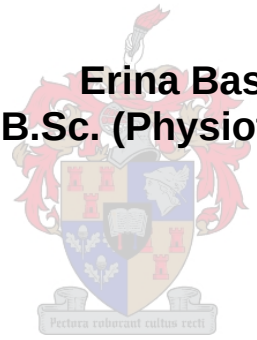
EFFECT OF ULTRASOUND ON TRANSDERMAL PERMEATION OF DICLOFENAC AND THE TEMPERATURE EFFECTS ON HUMAN SKIN

Thesis presented in partial fulfilment of the requirements for the degree of Master of Medical Sciences (Pharmacology) at Stellenbosch University

Erina Basson
B.Sc. (Physiotherapy)



Study leader: Prof. P van der Bijl
(Head, Department of Pharmacology)

Co-study leader: Dr. AD van Eyk
(Researcher and lecturer, Department of Pharmacology)

Tygerberg
August 2005

Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my original work and that I have not previously submitted it, in its entirety or in part, at any university for a degree.

Erina Basson

Signature: ..... **Date:** .....

Summary

During the last two decades the effects of ultrasound on the transdermal diffusion of a wide variety of drugs have been extensively investigated. Because there is much uncertainty regarding the efficacy of and mechanisms involved in this mode of permeation enhancement, the objective of the study was to investigate the effect of ultrasound on the transdermal permeation of the nonsteroidal anti-inflammatory drug, diclofenac. For this purpose a dual-stage experimental design and a continuous flow-through diffusion system was used. Therapeutic levels of continuous ultrasound of 3 MHz at an intensity of 2 W/cm^2 for 10 min, were used. It was clear from the present study that ultrasound enhanced the permeability of human skin to diclofenac released from a commercially available gel. These results were in contrast with those obtained for ibuprofen in an *in vitro* study across human skin, but in agreement with those obtained in two *in vivo* studies of the latter nonsteroidal anti-inflammatory drug. Steady state flux values of diclofenac remained approximately 1.26 times higher than those of controls during the 24 h of the experiment. These observations concurred with those made in two previous *in vivo* studies. Furthermore, the *in vitro* flow-through diffusion model was shown to have predictive value as an *in vivo* method for sonophoresis.

Temperature-dependent flux rates for ^3H_2O across human skin were also studied. The mechanistic effects of ultrasound on the permeability characteristics of human skin have been reported on in a number of studies. Although various mechanisms have been proposed, there is no consensus regarding their relative importance. In addition the temperature-dependent flux changes of ^3H_2O across human skin were investigated using a continuous flow-through diffusion system. The same ultrasound parameters as in the permeability experiments were used. The results obtained showed that temperature increases of approximately 10 °C occurred following sonication. The flux changes of ^3H_2O across human skin between 37 °C and 42 °C were shown to be reversible. The results from the present study do not support the sonication-heating theory in which permeability changes in skin are primarily attributed to thermally-induced changes in stratum corneum lipids. It was therefore concluded that the enhancement of diclofenac permeation by sonication could not be adequately explained primarily on a thermal basis.

Opsomming

Die effek van ultraklank op die transdermale diffusie van ‘n wye verskeidenheid middels is die afgelope twee dekades ekstensief nagevors. Daar bestaan heelwat onsekerheid rondom die effektiwiteit en meganismes van ultraklank om transdermale deurlaatbaarheid te verhoog. Daarom was dit die doel van hierdie studie om die effek van ultraklank op die transdermale deurlaatbaarheid van die nie-steroïed anti-inflammatoriese middel, diklofenak, te ondersoek. ‘n Twee-fase eksperimentsontwerp en ‘n deurlopende- vloei perfusie apparaat is gebruik tydens hierdie studie. Terapeutiese vlakke van ononderbroke ultraklank, met ‘n frekwensie van 3 MHz en intensiteit van 2 W/cm^2, is in hierdie studie gebruik. Die ultraklank is vir 10 minute toegedien. Die resultate het duidelik aangetoon dat ultraklank die deurlaatbaarheid van mensvel verhoog vir diklofenak vrygestel vanuit ‘n kommersiële jel. Die resultate was in kontras met dié van ‘n vorige *in vitro* studie met ibuprofen op mensvel. Dit het egter ooreengestem met twee ander *in vivo* studies. Die gemiddelde vaste vlak vloedwaardes vir diklofenak was ongeveer 1.26 keer hoër, as dié van die kontrole groep, gedurende die 24 h van die eksperiment. Hierdie bevindinge was in ooreenstemming met twee vorige *in vivo* studies. Verder is aangetoon dat die *in vitro* deurlopende-vloei apparaat ‘n effektiewe metode is om sonoforese in die *in vivo* situasie na te boots.

Temperatuurafhanklike diffusie vloedwaardes vir ^3H_2O oor mensvel is ook bestudeer. Verskeie studies het al gehandel oor die meganiese effekte van ultraklank met betrekking tot die deurlaatbaarheidseienskappe van vel vir middels. Alhoewel verskeie meganismes al voorgestel is, kon tot dusver geen konsensus bereik word oor hulle relatiewe belangrikheid en presiese rol nie. In hierdie studie is die temperatuur-toename in mensvel bestudeer. Die temperatuurafhanklike diffusie vloedwaardes van ^3H_2O oor vel is ook bestudeer. Dieselfde ultraklank parameters as die van die deurlaatbaarheidseksperimente is gebruik. Die resultate het aangetoon dat die temperatuur van mensvel met ongeveer 10 ^oC gestyg het na toediening van ultraklank. Die verandering in diffusie vloedwaardes van ^3H_2O oor mensvel, tussen 37 ^oC en 42 ^oC, was omkeerbaar. Die gevolgtrekking was dat die resultate van hierdie studie nie die ultrasonering-verhittingsteorie ondersteun nie. Die deurlaatbaarheidseienskappe van die vel kan dus nie hoofsaaklik toegeskryf word aan die temperatuurafhanklike veranderinge van die lipiede, van die stratum corneum, nie. Die verhoging in diklofenak deurlaatbaarheid a.g.v. sonikasie kan nie afdoende verklaar word primêr a.g.v. temperatuurverhoging van die vel nie.

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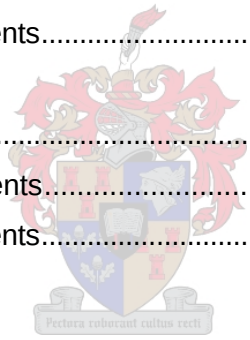

1. Introduction

1.1 Topical administration of nonsteroidal anti-inflammatory drugs (NSAIDs)

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most frequently prescribed drugs in the world, but also the cause of one quarter of all adverse drug reaction reports.[1] As many as 8 % of the global adult population are taking prescribed forms of these agents at any given time.[2] NSAIDs are frequently prescribed for rheumatic musculoskeletal complaints and are often taken, or applied topically, without prescription for minor aches and pains. These drugs relieve pain, reduce swelling and inflammation and increase mobility of muscles or joints. NSAIDs are also used for a variety of other conditions: postoperative pain, herpetic neuralgia, periodontitis, aphthous ulcers, actinic keratoses and cancer pain. Patients suffering from rheumatic disease, which often include the elderly, are at increased risk for serious gastrointestinal (GI) complications such as peptic ulcer disease and GI haemorrhage. The prevalence rate of rheumatoid arthritis in the adult population is 1 %.[3] The high incidence of serious gastrointestinal adverse events associated with the use of systemic NSAIDs, has prompted the development of growing numbers of topical nonsteroidal ant-inflammatory drugs. It is estimated that these drugs are responsible for approximately 25 % of all adverse drug reaction reports.[1] In this respect, NSAID-related gastrointestinal toxicity is the most frequently observed adverse reaction, and it is a significant cause of morbidity and mortality.[4] The minimisation of plasma concentrations of active drug associated with topical NSAIDs may result in fewer systemic adverse effects.

Topically administered NSAIDs penetrate slower and in smaller quantities into systemic circulation compared with the equivalent oral administration. Bioavailability and maximal plasma concentration after topical application are generally less than 5 and 15 % of oral administration respectively, but product formulation may have a dramatic impact on absorption as well as penetration depth. Topical administration leads to relatively high concentrations in the dermis, compared to oral administration. The concentrations in the muscle tissue underneath the skin area exposed to the drug vary, but are at least equivalent to concentrations reached when taken orally.[1] NSAIDs also reach the synovial fluid, but there is still dispute whether in this case the drug reaches the joint predominantly via the transcutaneous or systemic route. A number of studies have shown the efficacy of topical NSAIDs over placebo as well as

the similarity in efficacy with some oral NSAIDs. Minimal literature is available for athropaties.[5] Most NSAIDs are weak organic acids and tend to accumulate in inflamed tissues.[5] Wide ranges of tissue penetration depths, as well as inconsistency in patient response have been reported after topical administration. Individual variability in subcutaneous vasculature and individual skin differences such as hydration status of the skin may account for these findings. This significant intra-individual variability was found in both *in vivo* and *ex vivo* studies.

Percutaneous administration of drugs offers many advantages compared to traditional drug delivery methods, e.g. oral delivery and injections. Firstly, compared to oral delivery, it avoids gastrointestinal drug metabolism (since it circumvents the hepatic first-pass effect), reduces elimination by the liver, provides less chance of an over- or underdose, allows easy termination (e.g. removing the drug from the skin) and permits both local and systemic treatment of conditions. Secondly the pain, fear and the possibility of infections associated with injections, often results in low patient compliance. Transdermal drug transport seems to be an answer to all these problems. The research on methods to increase absorption of drugs transcutaneously constitutes a huge area of pharmaceutical research.

Potential adverse reactions of topical drugs can be divided into cutaneous reactions and systemic reactions. Compared to oral delivery of NSAIDs, topical administration of these drugs offers the advantage of local, enhanced drug delivery to the affected tissue while maintaining low serum concentrations and therefore reduces the incidence of unwanted systemic side-effects.[1] High concentrations in the alimentary tract may lead to nausea, vomiting, dyspepsia and diarrhoea. Adverse effects due to topical NSAID application are primarily cutaneous in nature and consist of a rash and pruritus at the site of application, but fortunately these cutaneous adverse reactions tend to be self-limiting.

Although topical drugs are usually applied at a convenient site, the target for the drug action may be local (e.g. cortisone creams, analgesics) or systemic (e.g. scopolamine to prevent motion sickness). Regardless of the target, all transcutaneous drugs that penetrate the skin and enter the vascular system will have some systemic effects.

For a drug to be a successful topical agent, it has to have efficacy at the target site and also have the ability to reach that site. This may involve delivery via the

systemic circulation and direct penetration. The topical agent consists of a vehicle or base, which often contains an active ingredient. Possible vehicles include: lotions, creams, gels, patches, ointments and pastes. The clinical effect is achieved through direct transport to the tissue or by systemic absorption and redistribution.[5]

1.2 Skin

The skin is an extensive organ covering the exterior of the body and varies in thickness, colour and the presence of hairs, glands and nails. It regulates heat and water loss from the body and also protects the body from the penetration of harmful chemicals or microorganisms, including agents applied to the skin. The human skin also plays a role in the absorption and blockage of radiation, temperature regulation, sensory perception and immunological surveillance. It therefore helps us to withstand a considerable range of environmental challenges. It has a surface area of approximately 2 m^2 and receives about a third of the body's blood circulation.[6] Since it is the biggest organ (that of an average adult male weighs 4.5 to 5 kg) and also easily accessible, it offers great opportunities for the administration of therapeutic compounds.[7]

Skin (*in vivo*) is metabolically active and in a state of continual regeneration. It has immunological and histological responses to different chemicals. Most transdermal drug delivery studies tend to use *in vitro* skin, due to ethical and experimental difficulties. Therefore it should be kept in mind that skin from *in vitro* studies serves only as a model and data obtained cannot always be translated directly to the *in vivo*-situation, since the abovementioned active processes are lost in these studies.

The skin is composed of three layers: an outer layer of epithelial cells called the epidermis, middle layer of connective tissue called the dermis, and a variable inner layer called the hypodermis (Figs. 1 and 2).[8,9]

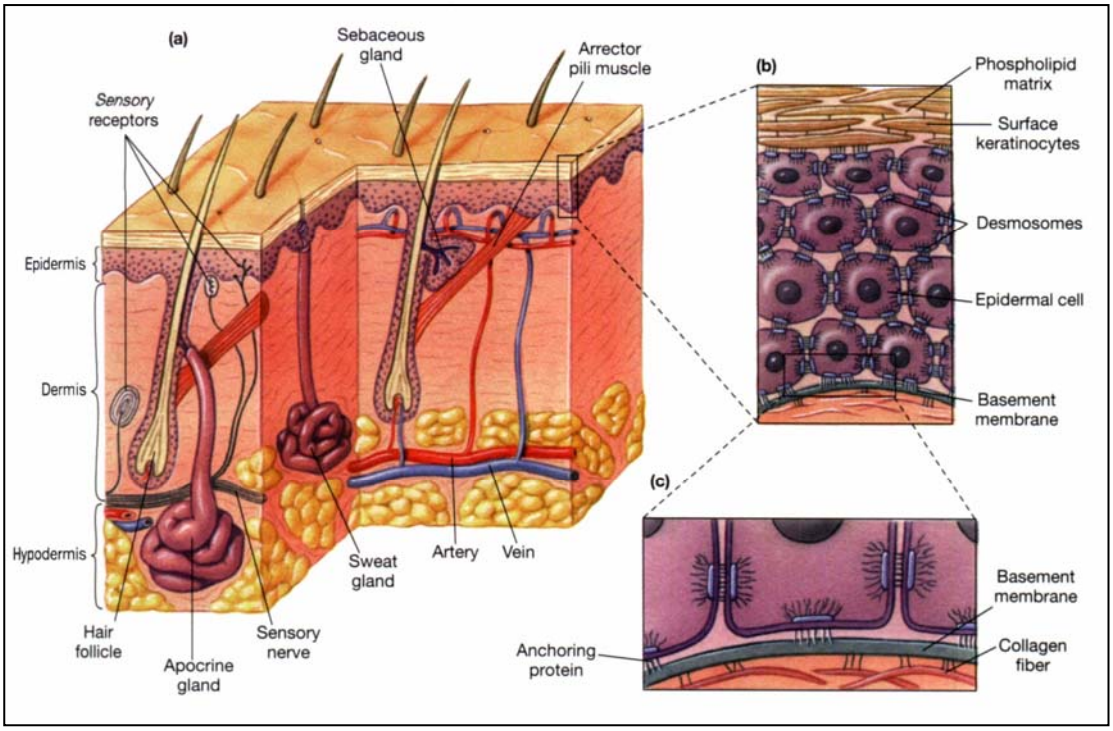

Fig. 1.

a. **The different layers of the skin**
b. **Epidermis**
c. **Basement membrane and the connection between epidermis and dermis**[8]

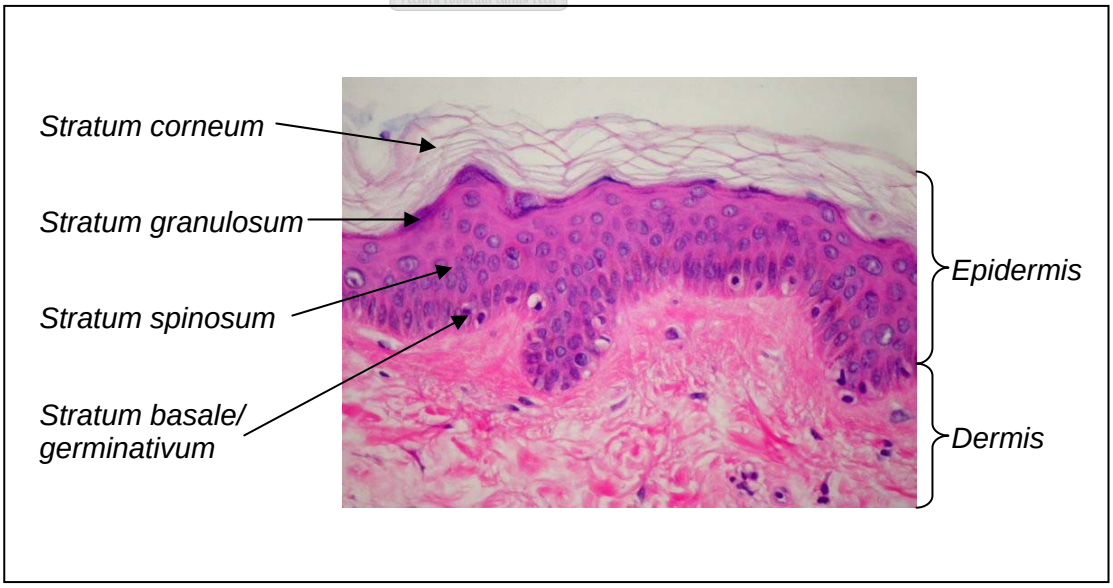

Fig. 2. Histology of human skin[9]

1.2.1 Epidermis

The epidermis, the outermost part of the skin, is a continually renewing, stratified, squamous epithelium. It varies in thickness from around 0.06 mm on the eyelids to around 0.8 mm on the load-bearing areas of the skin. The epidermis was first described by Marcello Malpighi (1628-1694), who believed that it was a gelatinous membrane, and he divided it into an inner layer of viable cells (now known as the stratum malphigii) and an outer one of anucleated keratinized cells (stratum corneum).

The epidermis consists of four histologically distinct layers, which represent the stages of maturation of keratin by keratinocytes. The stratum basale or germinativum, stratum spinosum, stratum granulosum and the outermost layer the stratum corneum (Fig. 2). Some literature describe a fifth layer, the stratum lucidum, but this layer is usually considered to be the lower layer of the stratum corneum.[7]

It does not contain any blood vessels and therefore molecules have to permeate to the dermis to be cleared or taken up by the systemic circulation. The main cell of the epidermis is the keratinocyte, which produces the protein keratin. Keratins are high-molecular-weight polypeptide chains and are the major constituent of the stratum corneum. The stratum corneum comprises keratin (65 %), along with soluble protein (10 %), amino acid (10 %), lipid (10 %) and cell membrane (5 %). The keratinocytes are tied to each other by an extensive system of desmosomes (Fig. 1b). As these cells mature, they synthesize numerous keratin fibres and secrete a hydrophobic phospholipid matrix that acts as the skin's main waterproofing agent. By the time the older keratinocytes are pushed to the surface of the epidermis by newer cells, their cytoplasm is thick with keratin fibres. At this point the cells die, and their nuclei and organelles disappear. The mats of keratin fibres remain behind still linked to each other by the protein fibres of the desmosomes. Dispersed between the keratinocytes are the melanocytes, the cells that produce melanin, and the cells of Langerhans, which have a defensive function.[7]

The stratum corneum seems to be the main permeability barrier. It comprises densely packed disc-like keratinocytes which are anucleate keratinized cells, and separated by multi-cellular lipid bilayers which function as cement.[10] These lipid bilayers consist of regions of ceramides, fatty acids, cholesterol and cholesterol esters and form regions of semi crystalline gel and liquid crystal domains. The

stratum corneum resembles a 'brick and mortar' structure, analogous to a wall, and this highly ordered construction makes the stratum corneum impermeable to many drugs. It forms the rate-controlling barrier for diffusion for almost all compounds.[11]

Most of the molecules penetrate through skin via the intercellular micro route and many of the transdermal enhancement techniques are aimed at disrupting this layer's molecular architecture. This layer is also lipophilic and therefore unionised drugs penetrate the stratum corneum more readily, while the rest of the epidermis is more hydrophilic.[12] A drug that exhibits both hydrophilic and lipophilic characteristics, will therefore have optimal penetration.

The epidermis does not contain any blood vessels or lymph nodes, but does contain a large number of nerve endings. Between the epidermis and the underlying layer of connective tissue is an acellular basement membrane. The cells of the epidermis and fibres of the dermis are anchored to each other by protein fibres that run throughout the basement membrane (Fig. 1c).

1.2.2 Dermis

The dermis is the layer below the epidermis. It is typically 3-5 mm thick and composed of a network of connective tissue, which consists primarily of collagen fibres embedded in a mucopolysaccharide gel.[13]

The dermis, in contrast with the epidermis, does contain blood vessels. The blood vessel supply plays an essential role in the regulation of body temperature whilst also delivering oxygen and nutrients to the tissue and removing toxins and waste products. The blood flow is responsible for removing molecules that have permeated the outer skin layers. This ensures a concentration gradient, and therefore a driving force, between the applied formulation on the skin surface and the vasculature. It also contains lymphatic vessels, nerve endings, pilosebaceous units (hair follicles and sebaceous glands), and sweat glands (eccrine and apocrine). The lymphatic flow seems to play a bigger role with the clearance of larger molecules.[7] Hair follicles are present over the entire body surface, except over the load-bearing areas and on the lips. The sebaceous glands are found together with the hair follicles and secrete sebum that plays a role in maintaining the skin's pH at approximately 5.[7]

This layer is often viewed as consisting of essentially gelled water and therefore provides a minimal barrier to the delivery of most polar drugs. The dermal barrier properties are more pronounced when delivering lipophilic compounds.[7]

1.2.3 Hypodermis

The hypodermis is the deepest layer of the skin. This subcutaneous fat layer forms the bridge between the overlying dermis and the underlying body organs. It serves as an insulator of the human body, protects it against physical shock and provides a reservoir of high-energy molecules. In most areas of the body it is a few millimetres thick, but in some areas, e.g. the eyelids, it is absent. The hypodermis contains blood vessels and nerves.[7] Below the hypodermis is the subcutaneous tissue, which forms the internal boundary of the skin.

1.3 Transmembrane diffusion processes

Compounds are thought to transfer through the skin by a predictable system of passive diffusion, which is defined by Fick's Law and the rate of permeation. Diffusion for most low molecular weight substances seems to occur uniformly through the stratum corneum. Percutaneous absorption of a topically applied drug involves a sequence of individual transport processes. A drug molecule has three possible pathways through skin tissue: through hair follicles with associated sebaceous glands, via sweat ducts or across the continuous stratum corneum (inter- and transcellularly).[14]

The area available for diffusion purposes from the skin appendages, is almost negligibly small, approximately 1 %.[11] This pathway may be important for ions and large polar molecules. Once diffusion through the stratum corneum has taken place, the molecules permeate the dermis and are absorbed by the capillary plexus and transported into the circulating bloodstream.[14] The molecule follows a tortuous route and has to cross, sequentially and repeatedly, a number of hydrophilic and lipophilic domains. The time necessary to reach C_{max} is approximately 10 times longer than that when the equivalent oral administration is given.[1]

The transmembrane diffusion process is passive in nature, requiring a concentration differential as the driving force and each molecule requires kinetic energy to effect a net movement down this gradient. The lipid-water partitioning characteristics of the

permeant play an important role in its penetration. Drugs that are either extremely hydrophilic or extremely hydrophobic are poorly absorbed. Molecular size also plays a role in penetration of the skin and so does the pH of the vehicle.[15] Other physiochemical properties of a drug include solubility/melting point and the state of ionisation. The governing factor for which pathway the permeant will follow when it diffuses through the tissue, is the partition coefficient. There is an inverse relationship between the permeant flux and its molecular weight. Therefore smaller molecules diffuse through the tissue at a faster rate than larger molecules. Topical medication is usually applied in an aqueous formulation and the permeant from the formulation should thus be somewhat water-soluble to minimise the depletion of the permeant from the formulation over the time course of the application. Most drugs are weak acids or weak bases which dissociate to various degrees depending on the pH of the formulation used and the pH of the membrane through which it must diffuse.[1,7]

For many years it was thought that percutaneous drugs all entered the dermal capillary network and reached the central blood compartment only to return to the local area above which they were applied. However, it now appears that there is a system of local delivery separate from systemic delivery via the central blood compartment.[14]

Permeability of the skin is also influenced by age, disease, the appearance of skin appendages (e.g. hair follicles, aprocrine sweat glands, erector pili muscles, sebaceous glands), the nature of its physiochemical condition (e.g. thickness, lipid structure, membrane capacity), as well as the viscosity and the extent of cross-linking of the collagen.[16] Hydration of the stratum corneum also plays a vital role. The amount of water in the stratum corneum increases the permeability of this layer to hydrophilic drugs. It is possible for the stratum corneum to absorb three to five times its own weight in water, resulting in a two- to threefold enhancement in the permeability to water and other polar molecules.[14]

1.4 Methods to breach the skin barrier

Scientists have looked at different means to enhance the transport of drugs through the skin and especially trying to decrease the barrier properties of the stratum corneum - the main barrier of the skin. The barrier properties of the skin are primarily attributed to the intercellular lipid bilayers of the stratum corneum. The ability of a

drug to penetrate the skin is closely related to its molecular weight and its affinity for the stratum corneum.[10,17]

Transdermal delivery is often only considered after a novel compound has been selected, shown to be active, but then was proven to be problematic for oral administration. The drugs that are available for transdermal formulation consequently seldom have ideal physicochemical properties for transdermal administration. Redesigning of the chemical is often too expensive and time consuming, after the compound's toxicological, pharmacological and pre-formulation studies have already been conducted.[7]

Many of the topically applied agents, e.g. the non-steroidal anti-inflammatory drugs (NSAIDs), do not penetrate skin optimally.[12,18,19] While product formulation may have a dramatic impact, not only on transcutaneous absorption rates, but also on depth of penetration into the underlying tissues, further optimisation may be desirable.[1] A number of approaches to enhance transport across skin and to expand the range of drugs delivered have been investigated.[20] Despite intensive research during the last three decades, there are still limited transdermal products available on the market for transdermal transport. Those available are mainly low-molecular weight lipophilic drugs (MW<500 Da). Expanding the range of topically available drugs, especially to include macromolecules, is primarily hindered by low skin permeability. Strategies for transdermal drug absorption are primarily targeted at enhancing the permeability of the stratum corneum, the main barrier of the skin. These strategies involve the selection of the correct drug or prodrug and chemical potential adjustment. Hydration of the stratum corneum and chemical methods can also be used. Chemical enhancers include surfactants, solvents, lecithin gels, liposomes and submicron emulsions. Surfactants may enhance partitioning by reducing the surface tension between the vehicle and the membrane surface, but also influence the barrier potential of the membrane.[21]

Physical methods, e.g., iontophoresis, electroporation, magnetophoresis, using a photomechanical wave and sonophoresis are other options.[11] These techniques are based on two principles: enhancing skin permeability and/or providing a driving force acting on the drug. Often combinations of methods are used because they are more effective than single techniques. Iontophoresis is the use of electricity to enhance transdermal transport and for this purpose a current between 0.5 and 20 mA is usually employed.[22] Skin electroporation creates transient aqueous pores in the lipid

bilayers by application of short (micro- to millisecond) electrical pulses of approximately 100-1000 V/cm. These pores provide pathways for drug penetration.[11] Magnetophoresis is the ability of magnetic fields to move diamagnetic materials through skin. Another technique which is likely to stay experimental is the use of a photomechanical wave. A drug solution, placed on the skin and covered by a black polysterene target, is irradiated with a laser pulse.[11] The resultant photomechanical wave stresses the stratum corneum and enhances drug delivery. Ultrasound, a physical treatment modality, may also enhance cutaneous uptake.[14] It is an especially helpful technique for physiotherapists, since ultrasound equipment are often present in physiotherapy practices.[23,24] Other advantages of using ultrasound in drug delivery include its non-invasive nature, relative convenience and comfort, as well as the ability to focus and control the spatial, along with temporal delivery, of the drugs.

1.4.1 Ultrasound

The use of ultrasound as a therapeutic modality was introduced over 5 decades ago and has since then been developed into one of the most widely available and frequently used electrophysical techniques by physiotherapists.[23,24] While the human ear can detect frequencies between 20 Hz and 20 kHz, ultrasonic waves have frequencies above 20 kHz. Ultrasonic waves are generated by applying a high frequency, alternating, electrical current, across a quartz or silicone dioxide crystal, or across certain polycrystalline materials such as lead-zirconate-tetanate (PZT) and barium titanate. Through a phenomenon known as the piezoelectric effect, the electrical current induces the crystal to undergo rhythmic deformation, producing ultrasonic vibrations. In the process of ultrasonic wave generation, electrical energy is converted into mechanical energy in the form of oscillations, which generate acoustic waves.[7,25]

Indications for therapeutic ultrasound in physiotherapy include musculoskeletal conditions, disorders of the peripheral nerves, disorders of the skin and open wounds. Despite the frequent use of ultrasound in practice, there is minimal scientific evidence to support its clinical beneficial effects.[26] Regarding ultrasound dosage, two trends are evident and these relate to either acute or chronic musculoskeletal conditions. Although a rationale exists for the usage of these dosages, clinical evidence for their efficacy is currently sparse.[23] Previous attempts at reviewing a dose-response relationship, using the information available from randomized

controlled trials (RCTs) of ultrasound have failed. Gam and Johannsen (1995) were unable to demonstrate even basic dose-response relationships in ultrasound after reviewing the literature extensively.[27] They identified 293 papers, published between 1950 and 1992 that investigated the use of ultrasound to treat musculoskeletal conditions. A relationship between dosage and treatment outcome could not be made due to the inadequacy of the treatment details provided by the studies. In 2002 Robertson performed a similar survey of the world-wide English literature.[24] Robertson analyzed dosage related variables from an existing set of randomized controlled trials published between 1975 and 1999 on the use of ultrasound to treat pain and soft tissue lesions. The results revealed that too few details were provided in most studies to identify a relationship between dosage and treatment responses.[24]

1.4.2 Phonophoresis

Phonophoresis (sonophoresis) is the use of ultrasound to enhance transdermal drug transport. This technique for improving drug delivery was first reported by Fellinger and Schmid, who demonstrated that polyarthritis of the hand could be successfully treated by driving hydrocortisone into inflamed areas with ultrasonic waves.[28] Since then the combination of ultrasound with steroids and analgesics has especially been used in a wide selection of musculoskeletal disorders (e.g. rheumatoid and osteoarthritis, tendonitis, as well as other joint, ligament, tendon and synovium pathologies). Furthermore, ultrasound has also been used to enhance the skin permeability to various other drugs, e.g. ketoprofen, lidocaine, benzydamine, acetylsalicylic acid, interferon, estradiol and insulin.[29] It has also recently been used in a process termed reverse phonophoresis, for monitoring “blood” glucose levels.[30]

The three most common types of topical drugs used in phonophoresis are:

1) Anaesthetics (substances such as lidocaine that block pain receptors by creating numbness).
2) Counterirritants (substances such as menthol).
3) Anti-inflammatories such as nonsteroidal medications (e.g. salicylates) or steroidal medications (e.g. hydrocortisone, dexamethasone).

The long term effects of ultrasound on the skin are still unclear. Although ultrasonic skin heating does occur, these increases in temperature of a few degrees, have not been considered to make large contributions to the increased permeability observed when compared to the cavitational effects caused by sonication.[30,32] However,

previous studies have shown double-digit temperature increases in tissues due to sonication.[33,34] While there may be other contributing factors such as acoustic streaming and mixing effects, cavitational channel-opening in the lipid layers, i.e. the porous pathway theory, is thought to play a major role in permeation enhancement.[35] The creation of these channels, which allows a freer passage of both hydrophobic and hydrophilic drugs of varying molecular weights, effectively indicates “damage” to the barrier lipid layer of skin. From *in vivo* evidence, this “damage” of the human skin caused by cavitational channel formation appears to persist for at least 15 h following sonication. Hereafter, the barrier regains normal, pre-sonication permeability status by 24 h.[36] In another, more recent *in vivo* study, the increased permeability to diclofenac was shown to last at least 3 h following application of ultrasound.[37]

Visual and microscopic examination of ultrasound-exposed skin, do not reveal any noticeable skin damage.[38-40] Other researchers, though, have reported noticeable skin damage from phonophoresis experiments.[33,41,42] Machet *et al.* did a histological examination of hairless mice skin after ultrasound was given at 1.5 W/cm^2, 1.1 MHz for 20 min, using a cooling coil.[42] The temperature was maintained at 31 °C. Histological examination showed no difference between control and sonicated skin. Scanning electron microscopy revealed holes at the surface of the corneocytes from the sonicated skin. Transmission electron microscopy showed no cellular abnormalities and well-preserved intercellular spaces without signs of intercellular disruption.[42] Cell lysis and membrane rupture may be considered a permanent change in permeability. However, there are reports in which membrane permeability was changed transiently, without cell disruption, and eventually the original permeability was restored. Furthermore, there appears to be an intensity threshold below which membrane permeability can be transiently perturbed. The skin eventually returns to its original permeability state without loss in cell viability or tissue damage. At higher intensities, cells are often permanently damaged, suggesting that holes in the tissue were formed that were too large to recover spontaneously.[43]

Many authors have reported on the effectiveness of phonophoresis *in vivo*.[30,44-47] Ultrasound has been shown to enhance transdermal permeation of compounds by factors ranging from 2 to 20, in treatments lasting a few minutes, at sound frequencies between 20 kHz and 3.6 MHz and at intensities between 0.15 and 4 W/cm^2. For instance, a 5-20-fold increase in the delivery of inulin and mannitol in

rats, within 1-2 h following ultrasound irradiation (1 MHz and 1.5 W/cm^2 for 3-5 min) was reported.[30] These authors also observed a reduction of the lag time usually observed during transdermal delivery of drugs. However, other workers could not demonstrate any significant flux enhancements after applying phonophoresis.[38,48,49] It therefore seems that phonophoresis is only effective under certain conditions. It was then concluded that the effect of ultrasonication depends on the nature of the drug, the formulation base, and the ultrasound parameters used.

Systematic reviews and research studies have repeatedly concluded that there exists insufficient evidence for the therapeutic value of ultrasound, as well as for the process of phonophoresis.[33,43] No clear consensus exists on the effectiveness of phonophoresis, or on the nature of the phonophoretic mechanism; consequently the foundation of this treatment modality is highly subjective and non-quantitative.[50] The conflicting data found in the literature are primarily attributable to the fact that different research groups have employed different ultrasonic parameters, i.e. frequency, intensity, duration and mode, as well as different skin membranes and vehicles. Furthermore, conclusions drawn from published studies performed using phonophoresis as a tool to enhance drug delivery have been limited by a lack of standardisation. The latter includes deficient controls, incomplete accounts of the dosimetry and protocols employed, and the non-calibration of the ultrasound equipment. Due to these reasons and the multiplicity of drugs as well as end-point evaluation techniques used, it is difficult to discern any trends between ultrasonic frequency, intensity, molecular structure and degree of permeation enhancement. In spite of these mechanistic uncertainties, there is no doubt that ultrasound has the ability to markedly increase percutaneous absorption for a number of molecules, under certain conditions. Without established dose-response relationships, users of therapeutic phonophoresis can only estimate effective dosages for patients. Considerably more controlled *in vitro* and *in vivo* studies are therefore necessary to evaluate the currently used treatment dosages properly, to clarify the mechanisms and to optimise the process.

1.4.2.1 Mechanisms of Phonophoresis

The propagation of an ultrasonic wave within the skin produces both thermal and non-thermal effects.[20] The latter effects are important in determining the therapeutic and phonophoretic efficacy, as well as potential deleterious effects of ultrasound.

1.4.2.1.1 Thermal effects

Heating

Ultrasound does not pass through tissues with 100 % efficiency. During its propagation, the ultrasound wave is partially scattered and absorbed by the tissue medium, resulting in attenuation of the emitted wave. The lost energy is converted into heat, while the remainder of the wave penetrates into and propagates through the medium. The quantity of heat absorbed depends on the absorption characteristics of the tissue being irradiated and the amount of ultrasonic energy passing through it.[20] Tissues with high protein content absorb energy more readily than those with a higher fat content. Once delivered, the heat is then dissipated by both thermal diffusion and local blood flow. Ultrasound is also known to cause deep penetrating hyperthermia, which may increase solubility of drugs, vasodilatation and blood flow, factors which may all facilitate drug permeation. Furthermore, phase transitions of the intercellular lipids of the stratum corneum may also occur close to physiological temperatures and may lead to increased diffusion of the drug.[51] In this respect it has been shown that percutaneous absorption of the hormone, estradiol, doubled when the temperature was increased by 10 ºC.[52]

It has been shown that the crystalline/gel barrier lipid domains undergo transitions (pre-melting) towards more disordered structures with increasing temperature.[53] Between 41 and 42 ºC, a clearly characterized phase transition of the intercellular lipid domains to an even more fluid and disordered state occurs.[54] Thermally-induced rearrangements of these systems change diffusion characteristics of permeants, because the compositions and physical structures of the lipid domains affect the barrier functions of skin. This has been demonstrated for both skin and mucosa.[17,55] Thermal energy also increases the molecular diffusion of the permeant molecules.[14,56]

Several authors have measured the *in vitro* increases in temperature at the skin surface following exposure to ultrasound waves. This elevation amounted to only a few degrees Celsius and could not explain the increase in percutaneous absorption observed.[52,57] If heating was the main cause of increased transdermal drug delivery, then clearly phonophoresis would be of no great therapeutic interest, since the same penetration enhancement effect could be obtained by applying any appropriate heat source. However, other studies have reported greater rises in skin surface temperature. Miyazaka *et al.* demonstrated a rise of 6 ºC with 1 MHz at a low intensity of 0.25 W/cm^2 and 12 ºC at a higher intensity of 0.75 W/cm^2.[58] A rise in

temperature may therefore be one of the major factors explaining the increase in percutaneous absorption in the frequency range 1-3 MHz in the continuous mode *(vide infra)*. Machet *et al.* therefore decided to control the heating effect of ultrasound *in vitro* with a cooling coil.[42] These researchers demonstrated that ultrasound was ineffective for the enhancement of percutaneous diffusion of drugs, when applied for 10-20 minutes. This concept was reinforced by some controlled *in vivo* studies, which failed to demonstrate any valuable effect of commonly used therapeutic ultrasound techniques.[38,49] Another study showed, that when heating was removed as an artefact of phonophoresis, the skin absorption of five different anaesthetic molecules was not affected by ultrasound.[59] Some workers have suggested 45 ˚C as a cut-off point for skin damage, with temperatures in excess of this value being potentially destructive to skin.[60]

The abovementioned studies do not imply that phonophoresis is ineffective, but indicate that the optimal ultrasound parameters have not yet been established. Ultrasound-enhanced delivery has also been reported in experiments where negligible ultrasonic heating developed.[61,62] This points to the existence of phonophoretic mechanisms other than heating. Merino *et al.* supported the concept that another mechanism (other than heating) exists which is responsible for the lowered skin barrier function after ultrasonification.[63] These researchers found that only about one-fourth of the skin permeability was attributable to the increased temperature induced by ultrasound. They therefore concluded that, at least for the permeant model they used, there existed another mechanism, most probably cavitation, responsible for the lowered skin barrier function they observed.[63]

1.4.2.1.2 Non-thermal effects

1.4.2.1.2.1 Cavitation

Cavitation occurs in a liquid medium owing to nucleation of small gaseous cavities during the negative pressure cycles of ultrasound, followed by the expansion of these bubbles throughout subsequent cycles. It thus involves the generation and oscillation of gaseous bubbles and their subsequent collapse. This cavitation leads to the disordering of lipid bilayers and formation of aqueous channels in the skin structures through which drugs can permeate. Cavitation may also generate violent microstreams, which may increase the bioavailability of drugs.[64] The occurrence of cavitation in biological tissue has been attributed to the existence of large numbers of gaseous nuclei. The latter are gas pockets trapped either intra- or intercellularly. It has been shown that cavitation within skin layers plays a dominant role in enhancing

transdermal transport during ultrasound exposure.[52] Cavitation inside the stratum corneum can potentially occur in the keratinocytes, or in the lipid regions, or both. Many drugs permeate the epithelial layer of the stratum corneum via the intercellular route (Fig. 3A). When ultrasound is applied, disordering of the lipid layers occurs and the transcellular route may become more prominent (Fig. 3B).

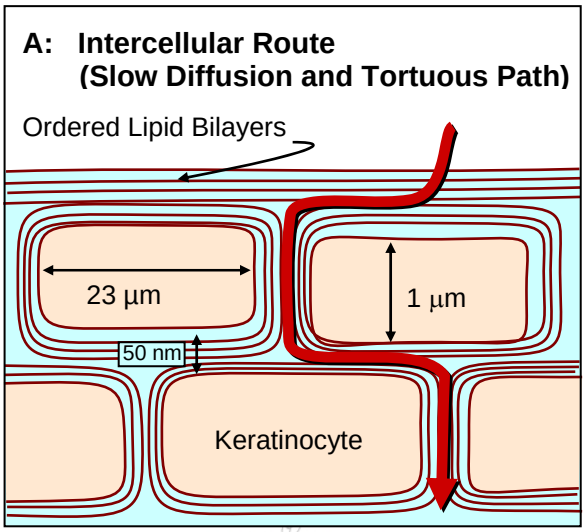

Fig. 3 A. Permeation of drugs through the epithelial layer of the stratum corneum via the intercellular route.

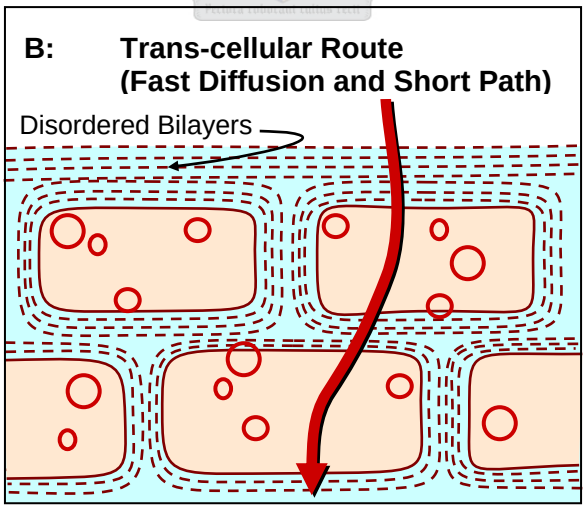

Fig. 3 B. Permeation of drugs through the epithelial layer of the stratum corneum via the transcellular route following disordering of the lipid layers by ultrasound (gas bubbles due to cavitation are shown).

Two types of cavitation occur in liquid media: stable and unstable. Stable cavitation corresponds to a bubble that slowly oscillates many times around its equilibrium radius, while unstable cavitation involves rapid growth and collapse of a bubble. The latter exists for less than one cycle during which it expands to at least double, and often to several times, its original size. Eventually it implodes, creating a shock wave, often followed by the creation of many smaller bubbles. In water, the occurrence of cavitation is facilitated by the presence of dissolved gas.[14,33]

A cavitation threshold exists, which is defined as the minimum ultrasound intensity required for the onset of cavitation, and it increases rapidly with ultrasound frequency. The most commonly used ultrasonic conditions for phonophoresis (frequency 1-3 MHz, intensity 0-2 W/cm^2) are termed therapeutic ultrasound conditions. However, as the number and size of cavitation nuclei are inversely correlated with the frequency of the applied ultrasound pulse, it was found that any frequency lower than that corresponding to therapeutic ultrasound was actually more effective in enhancing skin permeability.[10,65] At higher ultrasound frequencies it becomes increasingly difficult to induce cavitation owing to the short time-lapse between positive and negative acoustic pressures, because insufficient time is available for the dissolved gas within the medium to diffuse into the cavitation nuclei.[29]

Cavitation, as a mechanism for phonophoresis, has been studied widely and is supported by a series of experiments.[33] These include:

1) The importance of keeping dissolved gas in the medium to form nuclei of cavitation.[52]
2) The possibility of permeating cell membranes *in vitro* is enhanced in the presence of artificial cavitation nuclei.[42]
3) Demonstration of possible pores created by ultrasound on the skin surface and within the stratum corneum.[66]
4) Demonstration of multiple pits induced by bubble implosion on aluminium foil exposed to ultrasound and its correlation with intensity and skin conductivity.[65]

1.4.2.1.2.2 Radiation pressure force

Any medium or object that absorbs a beam of energy is subjected to a force, the radiation pressure force, which tends to push that material along the direction of wave propagation. Naturally this force will be greatest in a strongly absorbing medium. One hypothesis is that an induced radiation pressure, acting on the

penetrant molecules, can push the drug through the skin.[67] Another proposal is that ultrasound exerts a radiation pressure force on the stratum corneum, thus perturbing its barrier properties. Simonin used a 1 MHz, 1 W/cm^2 ultrasonic beam, and computed from theory that the stratum corneum would be subjected to a maximum pressure equivalent to that produced by 5 mg of weight distributed over a 1 cm^2 area of its surface.[68] At therapeutic intensities, the primary radiation pressures are therefore small and are unlikely to damage well-anchored soft tissues.

1.4.2.1.2.3 Acoustic Microstreaming

When a structure within an ultrasonic field is subjected to an unequal distribution of radiation pressure forces across its length, it is also subjected to a force known as the acoustic torque. This torque produces microscopic currents in a liquid or semi liquid medium, termed acoustic microstreaming. The latter phenomenon may also occur due to the oscillations of cavitation bubbles, which generate a liquid motion.[20] It has been postulated that shear forces generated by acoustic microstreaming can perturb the skin barrier and thus play a role in enhancing skin diffusion during phonophoresis.[69]

1.4.2.2 Dependence of phonophoretic skin permeabilisation on ultrasound parameters

1.4.2.2.1 Frequency

The most commonly used conditions for phonophoresis (frequency 1-3 MHz, intensity 0-2 W/cm^2) are termed therapeutic ultrasound conditions.[7] These parameters are often used in the physiotherapy practice. Attenuation of an acoustic wave is inversely proportional to its frequency, and thus the higher this parameter, the more energy is dissipated in the surface tissues, leaving less energy available for absorption in deeper tissues.[33]

1.4.2.2.1.1 High frequency phonophoresis (1-3 MHz)

High frequencies (1-3 MHz) were first investigated as physical enhancers for transdermal delivery of drugs.[14,30] Since the outer layer of the epidermis, the stratum corneum, is the main barrier to percutaneous penetration of drugs, it initially seemed logical to concentrate the ultrasonic energy in this skin layer using high frequencies. The first studies involved hydrocortisone[28] and anaesthetics[70] and the technique became very popular in the United States. An investigation performed at 52 military hospitals showed that 45 hospitals used phonophoresis.[14] Ultrasound was used at

continuous or pulsed frequencies, ranging from 0.7 to 1.1 MHz, with intensities from 1 to 2 W/cm^2.[29] The principal criterion for efficacy of the phonophoretic process was the subjective reduction of pain. These high frequencies are still being used in current treatments, even though previous attempts to use high frequency ultrasound to enhance transdermal drug delivery have produced inconsistent results that were found to vary significantly from drug to drug.[71]

1.4.2.2.1.2 Low frequency phonophoresis (20-100 kHz)

Subsequent to the research of Mitragotri and collegues in 1995, it has been clear that frequency is a major determinant in phonophoresis.[33,34] The latter researchers demonstrated that in contrast with previous assumptions, the use of low frequency ultrasound (20-150 kHz) was more effective than higher frequencies of ultrasound in enhancing transdermal transport.[29,30,40,57,61,72,73] Skin permeability increased as the frequency of ultrasound decreased, as well as with increased intensity and length of time of ultrasound application.[74] Mitragotri's research showed that cavitation played the dominant role in increasing skin permeability and that the number and size of cavitation nuclei showed an inverse correlation with the frequency applied.[10,65]

Low frequency sonophoresis, using a 20 kHz probe, showed increased transdermal diffusion of various molecules *in vitro*. Diffusion rates of low molecular weight molecules were increased 2-5000-fold across isolated epidermis *in vitro*.[75] Using human skin, a significant but moderate increase was also demonstrated for caffeine and fentanyl, the enhancement ratio being 4 and 34 during sonication, respectively.[75] Tachibana was the first to demonstrate transdermal diffusion of insulin *in vivo*.[30] Phonophoresis was performed on hairless mice using 48 kHz ultrasound for 5 minutes, the mice experiencing a marked decrease (80 %) in glycaemia. In hairless mice and in humans, reverse skin permeability of glucose measured after exposure to ultrasound at 20 kHz, was 100-fold enhanced compared with controls.[30,76] Significant results for large molecules such as insulin[57] and low molecular weight heparin[73] were also found.

1.4.2.2.2 Mode, Intensity, Time

Ultrasound waves can be emitted continuously (continuous mode) or in a sequential mode (discontinuous or pulsed mode). The rise in temperature is faster and more intense with the continuous mode. Intensity is directly dependent on the acoustic energy and the velocity of the sound waves in the medium.[33]

A threshold energy dose for phonophoresis exists.[74] Ultrasound has a large number of components that combine with each other to constitute the final dosage. *In vitro* studies with low-frequency ultrasound were performed to measure the dependence of percutaneous drug enhancement on ultrasound parameters, including mode, intensity and exposure time. While enhancement varied linearly with ultrasound intensity and exposure time, it was independent of the mode in the range of parameters studied.[74] The enhancement was also directly proportional to the ultrasound energy density once the threshold value was exceeded. For full thickness pig skin, the threshold value was about 222 J/cm^2, however, it must be kept in mind that this value may depend on the skin type itself and vary between different skin models.

1.5 Ultrasound before or after the application of the drug

Phonophoresis of mannitol across pig skin, *in vitro*, was reported to be identical whether ultrasound was applied before application of the compound, or simultaneously. This suggested the absence of significant convection processes during phonophoresis. It may also be assumed that the effect of ultrasound on skin structure was roughly similar in both cases.[74] Skin permeability in the form of skin conduction was measured after a close relationship between these two phenomena was established. The conductivity decreased by about 10 % after ultrasound was turned off (at 1.5 h), and thereafter remained nearly constant for the next 20 h. The skin conductivity 20 h after sonication was higher than that of control skin specimens. The data suggested that skin remained permeable well beyond the sonication time for ultrasound parameters used in that study.

Rosim *et al.* conducted a quantitative study of sodium diclofenac (Voltaren Emulgel®, Novartis) phonophoresis in humans.[37] Volunteers received ultrasound (continuous, 1 MHz, 0.5 W/cm^2, 5 min) on two 225 cm^2 areas on the dorsum (group A), followed by the application of the gel. Plasma diclofenac mass was measured at 1, 2 and 3 hours. The same procedure was repeated one month later with the same volunteers, but the ultrasound was switched off for this control group (group B). The plasma diclofenac mass was significantly higher up to 2 hours in group A. After 3 hours it was still higher, but the difference was not significant.

However, with a larger molecule such as insulin, pre-treatment with ultrasound was not effective in enhancing transdermal transport, even though effective transdermal

transport of this hormone could be achieved during sonication.[76] The increased skin permeability to a drug may therefore not always persist after the cessation of sonication.[30]

1.6 Coupling agent

An ideal coupling agent should exhibit an absorption coefficient similar to that of degassed water. The presence of air bubbles in the contact medium reduces ultrasound transmission and a good coupling agent will therefore exhibit a low capacity for dissolved gases. Even minute amounts of air can disrupt the flow of energy. The coupling agent should also retain a gel or paste consistency at body temperature, ensuring that the gel will remain on the skin and provide intimate contact between the transducer head and the epidermis in order to allow maximum energy transfer.[77]

General topical pharmaceutical products containing the active drug have often been used as the coupling material during phonophoresis. The problem with this approach is that, unlike contact media designed specifically for this purpose, topical pharmaceutical products are generally not formulated to optimize their efficiency as ultrasound couplants. Consequently, much of the ultrasound energy may be lost before it reaches the skin.[78] A group of investigators examined the transmission of ultrasound energy at frequencies of 0.75, 1.5, 3 MHz through 41 different common topical pharmaceutical preparations.[59] Large variations in ultrasound transmissions were observed between different products as well as, in some cases, between the same products at different frequencies. Benson and McElnay conducted a study in which ultrasound transmission through commercially available topical products containing non-steroidal anti-inflammatory drugs was investigated.[78] The percentage transmission of ultrasound was measured relative to deionised degassed water at a range of frequencies (0.75, 1.5, 3 MHz with total power output of 1.5 and 5 Watts). A Bio-tek® wattmeter was used to measure ultrasound transmission. Gel-based formulations were shown to transmit ultrasound efficiently at 1.5 and 3.0 MHz, but less efficiently at 0.75 MHz. This confirmed their findings in 1988 where a frequency-dependent attenuation was reported.[78] A possible rationale for these findings is that the higher frequencies ultrasound may cause some breakdown of the polymer chains of the viscosity-inducing agents used in topical formulations. This would have a fluidizing effect on the formulation, with less attenuation of the ultrasound wave.

Phonophoresis in animal studies most effectively increased hydrocortisone penetration, but also caused skin burns on the animals.[45] Such treatments can obviously not be applied in the clinical setting and skin damage alone is also known to increase transdermal drug penetration. A poorly transmitting coupling medium may convert ultrasound energy into heat and this may explain the findings of these and similar studies.

Although the ideal ultrasound dosimetry for clinical application of phonophoresis is unknown, irrespective of the intensity setting, a medium must be employed that transmits effectively to achieve predictable ultrasound transfer. When a poor transmission medium is used, much less ultrasound energy reaches the patient than that shown by the ultrasound parameter settings. Fortunately, a wide selection of media exists that do transmit ultrasound well and these should be used with the ultrasound power and frequency settings most appropriate to the pathology treated.

Degradation of drugs by ultrasound was studied *in vitro* and did not occur for insulin, fentanyl and caffeine.[76] The persistence of biological activity of insulin and low-molecular weight heparin *in vivo* was also in accordance with the absence of degradation under the phonophoretic conditions used.[40,73] It therefore appears as if most molecules are generally stable at the ultrasound intensities employed in phonophoresis.

1.7 Diclofenac

Diclofenac is a NSAID, available for oral, rectal, intramuscular and topical use. It is a phenylacetic acid derivate and has analgesic, anti-pyretic and anti-inflammatory activities.

Diclofenac, 2-[2,6-dichlorophenyl) amino] benzeneacetic is an acidic compound (pKa = 3.80 at 25^{o}C), has a high partition coefficient (log P = 4.0), with very low aqueous solubility (6 x 10^{-5} M at 25 ^{o}C) in the unionised form.[79] Because of these characteristics, it is often administered in a salt form.[79,80] Salt formation is a commonly employed method for enhancing the solubility and dissolution rate of poorly soluble weak acids or bases. Therefore diclofenac is mainly used as sodium or potassium salt, or as a salt with diethylamine (Fig. 4) or N-(2-hydroxyethyl) pyrrolidine for topical formulations. When the diethylammonium (diethylamine) salt of diclofenac is prepared, its water-solubility increases significantly (46 mM at 25 ^{o}C). It

has been suggested that, in solution, ion-pair species are formed between the cations and anions present in the diclofenac diethylammonium salt. These ion-pairs display a lower hydrophilicity than the two ions considered separately and offer unusual behaviour for ionic species, such as high solubility in non-polar solvents or increased partitioning into lipid phases. The ion-pairs of the diclofenac diethylammonium salt would therefore be able to pass through the lipid barrier of the stratum corneum into the dermis where the pH would cause a shift towards a more dissociated form of diclofenac, which is better able to travel through the hydrophilic dermal layers.[79,80]

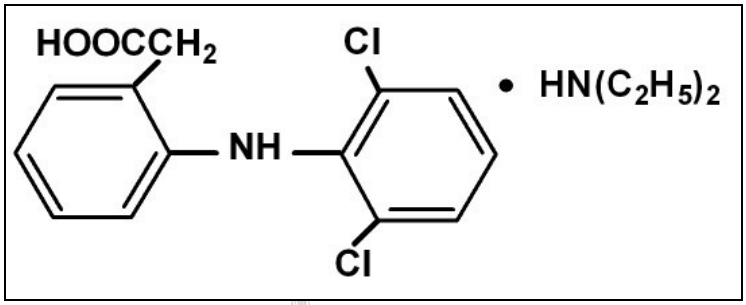

Fig. 4. Diclofenac Diethylammonium (diethylamine)

Voltaren Emulgel® (Fig. 5) is an anti-inflammatory and analgesic preparation designed for external application. Voltaren Emulgel® (100 g) contains 1,16 g diclofenac diethylammonium corresponding to 1 g diclofenac sodium. The base is a fatty emulsion containing isopropanol and propylene glycol, in an aqueous gel.[81] Both the isopropanol and the propylene glycol are classified as humectants and are thus capable of bringing about hydration and hydrophilisation of the stratum corneum, facilitating the absorption of diclofenac. Propylene glycol is also classified as a penetration enhancer, which causes increased penetration of diclofenac through the stratum corneum. The action of propylene glycol as a potential penetration enhancer has been questioned. Several authors have reported that its action is based on its cosolvent effect.[82] In this case, drug penetration is thermodynamically controlled. Only limited data demonstrate an enhancer effect in which drug penetration increases with increasing propylene glycol content of the formulation. This promoting effect is related to the solvent drag effect of propylene glycol.[83,84]

Fig. 5 Voltaren Emulgel®

Experimental studies have shown that when applied locally, the active substance penetrates the skin and the underlying tissue, and combats acute and chronic inflammatory reactions. It is an inhibitor of cyclooxygenase (COX 1 and 2) and therefore responsible for a reduction in prostaglandin, thromboxane and prostacyclin production, which are mediators of the inflammatory process. It is relatively effective in comparison with other NSAIDs and plasma concentrations of 100 $ng.ml^{-1}$ are associated with a therapeutic effect. Cutaneous application leads to the absorption of about 6 % of the dose administered, and is associated with serum drug concentrations well below those observed after standard oral or intramuscular dosage and below the range at which side effects usually occur.[85] It has been claimed that the topical application of diclofenac over an inflamed joint results in synovial fluid drug concentrations which exceed systemic plasma concentrations, suggesting a direct penetration of drug into the synovial fluid of the joint.[85] When given orally, GIT absorption is rapid and complete with an approximate first pass-effect of 50 %. Protein binding is 99.7 % and the mean terminal elimination half-life of the unchanged drug is 1 to 2 hours. Diclofenac and its metabolites are mainly excreted in the urine.[81]

1.8 *In vitro* permeability studies for drug testing

Different types of diffusion equipment are available for the study of *in vitro* tissue permeability. The different flow cells include the conventional static Franz cells (Fig. 6), where an accurate volume of sample must be removed with a simultaneous media replacement, and the Ussing chamber (Fig. 7), optimized for the use of high-frequency alternating current stimuli, as well as flow-through diffusion cells (Fig. 8).[86,87]

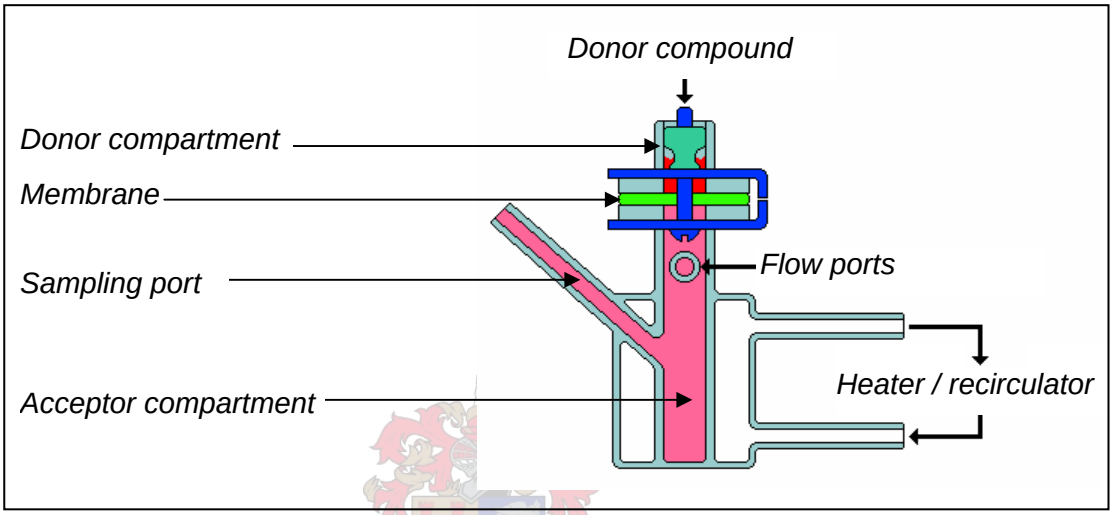

Fig. 6. Schematic drawing of a Franz cell

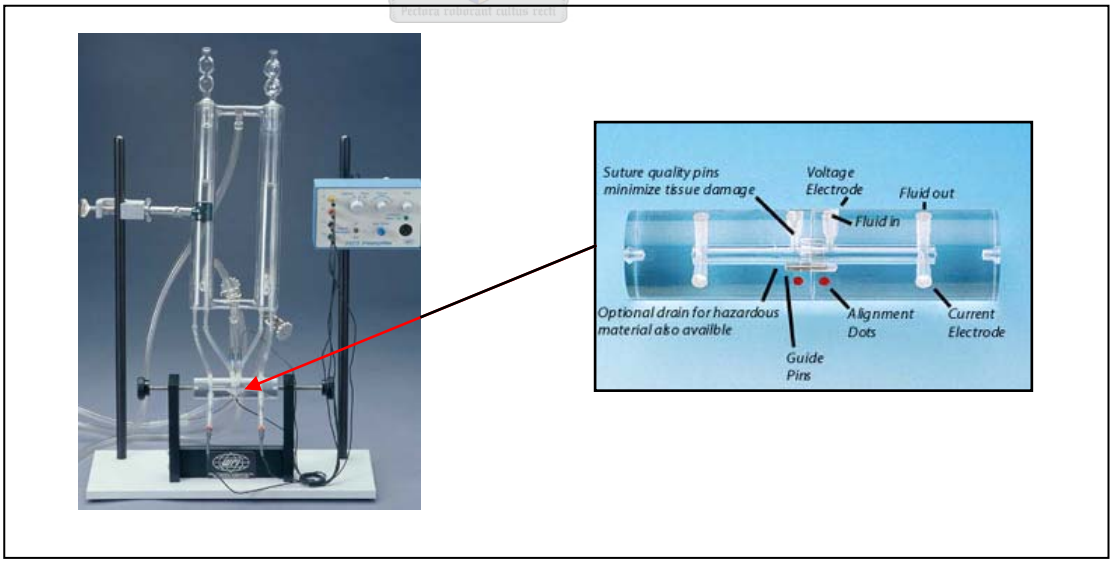

Fig. 7. An Ussing chamber

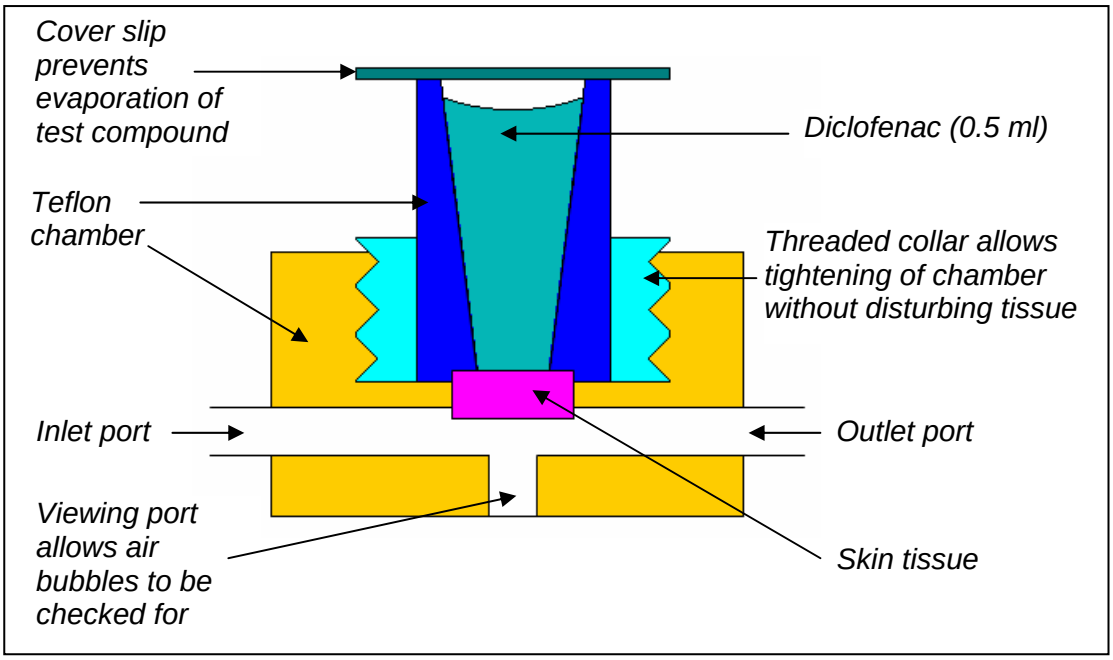

Fig. 8. Schematic drawing of a flow-through diffusion cell

A flow-through diffusion apparatus was used in the present study (Fig. 9). It contains 7 flow-through diffusion cells. Each cell contains an acceptor chamber through which there is a continuous flow of buffer. This ensures sink conditions throughout the course of the experiment. Therefore, at the completion of each run the concentration of permeant in the acceptor chamber never reached 10 % of that in the donor compartment. In contrast to the static Franz cells, the flow-through diffusion cells offers automation with the addition of a pump that offers an accurate, constant flow rate of buffer. The only other requirement is the addition of a fraction collector.[86] The drug is added to the donor compartment of the flow cell and collected by means of a fraction collector from the acceptor compartment of the flow cell. This is done for the required time period of the experiment, at a constant flow rate. The drug in the effluent may be detected by various means e.g. measuring labelled drug (radio-labelled, fluorescent-labelled) by means of scintillation counting, UV spectroscopy, fluorospectroscopy or high-performance liquid chromatography.

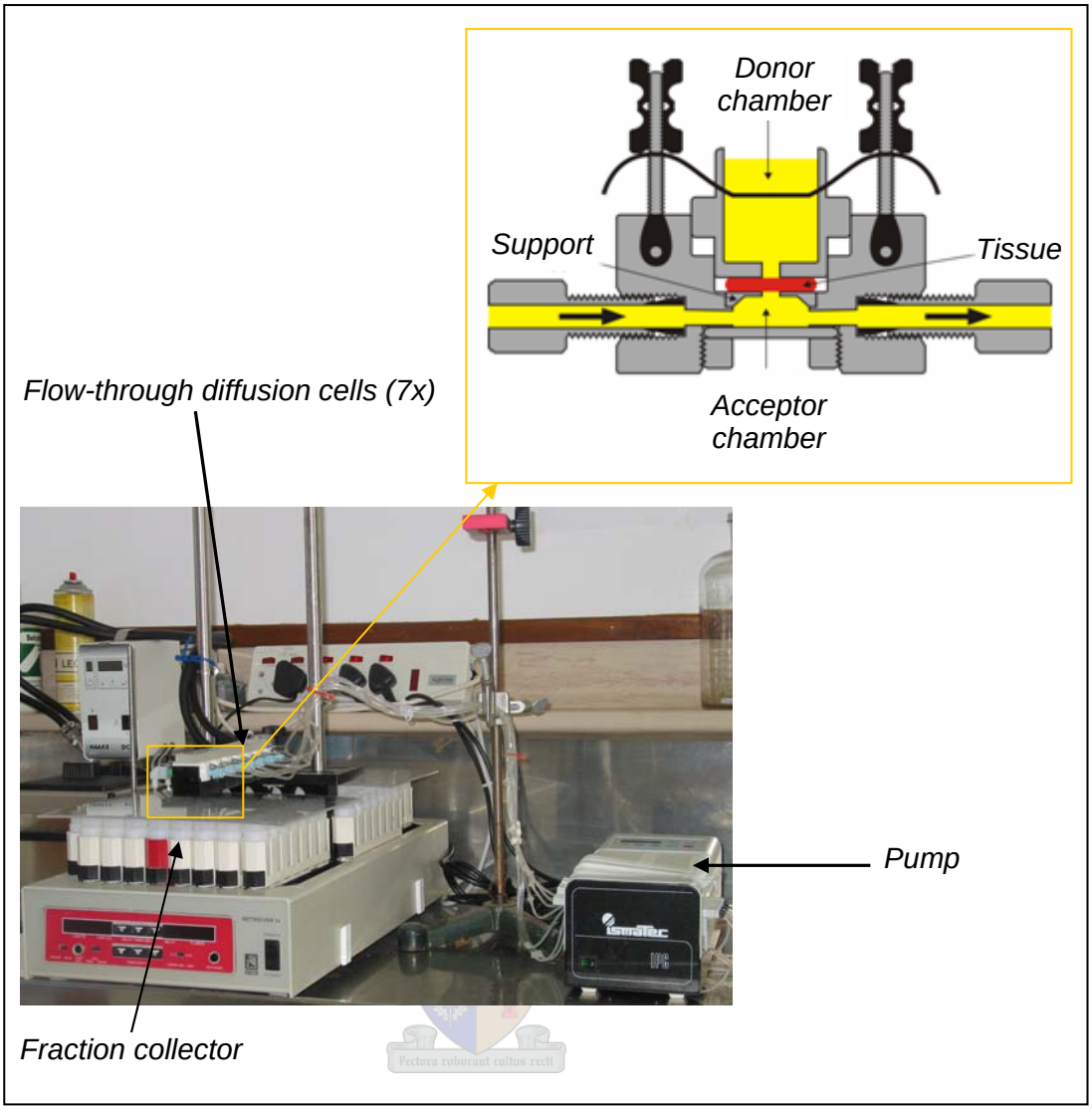

Fig. 9. Flow-through diffusion apparatus used in the present study

1.9 Aim

Because many of the agents employed topically, e.g. the non-steroidal anti-inflammatory drugs (NSAIDs) do not penetrate skin optimally, the aim of the current study was to assess the effects of therapeutic levels of ultrasound on the transdermal permeation of diclofenac. For this purpose a dual-stage experimental design and a continuous flow-through diffusion system were used. Researchers in the Department of Pharmacology, Stellenbosch University, have extensive previous experience with the continuous flow-through diffusion system to determine the diffusion kinetics of a wide variety of therapeutic agents and other chemical compounds across fresh and frozen human vaginal and buccal mucosa, skin, venous tissue and rabbit as well as

human corneas.[36,88-97] This prompted us to use the flow-through diffusion system to study the effects of ultrasound on the permeability of diclofenac across human skin. Furthermore, the aim of the study was to develop an *in vitro* flow-through diffusion model that could be used to predict values for *in vivo* studies. Ultrasonic heating and temperature variations in the skin were also studied to evaluate the sonication-heating theory in which permeability changes in skin are primarily attributed to thermally-induced changes in stratum corneum lipids.

2. Materials and Methods

2.1 Skin

Skin specimens were obtained from excess tissue removed from 18 females, mean age 41 ± 13 SD (range: 18-62) yr, during breast reduction procedures at the Louis Leipoldt Hospital, Bellville, South Africa. No specimens were obtained where there was clinical evidence of any disease that might have influenced the permeability characteristics of the skin.

All skin specimens were immediately placed in a transport fluid after removal and transferred to our laboratory within 24 hours. The transport fluid consisted of a stock solution of Eagle's Minimum Essential Medium (MEM) without L-glutamine and sodium bicarbonate (Gibco, Paisley, Scotland), to which the latter as well as an antibiotic (penicillin/streptomycin, 100 IU/ml) and an antimycotic (amphotericin-B, 2.5 µg /ml) were added prior to using it for the transport of tissue specimens. In the laboratory excess connective tissue was trimmed away and specimens from each patient (10 x 10 mm) were snap-frozen in liquid nitrogen and stored at –85 ˚C. Prior to use the frozen samples were thawed and hydrated in PBS for at least 24 hours at 4 ºC.

The study was approved by the Ethics Committee of Stellenbosch University and the Tygerberg Academic Hospital.

2.2 Diclofenac

Voltaren Emulgel® (Fig. 5), containing 1.16 g diclofenac diethylammonium equivalent to 1 g diclofenac sodium/ 10 g (i.e. 10 mg/g), was obtained form Novartis SA (Pty) Ltd, Rivonia, South Africa.

2.3 Sonication

Thawed skin specimens were placed on a gel-filled plastic bag which acted as a heat sink, and tightly covered with Parafilm® (Pechiney Plastic packaging, Menasha, WI 54952, USA) into which a fenestration (6 x 6 mm) was made yielding an exposed epidermal area of 0.36 cm^2 (Fig. 10).

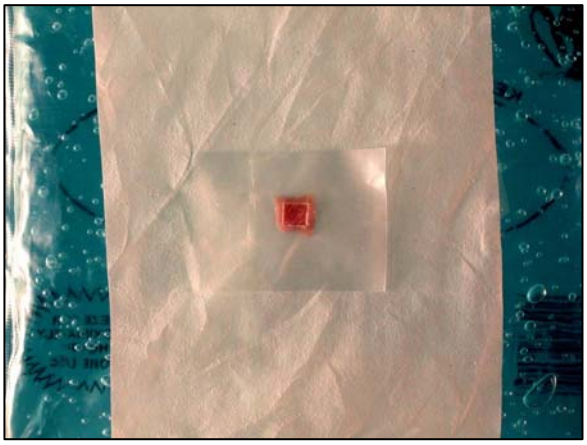

Fig. 10. Skin specimen covered by Parafilm® with a fenestration (gel-filled bag underneath the skin specimen serves as a heat sink)

A measured quantity (0.05 ml ~ 0.05 mg) of diclofenac was lightly applied to the exposed area and the transducer placed over the gel. This gel acted also as a contact medium (coupling agent). Ultrasound (2 W/cm^2, 3 MHz, continuous) was applied using a sonicator (Sonopuls 590®, DIMEQ, Delft Instruments, Delft, The Netherlands) for 10 minutes (Figs. 11 and 12). Control specimens, which were not exposed to ultrasound, were included in the permeability experiment. The exposed area of the control samples was also covered with diclofenac, but did not receive any ultrasound.

Fig. 11. Sonopuls 590® (Sonicator)

Fig. 12. Transducer head

2.4 Permeability experiments

Sonicated and control specimens of skin were mounted in flow-through diffusion cells (exposed areas 0.196 ± 0.002 SD cm^2) and permeation studies performed on 7 tissue replicates for each patient.[36] Voltaren Emulgel® (0.5 ml (~ 0.5 mg)) was placed in the donor compartment of each flow-through diffusion cell (Fig. 9).

The gels were covered with a Teflon disk and 0.5 ml of PBS. PBS at 37 ºC was pumped through the acceptor chambers at a rate of 1.5 ml/h and collected by means of a fraction collector, at 2-h intervals for 24 h. The permeability study was performed under sink conditions, i.e. at the completion of each run the concentration of NSAID in the acceptor chamber never reached 10 % of that in the donor compartment. The above experiments (sonication and controls) were repeated and fractions collected at 20-min intervals for 4 h (phase 1). The diclofenac in the acceptor chambers was quantified by means of HPLC analysis.

2.4.1 HPLC determination of diclofenac

Permeant-containing effluent samples, collected from the acceptor compartments of the perfusion apparatus over the 20-min and 2-h sampling intervals, were analyzed using an Hewlett Packard 1100 series high-performance binary liquid chromatograph (Agilent Technologies, Waldbronn, Germany) with an Agilent Eclipse (XDB-C18) Zorbax analytical column (5 μm particle size), 150 mm x 4.6 mm (ID) (Fig. 13). The latter column was preceded by a 30 x 2.1 mm (ID) C_{18} guard column (40 μm particle size). The temperature was maintained at 40 ^{o}C and flow rates of 1.0 ml/min were used. The mobile phase consisted of a mixture of two solvents, A (50 mM KH_2PO_4, pH 5.42) and B (acetonitrile-isopropanol; 4:1 v/v). An isocratic mixture of A:B of

65:35 was used for the determination of diclofenac. All reagents used for the mobile phase were HPLC grade (Burdick & Jackson, Honeywell International Inc, Muskegon, MI, USA) and were filtered through a 0.45 μm filter. Deionised water was used for preparing all aqueous standard and buffer solutions. Aliquots (50 μl) from each sample were injected directly into the column. Diclofenac was detected at 273 nm (retention time 2.3 minutes). Total run time was 3.5 minutes. Recording and integration of peaks was performed by means of an Agilent Chem Station. Spiked standards over the expected concentration range (0.5-20 μg/ml) were randomly included in each batch.

Fig. 13. HPLC used in the present study

2.5 Temperature experiments

In order to study heat-producing effects of ultrasound on skin, thawed skin specimens were sonicated as described in section 2.3.

Resistance (kΩ) of a waterbath-calibrated (20 °C to 42.5 °C) miniature glass bead thermistor (47 kΩ @ 20 °C, RS Components, UK) was measured using a digital multimeter (MASTECH®, Electrical and Electronic Measuring and Testing Equipment, Taiwan) immediately before (ambient temperature) and directly after cessation of sonication (Fig. 14).

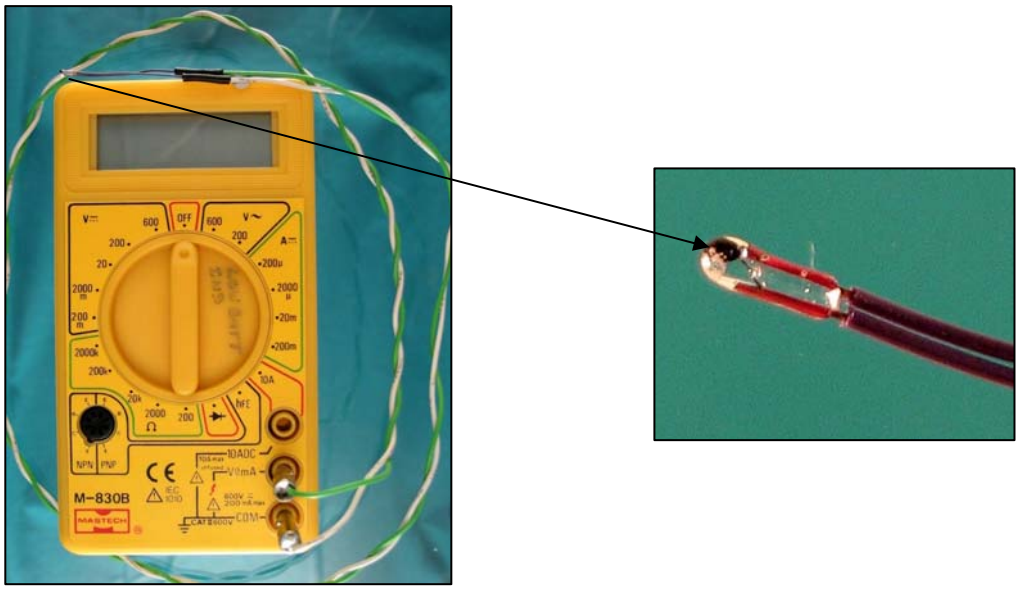

Fig. 14. Digital multimeter and Thermistor

To minimize heat loss during the measuring process, the thermistor was held tightly against the subdermal connective tissue sandwiched between two insulating polystyrene sheets. Tissue temperatures were read off directly from the previously constructed resistance vs. temperature calibration curve. After removing the insulating polystyrene sheets, times taken for the specimens to return to pre-sonication temperatures were also recorded.

For studying temperature-dependency of $^{3}H_2O$ flux rates, specimens of thawed, hydrated skin were mounted in flow-through diffusion cells (exposed areas 0.196 cm^2) and permeability studies performed on 7 tissue replicates, as previously described.[36] Prior to each permeability experiment, the thawed skin specimens were equilibrated for 10 min in PBS (pH 7.4) at 37 ^{o}C in the diffusion cells. The PBS was then removed from the donor compartment and replaced with 1.0 ml of PBS containing 1 µCi ^{3}H-water (Amersham Laboratories, Little Chalfont, Amersham, UK). Aliquots (100 µl) were removed within minutes from each of the seven donor compartments for the determination of donor cell concentration at time zero. A flow rate of 1.5 ml/h was maintained and fractions collected, by means of a fraction collector, at 2-h intervals. Temperatures were kept constant at 37 ^{o}C for the first 12 h of the experiment. Hereafter, they were increased to 42 ^{o}C for 6 h and then lowered again to 37 ^{o}C. The latter temperature (37 ^{o}C) was maintained for the remaining 6 h of the experiment. Temperatures were controlled by using a circulator water bath. All permeability studies

were performed under sink conditions, as described before (section 2.4). Scintillation cocktail (10 ml) (PCS Scintillation Cocktail; Amersham Biosciences, Uppsala, Sweden) was added to each sample collected and the radioactivity determined using a Beckman LS 5000TD liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA) (Fig. 15). The counting of the samples was continued until a 2-s value of 1 % was reached. Quenching for each sample was automatically corrected in the counter.

Fig. 15. Beckman LS 5000TD liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA)

2.6 Calculation of flux values

Flux (J) values of diclofenac across membranes were calculated by means of the relationship: $J = Q/A \times t$ ($\mu g \times cm^{-2} \times min^{-1}$), where Q = quantity of diclofenac crossing membrane (µg), A = Membrane area exposed (cm^2) and t = time of exposure (min). For the ^3H_2O experiments, flux values were calculated using the same relationship as above, but were expressed as dpm x cm^{-2} x min^{-1}, with Q = quantity of ^3H_2O crossing membrane (dpm).

2.7 Steady state kinetics

It was assumed that when no statistically significant difference at the 5 % level (t-test with Welch's correction) between flux values were obtained over at least 2 consecutive time intervals, a steady state (equilibrium kinetics) had been reached for a particular skin specimen and the diclofenac or $^{3}H_2O$.

2.8 Statistical Analysis

Non-linear regression analyses (third order polynomials) were performed using a GraphPad Prism, version 4, 2003 computer programme. A F-test was used to compare entire curves.[98] A t-test at steady state, was also performed for comparative purposes. A significant level of 5 % was used for all tests and comparisons.

3. Results

3.1 Permeability experiments

The overall mean flux values of diclofenac ex Voltaren Emulgel® across human skin versus time (24 h) are shown in Fig. 16. The graph exists out of 2 phases (dual-stage kinetic diffusion pattern) of which the first phase is encircled in green. During the first phase or 4 hours there is a decline in flux values and during the second phase there is an increase of flux values of both the sonicated and control groups. Steady-state fluxes were obtained after approximately 14 h. Significant differences, at the 5 % level, using a F-test and comparing entire curves could be demonstrated between flux values of diclofenac across sonicated and control skin specimens ($P = 4.16 \times 10^{-9}$). Flux values for diclofenac across sonicated skin were on average double those across control skin specimens.

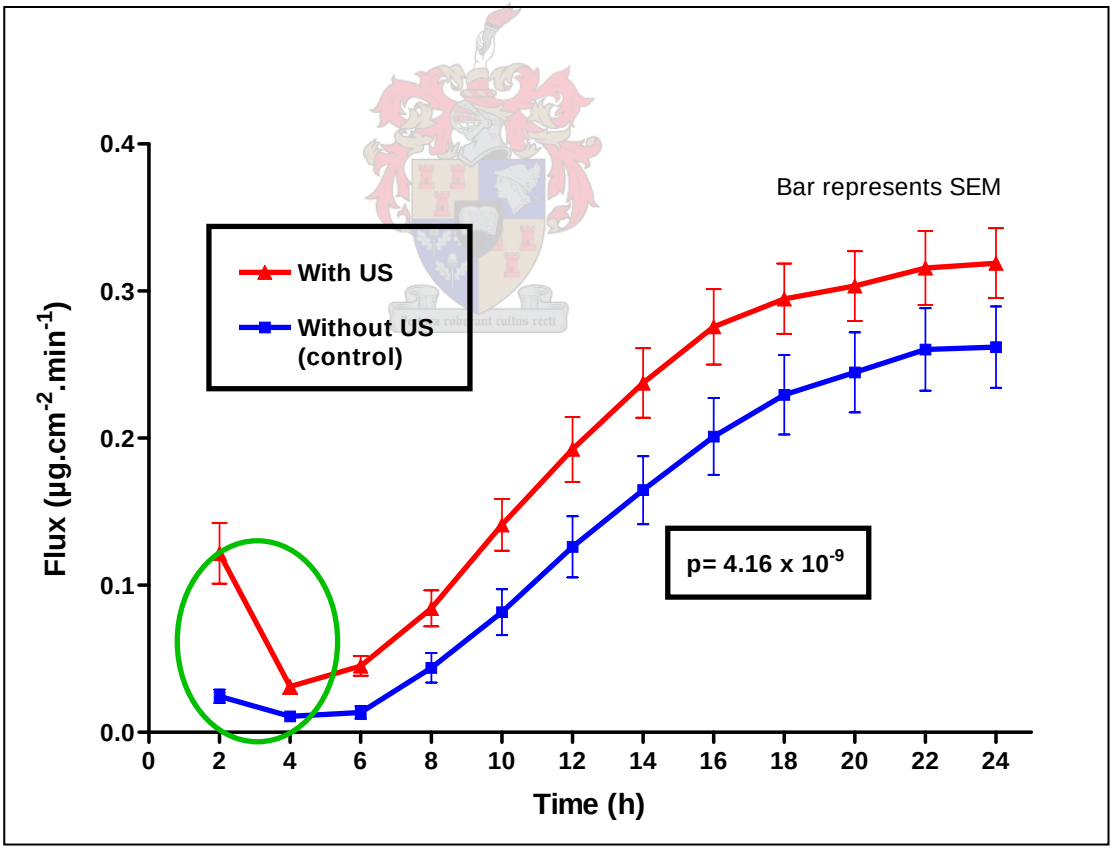

Fig. 16. Mean flux values for diclofenac ex gel across human skin with and without ultrasonication (US)

The overall mean flux values of diclofenac ex Voltaren Emulgel® across human skin versus time (4 h) are shown in Fig. 17. Significant differences, at the 5 % level (F-test, comparing entire curves), could be demonstrated between the flux values of diclofenac across sonicated and control skin specimens ($P = 3.05 \times 10^{-10}$).

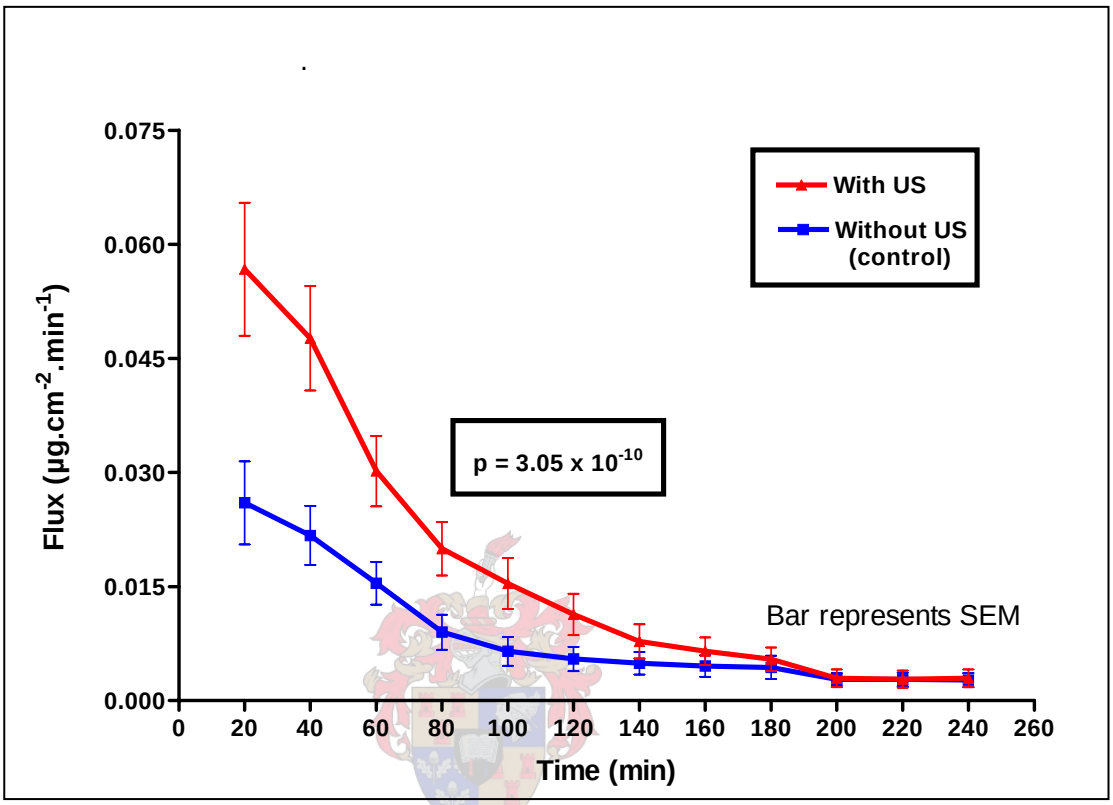

Fig. 17. Mean flux values for diclofenac ex gel across human skin with and without ultrasonication (US)

Fig. 18 shows the flux ratios for diclofenac gel across human skin with and without sonication (control) over 4 h with a regression analysis which clearly shows a linear relationship between these ratios ($r^2 = 0.7796$, $y = - 0.007x + 2.61$). At the start of the experiment the flux ratio between the sonicated and control specimens is 2.61, which declines over the 4 h period. This value is calculated by extrapolating the linear graph to the y-axis at time zero and is indicated by an arrow. The 95 % confidence intervals (CI) are also shown (Fig. 18).

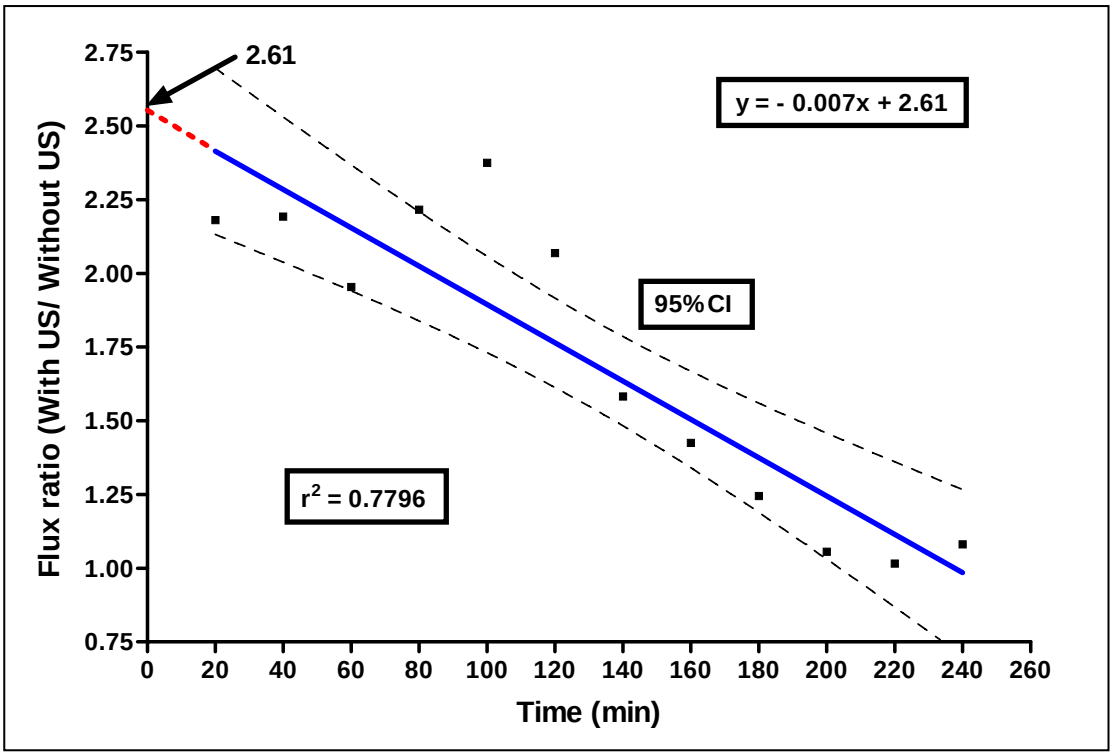

Fig. 18. Flux ratio for diclofenac ex gel across human skin with/without ultrasonication (US)

Using the Higuchi model, the cumulative amount of released diclofenac ($\mu g.cm^{-2}$) permeating the skin specimen per unit surface area was plotted against the square root of time ($h^{½}$).[99] Assumptions associated with the Higuchi model include that: (a) only single drug molecules are assumed to diffuse; (b) the drug is able to diffuse out of the membrane; (c) the drug reaching the acceptor chamber fluid is rapidly removed. Since the infinite dose technique was used in the present study, it was essential that less than 10 % of the amount of diclofenac introduced onto the skin surface in the donor chamber permeated through the skin in order to maintain sink conditions. Mean apparent release rates (slopes) and lag times (x-axis intercepts) were calculated by linear regression analysis of the plots of ($\mu g.cm^{-2}$) vs. ($h^{½}$) using the Higuchi equation (Fig. 19).[99] For the sonicated and control specimens apparent release rates were 168.3 ± 1.4 and 135.5 ± 1.5 $\mu g.cm^{-2}.h^{-½}$, lag times were 3.34 and 3.56 $h^{½}$ and r^2- values were 0.9998 and 0.9996, respectively. The non-linearly related portions of the data curves, i.e. <3 $h^{½}$, were excluded from the linear regression plots for both the sonicated and control specimens and hence have been omitted from Fig. 19. The graph of plasma concentration of diclofenac with and without sonication has been drawn using published data (Fig. 20).[37]

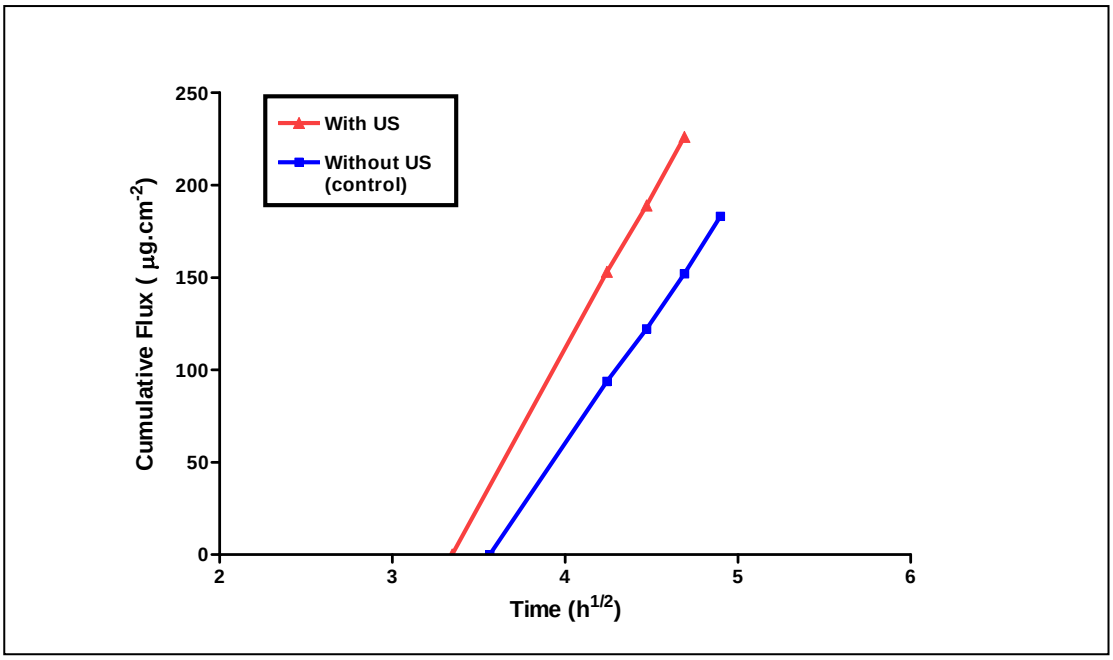

Fig. 19. Cumulative flux values for diclofenac ex gel with and without ultrasonication (US)

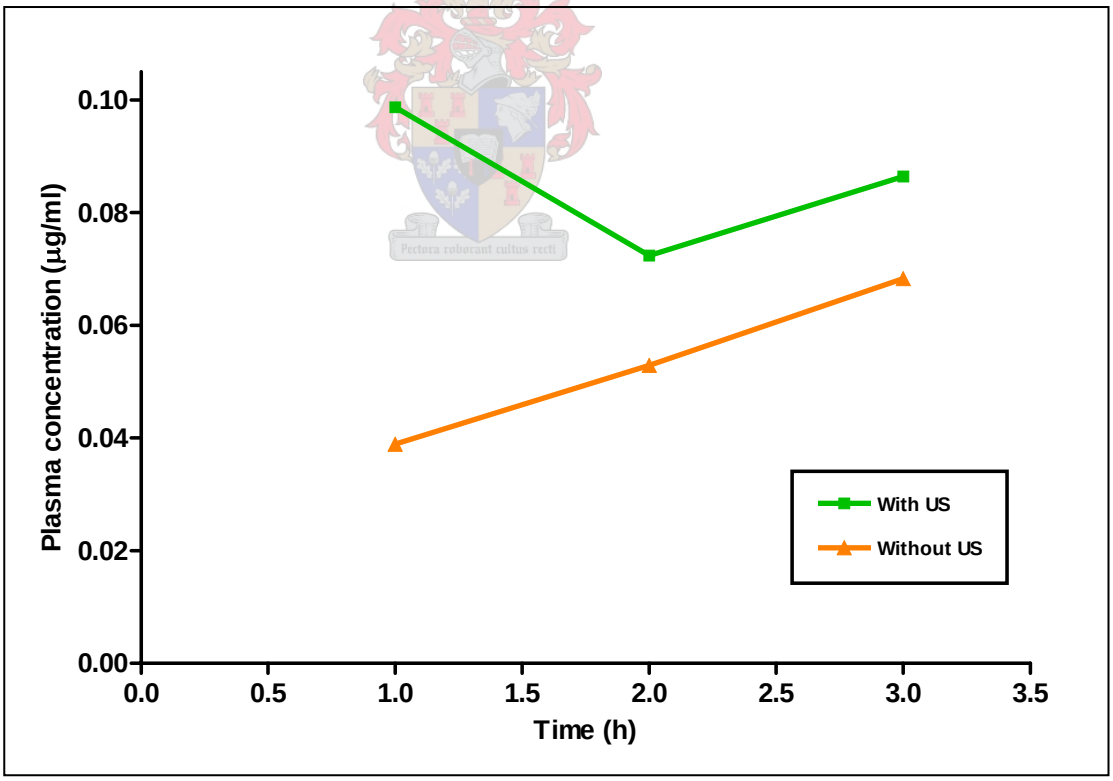

Fig. 20. Plasma concentration of diclofenac with and without ultrasonication (US) (using published data[37])

3.2 Temperature experiments

The calibration curve for the thermistor (Resistance (kΩ) vs. Temperature (°C)) is shown in Fig. 21. Average pre-sonication (ambient) skin temperatures were 20.8 ± 0.83 ^{o}C and rose to 31.0 ± 1.92 ^{o}C (N = 8) following sonication. Subsequent to cessation of ultrasound, these post-sonication temperatures returned to their pre-sonication values after approximately 3 min (Fig. 22).

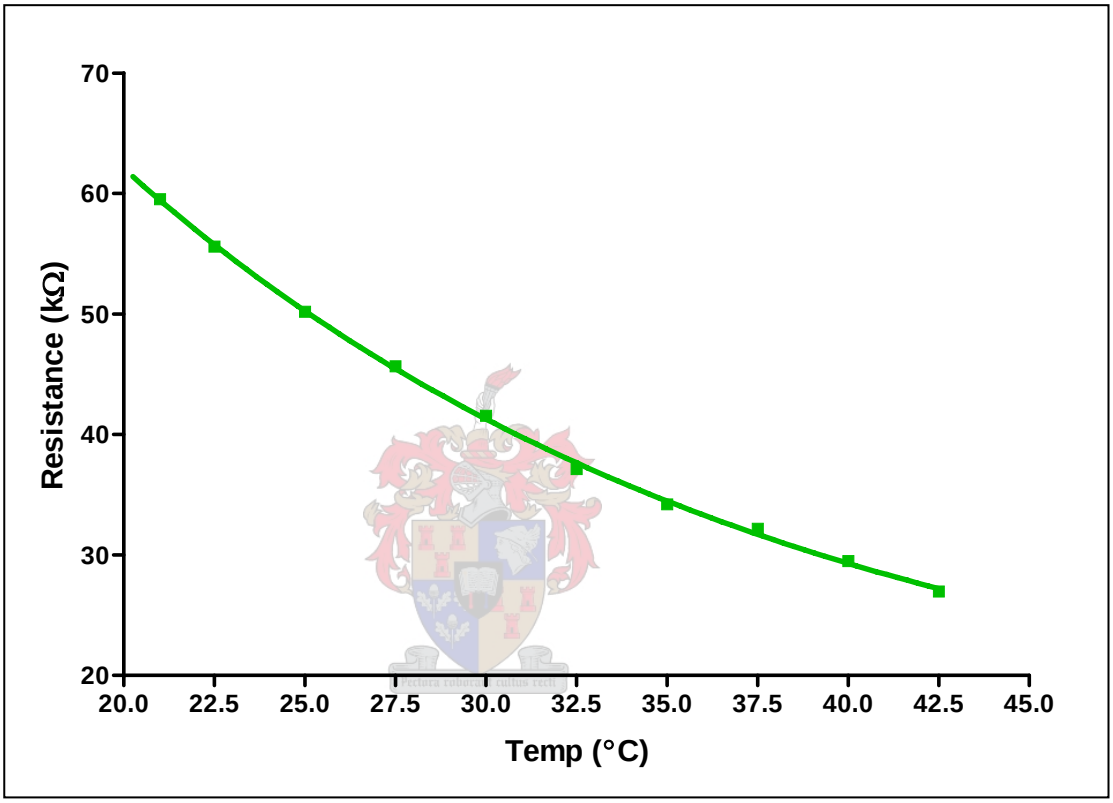

Fig. 21. Calibration curve of resistance vs. temperature for thermistor

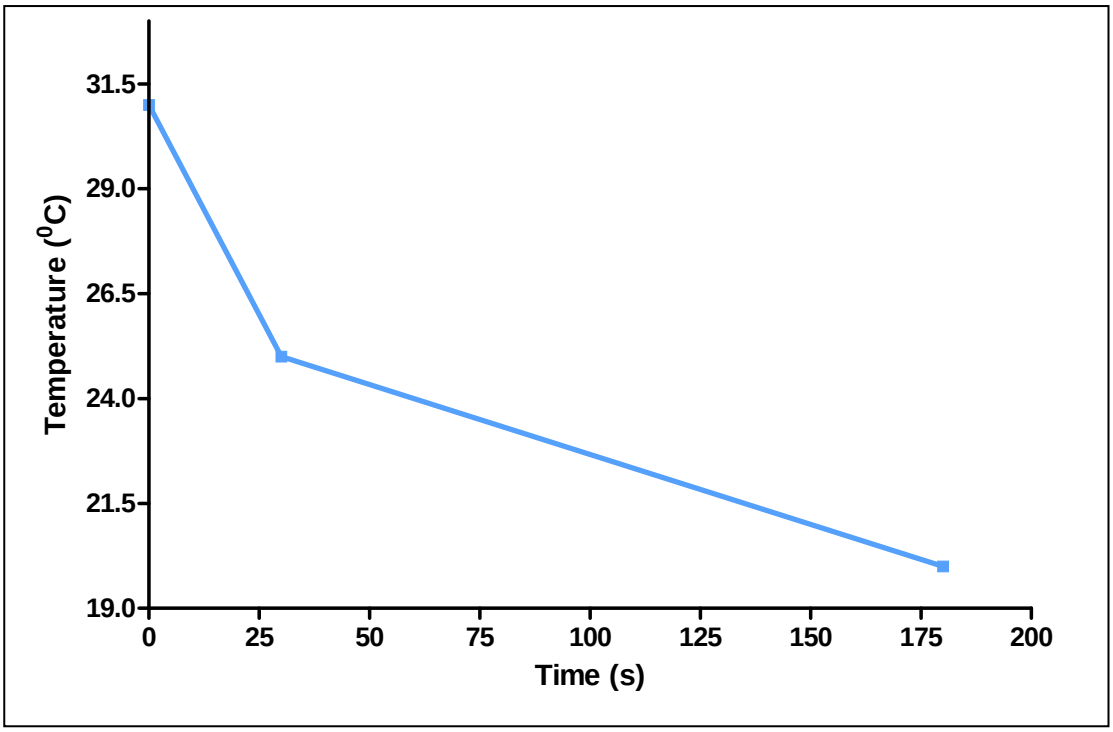

Fig. 22. Heat loss after cessation of ultrasound for human skin

Average flux values for $^{3}H_2O$ across skin rose significantly ($p<0.05$) from 186 ± 16 dpm.cm^{-2}.min^{-1} at 37 °C to maximally 316 ± 26 dpm.cm^{-2}.min^{-1} at 42 °C and decreased to approximately 235 ± 12 dpm.cm^{-2}.min^{-1} after the temperature was again lowered to 37 °C (Fig. 23). Steady state flux values at 37 °C were obtained between 8 h and 12 h (183 ± 17 dpm.cm^{-2}.min^{-1}) and again between 20 h and 24 h (243 ± 13 dpm.cm^{-2}.min^{-1}).

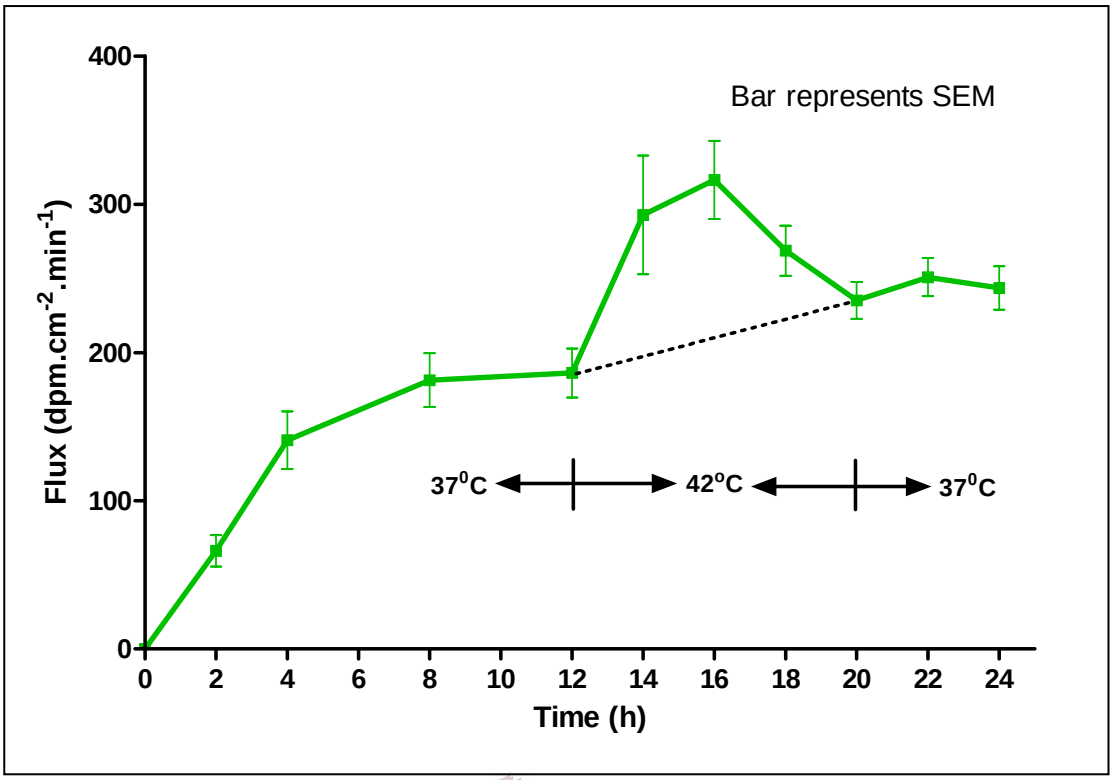

Fig. 23. Overall mean flux values of $^{3}H_2O$ across human skin at 37 °C and 42 °C

4. Discussion

4.1 Permeability experiments

Previous studies have shown that skin specimens can be frozen and banked without their permeability properties to a number of different permeants being changed.[36,100,101] In view of the foregoing, the assumption of using frozen/thawed skin for the current permeability study was considered to be a reasonable one.

From the results obtained in the present study, it is clear that ultrasound enhanced the permeability of human skin to diclofenac released from a commercially available gel, following the dual-stage kinetic diffusion pattern consistent with the design of the experiment (Figs. 16 and 17). This may be explained as follows. Subsequent to the application of ultrasound to the thin layer of diclofenac gel on the stratum corneum, which also acted as its own coupling medium, a certain quantity of the diclofenac was driven along its own concentration as well as the acoustic energy gradient into the underlying layers of skin. For the controls, the diclofenac diffused into the skin solely under its own concentration gradient. In the flow-through diffusion apparatus this primary quantity of diclofenac diffused out of the skin layers into the acceptor chamber. This occurred under the influence of the 10-fold higher concentration gradient created by the secondary amount of diclofenac gel applied to the skin before mounting the specimen in the donor chamber. The foregoing events are apparent from the decreasing flux values resulting from the depletion of this primary reservoir of diclofenac in the skin (sink conditions not satisfied), over the initial 4 h (first phase) of the experiment (Figs. 16 and 17). It can also be seen from the negative slope of the flux ratio plot (sonicated/control) over 4 h which, when extrapolated to its y-axis intercept at zero time, shows a flux enhancement ratio of 2.61 (Fig. 18). Hereafter, there occurred a gradual increase of flux as the secondary quantity of diclofenac from the donor chamber diffused across the skin following classical (Fickian) first order diffusion kinetics and reaching steady state after approximately 14 h. The latter values concur with the squares of the lag phase (x-axis intercepts) (Fig. 19). It is evident from the regression lines of the sonicated vs. the control specimens that ultrasound increases the release rate of diclofenac from the gel to the skin and shortens the lag phase (Fig. 19). This is in contrast with the observations made in a previous *in vitro* study on the sonophoresis of ibuprofen, a drug falling in the proprionic acid class of NSAIDs of which diclofenac is also a member, across human skin.[102] However, the results from the present study regarding release rate and lag

phase are in agreement of those made in two *in vivo* studies on rats and guinea pigs.[30,103] It is interesting to note that the steady state flux values of diclofenac across sonicated skin specimens remained approximately 1.26 times higher than those of the controls for the remainder of the experiment. While the skin was heated by approximately 10 ^{o}C following sonication, there was a return to pre-sonication temperature values after 3 min. Furthermore, the reversible temperature-dependent flux changes of $^{3}H_2O$ between 37 ^{o}C and 42 ^{o}C support the barrier lipid layer fluidity theory. In this theory the crystalline/gel barrier lipid domains undergo transitions (pre-melting) towards more disordered structures with increasing temperatures.[53] Between 41 ^{o}C and 42 ^{o}C, a clearly characterised phase transition of the intercellular lipid domains to an even more fluid and disordered state occurs.[54] Because the compositions and physical structures of the lipid domains affect the barrier functions of skin, thermally-induced rearrangements of these systems change diffusion characteristics of permeants. This has been amply demonstrated for both skin and mucosae.[104-107,108] Flux rates of drugs and other chemical entities across human skin are therefore expected to rise with increasing, and fall with decreasing temperatures. The fact that this did not occur for diclofenac for at least 24 h following sonication in the present study strongly suggests that ultrasonically increased skin permeability, and hence flux rates, cannot be primarily ascribed to heating phenomena. This concurs with data from a previous *in vivo* study in which increased permeability of the stratum corneum caused by sonication was shown to remain stable for 15 h following sonication and returned to its normal pre-sonication state by 24 h.[35] It was also demonstrated in another more recent *in vivo* study on 14 healthy human volunteers that diclofenac plasma levels were higher for at least 3 h following sonication than those in the same volunteers, acting as their own controls, one month later without sonication (Fig. 20).[37] However, it must be borne in mind that the "recuperative" properties of *in vitro* sonicated skin following ultrasonic insult may differ from those of the same tissue *in vivo.* The results from the present study support the concept that sonication causes profound and extended changes ("damage") of the stratum corneum, probably by perturbing its lipid barrier layer, and hence increase the skin's permeability to diclofenac. These sonication-induced changes may be indicative of cavitational channel-opening in the lipid layers, consistent with the porous pathway theory.[34] The observed permeability changes were irreversible during the 24 h over which the present study was conducted. Moreover, the effects of sonication-enhancement on the present *in vitro* transdermal permeation kinetics of diclofenac, concurred with those found in the previous *in vivo* study.[37]

4.2 Temperature experiments

It is an established fact that when skin is heated, its permeability increases and transport of a variety of chemical substances across this tissue is enhanced.[104,108-110] When ultrasound passes through biological tissues, a large fraction of the acoustic energy is attenuated by the dual process of scatter and absorption. The latter is primarily in the form of heat and is dependent on the type of tissue and the amount of energy passing through it. These thermal effects of ultrasound (sonication-heating) have been considered to be a major mechanism in the enhancement of fluxes of compounds across skin during sonication.[49,111] This increased permeability has been attributed to an increased fluidity of the stratum corneum intercellular lipids, which are thought to constitute the major barrier towards penetration of chemical substances across human skin.[17,55] Furthermore, thermal energy also increases the molecular diffusivity of the permeant molecules.[14,56]

From the results of the present study it can be seen that the temperature of the skin specimens increased by approximately 10 °C, from 20.8 ± 0.83 °C to 31.0 ± 1.92 °C, following exposure to continuous mode ultrasound of 3 MHz at an intensity of 2 W/cm^2 for 10 minutes (Fig. 22). These increased temperatures returned to pre-sonication (ambient) values approximately 3 min after cessation of ultrasound. Moreover, the reversible temperature-dependent flux changes of ^3H_2O between 37 °C and 42 °C support the barrier lipid layer fluidity theory (Fig. 23). The observation that the flux values between 20 and 24 h remained slightly higher than those between 8 and 12 h may possibly be attributed to “base-line drift” following minor degradative changes of human skin during *in vitro* exposure to elevated temperatures of 42 °C for several hours. Similar reversible temperature-dependent flux changes have also previously been reported for the permeation of ^3H_2O and 17β-estradiol through human mucosa.[105-107] While in the present study, the temperature-dependent flux changes were rapidly reversible, this was not found to be the case in a previously published study in which the permeability changes to skin following sonication were stable for 15 h and only returned to their normal pre-sonication state by 24 h.[35] The prolonged skin permeability in excess of 15 h seen in the latter study appears to indicate barrier perturbation due to ultrasound by mechanisms other than the short-lived and reversible changes caused by temperature fluctuations. The results from the study by Kost *et al*.[35], and the present study are therefore clearly at variance with the sonication-heating theory.[49,111] It can therefore be concluded that

the results in this study do not support the sonication-heating theory in which the permeability changes observed in skin, following sonication, are attributed primarily to thermally-induced fluidity changes in the barrier lipids of the stratum corneum.

5. Conclusions

In conclusion, it has been demonstrated in the present study that sonication of human skin with therapeutic levels of ultrasound caused an increased permeability to diclofenac which persisted for the entire course of the 24 h-experiment. Although sonication causes some tissue heating and hence an increase in permeability, the changes observed in the latter property of skin cannot be adequately explained primarily on a thermal basis. Furthermore, the *in vitro* flow-through diffusion model used in the present study has been shown to have predictive value for *in vivo* studies. However, considering the widely differing chemical and physical properties of agents on their diffusability across membranes, further validation of the above *in vitro* sonication model using a range of different compounds is warranted.

6. References

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