 mins, 20 mins, 30 mins, 1hour, 2hours, 4hours, 8hours, 24hours and 48 hours, and analyzed with HPLC method. The samples were analyzed for the decreasing in concentration of the prodrugs over time. The pseudo first order hydrolysis rate constants for all the prodrugs were determined.

Bioconversion Rate Determination

Diclofenac prodrugs solution with appropriate concentrations in DMSO is prepared, and the solution (about 5% of the plasma volume) was spiked into freshly prepared rat plasma, and then placed in 37^{o}C water bath shaker. Aliquots were taken from the plasma periodically into a methanol solution to stop the bioconversion. Then the samples were centrifuged and supernatants were analyzed by HPLC method. The bioconversion rates for all the prodrugs were determined.

In Vitro Flux Study of Diclofenac Acid and Its Prodrugs in Aqueous Solution

Saturated solutions of the prodrugs and diclofenac acid in 5% propylene glycol in water was made on the day of experiment, by adding excess drug to the solution (approximately 1% of the drug in the suspension) and sonicated in a water bath for 20 min and set aside for 1 hour before use. The HEM was mounted on the vertical Franz diffusion cell with the stratum corneum facing the donor cell. The diffusion area is 0.64 cm^2. The receptor cell was filled with 5 ml of 0.15 M phosphate buffer saline (PBS) solution at pH 7.4, and the donor cell was filled with 0.5 ml of the fresh made saturated drug solution. The donor cell was sealed with parafilm. The experiment was conducted at 37 ^{o}C. The whole 5 ml of receptor solution was taken, and fresh PBS solution was added at time 2, 4, 6, 8, 12, 24, 30, 36, and 48 hour. Samples were analyzed with HPLC method. The flux of each of the drug or prodrug was determined by the equation (Yan et al., 2008):

$$J = \frac{1}{A}\frac{\Delta Q}{\Delta t} \qquad (1)$$

Where J is the flux, A is the diffusion surface area, Q is the cumulative amount of the drug transported into the receiver chamber and t is the time. Under steady state, the ΔQ/Δt was the slope of linear regression cumulative amount of the drug verses the later experiment time points.

HPLC analysis method

The DA and its prodrugs were analyzed with a HP 1050 HPLC with quaternary pump and DAD detector. The column used was a *Zorbax* XDB-C18 4.6 x 150 mm 5.0 micron *HPLC column.* The mobile phases were acetonitrile and 0.02 M citrate buffer at pH 6.0 with a gradient method. The flow rate was 1 ml/min, and the detection UV wavelength was 280 nm. The retention time was 4.5 min for DA, 6.0 min for GD, 7.1 min for ED, 8.2 min for PD, and 9.1 min for MD.

Results and Discussion

Diclofenac and Its Prodrugs Physicochemical Properties

The solubilities of diclofenac acid and its prodrugs in a series of solvents are listed in Table 1. In the determination of the aqueous solubility of diclofenac acid (DA), 0.02 M phosphate buffer at pH 2.5 was used instead of purified water for other prodrugs. In this way, the solubility determined is the intrinsic solubility of the unionized DA. Methanol diclofenac ester (MD) showed lower solubility than that of the unionized diclofenac acid (DA) in water (more than 2 folds decrease). Propylene glycol diclofenac ester (PD) showed similar solubility to that of DA. On the other hand, ethylene glycol ester (EG) and glycerol diclofenac ester (GD) showed much higher solubility in purified water than that of the unionized DA (more than 10 and 100 folds increase, respectively). In addition, the addition of 5% propylene glycol (PG) into water showed a slight increase (20-30%) in the aqueous solubility of DA, ED, PD and GD; but basically no effect on the MD solubility. The addition of 5% PG in water is for the purpose of better wetting the prodrugs and increasing the dissolution rate of the prodrugs.

The solubilities of the four chemicals in a non-polar organic solvent hexane are also listed in Table 1. The formation of the ester prodrugs showed higher solubilities in hexane than that of DA, with MD being the highest. The solubilities of those five chemicals in isopropylmyristate (IPM), an organic solvent that shows similar lipophilicity as the lipid structure in stratum corneum are in the order of: GD > ED > MD > PD > DA. All five chemicals showed good solubilities in IPM, but the formation of ester prodrugs showed higher solubility than the parent drug in IPM.

The partition coefficients in octanol/water for the five chemicals follow the order of: MD > DA > PD > ED > GD. MD showed highest partition coefficient or highest lipophilicity, which is consistent with its lowest solubility in purified water. On the other hand, the formation of GD showed lowest partition coefficient or highest hydrophilicity, which is also consistent with its highest solubility in purified water. Our experimental DA partition coefficient value is 4.57, which is close to the Drug Bank website value of 4.9.

The hydrolysis rates of the diclofenac prodrugs in PBS at pH 7.4 are shown in Figure 2A, in which the intact prodrug concentrations in the PBS solution at different time points have been normalized by the initial starting concentration (in terms of percentage), and plotted against the experiment time. A linear relationship between the intact prodrug concentrations (in logarithm scale) vs. time is observed for each prodrug. This indicates that the hydrolysis was following a pseudo first order reaction mechanism. The half lives of the hydrolysis of the four prodrugs are listed in Table 1, based on the slopes determined from linear regression. PD showed the longest half life in PBS solution, followed by ED and MD, and GD showed the shortest half life. The shortest half life of GD could be due to the catalytic capability of its hydroxyl group to the hydrolysis of the ester. Overall, the half lives are in the range of one to three days at 37 ^{o}C, which indicates that these diclofenac prodrugs are not very stable in aqueous solution.

The diclofenac prodrugs have to convert to the parent drug in the body to exhibit pharmacology effect. Thus the bioconversion rates of the prodrugs in fresh rat plasma were determined. The intact prodrug concentrations (in terms of percentage normalized with the initial prodrug concentration) at different time points in rat plasma were plotted against the reaction time under the logarithm scale (see Figure 2B). A linear relationship

was also observed for each prodrug in the bioconversion reaction, and the half lives of the bioconversion are listed in Table 1. The bioconversion in rat plasma for the four prodrugs are much faster (half lives in minutes) than their hydrolysis in PBS, due to the presence of esterases in the plasma. MD and ED showed comparable bioconversion rates with half life only about 1.6 min, PD showed a half life of 2.4 min, and GD showed relatively slower bioconversion rate with half life of 11 min, possibly due to the bulky glycerol group in the GD partially hindering esterase enzyme access for its bioconversion.

In Vitro Flux Study of Diclofenac Acid and Its Prodrugs

The cumulative amount of DA, MD, ED, GD, and PD permeated through human epidermal membrane over time in the *in vitro* flux study with their saturated aqueous solutions are shown in Figure 3. GD showed the highest cumulative amount permeated across the HEM, followed by ED, DA and PD, but MD did not show any detectable amount permeated across HEM. The steady state fluxes of the drug or prodrugs are summarized in Table 2. In comparing the steady state fluxes with the octanol/water partition coefficients of the drugs, we can discover that the addition of polar group to the diclofenac molecule to form more hydrophilic ester prodrugs (ED and GD) with increased aqueous solubilities enhanced the transdermal drug permeation, and on the other hand addition of methyl group to form more hydrophobic ester prodrug (MD) with decreased aqueous solubility inhibited the transdermal drug permeation. This observation is in agreement with previous finding that there is a parabolic relationship between the transdermal flux and the octanol/water partition coefficient with maximum flux value observed at partition coefficient (LogP) values between 1 and 3 (Guy and Hadgraft, 2003).

It is generally believed that permeating through stratum corneum is the rate limiting step for transdermal delivery and the steady state flux across the human epidermis can be explained by the following equation(Guy and Hadgraft, 2003):

$$J = \frac{KD}{h} C_o \qquad (2)$$

Where J is the flux, K is the stratum corneum and formulation partition coefficient of the drug, D is the diffusion coefficient of the drug in stratum corneum, h is the path length in the stratum corneum, and C_o is the drug concentration in the formulation. The partition coefficients between the stratum corneum and the 5% PG water solutions for DA, MD, ED, GD and PD were reported in Table 2. There is a linear relationship between the octanol/ water partition coefficients ($LogP_{O/W}$) and stratum corneum/ PG water partition coefficient ($LogK_{SC/W}$) for the diclofenac drug and its prodrugs as demonstrated by Figure 4A. Based on Equation 2, if we assume the path length (h) in stratum corneum is the same for all four chemicals and they have similar diffusion coefficient (D), then there would be a linear relationship between the flux J and the value of KC_o, where C_o is the concentration of the drug or prodrug in the 5% PG solution. As we used saturated drug solution for the in vitro transdermal flux study, the concentration C_0 would be the drug or prodrug solubility in the 5% PG water solution as shown in Table 2. As we plotted the J verses KC_o in Figure 4B, the DA, ED, and PD fell into the linear relationship very well (as evidenced by the linear line passing the origin point), but MD and GD deviated from the linear line. GD is significantly more hydrophilic and has an extra hydroxyl group compared to DA, ED and PD. This extra hydrogen bonding group could lower the diffusion coefficient in stratum corneum by an order of magnitude (Guy and Hadgraft, 2003). The deviation from the linear relationship for GD could be due to its diffusion coefficient in the stratum corneum being

significantly higher than that of DA, ED, or PD. We observed there is some increase in flux with significant increase in aqueous solubility from ED to GD, but further increasing the aqueous solubility of diclofenac prodrug with additional hydroxyl group may not contribute to additional increase in the flux across the skin due to the offset in increase of the diffusion coefficient of the prodrugs.

MD has even one less hydroxyl group than DA, ED or PD, thus it would have a smaller diffusion coefficient value in the stratum corneum. Based on its KC_0, MD would have a flux value even higher than DA. But experimentally, it showed zero flux. As it is known, the permeability of a drug across the whole epidermis (P_{EP}) is dependent on the permeability across stratum corneum (P_{SC}) and the permeability across viable epidermis (P_{VE}) as described by Equation 3 (Guy and Hadgraft, 2003):

$$\frac{1}{P_{EP}} = \frac{1}{P_{SC}} + \frac{1}{P_{VE}} \qquad (3)$$

Usually, the permeability across epidermis is controlled by permeability across the stratum corneum ($P_{sc} << P_{VE}$). However, for water insoluble drug like MD with very high octanol/water partition coefficient, its permeability across the epidermis could be controlled by the viable epidermis (because of its extreme low aqueous solubility, its permeability across viable epidermis could be significant smaller than its permeability across stratum corneum). DA also has a very low aqueous solubility in unionized form, but its solubility under physiological pH condition is several orders of magnitude bigger, thus would have much larger viable epidermis permeability.

As showed in Figure 2A, the diclofenac prodrugs are prone to hydrolysis in aqueous solution. In the in vitro flux study, we removed the entire receiver solution for every

sample point and analyzed for both the diclofenac acid and the prodrug concentration in the samples. For PD flux study, no diclofenac acid was detected in all the receiver samples; and for ED and GD flux studies, diclofenac acid was detected in the receiver samples only at 24 hour and 48 hour time points, with the amount less than 20% of the ester prodrug forms. Only at those two time points, the interval between the sampling was 12 hours, and some of the prodrugs in the receiver chamber could hydrolyze to diclofenac acid in sufficient amount. There could be some prodrug hydrolyzed to diclofenac acid in the donor chamber, but the contribution of the diclofenac acid from hydrolysis in the donor solution to the flux across HEM would be small to negligible since we did not detect significant amount of diclofenac acid in the receiver samples in other time points during the flux study. We combined both the prodrug and the diclofenac acid in the receiver samples for cumulative amount permeated across the HEM in Figure 3. In addition, we suspect that the HEM used in the in vitro flux study would have little enzymes to convert the diclofenac prodrugs to the parent drug. This could be due to the skin used were not fresh (more than 1 year from the date obtained from its donor) and also the heat strapping separation procedure could deactivate the enzymes. Unpublished results of flux study with fresh full thickness rat skin did show most of the diclofenac prodrugs being converted to the parent drug in the receiver samples in the first 8 hours. Similar results was also reported in Naproxen prodrugs in vitro skin permeation study with rat skin(Rautio et al., 1998). Since the permeation across stratum corneum is the rate limited step for most drugs and the prodrug bioconversion usually occurs in the viable epidermis and dermis layers, it would be adequate for using HEM instead of full thickness skin in the in vitro flux evaluation in this study.

There is a linear relationship between LogP and the stratum corneum water partition coefficient as observed in this study (Figure 4A), thus drugs with higher LogP would have higher tendency to partition into the stratum corneum. However, the transdermal flux is also dependent on the solubility in the vehicle (5% PG water in this study) as showed in Equation 2. Conventionally, it is observed that drugs with octanol/water partition coefficient (LogP) in the range between 1 and 3 showed better flux across skin(Guy and Hadgraft, 2003). In this range, the drugs usually showed a balanced hydrophilicity and lipophilicity: adequate aqueous solubility and sufficient participation into the stratum corneum. For hydrophilic drug such as ketorolac ($LogP_{O/W}$ around 1), its prodrugs with increased lipophilicity improved the transdermal flux(Doh et al., 2003). On the other hand, for lipophilic drug such as diclofenac acid ($LogP_{O/W}$ of 4.5), a prodrug with lower $LogP_{O/W}$ would improve the transdermal flux. Indeed, we observed that converting DA to prodrug with even higher $LogP_{O/W}$ (MD) showed significant decrease in the flux across skin, converting DA to prodrug with a little lower $LogP_{O/W}$ but no improvement in aqueous solubility (PD) also showed lower flux than DA, only converting DA to prodrugs with lower $LogP_{O/W}$ but significant improvement in aqueous solubility (ED and GD) showed higher flux than DA.

A previous study also reported that ED showed better in vitro flux than the parent drug in its sodium salt form, but they used 5% PG in methanol as the vehicle and full thickness rats skin in their study(Jilani et al., 2003), where methanol could provide significant disruption to the stratum corneum and enhancing the molecules transport across skin. In another study(Bonina et al., 2001), diclofenac prodrugs formed with polyoxyethylene glycols with the polymer number from 2 to 6 was investigated and highest in vitro

cumulative amount was observed for the prodrug with longest chain length (molecular weight around 560) which has the lowest LogP and highest aqueous solubility. For comparison, the GD (molecular weight around 370) in our study showed similar LogP and solubility to the diclofenac polyoxyethylene glycol with the polymer number of 5 (molecular weight around 516). In our study, we noticed that GD deviated from the flux and KC_0 linear line as demonstrated in Figure 4B, possible from the much lower diffusion coefficient of GD in the stratum corneum. The formation of diclofenac prodrug with long polyoxyethylene glycol would decrease the diffusion coefficient even more as they demonstrate to have much higher molecular weight and more hydrogen bonds. In the previous study(Bonina et al., 2001), they used ethanol as the vehicle and 50% ethanol water as receiver media, which may cause significant skin disruption and therefore better flux for the diclofenac prodrug with longest polyoxyethylene glycol. We used 5% PG water, a more skin friendly vehicle for the in vitro flux study of diclofenac prodrugs, which could possibly provide better insight in the designing diclofenac prodrug for enhancing transdermal delivery of diclofenac.

Conclusion

Diclofenac prodrugs were synthesized and evaluated for the physicochemical properties and in vitro fluxes across human skin. The formation of less hydrophilic diclofenac acid prodrugs with alcohols like methanol and 1,3-propylene glycol rendered the prodrugs with lower aqueous solubility, higher partition coefficients, and lower fluxes across the skin in aqueous solutions. On the other hand, the formation of more hydrophilic diclofenac acid prodrugs with alcohols like ethylene glycol and glycerol rendered the prodrugs with higher aqueous solubilities, lower partition coefficients, and higher fluxes across the skin in aqueous solutions. In addition, the prodrugs all demonstrated the potential to be converted to the parent drug in vivo. In summary, diclofenac prodrugs with improved aqueous solubilities showed the potential in enhancing transdermal diclofenac delivery.

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Tables

Table 1. Summary of the physicochemical properties of the diclofenac acid and its prodrugs.

Drug	DA	MD	ED	GD	PD
Solubility in water (μmol/ml)	0.0034[a]	0.0019	0.0747	0.551	0.0033
Solubility in 5% PG water (μmol/ml)	0.0041[a]	0.0019	0.0918	0.662	0.0048
Solubility in Hexane (μmol/ml)	0.134	21.0	1.76	0.386	2.22
Solubility in IPM (μmol/ml)	12.6	43.0	49.3	69.7	13.6
Partition coefficient (LogP octanol/water)	4.57[a]	5.00	3.83	3.30	4.08
Hydrolysis in PBS (half life in hours)	--	34.6	50.2	25.9	63.9
Bioconversion in plasma (half life in min)	--	1.62	1.66	11.3	2.41

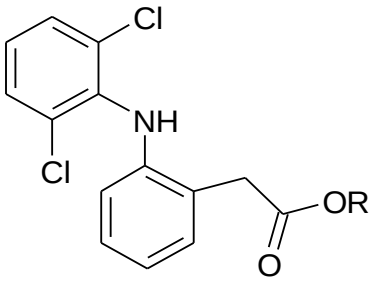
[a] Water was substituted with 0.02 M phosphate buffer at pH 2.5.

Table 2 Summary the relationship among the partition coefficient in stratum corneum, drug solubility, and the flux across the human epidermal membrane in aqueous solution (the value in the bracket is the standard deviation of the flux).

Drug	DA	MD	ED	GD	PD
Partition coefficient (LogK SC/ 5% PG water)	2.97[a]	3.56	1.74	1.56	2.58
Solubility in 5% PG water (C_0 in μmol/ml)	0.0041[a]	0.0019	0.0918	0.662	0.0048
$K \cdot C_0$ (μmol/g)	3.78	7.03	5.04	24.0	1.82
Aqueous solution flux ($nmol \cdot cm^{-2} \cdot hr^{-1}$)	2.57(0.29)	0	3.99(0.80)	5.54(0.78)	0.84(0.25)

[a] Water was substituted with 0.02 M phosphate buffer at pH 2.5.

Figures



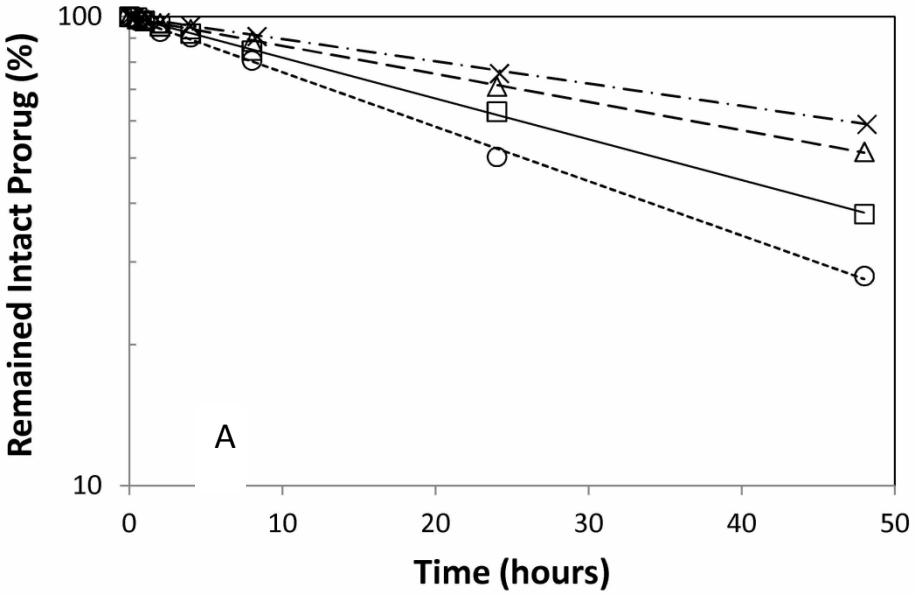
DA: R = -H

MD: R = -$^{a}CH_3$

ED: R = -$^{b}CH_2{}^{c}CH_2OH$

GD: R = -$^{d}CH_2{}^{e}CH(OH)^{f}CH_2OH$

Figure 1 The molecular structure of diclofenac acid and the prodrugs.



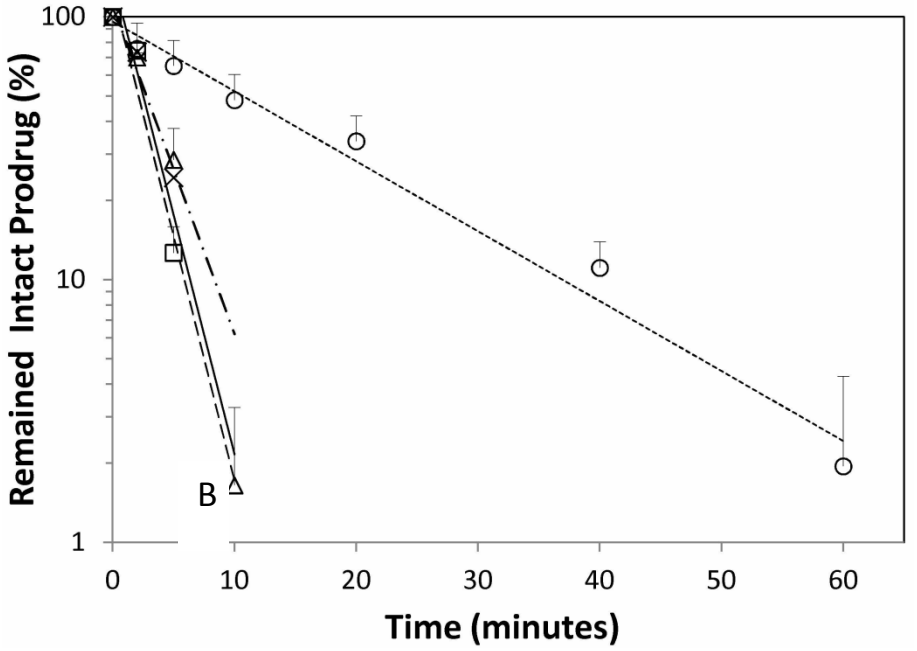


Figure 2 (A) The hydrolysis of the diclofenac prodrugs in pH 7.4 PBS solutions at 37 ^{o}C. (B) The bioconversion of the diclofenac prodrugs in rat fresh plasma at 37 ^{o}C. Symbols: (○) GD; (□) MD; (Δ) ED; (x) PD.

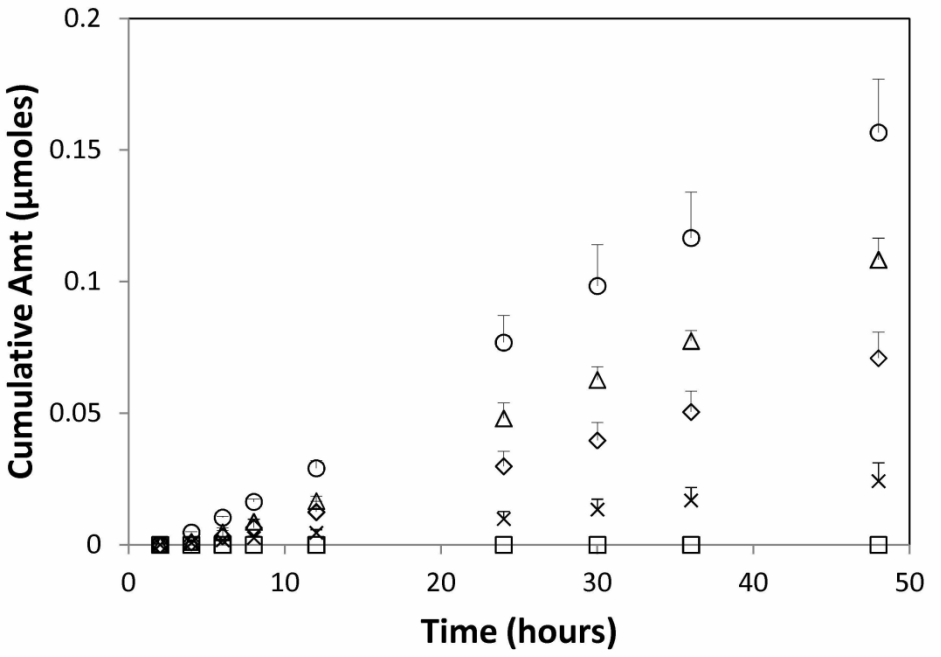
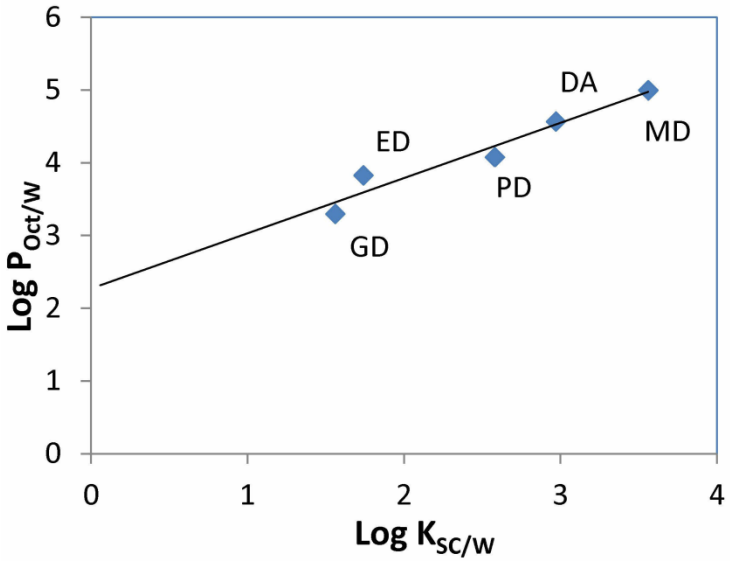


Figure 3 The in vitro flux across HEM of the diclofenac or its prodrugs in the 5% propylene glycol aqueous saturated solutions (y-axis is the cumulative amount of the drug detected in the receiver chamber). Symbols: (○) GD; (Δ) ED; (◊) DA; (□) MD; (x) PD.



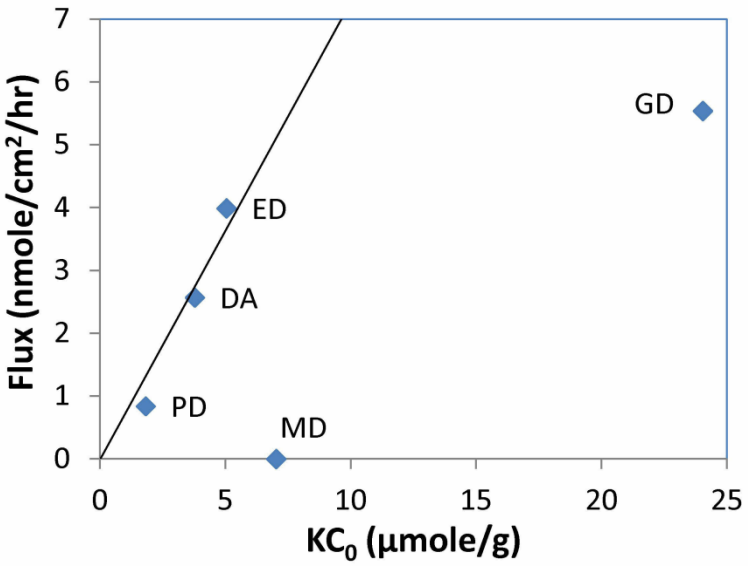


Figure 4 (A) The linear relationship between the stratum corneum water partition coefficients (Log $K_{SC/W}$) and the octanol water partition coefficients (Log $P_{Oct/W}$). Linear line is the linear regression of the data points with regression equation of y = 0.7598x+2.2701 and R^2= 0.9388. (B) The relationship between the in vitro fluxes across HEM in 5% PG solutions and the values of KC_0 (the stratum corneum water partition coefficient times the solubility of the drug or prodrug in 5% PG solution). The linear line is the linear regression of the data points of DA, ED and PD with forcing the linear line through the origin point, with a linear regression equation of y = 0.7284x and R^2 = 0.9248.

CHAPTER 3 Evaluation of the Direct Penetration of Diclofenac and Its Ester Prodrug across Live Rat Skin

This project was supported partly by the INBRE program, NIH Grant Nos P20 RR016454 (National Center for Research Resources) and P20 GM103408 (National Institute of General Medical Sciences)

Abstract

This research project was to determine the extents of direct penetration across live rat skin from topical application of diclofenac and its ester prodrugs. Diclofenac and its prodrugs were formulated into patches with different pressure sensitive adhesives. In vitro flux studies across the human epidermis and across hairless rat skin were conducted. Direct penetration across live rat skin from topical application of either a diclofenac acid patch or a glycerol diclofenac ester patch was evaluated with a dual agar gel *in situ* rat model. Diclofenac ester prodrugs showed higher *in vitro* fluxes from patches with polyisobutylene adhesives, while diclofenac acid showed higher fluxes in patches with polyacrylate adhesives. Direct penetration from diclofenac acid patch accounted for 0.12% of the drug absorbed into the body and 78% of the drug collected in the agar gel, and the numbers from glycerol diclofenac ester patch were 0.083% and 77%, respectively. Topical application of either diclofenac acid patch or its ester prodrug patch demonstrated some direct penetration across the live rat skin, but the extent of direct penetration across the live rat skin from the glycerol diclofenac ester patch was not advantageous over that from the diclofenac acid patch.

Introduction

Oral nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used in relief of pain from musculoskeletal diseases such as osteoarthritis and rheumatoid arthritis.However, oral NSAIDs usually cause significant adverse effects such as gastric intestinal bleeding and ulceration. Topical NSAIDs, by directly applying the medication to the skin of the affected area, usually showed much less systemic drug exposure [1], and thus much less systemic adverse effects. Topical diclofenac products (i.e., Voltaren gel and Pennsaid solution) are commonly prescribed for osteoarthritis.Based on a recent review on the clinical studies of topical diclofenac products for pain relief in knee arthritis, the pain relief effect from topical diclofenac products was only 10% better than that from their placebo in patients over a 12-week period (60% of the patients felt better from the use of topical diclofenac *vs.* 50% from the use of the placebo) [2].As the systemic drug concentration from topical diclofenac is very low, in order for topical diclofenac to be effective, sufficient direct penetration of diclofenac into the local tissues is necessary for its efficacy.

Mixed experimental results were reported on the drug concentrations in local tissues from topical application of NSAIDs. In one study, diclofenac gel was applied on one knee and there was no difference in diclofenac concentrations in synovial fluid between the drug-applied knee and the contralateral knee [3]. In a different study, compared to oral administration, topical ketoprofen plaster provided lower plasma, synovial tissue, and synovial fluid concentrations, but higher cartilage and meniscus concentrations [4].In another study, similar plasma concentrationsin human subjects were observed from topical diclofenac and oral diclofenac, but topical diclofenac led to significant higher

muscle concentration at the application site [5]. Such mixed results indicated big variations in direct penetration from topical NSAIDs.

It is of great interest to patients with arthritis that topical productsare capable of providing direct penetration to deeper tissue such as the subcutaneous, muscle or joint tissues underneath the skin at the application site. However, development of such topical products is challenging.One reason is that only limited amount of drug can be delivered into intact skin due to the skin barrier effect. But more importantly, it is because of the skin blood flow acting as a sink that clears away most of the drug entering into the skin before it is available for penetration into deep tissue [6]. It was shown in several studies that topical drug direct penetration into deep tissues could be enhanced by lowering the skin blood flow either with co-delivery of vasoconstrictors [7, 8]or applying a cold water jacket on the local skin [9]. There is not yet a device that can conveniently apply cold temperature to skin for a sustained period of time. The co-delivery of vasoconstrictors for lowering the skin blood flow was only achieved under iontophoresis conditions or passive diffusion on compromised skin such as stratum corneum stripped skin.

The direct penetration of topical drug into deep tissue is also dependent on the physicochemical properties of the drug molecules. Roberts's group investigated the direct penetration from dermal application of a range of drug molecules including NSAIDs [10], lidocaine [11], some bases and steroids [12] with different physicochemical properties. They discovered that the direct penetration of lidocaine, some bases and steroids could reach as deep as 1 cm into tissues, while the direct penetration of some NSAIDs could not exceed beyond 3 mm deep.They suggested that lidocaine and the unionized bases or steroids were more lipophilic at physiological pH and with lower

plasma protein binding, and thus, were to a less extent being cleared away by dermal blood circulation. On the other hand, the NSAIDs were ionized at physiological pH and with very high plasma protein binding, and thus were at a higher extent being cleared away by the dermal blood circulation. The less extent of NSAID direct penetration into deep tissues was also reported by other researchers [13, 14].

A majority of NSAIDs have a carboxyl group, are ionized at physiological pH (hydrophilic), and have very high plasma protein binding.With such physicochemical properties, topical NSAIDs would have very limited direction penetration into deep tissues.Such physicochemical properties could be modified with the formation of NSAID prodrugs, *i.e.*, forming of an ester prodrug between diclofenac acid and an alcohol. It is expected that the ester prodrug would be unionized and more hydrophobic at physiological pH, and have lower plasma protein binding. Indeed, it was demonstrated that methyl salicylate, a prodrug, was better than its parent drug salicylic acid in direct penetration into deep tissues [15, 16]. Would some diclofenac ester prodrug also show better direct penetration into deep tissue than its parent drug? If it is true, the topical product of such prodrug could improve the treatment efficacy for millions of arthritic patients. However, this important question has not been addressed by other researchers before. We previously synthesized several diclofenac ester prodrugs and discovered that some of the ester prodrugs showed better *in vitro* flux across the skin than that of the parent drug [17]. However, it was unknown whether the diclofenac ester prodrugs would also show better direct penetration into local tissue, which was one of the objectives to be investigated in this study. It is well known that skin blood flow clears most of the drug molecules penetrated into the skin and thus hinders topical drug direct penetration into

deeper tissues. A dual agar gels *in situ* rat model has been demonstrated its validity in the evaluation of topical drug direct penetration into local tissues across the skin [18, 19]. Thus this model was adapted in this study to evaluate the direct penetration of diclofenac and its prodrugs.In addition,saturated aqueous solutions of the diclofenac ester prodrugs were used in the evaluation of their flux across the skin in our previous study [17]. Since those ester prodrugs are fairly unstable in aqueous solutions, a non-aqueous formulation system would be more suitable for the delivery of those prodrugs into the body. Pressure sensitive adhesives such as polyacrylate and polyisobutylene are widely used in transdermal patches and such formulation system offers a non-aqueous environment. Thus another purpose of this study was to screen the pressure sensitive adhesives to obtain suitable patch formulations in the delivery of the diclofenac ester prodrugs into the skin.

Materials

Diclofenac sodium was obtained from Gallipot, Inc. Pressure sensitive adhesives Durotak 387-2287, Durotak 87-901A, and Durotak 87-608A were gifts from Henkel, Inc. Human cadaver skin was purchased from New York Firefighter skin bank. Transdermal backing film (CoTran 9722) and 1022 release liner were gifts from 3M. Female hairless rats were purchased from Charles River laboratory. The diclofenac prodrugs: glycerol diclofenac ester (GD), ethylene glycol diclofenac ester (ED), methanol diclofenac ester (MD) were synthesized as described in our previous paper [17]. Fresh human plasma was purchased from Zen-Bio, Inc.

Experimental

Preparations of the diclofenac and its prodrugs patches

Three pressure sensitive adhesives were investigated in the formulation of transdermal patches namely: Durotak 387-2287(polyacrylate adhesive with hydroxyl group), Durotak 87-901A (polyacrylate adhesive with no functional group), Durotak 87-608A(polyisobutylene adhesive). The drug or prodrug was added to obtain a final concentration of 10% w/w with respect of the drug to the final dry adhesive layer weight. 3% oleic acid was added as a permeation enhancer and 3% silicon dioxide was added as a suspending agent. The drug adhesive mixture was cast at a thickness of 0.254 mm on a release liner.

Evaluation of the in vitro flux across human skin

Human Epidermis (HE) was separated from human cadaver skin by a heat separation method. The HE with a size around 2 cm x 2 cm square was mounted on Franz diffusion cell with stratum corneum side facing the donor chamber. A circular patch with a

diameter of 1.2 cm was then applied to the HE surface. The Franz cell diffusion surface area was 0.65 cm^2. The receiver chamber was filled with 5 ml of 0.15 M phosphate buffer saline solution at pH 7.4 and maintained under stirring condition at 37 °C. At time 2, 4, 6, 8, 12, 24, 30, 36, and 48 hours, the whole 5 ml was taken and replaced with 5 ml fresh media. The drug or prodrug concentration in the receiver media was analyzed with a HPLC method. Triplicated experiments were conducted for each patch.

Evaluation of the in vitro flux across hairless rat skin

Female hairless rats were euthanized and then the abdominal skin was separated out. The fat tissue under the skin was scraped off with scissors. A 2 cm x 2 cm squares was mounted on Franz diffusion cells with stratum corneum side facing the donor chamber, a circular patch with a diameter of 1.2 cm was then applied to the skin surface. The Franz cell diffusion surface was 0.65 cm^2. The receiver chamber was filled with 5 ml of phosphate buffer saline solution. At time 2, 4, 6, 8, 12, 24, 30, 36, and 48 hours, the whole 5 ml was taken and replaced with 5 ml fresh media. The drug or prodrug concentration in the receiver media was analyzed with a HPLC method. In addition, the flux of diclofenac from a commercial available product (Voltaren gel) across the hairless rat skin was also determined here. This was to serve as a reference point for the flux results we obtained from our patch formulations. Triplicated experiments were conducted for each condition. The animal protocols used in this study were fully approved by the Institutional Animal Care and Use Committee (IACUC) at the Idaho State University.

Determination of plasma protein binding and bioconversion rate

The diclofenac prodrugs (GD, ED, MD) or diclofenac acid (DA) was dissolved in DMSO solution at suitable concentrations, then a small amount of the drug solution (20 µl) was spiked into 2 ml of 30 mg/ml human serum albumin in phosphate buffer solution

at pH 7.4 in a dialysis tube (MWCO = 5000) for dialysis against the same phosphate buffer for 6 hours at room temperature. The concentration of the drug in the dialysis tube (C_{total} = C_{free} + C_{bound}) and drug concentration outside the dialysis tube (C_{free}) was determined by HPLC. The drug plasma protein binding was determined by the ratio of C_{bound}/C_{total}.

The bioconversion rate of the diclofenac ester prodrugs in fresh rat plasma was determined in a previous study [17]. The bioconversion rate of the diclofenac ester prodrugs in human plasma was determined here with the same method. Briefly, diclofenac prodrugs in DMSO solutions with appropriate concentrations was spiked into human plasma and then incubated in 37°C water bath shaker. Aliquots were taken from the plasma periodically into a methanol solution to stop the bioconversion and also to precipitate out the plasma protein. Then the samples were centrifuged and supernatants were analyzed with an HPLC method.

Determination of the pharmacokinetic parameters from IV injection.

Diclofenac sodium in phosphate buffer saline solution was prepared at 500 µg/ml. 200 µl of the solution was injected into the hairless rat via the saphenous vein. 0.5 ml of blood sample was taken with the tail bleeding method at 10, 30, 60, 120, 180, and 240 min after the injection. The blood sample was centrifuged at 5000 rpm for 15 min to collect the plasma. The drug concentration in the plasma was analyzed with an HPLC method. The plasma concentrations at different time points were simulated with WinNolin 5.02 software, a bolus IV injection with two-compartmental model was used, the volume of distribution of the central compartment (V_d) and the total clearance (CL) from the central compartment were obtained from the software simulation.

Evaluation of direction penetration with a dual agar gel in situ rat model.

This study followed the method developed by Hasegawa *et al.*[18] with some modification (see Figure 1A). Specifically, hairless rats was anesthetized with isoflurane and maintained a body temperature of 36 to 37 °C. An incision (5 cm wide) was cut (horizontal) along the lower abdominal skin, the abdominal skin was carefully separated from the abdominal muscles to ensure no subcutaneous bleeding, then two pieces of agar gel (1.0% agarose in 0.15M phosphate buffer solution at pH 7.4), each filled in a polyethylene dish of 2.5 cm wide, 3.8 cm long, and 0.5 cm thick, were inserted into the space between the hypodermis and the abdominal muscles with the open surface facing dermis, one in the left side and one in the right side. A patch of the same size of the gel dish was applied exactly on the right abdominal skin where the gel was located, and at 2, 4, 6, 8, 10, 12 hrs time points, the two pieces of gel were taken out from the body, and at 2, 4, 6, 8, 10 hrs time points two fresh pieces of gel were inserted into the specified place, and then the incision was closed with Michel's clamps. The gel underneath the application site was served as a receptor to collect the drug penetrated through the skin from the patch. The other piece of gel on the contralateral site was served as a control for drug penetration into the gel through systemic blood circulation (assuming systemic circulation contributes to the same amount of drug to both gels). At the same time points, 0.5 ml of blood was taken from the rat tail. The amount of drug in the gel dish and the plasma drug concentration was analyzed by HPLC. Four rats were used for each patch formulation in the *in situ* rat study.

More schematic illustration of the dual agar gel model is shown in Figure 1B with modification from Hasegawa et al [18]. Drug from the patch permeates into the skin with part of the drug goes into systemic circulation and part of the drug penetrates directly to

deep tissue (here this amount of the drug is collected by the agar gel at the application site: $X_{gdirect}$). In addition, some drug from systemic circulation is redistributed to deep tissue (here the amount of the drug is collected by the gel dish at the contralateral site: X_{gc}). The gel dish at the application site also has similar contribution from systemic circulation, so the total amount of the drug in the gel dish at the application site can be expressed as: $X_{gtotal} = X_{gdirect} + X_{gc}$. In addition, based on CL and V_d determined from IV injection, the plasma drug concentration (C_b) after patch application and the amounts of drug in each of two gel dishes, the total amount of the drug absorbed into systemic circulation from time 0 to time t can be expressed:

$$\sum X_{b0-t} = CL \cdot AUC_{0-t} + C_b \cdot V_d \quad (1)$$

where $AUC_{0\text{-}t}$ is the area under the plasma concentration time curve from time 0 to time t calculated by trapezoidal rule. The total amount of the drug directly penetrated into the gel dish:

$$\sum X_{gdirect} = \sum X_{gtotal} - \sum X_{gc} \quad (2)$$

The percentage of the drug in the gel dish that was due to direct penetration:

$$\text{Direct\%} = \sum X_{gdirect} / \sum X_{gtotal} \quad (3)$$

In addition, the fraction (**f**) of the drug accounted for the direct penetration compared to the total amount of drug delivered into the body by the patch:

$$f = \sum X_{gdirect} / \sum X_{b0-t}$$

Sample analysis

For the plasma samples, 200 µl of plasma was taken and added 50 µl of 100 µg/ml ibuprofen as internal standard. 5 ml of Hexane : Tetrahydrofuran (90:10) was added to each sample, and then the sample was shaken in orbital shaker at 400 rpm for 1 hr, and then centrifuged to collect the upper organic layer. The organic layer was evaporated to dry in a vacuum oven and then redissolved in 200 µl of a solution (25/75 acetonitrile/0.02 M phosphate buffer at pH 7.4). 50 µl of sample was injected for HPLC analysis. For agar gel samples, The samples were mixed with 10 ml mixture of hexane : tetrahydrofuran (95:5), and then homogenized for 1 min, and then shake in orbital shaker for 1 hours, then centrifuged to collect the organic phase, the organic phase was evaporated to dry in vacuum oven, and then redissolved with 200 µl of the solution (25/75 acetonitrile/0.02 M phosphate buffer at pH 7.4) for HPLC analysis.

The HPLC assay method was the same as our previous study [17]. The DA and its prodrugs were analyzed with a HP 1050 HPLC with quaternary pump and DAD detector.The column used was a *Zorbax* XDB-C18 4.6 x 150 mm 5.0 micron *HPLC column.* The mobile phases were acetonitrile and 0.02 M citrate buffer at pH 6.0 with a gradient method. The flow rate was 1 ml/min, and the detection UV wavelength was 280 nm.

Results and Discussion

In vitro flux across human epidermis

We fabricated the patches for diclofenac acid (DA), glycerol diclofenac ester (GD), ethylene glycol diclofenac ester (ED), and methanol diclofenac ester (MD) with the three different adhesives: Durotak 387-2287, Durotak 87-901A, and Durotak 87-608A. The drug or prodrugs were uniformly dispersed in the adhesive layer of the final patches with no visible crystal formation over a 6-month period and all the patches showed sufficient tackiness (tested by finger).

In vitro flux study across human epidermis was conducted on those patches. The prodrugs maintained their ester form in the samples from the receiver chambers, especially in the first 12 hrs with no parent drug detected in the samples. However, some small amount of DA was detected in the samples in later time points, especially at the 24 hr and 48 hr time points with a span of 12 hrs in sampling time. Since the human epidermis was prepared with a heat separation method, most esterase present in the skin was probably destroyed, and thus little prodrug was converted to the parent drug in the skin and most the prodrug permeated through the skin in their ester form. However, the prodrugs are not very stable in aqueous solution with half lives from 25 to 50 hrs [17], thus some of the prodrugs could be possibly hydrolyzed to the parent drug in the receiver chamber between the sampling points. With this in consideration, we combined the amounts of prodrug and parent drug in the samples for plotting the cumulative amount of drug permeated across the skin and in the calculation of the flux across the skin.

For the patches made with the polyacrylate adhesive Durotak 387-2287, the cumulative amount of the prodrugs permeated through the human epidermis over the experiment period was much lower from all diclofenac prodrug patches than that from

the diclofenac patch (Figure 2A). In addition, among the diclofenac prodrugs, GD showed the highest amount permeated across the skin, and MD showed the lowest amount permeated across the skin. We also observed similar results from the patches made with another polyacrylate adhesive Durotak 87-901A, with DA showing the highest amount permeated across the epidermis, and MD showing the lowest penetration (Figure 2B). In the patches made with a polyisobutylene based adhesive Durotak 87-608A, the GD and ED patches showed similar amount of prodrugs permeated across the skin, and they were higher than that from the DA patch (more than double), but MD still had the lowest permeation across the skin (Figure 2C).

The fluxes and lag times for all the patches determined from Figure 2 were summarized in Table 1. DA showed the highest flux in Durotak 87-901A, while GD and ED showed the best fluxes in Durotak 87-608A. The inferiority in the fluxes of the prodrugs from the patches with polyacrylate adhesives was probably due to a strong interaction between the ester prodrugs and the ester type adhesives. On the other hand, the hydrophobic parent drug DA probably had stronger interaction with the polyisobutylene adhesive and thus showed much lower fluxes.

The purpose of this study was to screen the diclofenac and its prodrugs patch formulations with different adhesives, and to identify the ones with high in vitro fluxes for further studies. Human cadaver skin can be readily obtained from skin bank, and with that flux screening on large number of patch formulations can be done on the skin from the same donor. The using of skin from the same donor can potentially lower the variation of the flux study. We discovered that the diclofenac acid in Durotak 87-901A

patch and the Prodrugs (GD and ED) in Durotak 87-608A provided the best flux results, and thus they were chosen for further study.

In vitro flux across hairless rat skin

We further determined the flux behavior across hairless rat skin of the DA Durotak 87-901A patch and the ED and GD Durotak 87-608A patches. Unlike the flux study with human epidermis, the hairless rat skin had the full thickness dermis layer. This can significantly increase the lag time for the penetration to reach steady state. In addition, the skin was fresh excised from rats; some enzyme activity was retained in the skin, especially in the initial experiment period, which could help us understand how fast the prodrug converting back to the parent drug during the penetration across the skin. In addition, we also conducted an in situ study with rats and obtained the amount of drug absorbed in the body, the in vitro study with rat skin would also enable us to conduct an in vitro in vivo correlation check.

It is shown in Figure 3A that the DA flux across hairless rat skin from the DA Durotak 87-901A patch was almost two times of that from the commercial Voltaren gel (Lot#10071758), which indicated that the flux provided by the patch formulation was clinically significant. The flux across hairless rat skin was similar to the flux across human epidermis for the DA Durotack-901 patch, but the lag time was much bigger for the permeation across the hairless rat skin, due to the full thickness of the rat skin (see Table 1 and Table 2). The amount of Voltaren gel loaded in the Franz diffusion cells was 50 µl, and with a diffusion area of 0.64 cm^2, it was probably much more than the amount of gel used per cm^2 skin area for patient use. With this approach, the Voltaren gel also provided a relative stable steady flux over a long period of time. Unlike the patches, in

the case of applying such gel on patient skin, because of thinner application and fast drying of the gel material, a sustained steady state flux may not occur. The permeation across hairless rat skin from the GD Durotak 87-608A patch is shown in Figure 3B. Large percentage of the drug collected in the receive chamber was in the parent drug form, especially in the first 12 hours. This is contrary to the permeation across human cadaver epidermis skin, in which most of the drug in the receiver chamber was in the prodrug ester form.This was probably due to the hairless rat skin was freshly excised, which may retain some enzyme activity that converted the GD to its acid drug form either in the skin or in the receiver chamber. It would be very likely that the stratum corneum was the main permeation barrier and GD had to penetrate across the stratum corneum layer of the hairless rat skin before being converted, thus we calculated the flux of GD across the hairless rat skin by combining both the diclofenac acid form and the ester form in the receiver chamber.With that, the flux of GD delivered by the patch across the hairless rat skin was similar or even a little higher than that across the human skin (Table 1 and Table 2), and a larger lag time was also observed in the flux across the rats skin.

The permeation across hairless rat skin from the ED Durotak 87-608A patch is shown in Figure 3C. Most of the drug in the receiver chamber was also in the parent drug form in the first 12 hours, which indicated high bioconversion in the skin. Comparing to flux results from the GD patch, the fraction of the prodrug converted to the parent drug in the receiver chamber was even larger, which indicated the faster bioconversion of ED than GD in the skin. The total flux across the hairless skin for ED delivered by the patch was less than that across human epidermis (Table 1 and Table 2), and it was also less than half of that from the GD patch shown in Figure 3B. ED was more hydrophobic than GD and

less soluble in aqueous solution (almost 10 times less soluble than GD) [17], thus the dermis layer of the rat skin may pose a significant barrier for its permeation.

Plasma protein binding and bioconversion rate

The fraction of diclofenac or its prodrugs binding to plasma protein are listed in Table 3. The diclofenac ester prodrugs have significantly lower fraction of plasma protein binding than that of the parent drug, which means that the prodrugs may have almost 10 times higher unbounded free prodrugthan that of the parent drug.The bioconversion rates of the prodrugs in human plasma and in rat plasma were also listed in Table 3. In rat plasma, GD showed the slowest bioconversion rate. This is also consistent with the slower bioconversion of GD than that of ED we observed during penetration across the fresh excised rat skin in the flux study. But in human plasma, GD showed the fastest bioconversion rate. The bioconversion rates in human plasma of the ester prodrugs were much slower than those in the rat plasma. Our intention was to choose one diclofenac prodrug to compare with diclofenac acid for their direct penetration across live rat skin in the in situ rat study. Since GD showed higher flux across the rat skin and also slower bioconversion in rat plasma than those of ED, the GD Durotak 87-608A patch and DA Durotack 87-901A patch were selected for further *in situ* rat study.

Pharmacokinetic analysis of IV injection of diclofenac sodium

The rat plasma concentrations from the IV injection are shown in Figure 4. The data points were fit with a two-compartment IV injection model and the obtained pharmacokinetic parameters from the fitting are listed in Table 4. The volume of distribution (Vd) of the central compartment and the total clearance (CL) were similar to the values reported in a study on a subcutaneous injection of diclofenac to rats [20]. Those parameters are used for the calculation of drug absorbed into the body from the

transdermal patches in the *in situ* rat study. This is a part of the information needed in order to determine the in vitro in vivo correlation. In addition, this information is also needed to determine the fraction of drug went to direct penetration compared to the amount of absorbed in the body.

Direct penetration from in situ rat study

The cumulative amount of diclofenac collected in the agar gel dish under the skin applied with the DA Durotak 87-901A patch and also the amount of diclofenac in the agar gel disk under the contralateral skin site are shown in Figure 5A. Presumably, the amount of drug collected in the agar dish at the contralateral site (X_{gc}) was contributed from the redistribution of diclofenac from the systemic blood circulation. And the amount of drug collected in the agar dish at the patch application site (X_{gtotal}) was contributed from both the direct penetration across the skin from the patch and also the drug redistributed from the systemic circulation. The amount of the drug contributed from the direct penetration would be the difference of the drug collected between the two agar gel dishes. Based on the data in Figure 5A and Equation 3, roughly 78% (Direct%) of the drug in the gel dish at the application site was due to direct penetration from the DA patch.

The cumulative amount of diclofenac collected in the agar gel dish right under the skin applied with the GD Durotak 87-608A patch and also the amount of diclofenac in the agar gel dish under the contralateral skin site are shown in Figure 5B.No prodrug was detected in the agar gel dishes, which means all prodrug penetrated into the skin was converted back to the parent drug. In addition, the less amount of the drug collected by the agar gels from the GD patch than that from the DA patch was probably due to the less amount of drug delivered into the skin by the GD patch, as demonstrated from the *in vitro*

flux study (Figure 3A and 3B). Based on the data in Figure 5B, roughly 77% (Direct%) of the drug in the gel dish was due to direct penetration from the GD patch. The percentages of drug contributed from direction penetration were similar between the GD patch and the DA patch.

The diclofenac concentrations in rat plasma over the time from the DA patch or the GD patch applied on the skin during the *in situ* rat study are shown in Figure 6A. No GD was detected in the plasma samples. The DA patch provided higher plasma diclofenac level, which was almost double of that from the GD patch. Based on the diclofenac plasma concentrations and also the pharmacokinetic parameters from the IV injection study, the amount of drug absorbed into the systemic blood circulation was calculated from Equation 1 and plotted in Figure 6B for both patches. From the plot, the amount of drug delivered into the systemic blood circulation from the GD patch was only half of that delivered from the DA patch.

Based on the amount of drug absorbed into the body from Figure 6B for both patches and also the amount of drug penetrated through the hairless rat skin in the first 12 hours from the *in vitro* flux study (Figure 3A and 3B), we observed a very strong *in vitro in vivo* correlation as shown in Figure 7 for both the DA patch and the GD patch. This again supported that the amount of drug absorbed into the body we calculated in Figure 6B was valid. We also calculated the fractions of direction penetration to the agar gels compared to the total amount of drug absorbed into the body based on Equation 4 for both patches, which are 0.12% for the DA patch and 0.083% for the GD patch.

We showed in this study that both glycerol diclofenac ester (GD) and ethyl glycol diclofenac ester (ED) had lower plasma protein binding than the parent drug, and

therefore, they would be cleared away in a less extent by the blood circulation in the skin as compared to the parent drug. In addition, the direct penetration of drug molecules across a tissue layer is dependent on the concentration gradient of the free drug (unbounded molecules). Even though the fraction of unbounded drug in tissues and the fraction of unbounded drug in plasma protein are different, Roberts and Cross observed there was a direct correlation between the two fractions [21]. Based on that, we could probably assume that the fraction of unbounded diclofenac ester prodrugs was also higher than the fraction of unbounded diclofenac in the tissues, and thus topical application of diclofenac ester prodrugs could be advantageous in direct penetration into deep tissue.

We showed in a previous study that some diclofenac ester prodrugs can provide higher flux across the skin in a aqueous media [17]. Due to the diclofenac ester prodrugs being prone to hydrolysis in aqueous media, we here investigated the feasibility of formulating these prodrugs into non-aqueous pressure sensitive adhesive patch system. We showed that the polyacrylate based adhesives such as Durotak 387-2287 and Durotak 87-901A were suitable for the parent drug DA to provide high fluxes across the skin, but not for those ester prodrugs, possibly due to strong prodrug adhesive interactions. On the other hand, the polyisobutylene based adhesive (Durotak 87-608A) patches offered better fluxes for the diclofenac ester prodrugs, with the fluxes across the skin being similar or slightly better than those from their saturated aqueous solution as we observed previously [17].

An in-situ rat model was previously proposed by Sugibayashi group in investigation of topical drug direct penetration across the skin [14, 18, 19].This model retained an intact blood circulation in the skin and provided a mean to determine the contributions from

direct penetration and also from systemic circulation.We adapted the dual agar gel *in situ* rat model in this study to investigate the topical application of diclofenac or its ester prodrug in direct penetration across the skin. We observed that the amount of drug collected in the agar gel underneath the skin at the application site was consistently higher than the amount of drug in the agar gel at the contralateral site for both the diclofenac and its prodrug patches, with around 77% contributed from the direct penetration. However, the amount of the drug collected in the agar gel underneath the skin at the application site was very small compared to the total amount of drug absorbed into the circulation system, with only 0.12% contributed from the direct penetration from the diclofenac patch and 0.083% from the GD patch. These results indicate the skin blood flow cleared most of the absorbed drug with little drug available for direct penetration into deep tissues.

Contrary to our initial expectation, the topical delivery of glycerol diclofenac ester did not show advantage over the topical delivery of the parent drug diclofenac in terms of the direct penetration from our *in situ* rat study. This was probably due to the fast bioconversion of GD back to the parent drug diclofenac in the rat skin. The bioconversion of GD in rat plasma is fast with a half-life only 11.3 min, even though it is the slowest one among the three diclofenac prodrugs (GD, ED, MD). The bioconversion of GD in the rat skin could also be very fast, as we showed from the *in vitro* flux study across fresh hairless rat skin, with a significant amount of drug in the receiver chamber in the parent drug form, especially in the initial 12 hours when the enzymes in the skin may be still active. Such fast bioconversion of GD in the skin could render it to lose its

prodrug form in the skin and thus lose its advantage over the parent drug in direct penetration into deep tissues.

The bioconversion rates of the diclofenac prodrugs in human plasma are much slower, compared to those in the rat plasma (Table 4), which probably indicates a slower bioconversion in the human skin also. Because of such big differences in bioconversion rates for the ester prodrugs between the human and the rats, it is possible that the prodrug may still be advantageous over diclofenac in human application. To verify that, future study on human subjects with the topical application of diclofenac ester prodrugs may be needed.

As we discussed in the introduction section, the efficacy from current topical diclofenac products was only marginal because of insufficient direct penetration. In this paper, we confirmed that topical application of diclofenac provided very limited amount of drug (0.12%) for direct penetration, and the majority of the drug went to systemic circulation. We adapted a dual agar gel model proposed by Sugibayashi's group[14, 18, 19] for the direct penetration study. We showed that the direct penetration results from our diclofenac patch were similar to the results they obtained for flurbiprofen[18].These results further supported the validity of the dual agar gel *in situ* rat model for the direct penetration study. However, we discovered that the direct penetration from the diclofenac ester prodrug was not better than that from diclofenac, mainly due to the fast bioconversion of the prodrug in the rat, while such bioconversion was much slower in human. This indicates that the *in situ* rat model may not be suitable for investigation of direct penetration in drugs that have large difference in metabolic rates between that in the human skin and that in the rat skin.

Conclusions

Diclofenac ester prodrugs were more suitable to be formulated in patches with polyisobutylene adhesive, while the parent drug diclofenac was more suitable to be formulated in patches with polyacrylate adhesives. Diclofenac ester prodrugs showed lower plasma protein binding than diclofenac. The bioconversion of diclofenac ester prodrugs to diclofenac in human plasma was much slower than that in rat plasma. Topical application of diclofenac patch and its prodrug glycerol diclofenac ester patch showed direct penetration across rat skin, but the direct penetration only accounted a very small fraction of the drug absorbed into the body. Glycerol diclofenac ester did not show advantage over diclofenac in the direct penetration across live rat skin.

Tables

Table 1 A summary of the in vitro fluxes across human epidermis.

Drug/Prodrug	Adhesive	Flux ($nmol/cm^2/hr$)	Lag time (hr)
DA	DuroTak-2287	11.54 ± 0.80	0.39 ± 1.01
GD		2.75 ± 0.59	2.14 ± 0.94
ED		1.67 ± 0.14	- 4.06 ± 5.75
MD		0.57 ± 0.07	10.60 ± 1.49
DA	DuroTak-901A	15.06 ± 2.41	- 0.91 ± 1.38
GD		2.89 ± 0.86	- 0.13 ± 1.26
ED		1.99 ± 0.23	- 1.72 ± 0.85
MD		0.55 ± 0.08	0.36 ± 1.83
DA	DuroTak-608	3.55 ± 0.55	6.84 ± 0.29
GD		6.89 ± 1.93	1.99 ± 1.77
ED		5.97 ± 0.34	- 3.33 ± 2.10
MD		0.73 ± 0.14	4.94 ± 1.89

The number after ± is the standard deviation.

Table 2 A summary of the in vitro fluxes across hairless rat skin.

Drug/Prodrug	Flux ($nmol/cm^2/hr$) at 4 - 12 hr	Flux ($nmol/cm^2/hr$) at 12-48 hr	Lag time (hr)
DA in Durotak-901A	8.33 ± 0.94	12.84 ± 1.27	7.09 ± 0.73
GD in Durotak-608	5.97 ± 1.03	9.12 ± 0.42	6.08 ± 1.85
ED in DuroTak-608	2.38 ± 0.30	3.92 ± 0.34	7.90 ± 0.24
Voltaren gel	4.90 ± 0.93	6.69 ± 0.74	5.43 ± 1.03

The number after ± is the standard deviation.

Table 3 A list of the plasma protein binding and bioconversion results.

	DA	GD	ED	MD
Fraction Binding to Plasma Protein (%)	99.2%	91.0%	88.3%	--[a]
Bioconversion in Human Plasma (half life in min)	--	50.8	122	604
Bioconversion in rat plasma (half life in min)	--	11.3[b]	1.66[b]	1.62[b]

[a] Experiment was not conducted because of extremely low aqueous solubility of MD.

[b] Experiment results from previous study[17].

Table 4 The pharmacokinetics parameters from IV injection based on the WinNonlin analysis.

V_d (ml)	52.3 ± 1.4
K10 (min^{-1})	0.045 ± 0.005
K12 (min^{-1})	0.034 ± 0.005
K21 (min^{-1})	0.018 ± 0.006
CL (ml/min)	2.34 ± 0.31

The number after ± is the standard deviation.

Figures

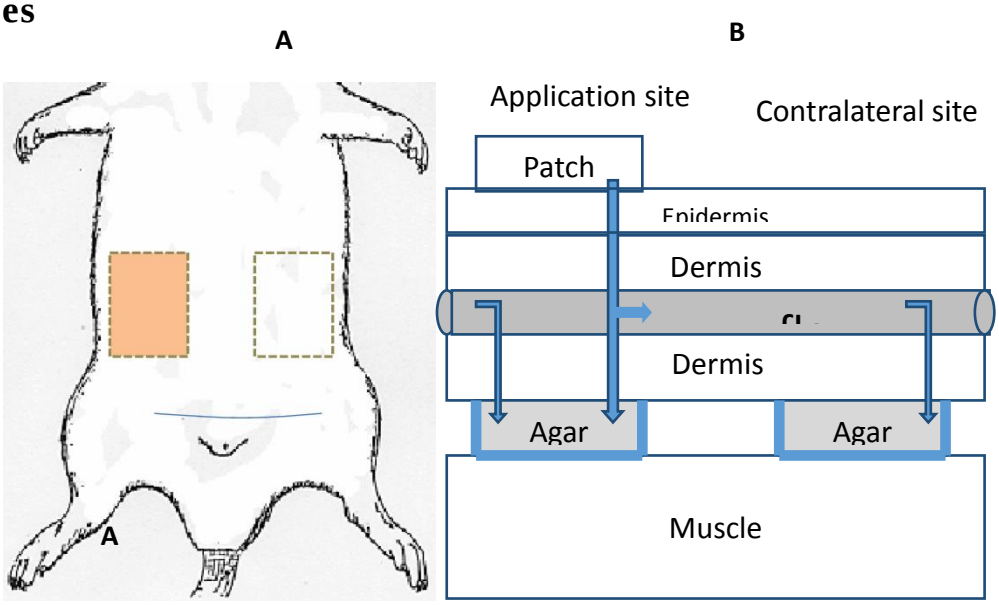


Figure 1 (**A**) Schematic diagram of a hairless rat being applied with a patch on the abdominal skin, an incision at the low abdominal skin, and two agar gels (dash line) were inserted under the abdominal skin. (**B**)Schematic diagram of the dual agar gel rat model where the topical patch provides penetration into the skin, direct penetration into the agar gel, and redistribution of drug into agar gel from blood circulation.

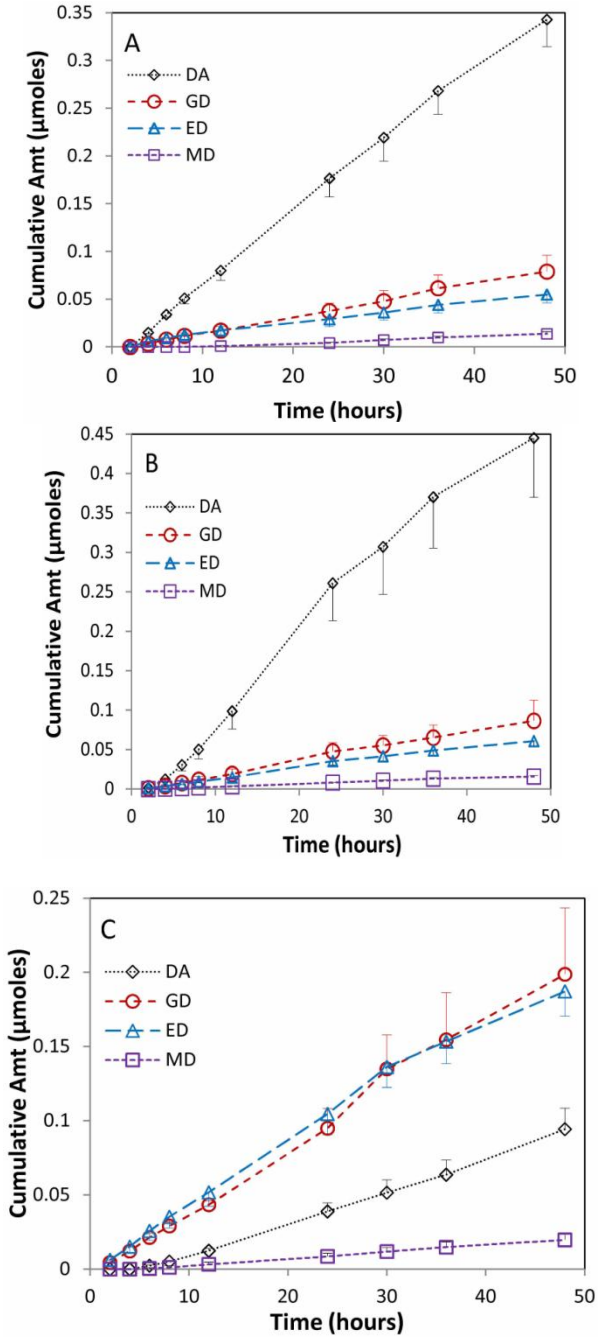


Figure 2 Cumulative amount of diclofenac or its ester prodrugs penetrated across the human epidermis from the *in vitro* flux study. **(A)** Diclofenac or its ester prodrugs in patches with adhesive Durotak 387-2287. **(B)** Diclofenac or its ester prodrugs in patches with Durotak 87-901A. **(C)** Diclofenac or its ester prodrugs in patches with Durotak 87-608A.

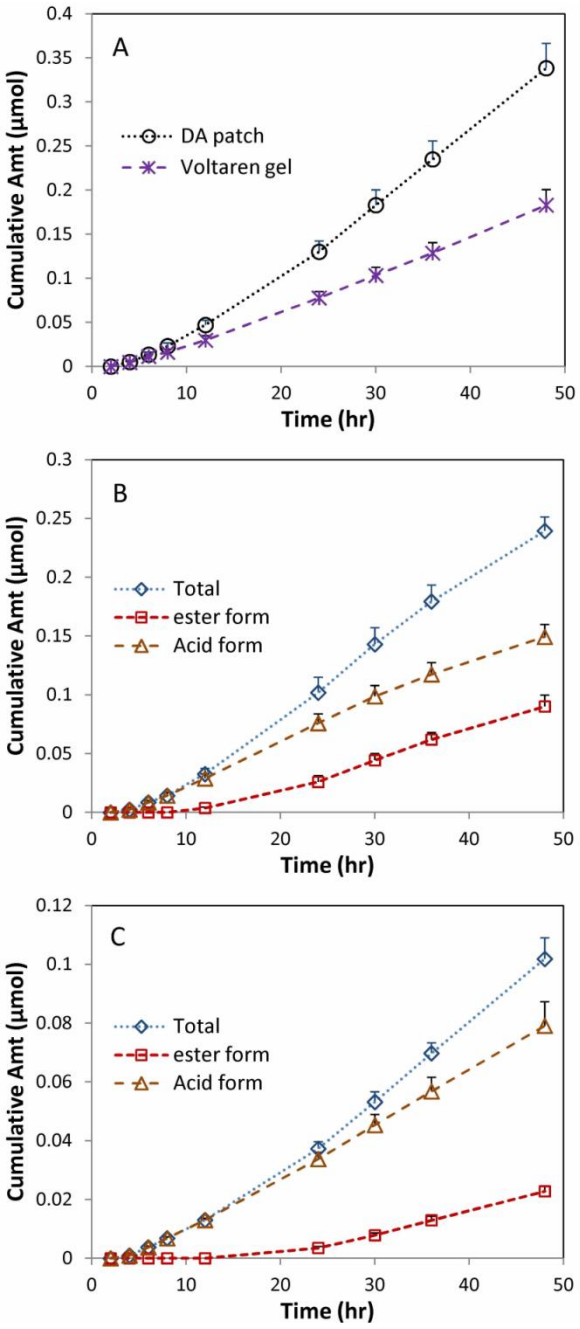


Figure 3 Cumulative amount of diclofenac or its ester prodrugs penetrated across the hairless rat skin from the *in vitro* flux study. **(A)** Diclofenac acid in Durotak 87-901A or Voltaren gel. **(B)** Glycerol diclofenac ester in Durotak 87-608A patch. **(C)** Ethylene glycol diclofenac ester in Durotak 87-608A patch.

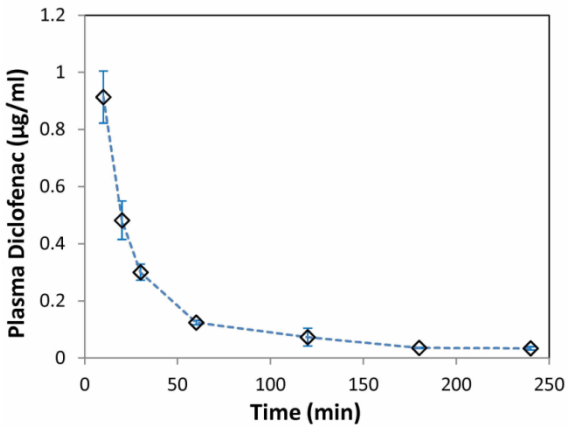


Figure 4 The diclofenac plasma concentrations over the time from I.V. injection of 100 µg of diclofenac sodium into hairless rats.

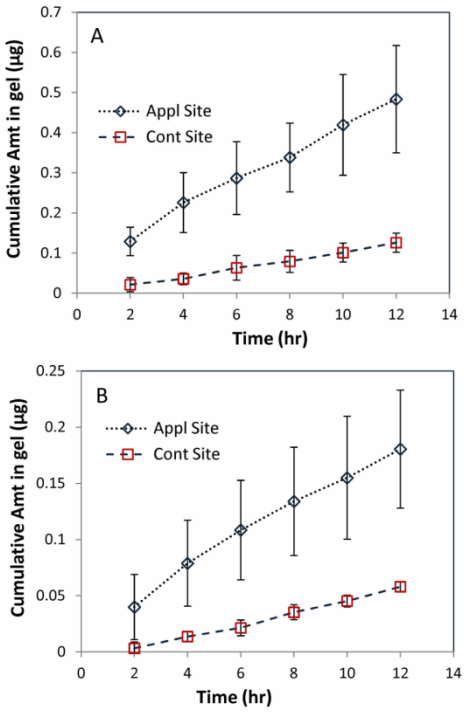


Figure 5 (A) The cumulative amount of diclofenac collected in the agar gels underneath the skin of the application site and the contralateral site from topical application of the diclofenac patch. (B) The cumulative amount of diclofenac collected in the agar gels underneath the skin of the application site and the contralateral site from topical application of the glycerol diclofenac ester patch.

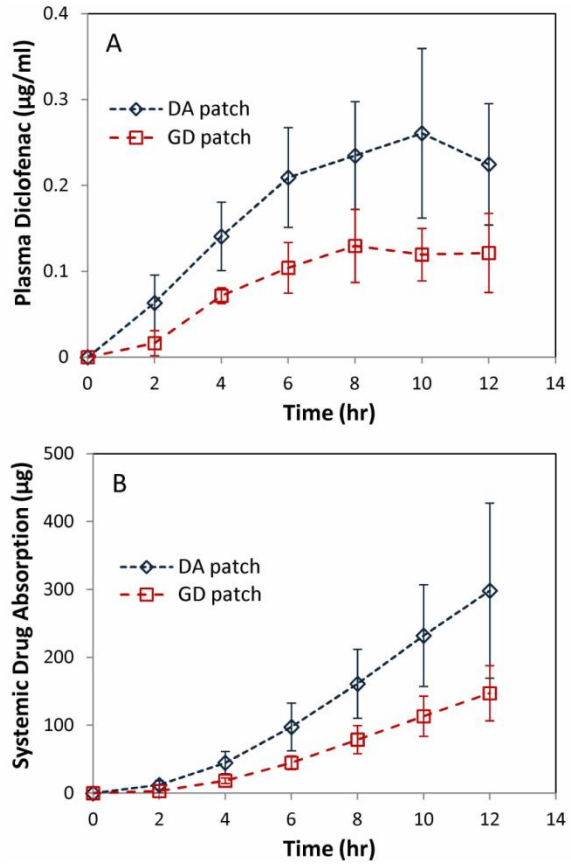


Figure 6 (A) Diclofenac plasma concentrations in hairless rats over the time from topical application of the diclofenac acid patch or the glycerol diclofenac ester patch. **(B)** The amount of diclofenac absorbed into the systemic blood circulation from the topical application of the diclofenac acid patch or the glycerol diclofenac ester patch.

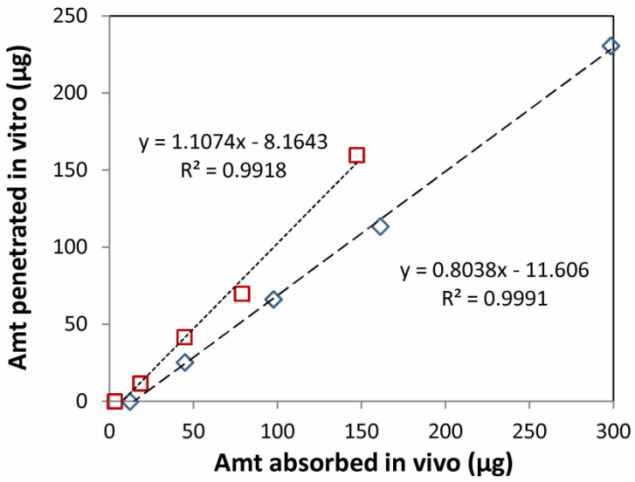


Figure 7 The correlation between the amount of drug penetrated across the skin from *in vitro* flux study demonstrated in Figure 3 and the amount of drug absorbed into the systemic blood circulation form the *in situ* study demonstrated in Figure 6. (◊) for diclofenac acid patch; (□) for the glycerol diclofenac ester patch.

CHAPTER 4 Cationic Prodrug for Iontophoretic delivery of NSAID and evaluation of skin disposition in Dual Agar Gel *In Situ* rat model

This project was supported partly by the INBRE program, NIH Grant Nos P20 RR016454 (National Center for Research Resources) and P20 GM103408 (National Institute of General Medical Sciences)

Abstract

Deep tissue penetration and localization of NSAIDs have already been thoroughly evaluated and studied, and the high protein binding of these drugs are found to be deterrents towards penetration into deeper tissue. We have successfully synthesized and evaluated cationic prodrug of ketoprofen and evaluated it for deep tissue penetration on a dual agar gel rat model. We have also gone ahead and co-delivered a vasoconstrictor (phenylephrine HCl) to evaluate the deep tissue penetration of the prodrug. The in vitro penetration of the prodrug was found to be much higher than the cationic iontophoreisis of the parent ketoprofen. The presence of the vasoconstrictor reduced the flux of the prodrug due to competitive sharing of charge transfer, but the flux was still much higher than that of ketoprofen. In the *in situ* study, the prodrug showed a drug transferred to the application agar gel 19 times that by cationic iontophoresis of ketoprofen. The systemic contribution of drug to the agar gel was higher for the ketoprofen iontophoresis at around 4-5%, whereas only 0.1% of the drug was contributed by systemic circulation in case of prodrug delivery. The presence of vasoconstrictor did not really increase the drug penetrating into the agar gel further and the contribution by the vasoconstriction effect was seen 2h after the start of iontophoresis was reflected in the invitro flux studies and because of the competitive ions of the vasoconstrictor, the drug appearing in the application side was lower than that when without vasoconstrictor. From this, the conclusion can be drawn that not only does the cationic prodrug transport more drug through the skin, it shows lesser appearance in the systemic circulation thus improving deep tissue penetration, and the presence of vasoconstrictor would not really improve the deep tissue penetration capability of the prodrug.

Introduction

The benefits of topical application of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) have been debated for quite a while on their superiority over their oral counterparts. Studies have discussed and contested over the drug penetration efficiency of the topical delivery route. There are various studies that show that in spite of the adverse effects, the oral route proceeds to give a higher tissue concentration of the drug than through the topical route. For instance, a study by Taburet (Taburet AM 1995) showed flurbiprofen to show maximum plasma concentration 140 times lower than that achieved by oral administration and the bioavailability was found to be just 3-5% that of oral.

Attempts have been made to increase the permeation of drugs through skin which includes studies involving preparation of prodrugs (Lobo S 2014), coadministration with penetration enhancers (Mikulak SA 1998), active transport methods such as iontophoresis (Peter C Panus 1997), and sonophoresis (Miyazaki S 1991). All of the mentioned processes aim either at increasing the diffusivity of the drug entity through the stratum corneum, or temporarily compromise the stratum corneum integrity for enhancing drug permeation. However apart from penetration enhancement, another aspect that demands attention in topical delivery systems and limits their proposed significance is the aspect of deep tissue penetration and localization. A study by Radermacher and group (Radermacher J 1991), experimented with Diclofenac gels on the knees of human patients, with a concluding statement reporting the concentration of unbound drug in the synovial fluid of both the treated and untreated knee were found to be same, and were also similar to the concentration in the blood. The study findings were also supported in the clinical parameter observations, in which both the knees showed a reduction in

swelling, but there was not a significant difference between the two. The blood microcirculation underneath the area of application plays an important part in how effectively a drug would be delivered locally at the site of administration. Studies have been done to show that the affinity of a drug for transport proteins in the circulating plasma dictates the residence time of the drugs in the blood microvasculature and in turn dictates how much of the drug is lost to the systemic circulation (Roberts MS 1999) (Wu ZY, Tissue and perfusate pharmacokinetics of melphalan in isolated perfused rat hindlimb. 1997). The more the drug that is lost to the systemic circulation becomes unavailable for deep tissue penetration and hence is an undesirable aspect in topical delivery. NSAIDs are a class of drug that are shown to have very high protein binding characteristics because of their carboxylic group, and hence show poor localization and deep tissue penetration properties when applied topically. There are studies that showed topical delivery of Ibuprofen to show 22.5 fold greater concentrations in the dermal tissue (4 to 5 mm) as compared to the oral route, but fail to show a similar superiority at the deeper levels at 25 to 30 mm (Tegeder I 1999). Conclusions drawn by Roberts et al (Singh P 1994), also showed the performance of six NSAIDs to be superior to a depth until the fascia (3-4mm), but at a depth below that, systemic blood supply was found to be the main source. Hence, there have been efforts to alter the microvasculature uptake of drugs when they penetrate the skin. Options include either to alter the protein binding of the drugs or to affect the blood flow at the site of application. The former includes approaches to mask these functional groups such as preparation of esters or amides to shield the negative carboxylic group (Bhandari KH 2007). Altering of the blood flow at the site of drug application can be brought about effectively by the co-administration of

vasoactive drug entities. Similar studies have experimented with co-administration of vasoconstrictors along with the drug to reduce loss of drug from the site of application (Higaki K 2005).

Another aspect that is addressed in this paper is the effectiveness of anodic iontophoresis vs cathodic iontophoresis. At a pH of 4 and above, both human skin and rodent skin are negatively charged. Hence, the contribution of ionic transport through electroosmosis would be observed to a much larger extent in case of cathodic iontophoresis, where the counterions would be cations with respect to the negatively charged skin. Panus et al, who studied the effect of anionic iontophoresis to show impact on permeation of ketoprofen (Peter C Panus 1997) as compared to passive delivery, showed that there was not a significant difference in the concentrations in the skin and fascia layers. He also went ahead to show that in swine median thigh tissue, there is no significant penetration enhancement of the drug to a depth below 1cm, as compared to that through passive diffusion (Panus PC 1999). A reason for this would be the counter movement and bulk association of the negatively charged drug along with the counter ions i.e. Na^+ ions through electroosmosis.

Hence, our current study aims to prepare cationic prodrug of the cationic drug Ketoprofen to ameliorate some of the above mentioned factors that would help in improving the effectiveness of the drug. Firstly, the aim is to increase the permeability of the drug through the skin by preparation of a cationic prodrug that would capitalize on the enhanced electroosmotic movement of the prodrug in a cationic iontophoresis setup. Secondly,we also intend to study and evaluate the effect of coadministration of a vasoconstrictor along with the prodrug and assess its effect on localization and deep

tissue penetration. The above factors should collectively tend to increase deep tissue penetration of the drug.

Materials and Animal Model

Ketoprofen was obtained from TCI ltd (Tokyo, Japan), Choline chloride was obtained from J.T.Baker (Phillipsburg, NJ), Phenylephrine Hydrochloride was obtained from Spectrum Chemicals (Gardena, CA), N,N'-dicyclohexylcarbodiimide (DCC) and 4-pyrrolidinopyridine were obtained from Sigma (St. Louis, MO), Agarose LF (PFGE grade) was obtained from Amresco (Solo, Ohio), Silica Gel ultra pure 40-60 µm was obtained from ACROS organics (Fair Lawn, NJ), Dichloromethane was obtained from Fisher Scientific (Fair Lawn, NJ) and Acetonitrile was obtained from EMD chemicals Inc (Gibbstown, NJ), Methanol was obtained from BDH chemicals (Radnor, PA), Trifluroacetic acid was obtained from Honeywell Riedel-de-Haen (Seelze, Germany) and Human plasma was obtained from Interstate Blood Bank Inc. (Memphis, TN). The direct current source used in the experiments was Activa Dose® II Controller Ionto Device purchased from ActivaTek Inc (Salt Lake City, Utah). The rats used in the study were Sprague Dawley rats bred and reared at the Animal Care Facility at Idaho State University and ranged between 280 and 350g weight.

Experimental

Preparation of Prodrug of Ketoprofen

The esterification of Ketoprofen drug was carried out based on the procedure referenced by Lobo et al[4], with certain modifications in the solvent used. Specifically, choline chloride (0.00353 moles) was added to 50ml of anhydrous acetonitrile, and dissolved by sonication at 50°C. Upon solubilization, ketoprofen (0.00118 moles) was added and solubilized, followed by 0.000054 moles of 4-pyrrolidinopyridine. Reaction was initiated by addition of N,N'-dicyclohexylcarbodiimide (0.00146 moles), and continuously stirring at room temperature for 18 hours. A white precipitate was formed which was separated by filtering through non-absorbent cotton. The reaction vessel and the precipitate was further washed with 10ml of anhydrous acetonitrile and filtered and combined with the initial filtrate. The acetonitrile was evaporated under vacuum at room temperature, and a thick oil results in the flask which was the crude ester product. The oil was dissolved in 1ml of dichloromethane and was added to a flash chromatographic column. A combination of methylene chloride, acetonitrile and methanol (60:20:20) was used for eluting the pure prodrug.

Determination of Degradation rate

The hydrolysis rate of KCC was evaluated both in purified water and in PBS pH 7.4 at 37°C. The prodrug solutions were placed in a water bath shaken at 37°C, and agitated at 150 rpm. Samples were taken from the solutions at time intervals of 0min, 15mins, 1h, 2h, 4h, 6h, 12h, 24h, 30h and 48h. The samples were analyzed for the concentrations of the prodrug remaining unhydrolyzed and the pseudo first-order hydrolysis rate constant was determined. The degradation studies were conducted in triplicate.

Determination of Bioconversion rate

The bioconversion rate of the prodrug was determined both in rat plasma and in human plasma at 37°C. Specifically, KCC prodrug aqueous solution of appropriate concentration was prepared and spiked into freshly collected rat plasma such that the final drug concentration in the plasma was around 45 µg/mland the spiked volume was around 5% of the plasma volume. Immediately after spiking, the plasma was kept in a constant temperature water bath shaker maintained at 37°C. 100µl of sample was taken from the plasma at 0 mins, 2 mins, 5 mins, 15 mins, 30 mins, 1h and 2h, and spiked into 1ml methanol. Then 10ul of a 1M solution of Trifluoroacetic acid in methanol was added. The samples were centrifuged and supernatents were analyzed for amount of prodrugs remaining over time by HPLC method. The same procedure was followed for determining the bioconversion rate of the prodrug in Human plasma. All the bioconversion experiments were conducted in triplicate.

Determination of plasma protein binding

The plasma protein used for the determination of the binding properties of the drugs was Bovine Serum Albumin. A 4% bovine albumin solution was prepared in PBS buffer pH 7.4 for the study. Initially, drug solution prepared in purified water was spiked into the above prepared BSA solution. 5ml of this solution was then enclosed in a dialysis tube (MWCO 2000 – Spectra/Por®) and sealed tightly. The study was initiated by submerging this pouch into 5ml of PBS buffer, and followed by continuous agitation. Aliquots were taken after 5 hours of incubation, and the concentration of the drug, inside (C_{in}) and outside (C_{free}) the pouch were determined by HPLC. .

The C_{in} value would be the summation of drug concentration bound to the protein and free drug, i.e.

$$C_{in} = C_{bound} + C_{free}$$

Hence, the % protein bound of the drug could be calculated as

$$\%\ drug\ bound = \frac{C_{bound}}{C_{in}} = \left(C_{in} - C_{free}\right) * \frac{100}{C_{in}}$$

In vitro flux study of KCC vs ketoprofen across Human Epidermal Membrane through passive diffusion and iontophoresis

The passive diffusion permeation study of KCC and ketoprofen was done on vertical Franz diffusion cells (0.63585 cm^2 surface area) maintained at a temperature of 37°C. Specifically, HEM was mounted on the diffusion cells and donor solutions at 26.58mM concentrations were added to the donor cell. For the ketoprofen donor solution, the pH was adjusted to pH 7.4 to obtain a clear solution, and in the case of KCC donor solution, the requisite quantity of the drug was weighed and dissolved in purified water. 300 µl of the solutions were added to the donor cell. The entire 5 ml of the solution from the receiver chamber was removed at 1, 2, 3, 4, 5 and 6hrs, freeze dried, and reconstituted appropriately to analyze in HPLC.

In case of iontophoretic *in vitro* permeation through HEM, the study was done in a water jacketed side-by-side diffusion cell with a diffusion area of 1 sq cm. The HEM was mounted on the diffusion cells, and PBS buffer pH 7.4 added to the receiver chamber. For the donor chamber, 26.58 mM concentration of drug solutions were prepared for each of

the drug entities as mentioned above. 2ml of these donor solutions were added to the donor chamber.

The direct current source device and Ag/Agcl electrode system was used for carrying out the iontophoresis in the study. A cathodic electrophoresis setting was followed for permeation of ketoprofen and an anodic setting for KCC. A current of 0.2mA was applied across the HEM for a period of 6 h. The entire 2ml of PBS from the receiver chamber was removed at 1, 2, 3, 4, 5 and 6hrs, the chamber washed once and replaced with fresh PBS. The samples were taken and analyzed with HPLC method. Based on the amounts of the parent and the prodrug diffused per time, the flux values of each of these were calculated from the formula

$$J = \Delta Q/(A.\Delta t)$$

Where J is the flux value, A is the diffusion surface area, Q is the cumulative amount of the drug diffused into the receiver chamber and t is the time.

In vitro flux study of KCC vs ketoprofen across Rat Skin through iontophoresis

The skin was obtained from abdominal side of rat which was depilated using clippers and depilatory cream 18h before the study. The subcutaneous fat was thoroughly removed before using in the study. The iontophoretic permeation through rat skin was done on vertical Franz diffusion cells maintained at a temperature of 37°C. The rat skin was mounted on the cells and the donor solutions were used as mentioned. The donor solutions were prepared as mentioned above. Another set of solution was prepared with same formula as that of the KCC above with 1% phenylephrine. 600 µl of the solutions were added to the donor cell. The donor solutions were replaced every 2h. The entire 5

ml of the solution from the receiver chamber was removed at 1, 2, 3, 4, 5 and 6hrs and replaced. The solutions were analyzed as is using HPLC method.

In situ dual agar gel permeation study of ketoprofen vs KCC in Rat model through iontophoresis

The basis for the experimental set up was similar to the model developed by Sugibayashi[10], with a few minor modifications. Specifically, the agar gel plates were prepared using 0.5% agar dispersed in PBS pH 7.4, followed by microwaving in a semi closed scintillation vial, and pouring the clear matrix into vinyl disposable cryomolds with dimensions 25mm x 20mm x 5mm. The gels were then cooled at room temperature to obtain the finalized gel plates. Before using in the study, the plates were equilibrated at a temperature of 37°C.

The rat for the study was clipped and treated with depilatory cream on the ventral abdominal side, 12 hours before the study began. This time allows for the recovery of the skin integrity that might be lost to the hair removal process. An incision of 4 – 5cm length was made on the skin of the lower abdominal side, taking care that no major blood vessels are damaged in the process. The skin was separated carefully from the lower muscle tissues, and the subcutaneous fat removed as thoroughly as possible. The cavity was observed for the absence of any blood accumulation that might result due to the surgery. Two gel plates were inserted underneath the skin side by side, taking care that a distance of 15 to 20 mm was maintained between the plates. The incision was finally sealed with the help of Kelly forceps.

A glass donor cell with a diffusion area of 0.636 sq cm was attached to the skin just above one of the implanted gel plates using cast polyacrylate adhesive. 600µl of the donor solutions (26.58 mM ketoprofen with pH adjusted to 7.4; 26.58 mM for KCC in purified water and 26.58 mM for KCC in purified water with 1% phenylephrine) was added to the donor cell. The current carrying electrode was dipped into the donor solution and the return electrode was attached to the hind limb. For the iontophoresis of ketoprofen, the cathodic iontophoresis set up, and for KCC, anodic iontophoresis setup was used. A current of 0.2 mA was applied for 6 h, and the agar gels were collected every hours, diced and stored in glass scintillation vials for extraction. 0.5ml of blood was also collected at the hour, and centrifuged to obtain 0.2ml of plasma which was frozen till extraction. The donor solution was replaced every 2 hours.

Sample extraction and analysis

Plasma Extraction. The plasma samples were first taken and 100µl of 0.2M Trifloroacetic acid in purified water was added. The sample was frozen at -80°C and freeze dried. 5ml of methanol was added and kept on orbital shaker for 1 h. After agitation, the samples were centrifuged and maximum methanol was collected. The methanol extract was then dried and reconstituted with ACN : phosphate buffer pH 3.0 (30:70) and analyzed using HPLC method.

Agar Gel Extraction. The diced agar gel samples were taken and 500µl of 0.2M Trifluroacetic acid in purified water was added to it. These were then frozen at -80°C and freeze dried. After drying, 8ml of methanol was added to it, and shaken on an orbital shaker for 1h. These were then homogenized thoroughly, and further shaken on orbital shaker for 12 hrs. These samples were taken and filter centrifuged through paper filter

and the supernatant collected in glass vials. The precipitate was further washed with additional 1ml methanol and filtered and the methanol extracts combined. The extracts were dried and reconstituted with ACN : phosphate buffer pH 3.0 (30:70) and analyzed using HPLC method.

Results and Discussion

Hydrolysis rate, Bioconversion rate and Protein Binding

The Ketoprofen choline ester (KCC) prepared with the above mentioned procedure was found to have a purity of >99%, as tabulated from the HPLC chromatograms which showed no other peaks from impurities. In the chromatogram, the ester showed a significantly reduced retention time on a Polar RP column, which is probably due to the ionized quaternary ammonium group on the compound.

The degradation rate of KCC is shown in figure 1(A), where the intact drug concentration (normalized with initial concentration) is plotted over the experiment time. The linearity of the concentration observed on a Log concentration scale, indicates that the hydrolysis followed a pseudo first order dynamics in both the media. Table 1 indicates the $T_{1/2}$ of KCC in purified water and in PBS, tabulated from the slope of the concentration – time plot. The prodrug showed a significant amount of stability in both purified water and in PBS 7.4 at 37°C as indicated by the half-lives of > 2 days in these media, which suggests stable reliability of the solutions when used in *in vitro* and *in vivo* studies. As the de-esterification process of the ester bond might be due to nucleophilic substitution, there was a comparative stability of the prodrug in the slightly acidic purified water than in PBS pH 7.4.

The bioconversion rate of KCC was assessed, as being a prodrug, KCC is expected to convert back into its parent, ketoprofen, to show an activity profile. As seen in Figure 1(B), the profile shows a relatively susceptible prodrug when in the Human plasma, where not a trace of intact drug was observed at 2mins, whereas when in Rat plasma, it

showed relatively higher half-life of 9 mins. Even though the activity of rat plasma esterase enzymes are reported to be higher than that of the Human counterparts, in this particular case the choline ester prodrug might show structural affinity to the Human esterase isozymes than the rat isozymes, which is reflected in the < 2mins half-life in Human plasma. The 9 min $T_{1/2}$ ensures relative stability of the drug during the transit phase while permeating the rat skin, and would be converted to ketoprofen when in the deep tissues. The bioconversion profile was also shown to be linear on a Log concentration versus time plot, which indicates that the concentration of the drug did not saturate the plasma esterase enzymes in the study.

Invitro diffusion of KCC Vs Ketoprofen through HEM; Passive and Iontophoresis

The cumulative amount of the prodrug and ketoprofen were evaluated and contrasted both passively and through iontophoresis across Human Epidermal Membrane (HEM). The permeation profile of the passive delivery of the drugs are show in figure 2(A). In absence of influence of electric current, the permeation of ketoprofen was found to be much higher than that of the prodrug through HEM. In fact, no trace of the prodrug was observed in the receiver chamber until the 6^{th} hour. The reason for the insignificant values of drug permeated for KCC is due to the cationic charged moiety on the prodrug. With a reported pKa value of 13.9[11], choline would be 10^{-3}% unionized at a solution pH of 9. Whereas ketoprofen is stated to have a pKa of 4.45[12], hence it would be 0.1% unionized at a pH of 7.4. Hence, the higher permeation profile of ketoprofen might be due to the presence of greater percentage of permeable unionized species in the donor solution, as compared to that of KCC. Another factor that could add to the decreased flux

would be the attachment of the quaternary ammonium choline moiety that makes the molecule large and bulky, and in the absence of electric current, the added weight is only further slowing down the molecule.

However, on observing the permeation profile of the drug at iontophoretic conditions, a completely contrasting trend was observed. The flux values of ketoprofen increases 60 folds on application of iontophoretic current (Table 2), which can be attributed to the movement of ketoprofen cations in the electric field. However, the permeability of the prodrug KCC through the HEM in the anionic iontophoresis setup showed a 6 times greater flux value than that of ketoprofen. The reason for the magnified increase in the KCC flux as compared to ketoprofen would be found in the mechanism of movement of ions in the anionic setup. The ions not only move in the influence of the electric potential created, but there is also movement of ions due to electroosmotic migration, which is particularly profound for bulky groups as that of the cationic quaternary ammonium prodrug. Another aspect that would explain the higher flux of the prodrug would be absence of competing ions, which otherwise are generated as Cl^- ions in the cationic setup during delivery of ketoprofen. In case of flux of KCC, the prodrutg was found predominantly in the receiver chamber as the HEM lacks the esterase enzymes required to convert the prodrug to ketoprofen. The lag times for the appearance of KCC and ketoprofen are found to be comparable under iontophoretic conditions. This might be due to the restricted movement of the bulky quaternary ammonium prodrug in the solution medium. As compared to the passive diffusion, the lag time of ketoprofen did not change much from the lag time observed in the iontophoretic condition.

In the flux studies on rat skin, the permeation profile of KCC, with (figure 3(A)) and without vasoconstrictor (figure 3(B)), was compared to that of ketoprofen after cationic iontophoresis. Here, the iontophoresis of KCC alone, without the vasoconstrictor showed a 2.5 times higher flux than iontophoresis of ketoprofen. The data also shows a large majority of the drug on the receiver side to be the converted acid. This is due to the abundance of esterases present in the excised rat skin. However, the presence of the vasoconstrictor reduced the flux of the prodrug by 30% (Table 2). A valid explanation would be the cationic vasoconstrictor acting as a competing ion to the cationic prodrug, thus interfering with the flux of the ions.

In situ dual agar gel study of Ketoprofen vs KCC – with and without vasocontrictor

The cumulative amount profile of ketoprofen and KCC (with and without vasoconstrictor) observed in the agar gels after iontophoresis are shown in Figure 4. The data shown are obtained as the cumulative amount of the total drug in the agar gel over time. On plain observation, the total amount of drug obtained in the application gel in the anionic iontophoretic study with prodrug, was found to be 19 times higher than that in the

...Eqn 1

cationic iontophoresis of ketoprofen.. of the drug appearing in the application gel plates in both the iontophoretic conditions, the amount of drug observed in the application site agar ($\sum Tot_A$), is the summation of the prodrug amount ($\sum Kcc_A$), and the amount of converted acid ($\sum kt_A$).

$$\sum Tot_A = \sum Kcc_A + \sum Kt_A$$

The value for the amount of drug observed in the contralateral site was found to be close to 7 times higher in case of Ketoprofen iontophoresis as compared to KCC iontophoresis. The cumulative amount of drug observed in the contralateral gel ($\sum Tot_C$) for KCC iontophoresis, did not show any presence of KCC and consisted of only the parent drug ketoprofen ($\sum kt_C$).

$$\sum Tot_C = \sum Kt_C$$

The total amount of drug observed in the application gel would consist of drug directly penetrating from the donor cell ($^{DIR}\sum Tot_A$) as well as drug absorbed from the systemic circulation. No prodrug KCC was observed in the systemic circulation, and all the plasma concentration of the drug was contributed by converted ketoprofen. Hence, the drug in the application gel that is absorbed from the systemic circulation would be ketoprofen only, and would be comparable to the drug observed in the contralateral gel.

Hence, $^{Dir}\sum Tot_A = \sum Tot_A - \sum Kt_C$

Or, % of Total drug direct penetration (% Tot_{Dir}) = $\sum Tot_A - \sum Kt_C / \sum Tot_A$ …Eqn 2

And, % of free acid direct penetration (% Kt_{Dir})= $\sum Kt_A - \sum Kt_C / \sum Kt_A$ …Eqn 3

Table 3 shows the % contribution of drug in application gels by direct penetration. The data is calculated using the equations 1, 2 ad 3 above. It also shows the % of free parent drug in the application gel plates that is contributed by direct penetration. From the data, it can be observed that, almost 4 – 5 % of the drug amount observed in the application gel in the ketoprofen cationic iontophoresis study, was from systemic circulation. Where as in case of the prodrug iontophoresis (with and without vasoconstrictor), only 0.1% of the total drug observed in the application gel was from systemic circulation.. The increased level of acid in the contralateral side in case of ketoprofen iontophoresis can be explained by the protein binding results that are mentioned in Table 1. With a protein binding value of > 99%, ketoprofen is taken up by the microcirculation in the lower epidermis and dermis layer, and is tightly bound to the transport proteins. As a result, rather than being released into the deeper tissues or the gel, the drug is held tightly and circulated throughout the body. This is how, a large proportion of it was observed in the contralateral gel. On the other hand, KCC has a very low protein binding value, and even if taken up by the microcirculation, the drug would be released immediately, and would end up in the application gel. The only component that would be held tightly, would be the converted acid, which is the only entity found in the contralateral gel, and shows no initial intact prodrug.This data is also supported by the plasma drug content observed in both the cases, where ketoprofen, inspite of showing lower drug penetration, showed a higher plasma concentration as compared to that of KCC iontophoresis. Again, there was no parent intact prodrug observed in the plasma. . However, the presence of vasoconstrictor along with the prodrug was not found to increase the penetration of the prodrug (Figure 4 (C)). This suggests that vasoconstriction in this case did not actually

contribute to lower the systemic uptake of the prodrug. In fact, the appearance of the drug in the application side agar gel in this case was found to be lower than that in the study without vasoconstrictor. It can be noted that the ionic competition between the cationic vasoconstrictor and the prodrug plays a role in decreasing molecule transfer under electric potential as it did in the in vitro study. The plasma concentrations however did not show a contrast between the prodrug iontophoresis, with and without the vasoconstrictor, even though there was a distinct difference in the amount of drug appearing at the application agar gel site (figure 5). An explanation for this could be that the amount of drug taken up into the systemic circulation is too small an amount and gets further diluted into the volume of the total plasma and interstitial fluid. The analysis method is incapable of discriminating in the subtle difference in these concentrations in the plasma. Again, in the case of prodrug iontophoresis with vasoconstrictor, only the free drug was found to appear in the plasma and gel on the contralateral gel.

From the above results, it can be concluded that even though the iontophoresis process was found to increase the permeability of the parent drug ketoprofen from passive delivery, it did not increase the permeability as effectively as it did for the prodrug KCC. Anionic iontophoresis of the prodrug was found to show a more substantial drug delivery capability. In addition, the in situ studies showed that when delivering ketoprofen, a larger proportion of the drug was also observed in the contralateral side, which was also supported by the plasma protein binding study. This showed that, rather than being delivered and contained at the local site of application, ketoprofen had a greater tendency to being cleared away by the microcirculation and being diffused into other parts of the body. However, the delivery of KCC showed majority of the drug being retained at the

site of application and not being lost to the systemic circulation. The co-delivery of a vasoconstrictor however did not have the intended effect of further enhancing the drug penetration. Work on explaining this has been attempted, and preliminary studies have shown the prodrug to have inherent vasoconstrictor activity (Data not shown). A study with analyzing methylene blue as a marker for blood flow has shown less blood observed in the tissues treated with KCC iontophoresis. The same was not observed for ketoprofen. Hence, the anionic iontophoresis of the prodrug was simulataneously accompanied by vasoconstriction in the skin on the application side which is responsible for the negligible concentration in the plasma and at the donor side. Hence, due to this intrinsic property, the study with the co-administered vasoconstrictor did not show any difference.

The current dual agar gel rat model was chosen as an effective avenue to gauge the deep tissue penetration and systemic distribution of drugs after topical administration. However, the results obtained in this study do not really reflect the magnitude of the affect the prodrug has on deep tissue penetration. For one, the parent drug also showed a 95% contribution to the agar gel by direct penetration, but in the actual clinical scenario, the site of action is the synovial fluid which has to be reached after traversing a much longer treacherous path of skin, muscle and cartilaginous tissue. Here, the contributions of the prodrug would be realized to a much greater magnitude.

Hence in conclusion, the derivatization of the ketoprofen to a cationic prodrug not only enhanced the permeation rate of the drug under iontophoretic conditions, but the cationic charge also helped contain the drug at the site of application. The coadministration of a vasoconstrictor did not prove to be beneficial due to the potential vasoconstrictor activity of the prodrug itself which contributed to its deep tissue penetration properties. Thus the

derivatization to a cationic prodrug was found to be a viable approach for localized drug delivery for NSAIDS, showing a potential in reducing the adverse effects associated with higher plasma levels of the drug usually ecncountered in NSAID therapy. This ground work creates further avenues to explore this prodrug and other prodrugs with varying pharmacokinetic and physicochemical properties in a model that would reflect the drug performance in a more closer to clinical setting.

Conclusion

The conversion of ketoprofen to prodrug greatly reduced the protein binding property of the parent drug, and also greatly reduced the probability of the drug to be taken up by the microcirculation at the site of application. In addition, the cationic charge rendered to the drug, made it more efficient in being delivered through the stratum corneum and skin, under the influence of anionic iontophoresis conditions. The prodrug was also found to be converted to its parent drug in an in vivo setting and would potentially show the desired pharmacological effect.

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Tables

Table 1 Hydrolysis and Bioconversion rates of Ketoprofen choline Ester (KCC)

Study	Conditions	Values
Hydrolysis Half-Life of KCC at 37°C (in Hours)	In Purified water	81.6 (10.4)
	In PBS pH 7.4	53.7 (3.7)
Bioconversion Half -Life of KCC at 37°C (in Mins)	In Rat Plasma	8.7 (1.1)
	In Human Plasma	< 2
% Plasma Protein Binding	Ketoprofen	99.16 (0.003)
	KCC	8.87 (0.99)

Table 2 Summary of lag times and Flux of ketoprofen and KCC (with and without vasoconstrictor) through HEM and shaved rat skin when diffused passively through iontophoresis

Condition	Property	Drug		
		Ketoprofen	**KCC**	**KCC with Phenylephrine**
Passive Diffusion	Flux (umoles.h^{-1}.cm^2)	0.0025	8.5 x 10^{-5}	-
	Lag time (min)	22.5	> 300	-
Iontophoresis through HEM	Flux (umoles.h^{-1}.cm^2)	0.1463	0.8737	-
	Lag time (min)	27.9	28.1	-
Iontophoresis through rat skin	Flux (umoles.h^{-1}.cm^2)	0.348	0.844	0.600
	Lag time (min)	11.1	26.1	23.65

Table 3 % of cumulative drug in agar gel attributed to by drug from systemic circulation.

Time (h)		1	2	3	4	5	6
% drug in Cationic iontophoresis		97.73	97.05	96.39	95.68	95.02	94.13
% drug in Anionic iontophoresis without vasoconstrictor	% Tot_{Dir}	99.88	99.95	99.94	99.95	99.95	99.95
	% Kt_{Dir}	99.13	99.66	99.68	99.76	99.77	99.76
% drug in Anionic iontophoresis without vasoconstrictor	% Tot_{Dir}	99.64	99.76	99.76	99.77	99.80	98.18
	% Kt_{Dir}	98.27	98.91	99.06	99.16	99.26	78.46

Figures

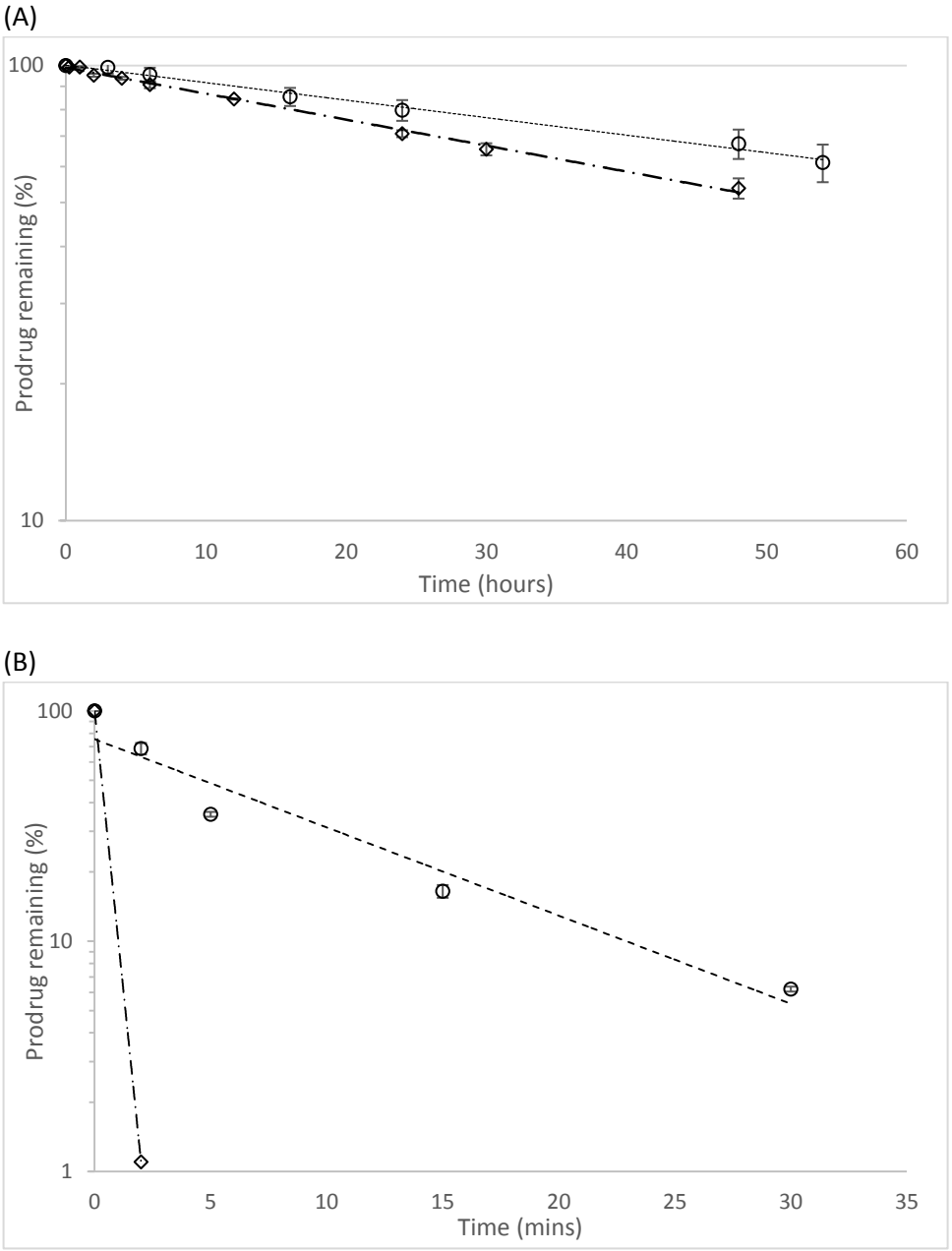
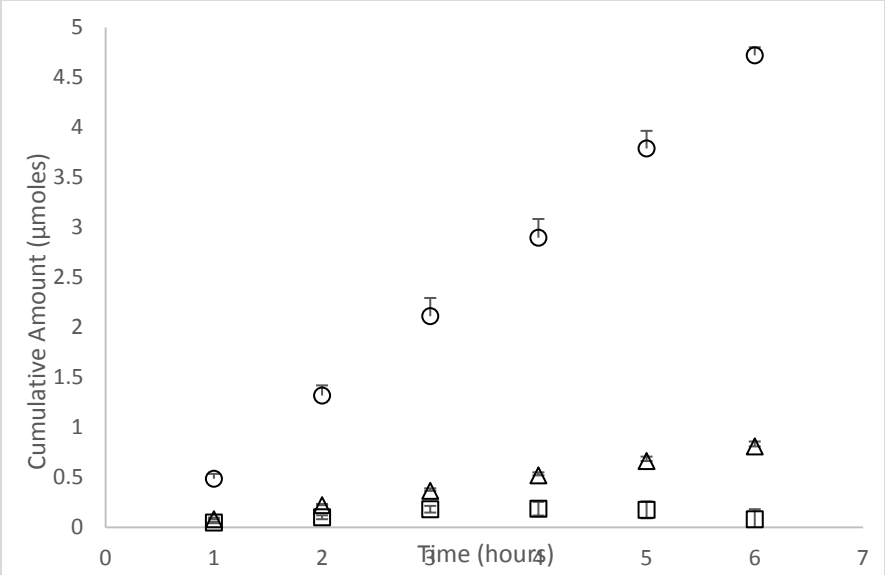


Figure 1 (A) The hydrolysis pattern of ketoprofen choline ester (KCC) in purified water (○) and in PBS 7.4 (◊) at 37°C; (B) The bioconversion rate of KCC in Rat Plasma (○) and in Human plasma (◊) at 37°C

(A)



(B)

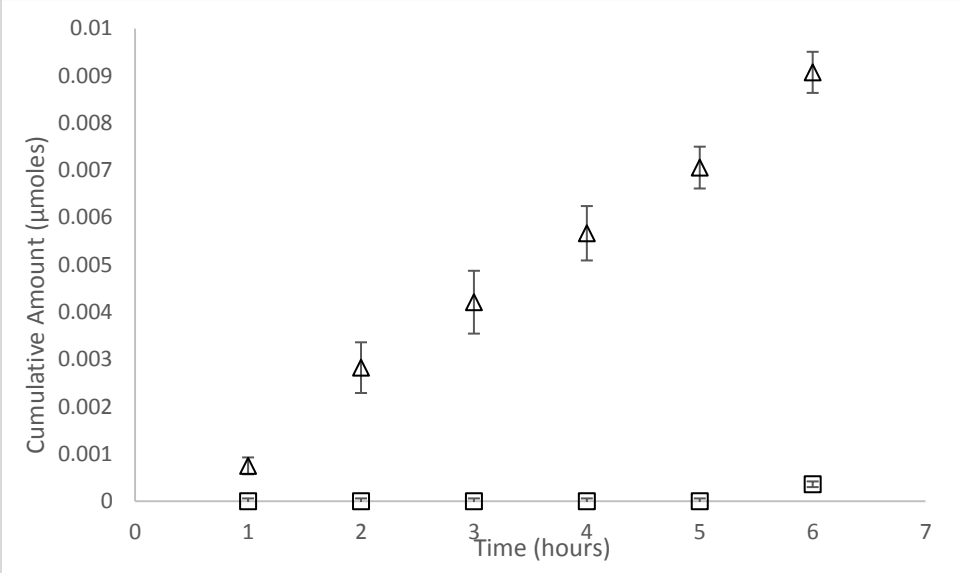
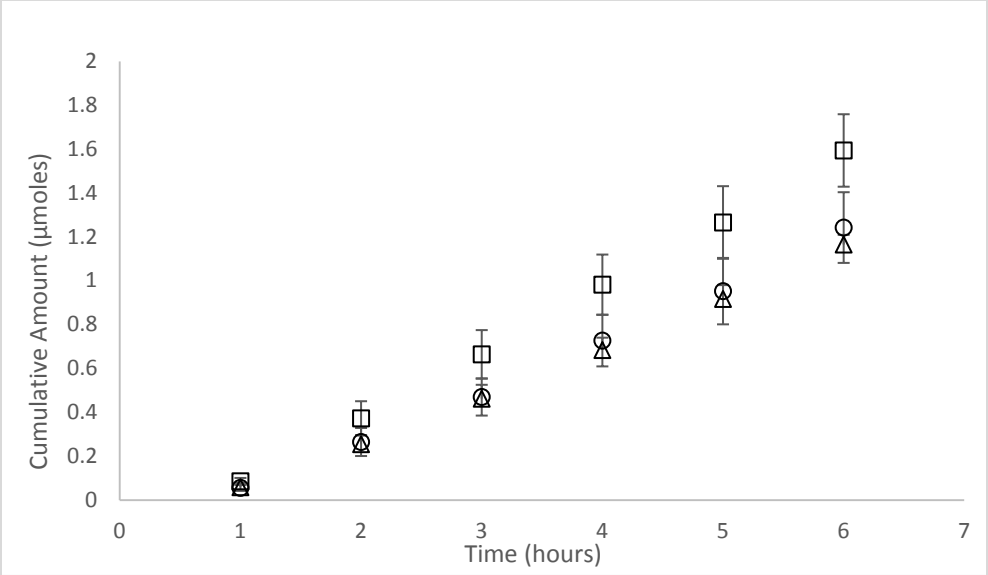


Figure 2 In Vitro diffusion profile of KCC (○) and converted acid (□) Vs Ketoprofen (◊) across HEM (Human Epidermal Membrane) (A) by Iontophoresis (B) By Passive Diffusion.

(A)



(B)

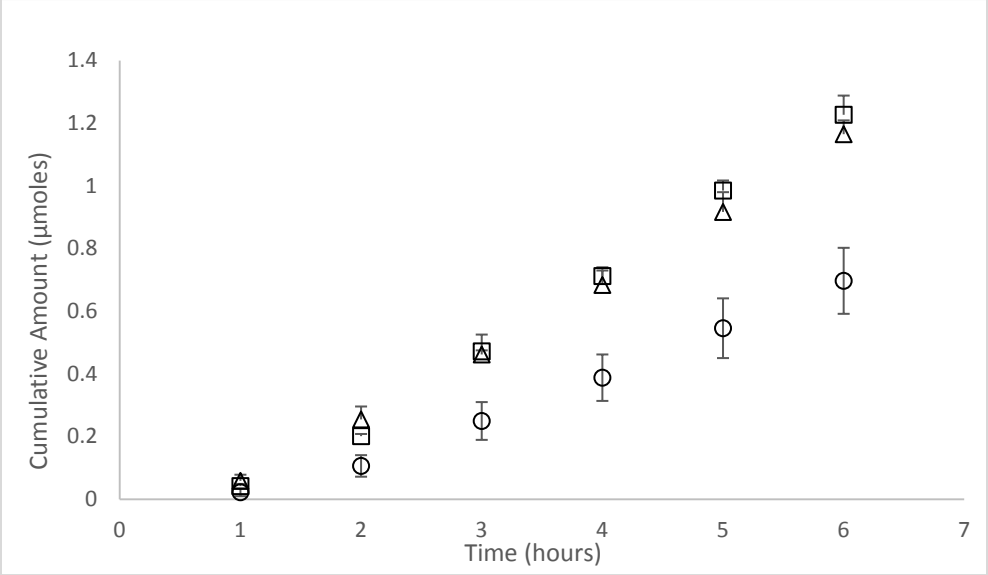
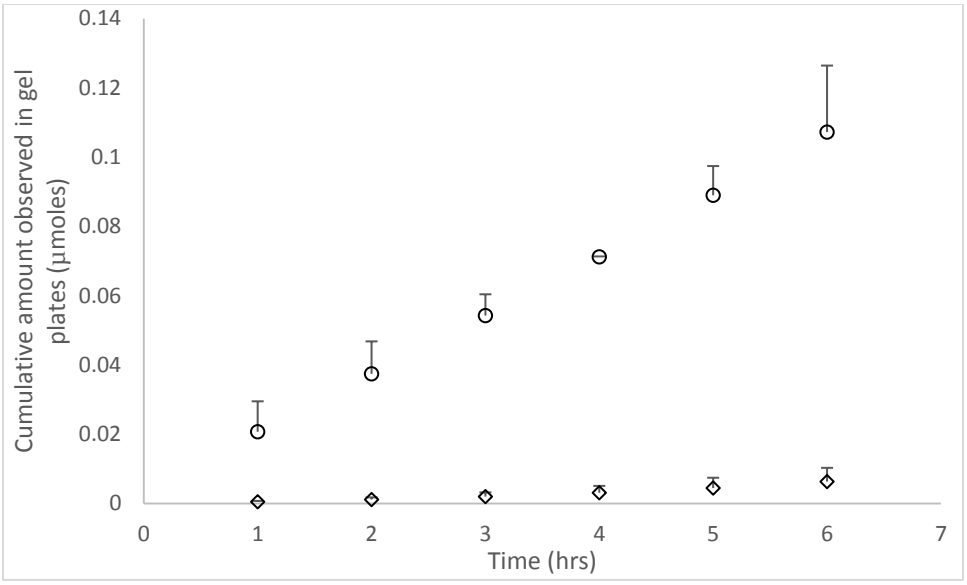
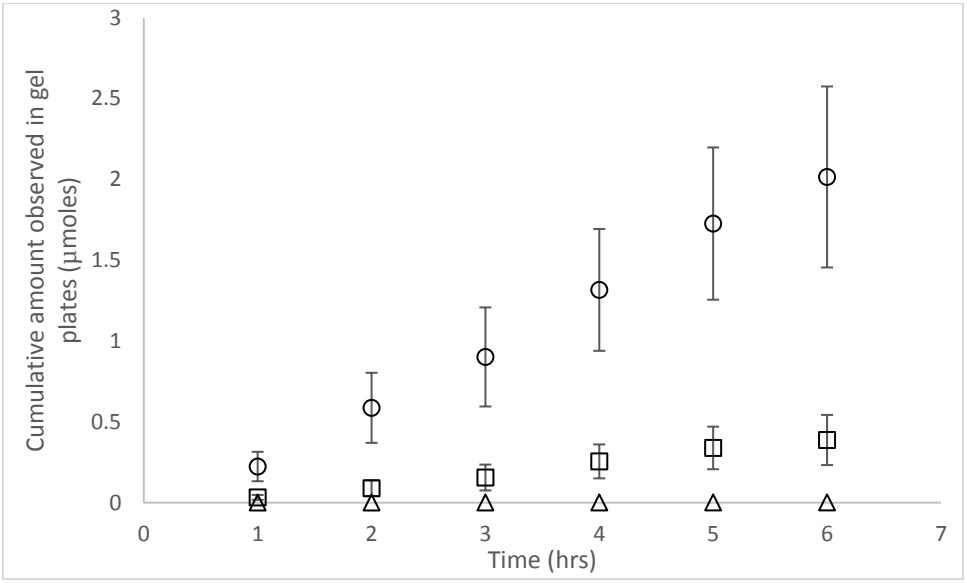


Figure 3 (A) In Vitro diffusion profile of KCC without vasoconstrictor (A) and with vasoconstrictor (B) showing prodrug KCC (○), converted acid (□) vs ketoprofen diffusion profile (Δ) across rat skin through iontophoresis at 0.2mA for 6h.

(A)


Cumulative amount observed in gel plates (µmoles)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
1
2
3
4
5
6
7
Time (hrs)

(B)


Cumulative amount observed in gel plates (µmoles)
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
6
7
Time (hrs)

(C)

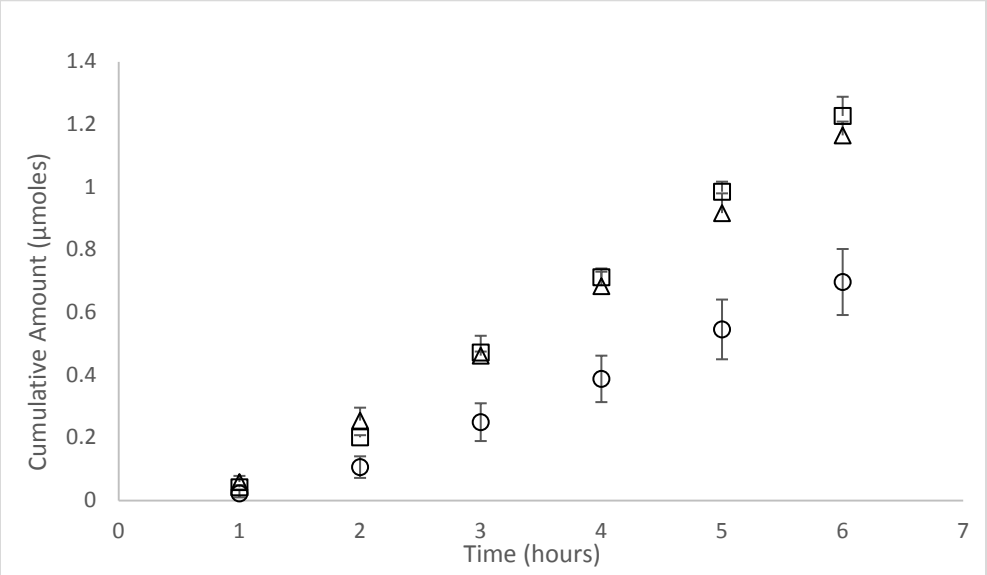


Figure 4 Time course of total amount of drug observed in the application gel showing KCC at donor site (○), ketoprofen at donor site and ketoprofen at contralateral site (Δ), during cationic iontophoresis of ketoprofen (A), anionic iontophoresis of KCC without vasoconstrictor (B) and anionic iontophoresis of KCC with vasoconstrictor (C).

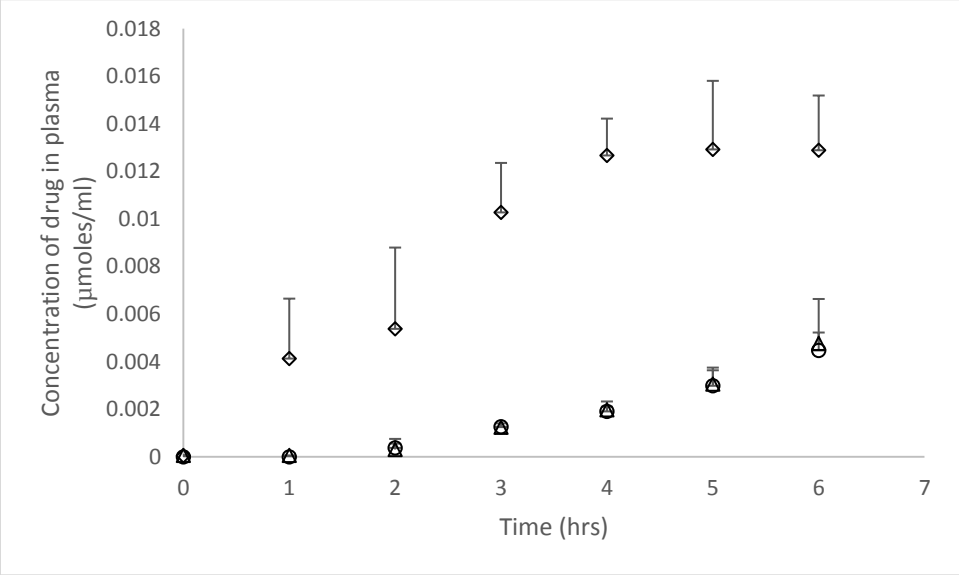


Figure 5 Plasma concentration of drug observed during the cationic iontophoresis of ketoprofen (◊), and anionic iontophoresis of KCC with vasoconstrictor (○) and without vasoconstrictor (Δ).

CHAPTER 5 Skin Disposition and Deep Tissue Penetration of Cationic Prodrugs of ketoprofen after Iontophoretic Delivery in Anaesthetized Rats.

This project was supported partly by the INBRE program, NIH Grant Nos P20 RR016454 (National Center for Research Resources) and P20 GM103408 (National Institute of General Medical Sciences)

Abstract

Our earlier study already showed a cationic prodrug KCC (ketoprofen choline Chloride ester) of ketoprofen to show improved drug delivery properties as compared to the parent through iontophoresis. In this study, we have prepared other cationic prodrugs KBCC (ketoprofen β-methyl choline chloride ester) and KPCl (ketoprofen 1-(dimethylamino)-2-propanol Hcl ester) with varied bioconversion rates to see how this parameter effects deep tissue penetration of the drugs. KBCC was shown to have a bioconversion half-life 100 times higher than KCC and KPCl showed a half-life 4 times higher than KCC. Between the prodrugs themselves, there was a difference in the performance in deep tissue penetration, and this was mainly attributed to the different bioconversion rates. KCC due to its faster bioconversion rate, could show sufficient deep tissue penetration as good as the other prodrugs when the tissues were collected at 2h, but on the longer wait period of 4h, the prodrug was able to show a majority deep tissue penetration only up to the depth of shallow muscles. A similar trend was also seen for the iontophoresis at lower current of 0.1mA, where a deep tissue penetration efficiency was shown only till the shallow muscle in case of KCC. KPCl also performed similar to KCC, where deep tissue penetration was observed in the tissues at the 2h time point, but after waiting for 4h, the deep tissue penetration was only restricted to the upper shallow muscles. However, because of its better resistance to enzymatic attack, KBCC showed better deep tissue penetration across all the iontophoretic conditions. But overall, all the prodrugs were found to show deeper tissue penetration as compared to the cationic iontophoresis of the parent drug.

Introduction

The Topical route has been explored for a long time as an alternative route to delivery of NSAIDs in treatment of ailments such as arthritis and muscle spasms. The biggest challenge in achieving this has been the skin itself, which is a very efficient barrier against influx of xenobiotics. The outermost hydrophobic layer called the Stratum Corneum is accredited with providing this protective property to the skin. However, as of today, various techniques have been explored to breach this barrier function of the skin, and one of these techniques that has been studied extensively is iontophoresis (Dixit N 2007). Our study in the earlier chapter has already explored the applications of this delivery system in delivering cationic prodrugs of an NSAID. The technique basically uses an externally applied potential across the skin to drive a charged molecule. Various NSAIDs have already been studied to evaluate their effectiveness when using iontophoresis as a delivery technique. However, these studies have evaluated the effectiveness of iontophoresis as a delivery method for NSAIDs by measuring the systemic concentration of the drug rather than at the local tissue (Tashiro Y 2001), or have compared effectiveness of iontophoresis on NSAID delivery against a control that had no drug application at all, and have used evaluation methods such as the Skin prick test which is not particularly a reliable method (S Hariharan 2006). Our earlier study was involved in the actual measurement of the drug that permeated through the skin into agar gels, which is a surrogate technique to measure drug retention in body tissues.

Another facet that we have tried to explore and address in the study is the aspect of localization of the NSAIDs. A majority of the NSAIDs used widely in ailments such as arthritis have a plasma protein binding value of greater than 95% (Borgå O 1997). And as

per extensive research conducted in the 1990's by a group in Queensland (Singh P 1996) (Roberts MS 1999), protein binding property of a drug is a major determinant in the uptake of the NSAID from the site of application, to the systemic circulation. They have also evaluated drug with different protein binding levels and have concluded to suggest that protein binding levels directly correlate to the drug concentrations at tissues away from the application site and thus indicate lower deep tissue penetration at the site of delivery (Cross SE 1996). They also have detailed studies done where they have related washing out of Diclofenac acid from tissues at the site of application with increasing flow rate of medium in blood vessels and increasing carrier proteins in the circulatory system (Wu ZY 1997). In our earlier study evaluating the permeation of our cationic prodrugs, we had observed that by altering the blood flow by the coadministration of vasoconstrictors, the systemic uptake and deep tissue penetration of the cations was not impacted in a major way. An explanation for this was the inherent vasoconstrictor activity of the cationic prodrugs itself. But we also saw that the amount of drug observed in the application side gel was many folds higher (18 times) for the prodrug than the parent.

This earlier study proves the cationic prodrugs to show greater capacity to be delivered through the skin, and also show higher localization tendency. In our attempt to further understand the localization process and deep tissue penetration, we have explored more prodrugs, which differ in their stability in the biological environment, and studied the impact of bioconversion rate on deep tissue penetration. In addition, our earlier method to evaluate deep tisuue penetration was a surrogate method (dual agar gel) to measure drug penetration. Here, despite it being an effective method to study drug localization and

deep tissue penetration, the path of penetration for the drug was only the epidermis and dermis, which is merely a few mms. So in this study here, we will evaluate the performance of the drug in a more real scenario, evaluating the penetration of the prodrugs through the different layers of tissue underneath the skin. The penetration of the prodrugs through these different heterogeneous tissues will reflect better of their performance when the drug has to penetrate to the deeper tissues of the joints and muscles in a realistic clinical situation.

Materials and Animal Model

Ketoprofen was obtained from TCI ltd (Tokyo, Japan), Choline chloride was obtained from J.T.Baker (Phillipsburg, NJ), β-methylcholine Chloride was obtained from TCI (Portland, OR), N,N'-dicyclohexylcarbodiimide (DCC) and 4-pyrrolidinopyridine were obtained from Sigma (St. Louis, MO), Silica Gel ultra pure 40-60 µm was obtained from ACROS organics (Fair Lawn, NJ), Dichloromethane was obtained from Fisher Scientific (Fair Lawn, NJ), Acetonitrile was obtained from EMD chemicals Inc (Gibbstown, NJ), Methanol was obtained from BDH chemicals (Radnor, PA), Trifluroacetic acid was obtained from Honeywell Riedel-de-Haen (Seelze, Germany) and Human plasma was obtained from Interstate Blood Bank Inc (Memphis, TN). The Bovine γ-globulin was obtained from Pel-Freez Biologics (Rogers, AR). The Rats used for the study were Sprague Dawley Rats bred and reared at the Animal Care Facility at Idaho State University.. The direct current source used in the experiments was Activa Dose® II Controller Ionto Device purchased from ActivaTek Inc (Salt Lake City, Utah)

Experimental

Preparation of Prodrugs

The prodrug KCC was prepared as mentioned in the earlier chapter and was based overall on an earlier research work done based on preparation of prodrugs of Diclofenac (Lobo S 2014). Ketoprofen β-methylcholine Chloride ester (KBCC) was prepared similarly but with the following changes. Specifically, β-methylcholine (0.00353 moles) was weighed accurately and dissolved in acetonitrile at room temperature. This was followed by addition of 0.00118 moles of ketoprofen. 0.054 mmoles of 4-pyrrolidinopyridine was added after ketoprofen was dissolved. The reaction initiated by addition of 0.00146 moles

of N,N'-Dicyclohexylcarbodiimide and the reaction mixture was kept under continuous stirring for 18 hours. The white precipitate formed was filtered by passing through non-absorbent cotton. The reaction vessel was further washed with 10ml anhydrous acetonitrile and combined with initial filtrate. The acetonitrile was evaporated under vacuum and the resultant oil was solubilized in dichloromethane and purified by flash chromatography using a combination of Dichloromethane, acetonitrile and methanol at a ratio of 70:20:10

For the preparation of ketoprofen 1-(dimethylamino)-2-propanol HCl (KPCl), 1-(dimethylamino)-2-propanol (0.00353) was taken and dissolved in 3ml of dichloromethane. This was followed by 0.00118 moles of ketoprofen. 0.054 mmoles of 4-pyrrolidinopyridine and 0.00146 moles of N,N'-Dicyclohexylcarbodiimide were added and kept for stirring for 18 hours. The white precipitate was filtered and the filtrate was dried under vacuum. The resultant oil was purified on normal phase silica gel using Dichloromethane, Acenitrile and methanol at a ratio of 70:20:10. After purification, the oil was acidified to a pH of 4 using cold dilute HCl, and then freeze dried to obtain the pure KPCl salt.

Determination of degradation rate

The degradation rate of KCC was already determined in the earlier paper. In a similar way, the degradation rate of KBCC and KPCl was also determined in PBS pH 7.4 at 37°C. Samples were withdrawn and analyzed for prodrug remaining at 3h, 6h, 12h, 24h, 36h, 48h and 84h. The results for the prodrug at different time points were scrutinized for the determination of the pseudo first-order hydrolysis rate of the ester. The study was conducted in triplicate.

Determination of Bioconversion rate

The bioconversion rate of KCC in rat plasma and human plasma was already determined in the earlier chapter. In a similar way, the bioconversion rate of KBCC and KPCl were also determined in the two plasma milieu. Specifically, a solution of KBCC/KPCl in purified water was spiked into the plasma such that the final drug concentration in the plasma was 45µg/ml. In addition, care was also taken that the spiked volume did not exceed 5% of the plasma volume. The reaction mixture was kept in a constant temperature water bath and continuously agitated on a reciprocating platform. Aliquotes were taken at 0m, 5m, 15m, 30m, 60m, 2h, 12h, and 24h, and spiked into methanol. The methanol was also acidified with 10ul of 1M methanolic TFAA solution. The samples were then centrifuged and taken for analysis on HPLC. The samples were analyzed for the amount of intact ester remaining in the plasma over time, and the data was used to plot a semi log plot of the bioconversion profile to give the degradation constant and half-life. This procedure was used to analyze the concentrations in both rat and human plasma.

Determination of Protein Binding in both Bovine serum albumin and bovine γ-globulin

The plasma protein binding evaluation procedure was the same as carried out in the earlier paper for KCC using 4% bovine albumin solution in PBS buffer and 4% bovine γ-globulin in PBS. The procedure was followed and the C_{in} and C_{out} concentrations were analyzed using HPLC. The % protein binding was calculated in a similar way using the formula,

$$\%\ drug\ bound = \frac{C_{bound}}{C_{in}} = \left(C_{in} - C_{free}\right) * \frac{100}{C_{in}}$$

Iontophoretic delivery of drugs in rat

The blood for the degradation studies were collected from Female haired rats by cardiac puncture. The in vivo studies were performed on 9 female haired rats weighing between 250 – 370 g. During the iontophoresis procedure, the rats were anaesthetized, and after the application, the rats had free access to food and water throughout the duration of the study. 12 hrs before the actual study, the rats were shaven on the dorsal side and the hairs were reduced to a minimum taking care that the skin was not damaged during the procedure. Depilatory cream was applied and any remaining hair removed and the area washed away thoroughly. The rats were anaesthetized before the study and kept on a constant temperature blanket maintained at 37°C. A glass cell of diameter 15mm was attached to the dorsal side of the rat 1 – 1.5 cm off the vertebral line. For iontophoresis, the cells were filled with either the ketoprofen solution (26.58 mM, solution adjusted to pH 7.4) or the ester solutions (26.58 mM aqueous solutions for KCC, KBCC, and KPCl in purified water). The return electrode was attached to the fore limb on the same side as the side the donor cell is attached. Three different iontophoretic conditions were studied on the rats, one at 0.7mA for 2h followed by separation of the tissues, the second at 0.7mA for 2h and collection of tissues at the 6h time point from start of study, and the third at 0.1mA for 6h followed by collection of tissues. Blood samples were collected at appropriate time points by tail bleeding, and centrifuged at 6000 rpm for 6 mins before collection of plasma.

Collection of Tissues

Before the collection of tissues, the area of drug application was treated as follows. Firstly, the area of application was washed thoroughly with wet Kim wipes, and tape

stripped once with standard 3M Scotch® Magic™ tape. The rats were then sacrificed by cervical dislocation. They were then placed in a clean cloth in a box filled with finely crushed solid ice. After the partial freezing of the rat tissues, a 16mm biopsy punch was used to punch out the different layers of tissues underneath the area of application. The freezing process was done to ease the excision process and separation of the tissues. The punch was forced gradually and tissue separated sequentially through the different tissue layers to prevent contamination of the lower layers with tissue from the upper layers. Similarly, tissue excisions were also removed from the contralateral side. The samples collected were in four levels – the Dermis, subcutaneous layer + fascia, shallow muscle and deep muscle. After collection the samples were frozen until further processing.

Extraction of tissues and analysis

The tissues collected were first frozen in liquid nitrogen, and then crushed in a cold S.S mortar and pestle. The crushed tissues were then collected in a large test tube and weighed for the amount of tissue crushed and transferred. The crushed tissue was then extracted with 10ml of methanol over 18 h with continuous shaking on an orbital shaker. This whole procedure was done in the cold room at 2-8°C. After the extraction, the clear methanol was separated into test tubes and evaporated to dry. This residue was then further extracted with a Dichloromethane: methanol mixture (80: 20) over 3 h on a multi-tube vortexer mixer. This clear extract was then evaporated to dry. The residue was reconstituted with mobile phase and analyzed for amount of drug by HPLC method.

Results and Discussion

Synthesis

The preparation and purification of the KCC salt is mentioned in the earlier paper. In a similar way, the the ketoprofen β-Methylcholine ester and the Ketoprofen 1-(dimethylamino)-2-propanol was obtained with a purity of > 99% as seen from the chromatogram that showed no other peaks of parent drug or impurities. The retention time of quaternary salt prodrug prepared was also found to be considerably reduced on the Polar – RP column as was seen for the KCC salts, but showed a slightly higher retention time of 4.6 mins as compared to the 4.2 min retention time showed by KCC. The slightly higher retention time could be attributed to the comparatively higher molecular weight of the prodrug. The retention time of KPCl was also found to be comparable to that of KBCC.

Degradation Rate of esters

A comparison of the hydrolysis rates for the newly synthesized KBCC molecule and the earlier prepared KCC molecule is represented in Table 1. A distinct increase in the half-life (6 times) is observed for the KBCC molecule in PBS. An apparent account for this increase would be the branched methyl group at the α- position to the ester bond.

The electron donating methyl group at the α-Carbon would make the adjacent O- more basic and thus the ester bond becomes less prone to nucleophilic substitution, which is the predominant path for de-esterification for the molecule. Figure 1 (A) indicates the trend of hydrolysis observed during the study. The amount of ester remaining unchanged shows a linear trend on an exponential scale versus time for the ester as was the case

earlier with KCC. This indicates that the hydrolysis process is a pseudo-first order, as was observed in the case of the degradation of KCC.

A similar trend is also observed in the plasma degradation results for KBCC, where the molecule showed a half-life much higher than that of KCC [figure 1(B)]. In both the rat plasma and the human plasma, the half-life for KBCC was found to be more than a 100 times longer as compared to KCC. The steric hindrance due to the branched methyl group apparently makes the ester bond less prone to the enzymatic attack of esterases in the plasma. In case of KPCl, the reduced half-life to that of KBCC could be due to the reduced steric hindrance of the tertiary salt. Comparatively, amongst the plasma media, for KBCC, the human plasma showed the ester to have a shorter half-life as compared to the rat plasma. A similar trend was also observed in the case of KCC, and the affinity for the human plasma esterases for these choline moieties would be a rational explanation for the relatively lower half-lives. On observing the bioconversion trend in both the plasma media, it is evident that the bioconversion process follows a pseudo first order pattern.

Protein binding of esters

The bovine albumin used in the study is the predominant transfer protein in the plasma, and accounts for 3.5 to 5% of the total composition of blood and 55% of the total blood proteins. The bovine serum albumin used in our study is comparatively very similar to the human serum albumin, and only differs in the presence of one single additional tryptophan residue in the BSA and is used as a model protein for this study due to its cost viability and ready availability. As was expected for a large number of NSAIDs and more specifically for ketoprofen, the molecule in our earlier study showed a protein binding value of close to 99% as was mentioned in literature. The KBCC molecule showed a

protein binding much lower and was found to be comparable to the protein binding value of KCC. A similar justification could be attributed to this reduction in protein binding tendency i.e. the masking of the carboxyl group and attachment of a quaternary ammonium moiety.. Hence, albumin is the predominant transport protein for NSAIDs such as ketoprofen.

Iontophoretic delivery of drugs on hairless rats

This study was carried out on the dorsal side of the rat due to the presence of substantial muscle tissue in the region, which makes it viable for adequate tissue collection. The contralateral tissue collected too was collected from a similar position off the spine as that of the donor position to minimize for any regional variability in blood supply and tissue composition. In addition, special care was taken in the study, in that every sequential tissue was punched one at a time and removed, to minimize any tissue carry over to the subsequent layers due to the punch penetration. Also, the punch was thoroughly washed and cleaned before every punching process. The freezing of the carcass in dry ice was done to make the punching of the dermis easier and manageable.

The iontophoresis conditions were planned taking care that the $0.5mA/cm^2$ threshold for the pain tolerance to the human skin was not crossed. The tissue concentrations for the three conditions are elaborated in figure 2. At a direct current of 0.7mA, when the current was applied for 2h, the dermal layer concentration for the prodrugs were found to be considerably higher than the concentration of ketoprofen iontophoresis. The dermal concentrations were found to be approximately 20 times higher than that observed in iontophoresis of ketoprofen itself (figure 2(A), 3(A), 4(A)). The iontophoretic delivery of the prodrugs to a depth to the subcutaneous and fascia layer was found to be even more

efficient, where the concentration in the tissues were found to be 60, 90 and 20 times higher than the parent for KCC, KBCC and KPCl respectively. However at a depth of the shallow muscles, the contrast in the tissue concentrations becomes less significant, where both KCC and KPCl showed a 2 fold higher and KBCC showed a 4- fold higher concentration than that observed in cationic iontophoresis of ketoprofen. When the donor side deep tissues were evaluated, there was no significant difference observed in the concentration between the parent and the prodrugs at this iontophoretic condition. However, when observing the tissue concentrations on the contralateral side, an account of the percentage of the drug contributed to these tissues by direct penetration (equation 1) versus through systemic circulation was tabulated (Table 2).

Or, % of Total drug direct penetration (% Tot_{Dir}) = $\sum Tot_A - \sum Kt_C / \sum Tot_A$

where $\sum Kt_C$ is the concentration of drug in nmoles/gm of tissue on contralateral side and $\sum Tot_A$is the total drug concentration at the site of delivery.

It can be observed that a majority of the drug reaching the shallow and deep muscle tissue in the case of ketoprofen iontophoresis at 0.7mA for 2h is through systemic circulation. However, in case of the esters, KCC shows more than 75% of the drug in the shallow and deep muscles through direct penetration and in case of KBCC, all of the drug here reaches through direct penetration. KPCl too showed more than 90% of drug through direct penetration, and close to 70% of direct penetration in the deep muscle. In the case of KCC, the drug that is attributed to systemic circulation is only contributed by the free acid because only the free acid is observed on the contralateral site, and this is the only way the drug would ester the systemic circulation. This could also be an explanation for

the absence of drug in contralateral site in case of KBCC, because an insignificant amount of the prodrug is converted to the free acid in the study period of 2h. This clarification is further supported by the plasma levels of the drug (Figure 3(A)) for 0.7mA for 2h, which shows no presence of free acid in the case of KBCC, however shows a 6-8 times higher ketoprofen concentration for ketoprofen iontophoresis as compared to KCC iontophoresis.

In the second iontophoretic condition, i.e. 0.7mA iontophoresis for 2h followed by a wait period of 4h (Figure 2(B), 3(B), 4(B), 5(B) a contribution to deeper tissues by passive diffusion was anticipated from the drug rich dermis and subcutaneous tissues. However, in case of iontophoresis of ketoprofen, a decrease in the drug concentrations was observed in all the tissues as compared to the study without wait period. An explanation for this could be obtained from the plasma drug concentration data, where in case of ketoprofen iontophoresis, a decrease in drug concentration was observed after 2h due to drug metabolism in liver subsequent to continuous washing away of drug from site of application. In case of KCC, an increase in plasma concentration of ketoprofen was observed probably because of the continuous degradation of the depot KCC at the site of application, especially dermis, which was also reflected in the increased contralateral concentration of the drug as compared to the 2h study. The table 3 also supported this explanations as the systemic contribution to deeper tissues in case of KCC delivery was mainly through systemic circulation at end of 6h, close to 50% of the drug in the shallow muscles was attributed to direct penetration. However, in case of KBCC, there was a 4 fold and 2.4 fold increase in the subcutaneous and shallow muscle concentrations of drug in the delivery site as compared to 2h results. This can be attributed to the considerably

longer half-life of the prodrug, which prevents the bioconversion and subsequent washing away of the free drug into the systemic circulation, which prolongs the depot effect at site of application. This is also reflected in the plasma level data for the free drug, as well as the % systemic contribution which shows a more than 90% direct penetration to till the shallow muscles. KPCl also showed a trend similar to KCC, where a depot effect from the 2h ionotophoresis process could not maniest into higher tissue concentrations after waiting for 4h, again due to the short half-life of the prodrug.

When the iontophoretic conditions were reduced to a level of 0.1mA, ketoprofen showed direct penetration only till the upper dermis, and showed majority of the drug below the dermis to be contributed by systemic circulation (Figure 2(C)). KCC at this current level, showed a 3.2 times higher concentration of drug in the dermis as compared to the tissue concentration at 6h after 2h of 0.7mA iontophoresis. However, there was no real distinction between the concentrations in the deeper tissues between these two conditions. The reason for this could be because after delivery of drug at 0.7mA and a wait period of 4h, the larger depot load of KCC at the dermis and subcutaneous tissue, allowed for the bioconversion and transfer of free acid to the deeper tissues by systemic circulation, as can also be deciphered from Table 2, where the contribution to deeper tissues was larger in this case, as compared to when delivered at 0.1mA. KBCC, on the other hand showed deep tissue penetration even at this current level up to a depth of deep muscles where less than 15% of the drug was attributed to by systemic circulation. The blood plasma levels of ketoprofen in the KBCC iontophoresis study showed comparable levels when delivered at 0.7mA for 2h and a wait of 4h, or 0.1mA for 6h. The reason for this is that

the enzymatic degradation is the major determinant in the diffusion of the drug through the body, and is independent of the prodrug concentrations in the tissues.

In the earlier chapter, the study on the dual agar gel model showed a considerable increase in the drug collected in the application gel for the cationic prodrug as compared to that of the parent drug iontophoresis. In the current study, where the drug had to penetrate across a heterogeneous medium of tissues, the distinction in the drug concentrations from the application side to the contralateral side diminished with the depth. And this distinction varied hugely depending on the bioconversion rate of the cationic esters and the current applied.

From the tissue concentration data, all the prodrugs showed a capability to perform better than the parent drug in terms of deep tissue penetration. This enhancement was comparable for KCC, KPCl and KBCC at a current of 0.7mA for 2h, as the iontophoresis conditions was strong enough to push the prodrugs deeper in to the tissues, and the duration of 2h was short enough for the KCC prodrug to evade bioconversion and subsequent transfer of converted acid to the contralateral site. However, the wait period of 4h allowed for sufficient prodrug to be degraded and appear in the contralateral site, and showed deep tissue penetration only to a depth upto the shallow muscle. The 0.1mA current was a little lower for showing sufficient deep tissue penetration, and thus showed only accounted for 50% of the drug at shallow muscle by direct penetration. The KBCC prodrug showed deep tissue penetration potential across all the iontophoretic conditions, and proved to be a very effective way of localizing the drug at the site of application.

KPCl, inspite of its comparatively faster degradation than KBCC, still showed good deep tissue penetration properties in the 2h study.

From the above data, evaluating the results of the different types of cationic prodrugs, it can be concluded that the bioconversion rate of the cations dictated greatly, the deep tissue penetration of the drug. The deep tissue penetration of the drugs were unaffected by the nature of the cations i.e. if it was a tertiary or a quaternary amine. However, the tertiary amine was found to show a lower stability in blood plasma, which affected its deep tissue penetration properties. The quarternary cation (KBCC) showed a greater deep tissue penetration property as compared to the tertiary amine (KPCl).

Overall, this approach of derivatization and iontophoretic delivery seems to be a very promising solution to counter adverse effects associated with topical therapy using NSAIDs. The prospects of these prodrugs looks very promising in later studies to be done on higher mammals to further support their benefits. Future studies will revolve around evaluating the performance of the prodrugs in an actual arthritic model, and see how these prodrugs would work on alleviating the pain symptoms of the disease.

Conclusion

The prodrugs were found show an enhanced deep tissue capability when delivered under an electric current as compared to the parent drug. In addition, considerably lower amounts of the drug were observed in the plasma in the rat model, which was also reflected in lower drug concentrations in the tissues on the contralateral site. This shows that not only was the prodrug derivatization of the NSAID to a quaternary compound effective in delivering more drug to the tissues at the application site, but also reduced the systemic presence of the drug, hence showing potential for lesser adverse effects.

Tables

Table 1 Hydrolysis, Bioconversion rates and plasma protein binding values of Ketoprofen choline Ester (KCC), ketoprofen β-Methylcholine ester (KBCC) and Ketoprofen

Study	**Hydrolysis rate at 37°C in PBS pH 7.4**	**Bioconversion rate at 37°C**		**Plasma Protein Binding**	
		In rat plasma	**In Human Plasma**	**Bovine serum albumin**	**bovine γ-globulin**
	(in hours)	(in mins)	(in mins)	(%)	(%)
Ketorofen*	-	-	-	99.16 (0.003)	0.57 (0.2)
KCC*	53.7 (3.7)	8.7 (1.1)	< 2	8.87 (0.99)	1.69 (0.26)
KBCC	306.7 (16.3)	1332 (186)	216 (10.2)	8.4 (2.6)	2.30 (0.12)
KPCl	174.2 (80.6)[#]	34.4 (0.3)	30.5 (2.7)	-	-

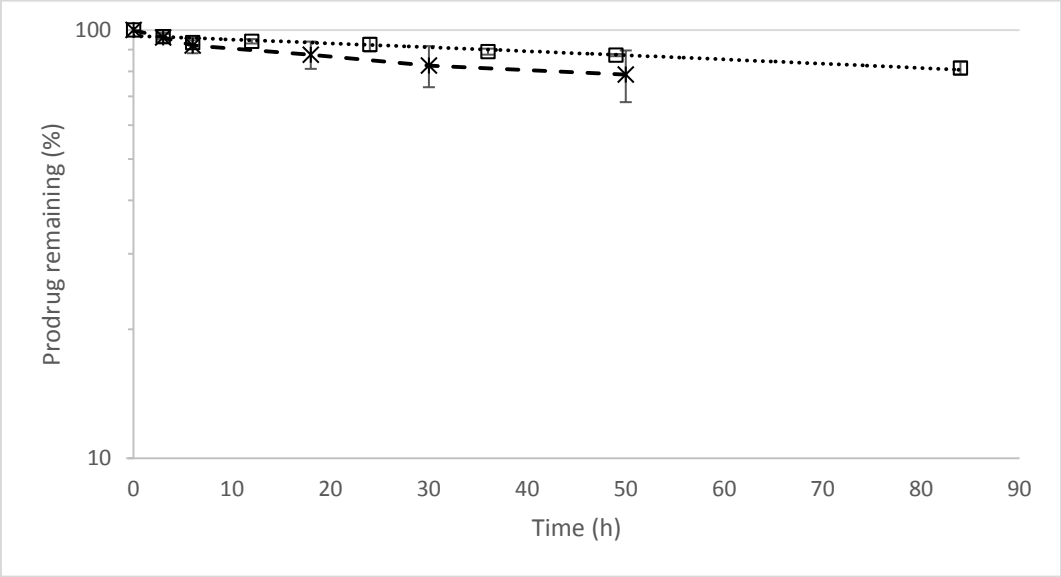
* Data from the previous study[#] done in pH 4.5 acetate buffer

Table 2 Percentage of drug concentration in tissues attributed to direct penetration

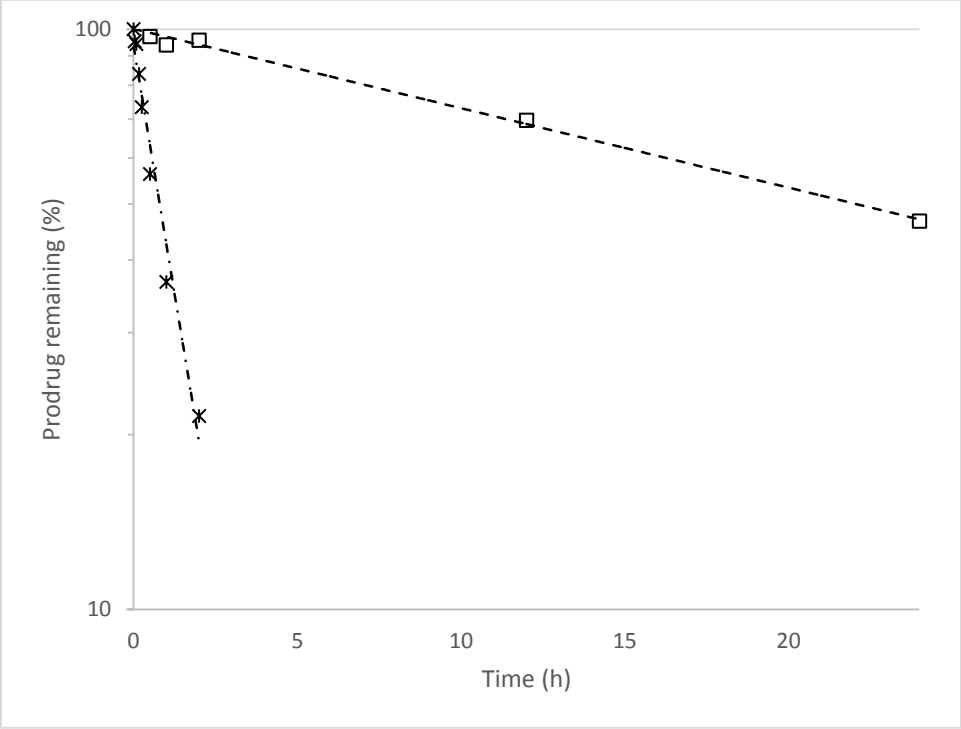
% of drug in tissues attributed to direct penetration											
Iontophoretic condition	**0.7mA for 2h**				**0.7mA for 2h, followed by wait period of 4h**				**0.1mA for 6h**		
	Keto	**KCC**	**KBCC**	**KPCl**	**Keto**	**KCC**	**KBCC**	**KPCl**	**Keto**	**KCC**	**KBCC**
Dermis	97.9	100	100	100	97.0	99.2	100	99.9	97.8	99.9	100
Subcutaneous + Fascia	5.0	99.8	100	99.6	54.4	95.3	99.9	99.3	3.7	98.3	99.9
Shallow muscles	0.0	86.4	100	97.3	0.0	46.5	93.1	80.7	32.7	50.2	96.4
Deep muscles	0.0	76.9	100	69.8	0.0	7.0	68.9	49.9	4.2	29.4	85.9

Figures

(A)


100
10
Prodrug remaining (%)
0
10
20
30
40
50
60
70
80
90
Time (h)

(B)


100
10
Prodrug remaining (%)
0
5
10
15
20
Time (h)

(C)

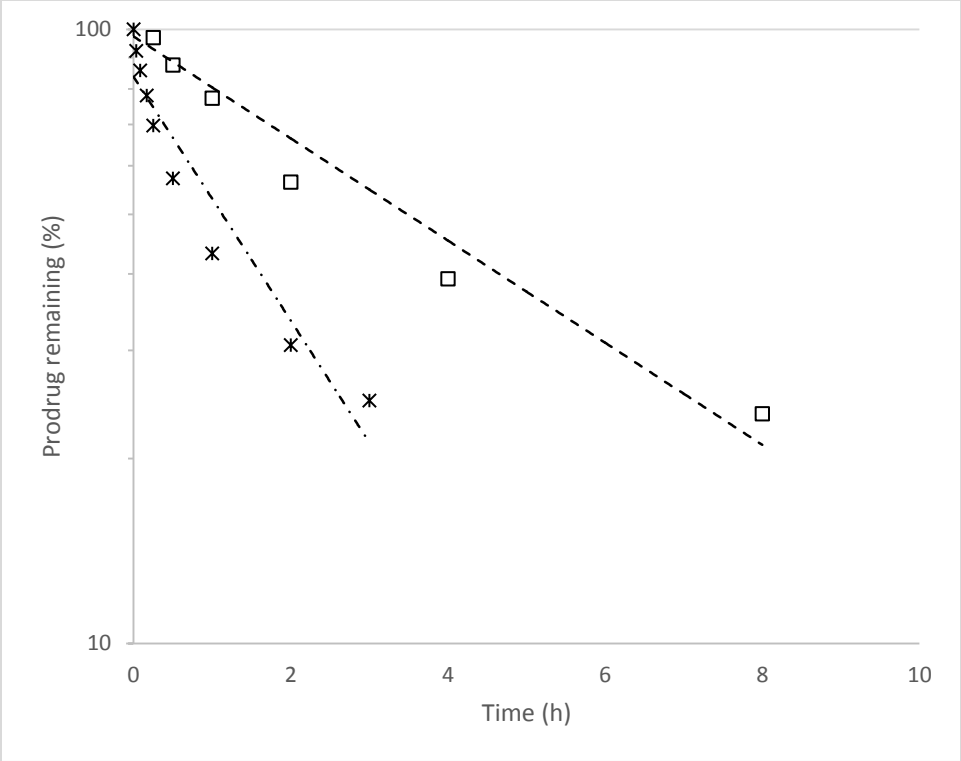
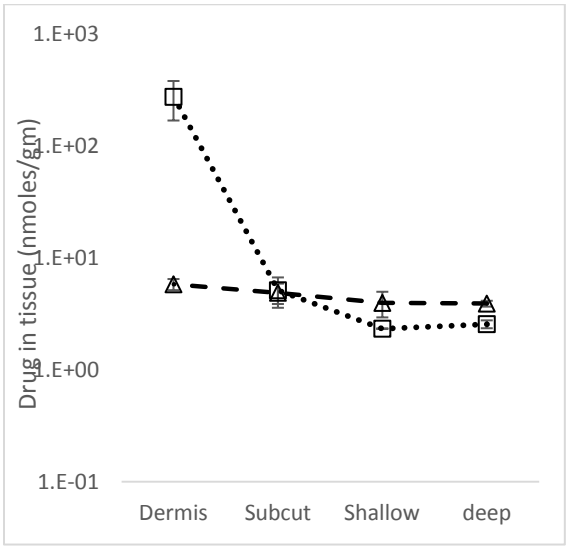
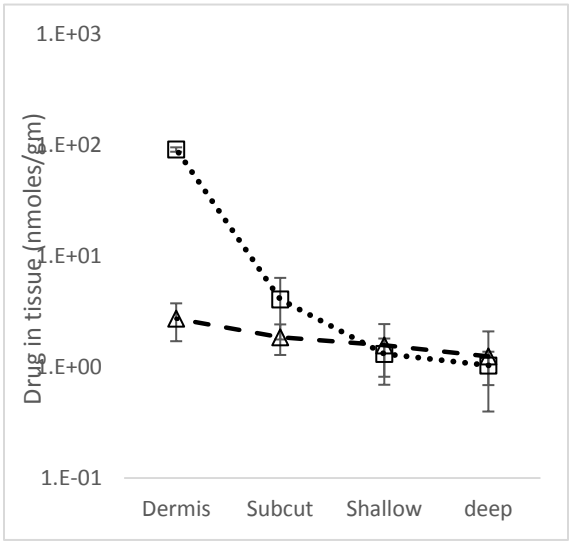


Figure 1 (A) The degradation pattern of Ketoprofen β-Methylcholine ester (KBCC) in phosphate buffer pH 7.4 (□), and Ketoprofen 1-(dimethylamino)-2-propanol ester (KPCl) (ж) in pH 4.5 (B) The bioconversion rate of KBCC (□) and KPCl (ж) in Rat Plasma at 37°C; (C) The bioconversion rate of KBCC (□) and KPCl (ж) in Human Plasma at 37°C.

(A)



(B)



(C)

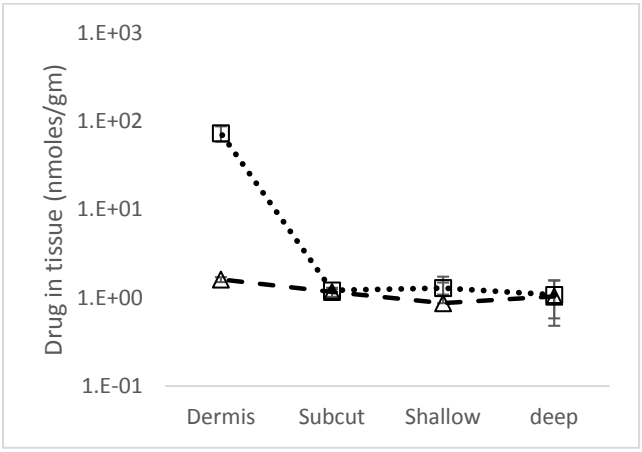
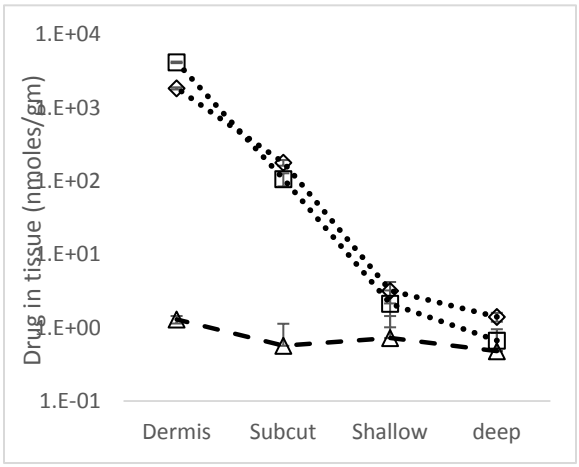


Figure 2 Tissue Concentrations of ketoprofen - Donor side (□), and contralateral side (Δ) after cationic iontophoresis of ketoprofen at 0.7mA for 2h and immediate tissue separation (A); iontophoresis at 0.7mA for 2h followed by 4h wait and tissue separation (B); iontophoresis at 0.1mA for 6h and tissue separation (C).

(A)



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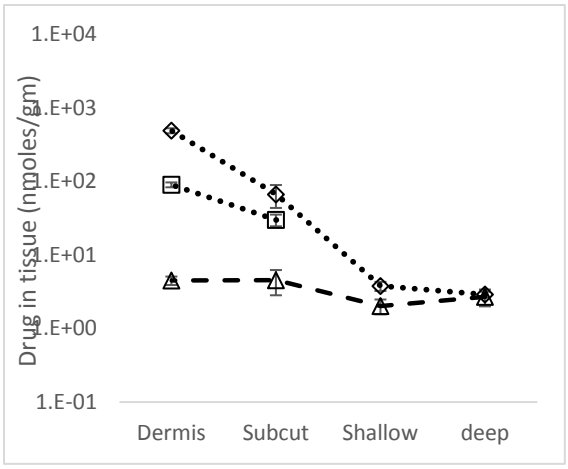

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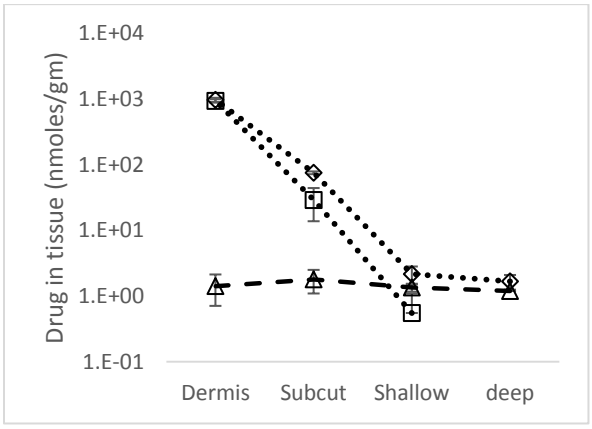
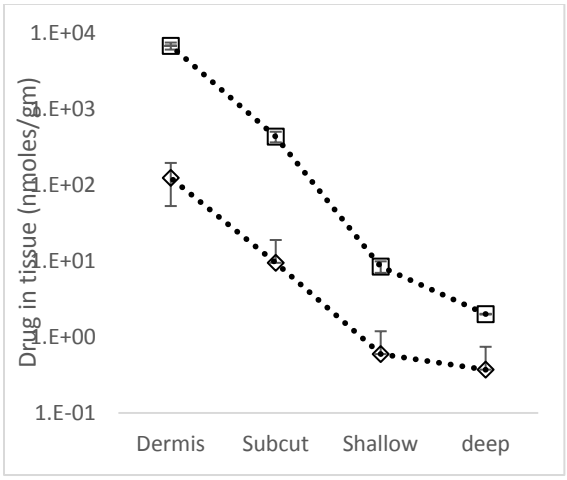
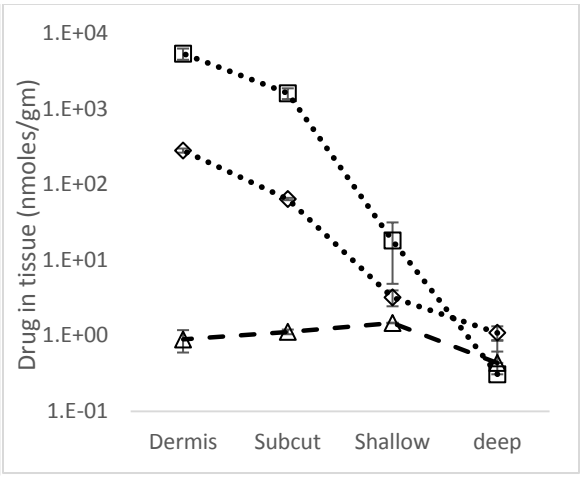


Figure 3 Tissue Concentrations of KCC - Donor side (□), converted acid – Donor side (◊)and contralateral side (Δ) after anionic iontophoresis of KCC at 0.7mA for 2h and immediate tissue separation (A); iontophoresis at 0.7mA for 2h followed by 4h wait and tissue separation (B); iontophoresis at 0.1mA for 6h and tissue separation (C).

(A)



(B)



(C)

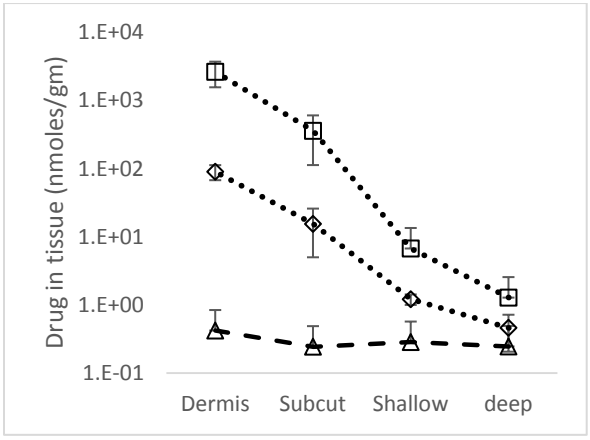


Figure 4 Tissue Concentrations of KBCC - Donor side (□), converted acid – Donor side (◊)and contralateral side (Δ) after anionic iontophoresis of KBCC at 0.7mA for 2h and immediate tissue separation (A); iontophoresis at 0.7mA for 2h followed by 4h wait and tissue separation (B); iontophoresis at 0.1mA for 6h and tissue separation (C).

(A) (B)

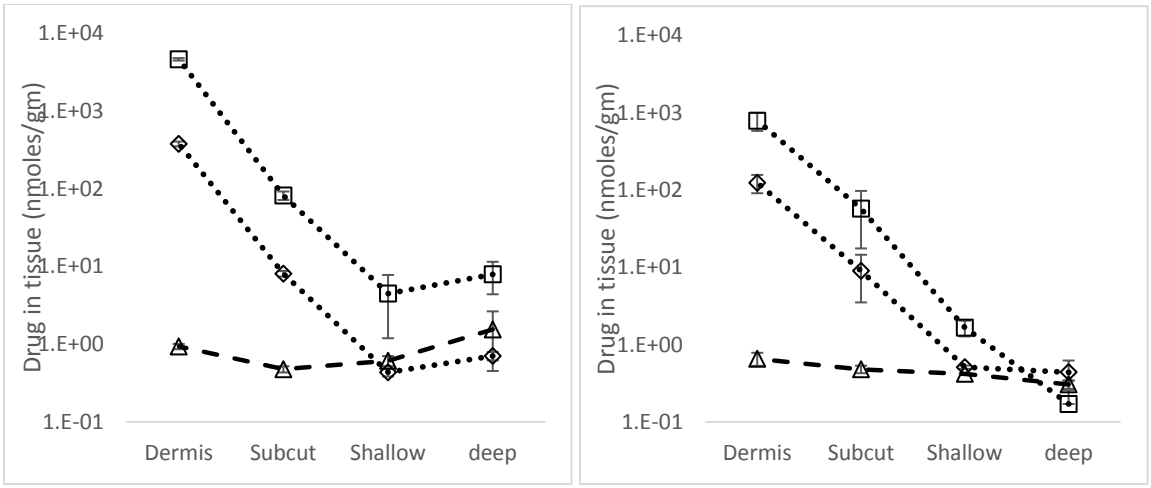
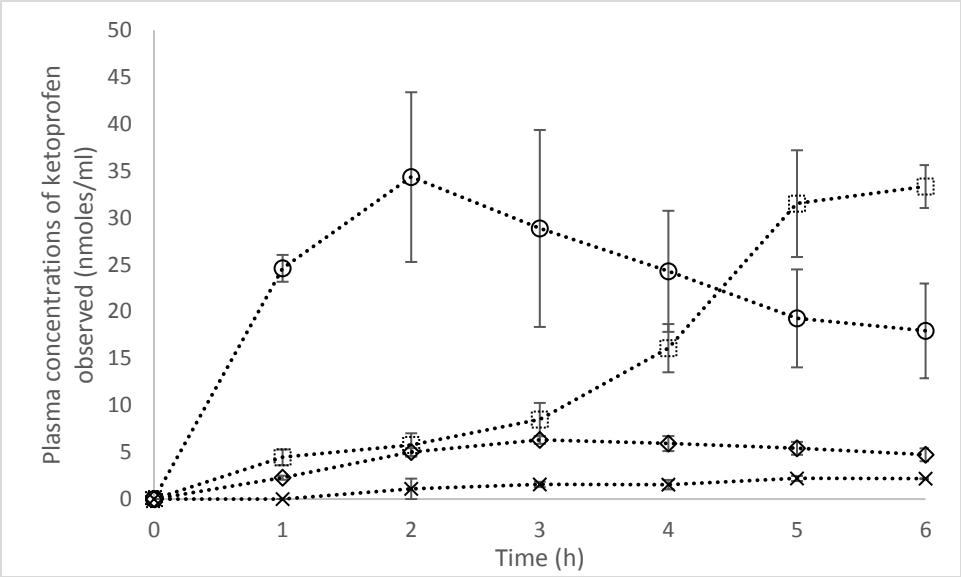


Figure 5 Tissue Concentrations of KPCl - Donor side (□), converted acid – Donor side (◊)and contralateral side (Δ) after anionic iontophoresis of KPCl at 0.7mA for 2h and immediate tissue separation (A); iontophoresis at 0.7mA for 2h followed by 4h wait and tissue separation (B); iontophoresis at 0.1mA for 6h and tissue separation (C).

(A)



(B)

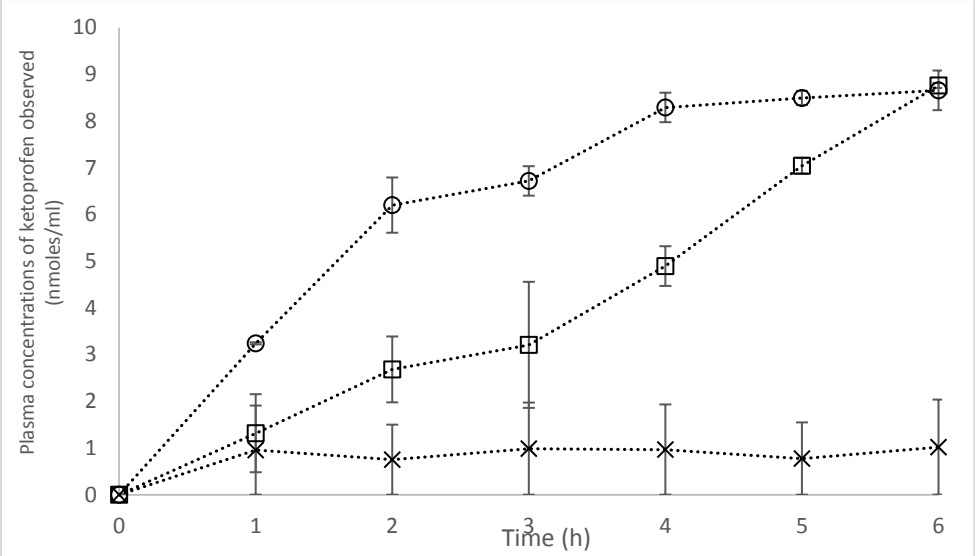


Figure 6 Plasma concentrations of drug indicating concentration versus time at 0.7mA for 2h followed by wait period of 4h (A); and at 0.1mA for 6hrs (B); for cationic iontophoresis of ketoprofen (o); anionic iontophoresis of KCC (□), anionic iontophoresis of KBCC (X), and anionic iontophoresis of KPCl

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CHAPTER 6Efficacy measurement of Cationic Prodrugs in treating nociceptive symptoms in MIA induced osteoarthritic rat model

This project was supported partly by the INBRE program, NIH Grant Nos P20 RR016454 (National Center for Research Resources) and P20 GM103408 (National Institute of General Medical Sciences)

Abstract

In our earlier studies, anionic delivery of positively charged choline and analogous ester prodrugs of ketoprofen was shown to have much better deep tissue penetration properties than the parent drug itself. In this study, we went about demonstrating the effectiveness of these prodrugs on an MIA (Monosodium IodoAcetate) induced arthritis rat model. The osteoarthritis induction was successful and the parent and prodrugs were delivered to the knee by iontophoresis. On observing the concentrations of the prodrug in the knee after iontophoresis, KCC was shown to have higher concentration of the drug than the parent drug till the first 2h. This increased drug concentration is reproduced in the MIA arthritic model where the prodrug treated rats showed reduction in the diameter of the arthritic knee at the second day of treatment. The prodrug was even found to reduce the diameter of the knee better than the parent drug at the 2^{nd}, 3^{rd} and 6^{th} day. Similar pattern was also seen in the hind paw weight distribution study where the prodrug iontophoresis improved the arthritic knee weight bearing efficiency much better than it did for the parent drug or the control. The improved activity in the prodrug group started right at the 2^{nd} day and lasted upto the 4^{th} day after start of treatment. From the observations in this study, it can be said that the prodrug iontophoresis treatment of the arthritic knee were much more effective than the parent drug iontophoresis and definitely more than the control, and were surely found to reduce the transient synovial inflammation caused due to the injection of the MIA.

Introduction

Osteoarthritis has come up to be the most widely encountered chronic disease affecting the joints especially of patients above the age of 65. As a result, NSAIDs have turned out to be a routine regiment in the medication of the elderly. However, with the widely identified gastrointestinal complications associated with NSAID therapy have limited their use in patients prone to GI events (Soll AH 1999) and anticoagulation (Davidson, et al. 2014). An alternate therapy suggested to these high risk patients is the use of COX-2 inhibitors which have highly reduced GI complications. But with the withdrawal of Rofecoxib and Valdecoxib from the market (Sun SX 2007), celecoxib has been the major therapy for osteoarthritis, but has also been encountering some backlash on its problems of myocardial infarction (Caldwell B 2006).

There have been studies performed to evaluate the effect of oral ketoprofen vs topically applied ketoprofen on an osteoarthritic rat model, and they did not manage to significantly increase the joint concentration of the drug in the donor side as compared to the oral route (Shinkai N 2008). As a result, there needs to be an emphasis of increasing the penetration of the drug through the skin, and also at the same time to reduce the systemic exposure of the drug. Various approaches have been identified and validated for increasing not only the permeability of NSAIDs through the skinusing chemical enhancers (Smith EW 2006), microneedles (Jessica Stahl 2012), iontophoresis (Curdy C 2001) and sonophoresis (Cagnie B 2003), but also on enhancing the deep tissue penetration and localization of drugs at the site of application by using techniques such as use of vasoconstrictors (Higaki K 2005) and cold temperatures. To expand and improve on some of these approaches, our laboratory has worked on evaluating prodrugs of

Diclofenac to improve the skin permeability properties of the drug (Lobo S 2014), and also to evaluate their potential to be localized at the site of application. Our most recent work has been in the synthesis of cationic prodrugs of ketoprofen for enhanced delivery through iontophoresis. The prodrugs have not only proven to deliver more drug through the skin, but have also shown to have localization and deep tissue penetration properties in vivo in rats.

Our study here proposes to build further on the potential benefits of these prodrugs by evaluating their performance on an arthritic model. Rats have been used as an osteoarthritic model for studies to evaluate anti-osteoarthritic therapies using various methods to induce there arthritis in the rodents (Bendele 2001). One of the methods that has been used extensively is the iodoacetate injection method which includes the injection of the compound directly into the joint. Our proposed approach is to evaluate the weight bearing imbalance between the hind limbs on a single joint inflammation model as a measure of the efficacy of the prodrugs. This method has already been used and validated (Bove SE 2003) and is an efficient method to evaluate the progress of arthritic pain. Along with this parameter, the diameter and circumference change would also be tabulated to corroborate the results.

Materials

Ketoprofen was obtained from TCI ltd (Tokyo, Japan), Choline chloride was obtained from J.T.Baker (Phillipsburg, NJ), Phenylephrine Hydrochloride was obtained from Spectrum Chemicals (Gardena, CA), N,N'-dicyclohexylcarbodiimide (DCC) and 4-pyrrolidinopyridine were obtained from Sigma (St. Louis, MO), Agarose LF (PFGE grade) was obtained from Amresco (Solo, Ohio), Monosodium Iodoacetate was obtained from Amresco (Solon, OH), Silica Gel ultra pure 40-60 µm was obtained from ACROS organics (Fair Lawn, NJ), Dichloromethane was obtained from Fisher Scientific (Fair Lawn, NJ) and Acetonitrile was obtained from EMD chemicals Inc (Gibbstown, NJ), Methanol was obtained from BDH chemicals (Radnor, PA), Trifluroacetic acid was obtained from Honeywell Riedel-de-Haen (Seelze, Germany) and Human plasma was obtained from Interstate Blood Bank Inc. (Memphis, TN). The direct current source used in the experiments was Activa Dose® II Controller Ionto Device purchased from ActivaTek Inc (Salt Lake City, Utah) and the Incapacitance tester was from Linton Instrumentation (UK). The rats used in the study were female CD® Hairless Rats obtained from Charles River Laboratories (Raleigh, NC).

Experimental

Tissue concentration of drug after iontophoresis

The rats were housed in cages with bedding and allowed free access to food and water through the duration of the study. The ketoprofen drug solution and the KCC solution were prepared as is mentioned in the earlier chapters. Specifically, a 26.58 mM solution of ketoprofen was prepared in purified water with pH to 7.4, and a 26.58mM solution of KCC was prepared in purified water. The rats used to measure the tissue concentrations were healthy rats and did not have any MIA injected into them.

Iontophoresis of drugs - The rats were anaesthetized and the knees were wiped thoroughly with wet Kim wipes before start of the study. A return electrode was attached to the upper tail of the rat and connected appropriately. 5ml of the solution was loaded on to a plastic receptacle of surface area 1.2cm^2 containing a sponge. An electrode was connected to this donor cell, and placed carefully on the right knee of the rat. Iontophoresis was started at 0.5mA for 20 mins, following which the skin was wiped thoroughly with wet Kim wipes. The tissues (skin, muscle and joint) were collected at 4 different time points, 30min, 2h, 4h and 6h after the start of iontophoresis. Blood samples were also collected at appropriate time points. The tissue and blood samples were stored at -80°C until further processing.

Extraction of samples – The tissues samples collected were first frozen in liquid nitrogen. After being cooled the tissue were then crushed in a cold S.S mortar and pestle. The crushed tissues were then collected in a large test tube and weighed for the amount of tissue crushed and transferred. The samples were first acidified with Trifluroacetic acid. 10ml of methanol was then added to the samples and were extracted over 18 h with continuous shaking on an orbital shaker. This whole procedure was done in the cold room at 2-8°C. After the extraction, the clear methanol extract was separated into test tubes and evaporated to dry. This residue was then further extracted with a Dichloromethane: methanol mixture (80: 20) over 3 h on a multi-tube vortexer mixer. This clear extract was then evaporated to dry. The residue was reconstituted with mobile phase and analyzed for amount of drug by HPLC method.

Induction of Osteoarthritis

The procedures used in this study were conducted in accordance with the Idaho State University – Institutional Animal Care and Use Committee (IACUC) guidelines. The rats were weighed and found to be between 250 – 300 gms. For induction of osteoarthritis, a solution of Monosodium Iodoacetate was prepared in PBS buffer in a concentration of 60 mg/ml. The rats were first anaesthetized and the left knee was wiped thoroughly with rubbing alcohol. A single intra-articular injections of 60µl was injected in through the intrapatellar ligament.

Assessment of change in knee dimensions and Hind paw weight distribution after drug treatment

Measurement of Knee diameter - The extent of osteoarthritic progress was evaluated by comparing the change in diameter and weight distribution between the left (Osteoarthritic) and the right (Contralateral) knee in the same animal. The change in knee diameter was measured using a Vernier caliper, while keeping the knee extended and measuring the diameter sideways along the center of the joint.

Measurement of Hind paw weight Distribution - The hind paw weight distribution was measured on the Incapacitance Tester with an improvised acrylic housing to accommodate the larger size of the rat. The rat was placed in the housing such that each of the hind limbs were rested on either of the balance plates, and the forelimbs on the inclined Plexiglas wall. Before measuring the weight distribution, the rat was allowed to get acclimatized to the Plexiglas housing and when the weights were measured when all the limbs were at their appropriate positions as mentioned above. The weight measured by each of the balance plates were averaged over a 3s period. The reading measured were taken as a mean of 3. The % of weight bearing on the left paw can be indicated by

$$\%\ left\ knee\ weight\ bearing = \frac{W^{left}}{(W^{left} + W^{right})} * 100 \qquad \textit{Equation 9}$$

Treatment with drugs – The rats were divided into three groups, a control group, ketoprofen group and KCC group. Each group consisted of four rats. In these groups, the rat knees were treated with NaCl solution (0.15M), ketoprofen and KCC respectively by iontophoresis. The ketoprofen and KCC were prepared as mentioned above. The study starts with the induction of osteoarthritis, but before this, the initial parameters of the knee i.e. the knee diameter and the Hind paw distribution were noted. The injection of MIA were given to the left knee of the rats and this was noted as time 0 (T_0). The knees were then treated with 0.5ml of the drug solutions (NaCl soln in case of control) 1d, 2d and 3d after osteoarthritis induction. The iontophoretic parameters maintained was same as that maintained in the tissue concentration study i.e. 0.5mA for 20 min. The knee parameters were monitored at 1d, 2d, 3d, 4d, 6d and 10d after induction of osteoarthritis. At 1d, 2d and 3d, the parameters were measured right before the treatment with the drugs.

Results and Discussion

Tissue concentration of drug after iontophoretic treatment

Work on deep tissue penetration of ketoprofen and KCC have already been done on the dorsal region of rat and has been elaborated on in the previous chapter. These results had shown that KCC was shown to have better deep tissue penetration properties as compared to that of ketoprofen. The performance of the drug on the knee joint is shown in figure 3. For the parent drug ketoprofen, it can be seen that there was a significant difference in the skin concentration (Figure 1) of the drug in the donor side versus the contralateral side in the joint tissues after 2h and 4h of treatment (Figure 2). However, the concentration of the drug in the joints was only 2.3 times higher in the joint in the application site as compared to that in the contralateral side at 2h after treatment and 2.1 times as high after 4h. The joint ketoprofen concentrations were not found to be significant at 30 mns and the reason for this could be that 30mns was not sufficient for the drug to penetrate the synovial tissue quickly only through diffusion, but was sufficient for the drug to be taken up by the systemic circulation and end up in both the joints.

On the other hand, in case of the anionic iontophoresis of the ester KCC, the drug concentration was found to be significantly higher in the joint tissue on the donor side at 30mns and 2h after application. At 30 mns, the joint concentration at application side after KCC iontophoresis was found to be 54 times higher than that on the contralateral side and 10 times as higher at 2h. However, at 4h there was no significant difference observed in the concentration of the joints at contralateral and donor side. A reason could be that the quaternary prodrug does not show sufficient passive diffusion properties through the joint tissue, and only showed effective deep tissue penetration during

iontophoresis. As a result, the contrast in the joint concentration at application side to that of contralateral diminished at the latter hours. Quite the opposite was observed in case of ketoprofen, which showed comparatively better passive diffusion properties through the fibrous synovial capsule, but poor concentration during iontophoreisis. At 30mns, KCC iontophoresis was found to deliver 9 times higher concentration of drug into the joint under the donor site as compared to that of ketoprofen iontophoresis, and at 2h, the contrast was 5 times as high. The joints concentrations at the application side levelled out for both iontophoresis trials at the 4h time points and later.

From the graph (figure (3)), the plasma concentrations of the drug showed cationic ketoprofen iontophoresis to show a C_{max} of 9.2 nmoles/ml at 30mins whereas the anionic iontophoresis of KCC showed a C_{max} of 5.8 nmoles/ml at 2h. The delayed C_{max} in case of the prodrug is probably due to the gradual bioconversion of the prodrug to the parent and uptake into the systemic circulation. Both the ketoprofen and the KCC show a similar trend in the plasma concentriaon after 2hrs. This fact is also reflected in the tissue concentrations, where no difference is observed in the joint and muscle concentrations in the cationic and anionic delivery trials.

Osteoarthritic inductions and effect of iontophoretic treatment

Induction of Osteoarthritis - The MIA solution at 60µl of a concentration of 60 mg/ml, was adequate to induce osteoarthritic manifestations in the knee of the rats. There were measurable changes in the knee diameter as well as the weight bearing distribution in the hind limbs of the rats as can be seen comparing the parameters at day 0 (before MIA injections) and day 1 (24h after MIA injection and before start of treatment regimen in figures 4 and 5). A decrease from even weight distribution (48.7 %) to 39% was observed

in the first 24h. The thickness was found to increase by 50% from an average of 8.2mm to 12.3mm 24h after injection of MIA and the circumference increased 37% from 5.1cms to 7cms.

Changes in knee dimensions after treatment – The thickness and circumference of the left knee of the rats (Treated knee) were measured and contrasted with the right knee (control knee) and the data is presented in figure 3. In case of the thickness of the knee, a significant difference was observed in the improvement of the arthritic condition at 2^{nd}, 3^{rd} and 4^{th} and 6^{th} day after treatment with KCC as opposed to the control. Both Ketoprofen and KCC were found to do better than the control at the 3^{rd} and 4^{th} day after treatment but KCC was shown to have a significant improvement at 2^{nd} day itself and last up to the 6^{th} day. At the 2^{nd}, 3^{rd} and 6^{th} day, the prodrug was found to perform significantly better than ketoprofen too. The circumference of the knee however was not found to show significant differences in the performance of the prodrug and parent drug versus the control. The process might not be sensitive enough to measure the changes in the girth of the knee.

Changes in hind Paw weight distribution – In evaluation of the weight distribution, ketoprofen was not found to show any improvement at all across the whole duration of the study of 10 days (figure (6)). However, the prodrug showed an improved performance just after 2 days of treatment. The trend in the improvement lasted upto the 4^{th} day, and the improvement was significantly more than the control. At the 2^{nd} day, KCC was shown to be significantly better than both the control as well as the ketoprofen treatment. At the 3^{rd} and 4^{th} day, a significance in the improvement as compared to ketoprofen could not be achieved, but it definitely showed a significant improvement from the control.

An understanding of why the performance was strong for the prodrug in the beginning of the study can be understood by studying the tissue concentrations of the drug. The prodrug was found to show a significant increase in the donor joint concentration shortly after the end of the iontophoresis, without much penetration at the 4^{th} hour. Since the treatment was given on the first three days of the study program, the treated knees of the rats were prevented from deteriorating further as was seen in the case of the control and ketoprofen. At the 6^{th} and the 10^{th} day, the control rats showed a natural route of recovery and the balance in the weight distribution was reached.

Ketoprofen iontophoresis was also shown to have a significant effect as compared to the control in case of the nociceptive study. However, looking at the data, both the joint in the application side and the contralateral side received the same amount of the drug, and the improvement that was probably due to the drug that arrived at the synovial joint through the systemic circulation.

From studying the results above, it can be seen that the prodrug was found to be more effective in alleviating the symptoms of arthritis for the duration of the study. On evaluating the MIA model of arthritis, the initial indications of swelling and pain sensations in the knee of the rat can be attributed to the transient synovial inflammation (Beyreuther B 2007). This initial stage is manifested as a result of the inflammation brought about by macrophage infiltration into the synovial cavity (Haywood L 2003). The prodrug was found to be very effective in reducing the inflammation incited due to this initial macrophage infiltration. However, a week after the initial induction of osteoarthritis in the MIA model, the inflammation is mostly resolved and the later pain sensation is due to the mechanical friction at the articular cartilage and the subchondral

bone. This effect can be seen in the inflection in the graphs in both the knee diameter data and the weight distribution data. Further studies would have to be done with the prodrug, with chronic treatment of the knee lasting more than a week to show its effect on this secondary manifestation of the MIA model.

Moreover, the prodrug showed to have a significant difference in the activity studies as compared to that of control and showed a significance on a majority of the time points as compared to the ketoprofen iontophoresis. A plan for a study in the future with a larger sample size than the one used in the study could further unanimously signify the difference in the two treatments.

Ketoprofen iontophoresis was also shown to have a significant effect as compared to the control in case of the nociceptive study. However, looking at the data, both the joint in the application side and the contralateral side received the same amount of the drug, and the improvement that was probably due to the drug that arrived at the synovial joint through the systemic circulation. Looking at some earlier studies done in studying inflammation in rats, 8-12 nmoles/ml of plasma level of ketoprofen seemed to show adequate anti-inflammatory properties in carrageenan induced rat paw oedema and were shown to reduce swelling (Fumitoshi H 2002). In our studies involving cationic iontophoresis of ketoprofen, a C_{max} of approximately 9nmoles/ml was achieved and hence the plasma ketoprofen might have been a major contributor in reducing the swelling and pain.

Hence, on further speculation, the results obtained are that in a rat, a 300g individual of which would have a blood volume of approx. 20ml. With a smaller blood volume and a volume of distribution of 126ml for a 300g individual, the contrast in the local delivery

and systemic appearance is reduced as the distribution volume is too low for the dose (Berry LM 2011). Whereas potentially, the contrast in the application tissues and the contralateral tissues would be greater and more significant in a larger mammal, for instance, a human who has a volume of distribution of around 9 liters for a 70kg individual. Further studies can also be planned using large mammals such as a mini pig that would have a blood volume of 300 – 400ml, and hence would show a better representation of drug dynamic in humans.

Hence, from this study it can be concluded that the prodrug is capable of not only increasing the penetration of the drug into the osteoarthritic knee, but also in converting to sufficient amounts in the knee joint to the parent drug to show a therapeutic response as can be seen in the changes in dimensions and paw weight distribution in the rats. It not only showed a significant improvement in alleviating the discomfort of arthritis when compared to the control, but also showed to be significantly better than that of ketoprofen iontophoresis.

Conclusion

As was concluded in the earlier chapters, here too, the prodrug was found to show many folds increased deep penetration into the joint tissue as compared to that of ketoprofen. It now only showed increased concentrations, but also showed its physiological affects by converting into the acid at an appropriate rate to show relief from the discomfort of arthritis as can be concluded from the weight distribution and knee diameter study.

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Figures

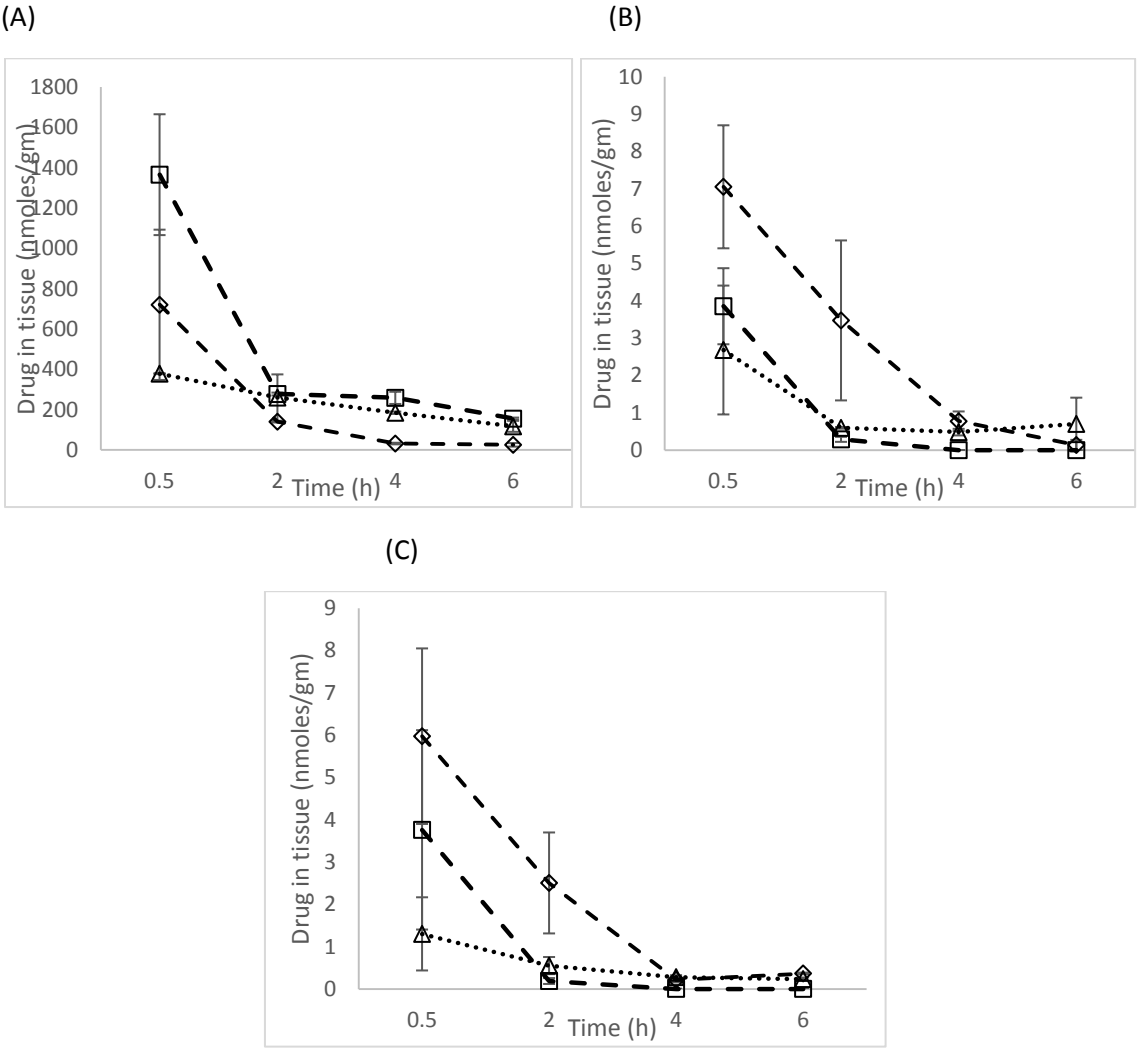

Figure 1 Graphs indicating the application site drug concentrations ofskin (A), muscle (B) and joint (C) after cationic iontophoresis of ketoprofen (Δ), prodrug concentration after anionic iontophoresis of KCC (□) and converted acid concentration after anionic iontohoresis of KCC (◊)

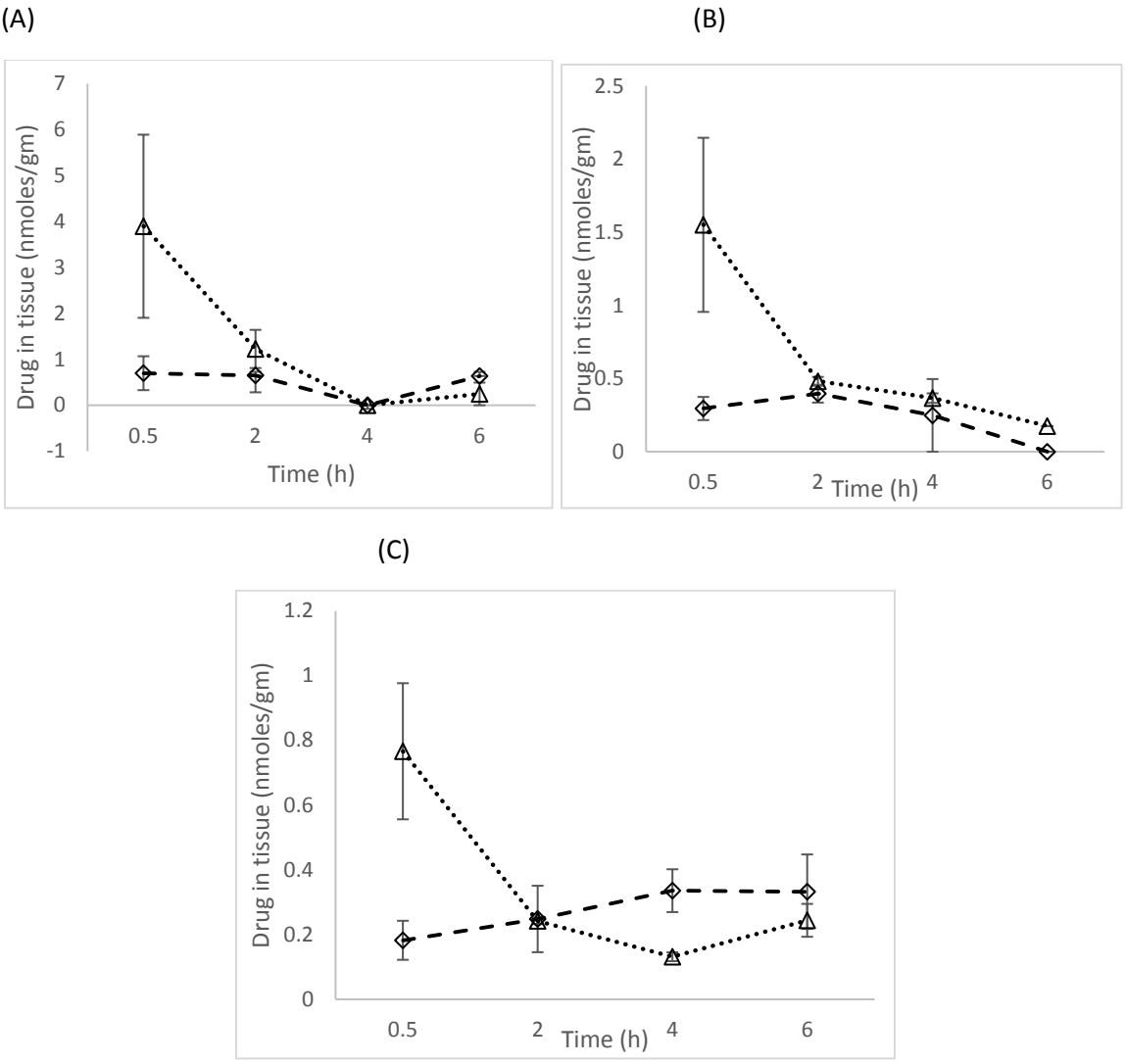

Figure 2 Graphs indicating the contralateral site drug concentrations ofskin (A), muscle (B) and joint (C) after cationic iontophoresis of ketoprofen and converted acid concentration after anionic iontohoresis of KCC (◊)

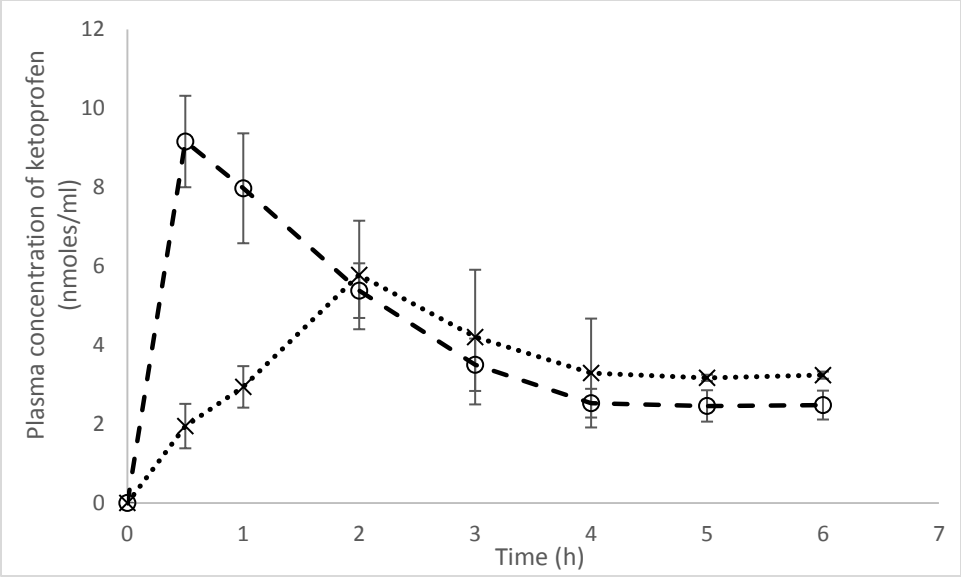

Figure 3 Plasma concentration of drug* after cationic iontophoresis of ketoprofen (○) and anionic iontophoresis of KCC (×)

* only ketoprofen observed in the plasma in case of KCC iontophoresis

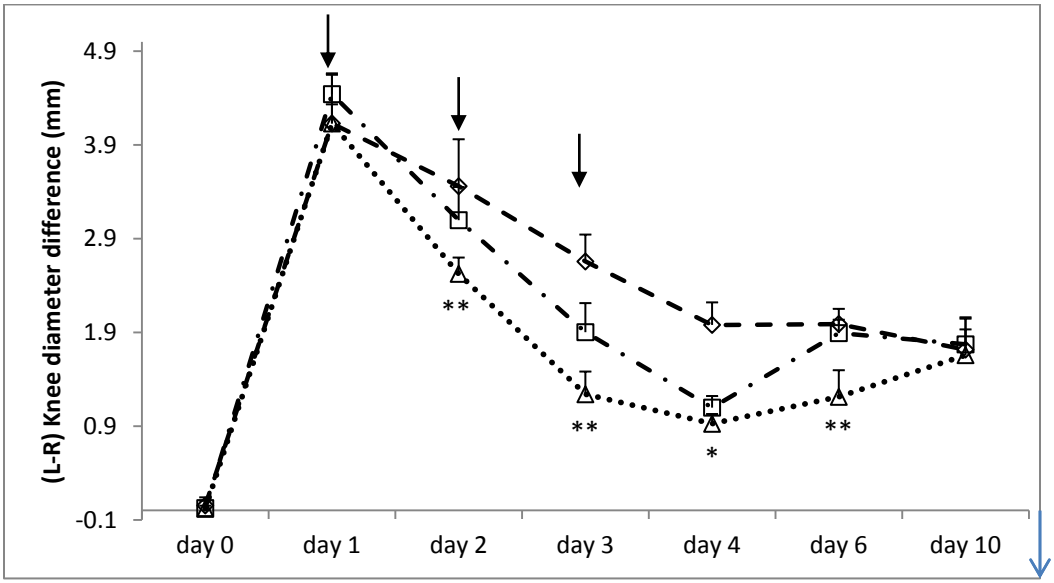

Figure 4 Plot of difference in knee diameter in between the left and right knee after treatment with anionic iontophoresis of control – NaCl (◊), cationic iontophoresis of ketoprofen (□) and anionic iontophoresis of prodrug KCC (Δ); ‘ ‘ indicates treatment

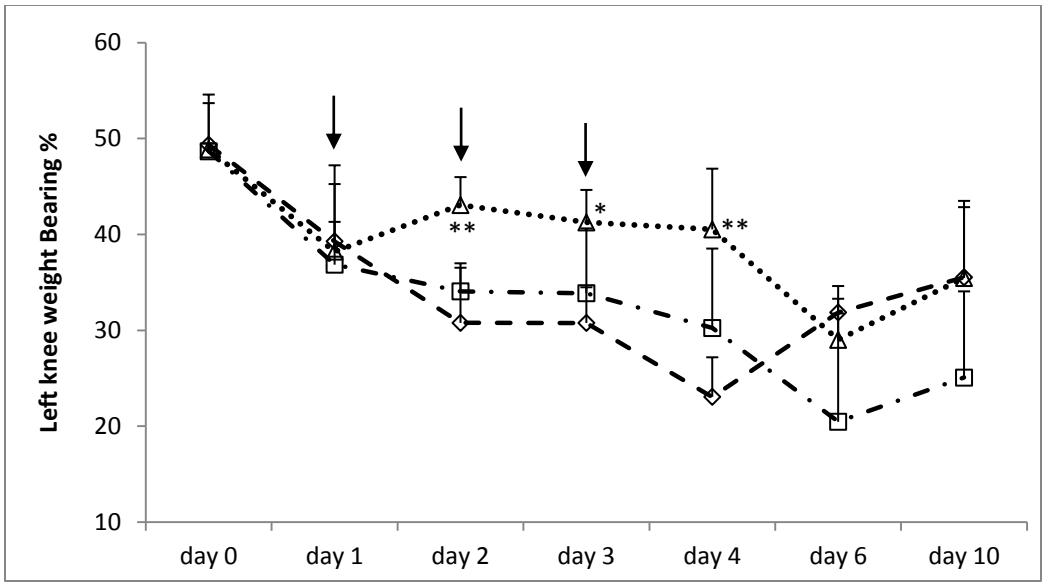

Figure 5 Plot of percentage of the weight bearing on the left knee (osteoporosis induced) after treatment with anionic iontophoresis of control NaCl (◊), cationic iontophoresis of ketoprofen (□) and anionic iontophoresis of prodrug ~~K-G~~C (Δ); ‘ ‘ indicates treatment

General Discussion

In our attempt to prepare prodrugs of the two model drugs, we were able to utilize the specified method and obtain pure esters throughout. In case of the diclofenac prodrugs, we were able to obtain pure esters, and also were able to obtain an array of prodrugs varying in their Log P values and solubilities. These factors were also found to be the major determinants in the flux of the prodrugs through the Human Epidermal Membrane. Both GD and ED were shown to have greater solubilities and a lower Log P value than the parent drug. The Log P value of GD was found to be the ideal in terms of Log P value for optimum flux through the membrane. The combinations of the optimum Log P along with the highest solubilities amongst the prodrugs made it the most efficient in terms of HEM flux. The MD did not show any flux through the HEM for the whole 12h of the study. The reason for this was the high log P value, which made it highly insoluble in the aqueous receiver medium. However, from the partition coefficient value in the stratum Corneum/water system, it can be hypothesized that the prodrug permeated the stratum Corneum adequately, but couldn't traverse the lower viable epidermis.

Transdermal Patches of the prodrugs were prepared in three different patch bases, acrylic polymers without functionality, acrylates with Hydroxy- functionality and polyisobutylene based. In these adhesive bases, GD performed best in the polyisobutylene patch, and in the acrylate patches, it was not able to release the drug adequately for diffusion. All of the prodrugs perfrmed best when in the polyisobutylene patch. The patches in the dual agar gel model i.e. the GD patch and the DA patch did not show a difference in the direct penetration and the amount absorption into the body. Both the parent drug as well as the ester showed around 75% of the drug directly penetrating

the gel. The reason the prodrug did not perform better than the parent is probably due to the rapid bioconversion of the prodrug before it reaches the agar gel plates.

The cationic ketoprofen prodrugs prepared (KCC, KBCC and KPCl) were all found to show a varied bioconversion rate as expected with KCC showing the shortest half-life and the branched KBCC and KPCl showing longer. Having compounds with varied bioconversion rates gave us different perspectives in deep tissue penetration studies as will be discussed. In case of the flux studies, the passive diffusion condition showed the prodrug to be completely incapable of passing through the HEM, the charged moiety being the group rendering it impenetrable through the hydrophobic stratum Corneum. However, on application of electric current, the flux of ketoprofen increased 60 times that at passive diffusion, but the prodrug showed a flux value 6 times higher than the cationic iontophoresis of ketoprofen. This can be attributed to not only the ionic movement of the anionic prodrug in the electric current, but also through electro-osmosis, a phenomenon only observed in case of anionic iontophoresis. When studied for localization and deep tissue penetration evaluation in the *in situ* rat model, a similar trend was observed where at the end of the study, the cumulative amount of drug collected on the donor side for the prodrug was found to be 19 times higher than that collected for the cationic iontophoresis of the parent drug..In addition, even though the amount was more for the prodrug, the content from systemic circulation was less than 0.1%, whereas 4-5% of the drug in case of the cationic iontophoresis of the parent. The less systemic appearance of the prodrug can be attributed to its lower protein binding affinity and hence a lesser tendency to be taken up by the systemic circulation. The trials with iontophoresis of prodrugs along with vasoconstrictor showed no improvement in drug penetration, in fact lowered the

drug captured in the gel plates. A reason for no difference can be attributed to the potential vasoconstrictor effects of the cationic prodrugs itself, that over shadows the effects of the vasoconstrictor.

In the deep tissue penetration study, all the three prodrugs (KCC, KBCC and KPCl) showed better penetration properties than the parent drug. Amongst the prodrugs themselves, there was a difference in the performance and this was mainly attributed to the different bioconversion rates. KCC due to its faster bioconversion rate, could show sufficient deep tissue penetration as good as the other prodrugs when the tissues were collected at 2h, but on the longer wait period of 4h, the prodrug was able to show a majority deep tissue penetration only up to the depth of shallow muscles. A similar trend was also seen for the iontophoresis at lower current of 0.1mA, where a deep tissue penetration efficiency was shown only till the shallow muscle in case of KCC. KPCl also performed similar to KCC, where deep tissue penetration was observed in the tissues at the 2h time point, but after waiting for 4h, the deep tissue penetration was only restricted to the upper shallow muscles. However, because of its better resistance to enzymatic attack, KBCC showed better deep tissue penetration across all the iontophoretic conditions. But overall, all the prodrugs were found to show deeper tissue penetration as compared to the cationic iontophoresis of the parent drug.

The better penetration performance of the drugs were also reflected in the activity study on the MIA induced rat model. On observing the concentrations of the prodrug in the knee after iontophoresis, KCC was shown to have higher concentration of the drug than the parent drug till the first 2h. This increased drug concentration is reproduced in the MIA arthritic model where the prodrug treated rats showed reduction in the diameter of

the arthritic knee at the second day of treatment. The prodrug was even found to reduce the diameter of the knee better than the parent drug at the 2^{nd}, 3^{rd} and 6^{th} day. Similar pattern was also seen in the hind paw weight distribution study where the prodrug iontophoresis improved the arthritic knee weight bearing efficiency back to 45% of performance. This activity study corroborated all the deep tissue penetration data that is derived in the earlier studies, and substantiate the fact that reducing the protein binding capacity of a drug and the cationic prodrugs of the acidic NSAIDs increases its deep tissue penetration properties and hence render better topical delivery of the drugs.

Conclusion

Based on our findings in the chapter above, we found that the performance of Diclofenac prodrugs were as was expected and in line with the research conducted as of now. The prodrugs were found to show much higher diffusion rate across skin and the rate showed an optimum Log P value above which flux diminished. The bioconversion rate of the prodrugs were vital in determining the deep tissue penetration of the prodrugs, and hence were insignificant to show the desired penetration properties.

The Cationic prodrugs of Ketoprofen showed surprisingly much higher iontophoretic skin fluxes as compared to that of the parent drug. Not only did the fluxes increase, but the prodrugs did also show localization and deep tissue penetration *in vivo*. The presence of vasoconstrictor was proven to not show a higher penetration effect to the prodrugs. The systemic presence after anionic iontophoresis of the prodrugs were negligible as compared to that after cationic iontophoresis of the parent. These improved deep tissue penetration properties of the prodrugs were also reflected in the pain relief and alleviation of nociceptive symptoms of arthritis *in vivo*, where the prodrug not only performed better than the control but showed significant results to that of ketoprofen.