APPLICATION OF DERMAL MICRODIALYSIS AND TAPE STRIPPING METHODS TO DETERMINE THE BIOAVAILABILITY AND/OR BIOEQUIVALENCE OF TOPICAL KETOPROFEN FORMULATIONS

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by

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This dissertation is dedicated to the following:

To Professor Isadore Kanfer, Mrs Josephine Kanfer, Professor John Haigh, Mrs Lil Haigh and Professor Beverly Wilson.

In loving memory to the late Theodore Tei-La Darko aka Uncle Theo (61) and the late Ashanti Kakaza aka Shasha (24).
I miss you and may you both rest in perfect peace.

To the Tettey-Amlalo family of Odumase-Krobo, Eastern Region, Ghana.

ABSTRACT

The widespread acceptance of topical formulations intended for local and/or regional activity has prompted renewed interest in developing a model to determine the bioavailability of drugs in order to establish bioequivalence as a means of evaluating formulation performance of multisource products and also for use during formulation development.

Current *in vivo* techniques such as blister suction and skin biopsy amongst others used to determine the bioavailability and/or bioequivalence of topical formulations are either too invasive to generate appropriate concentration-time profiles or require large numbers of study subjects thereby making the study expensive and time-consuming. Moreover, there are currently no sampling techniques that can demonstrate dermal bioavailability and/or bioequivalence of topical formulations intended for local and/or regional activity.

Dermal microdialysis is a relatively new application of microdialysis that permits continuous monitoring of endogenous and/or exogenous solutes in the interstitial fluid. The technique is involves the implantation of semi-permeable membranes which are perfused with an isotonic medium at extremely slow flow rates and collection of microlitre sample volumes containing diffused drugs.

Tape stripping, a relatively older technique, has been extensively used in comparative bioavailability studies of various topical formulations. However, due to shortcomings arising from reproducibility and inter-subject variation amongst others, the published FDA guidance outlining the initial protocol was subsequently withdrawn. The incorporation of transepidermal water loss with tape stripping has garnered renewed interest and has been used for the determination of drug bioavailability from a number of topical formulations.

Hence the primary objective of this research is to develop and evaluate microdialysis sampling and tape stripping techniques, including the incorporation of the determination of transepidermal water loss, to assess the dermal bioavailability of ketoprofen from topical gel formulations and to develop models for bioequivalence assessment.

A rapid UPLC-MS/MS method with requisite sensitivity for the analysis of samples generated from dermal microdialysis was developed and validated which accommodated the microlitre sample volumes collected. An HPLC-UV method was developed and validated for the analysis of samples generated from the *in vitro* microdialysis and *in vivo* tape stripping studies.

The work presented herein contributes to a growing body of scientific knowledge seeking to develop a model for the determination of bioequivalence of pharmaceutically equivalent topical formulations intended for local and/or regional activity in human subjects.

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LIST OF ABBREVIATIONS

APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
APPI	Atmospheric pressure photo-ionisation
AUC	Area under the plasma concentration versus time curve
BEH	Ethylene Bridged Hybrid
CE	Capillary electrophoresis
CI	Confidence interval
CID	Collision-induced dissociation
C_{max}	Maximum concentration
DMD	Dermal microdialysis
DPK	Dermatopharmacokinetics
ECF	Extracellular fluid
EE	Extraction efficiency
ESI	Electrospray ionization
FDA	Food and Drug Administration
GC	Gas chromatography
GIT	Gastrointestinal tract
HETP	Height of the column equivalent to a theoretical plate
HHS	High Strength Silica
HILIC	Hydrophilic Interaction Chromatography
HPLC	High performance liquid chromatography
HTLC	High temperature liquid chromatography
IG	Instrument grade
IR	Infra-red
IS	Internal standard
ISF	Interstitial fluid
LBP	Lower back pain
LC	Liquid chromatography
LLOQ	Lower limit of quantification
MD	Microdialysis
MP	Mobile phase
MRM	Multiple reaction monitoring
MS	Mass spectrometer
MS/MS	Tandem mass spectrometry
MWCO	Molecular weight cut-off
NI	Negative-ion
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
OTC	Over-the-counter
PDA	Photo Diode Array
PI	Positive-ion
RA	Rheumatoid arthritis

RSD	Relative standard deviation
RT	Retention time
SC	*Stratum corneum*
SPE	Solid phase extraction
T_{max}	Maximum time
TEWL	Transepidermal water loss
TQD	Triple Quadrupole Detector
TS	Tape stripping
UHPLC	Ultra-high pressure liquid chromatography
ULOQ	Upper limit of quantification
UPLC	Ultra Performance Liquid Chromatography
UV-Vis	Ultraviolet-visible

CHAPTER ONE

BIOAVAILABILITY AND BIOEQUIVALENCE OF TOPICAL FORMULATIONS

1.1. Introduction

The skin, despite its function as an efficient barrier, has beneficial properties which permits it to be considered as an alternate route for drug application. The merits of topical application have been well documented (1). These benefits include the ability of the skin to control the *rate* at which drugs reach the target site within the body, i.e., if the drug is intended for a systemic effect. For local and/or regional effects, topical application permits localised and/or regionalised drug delivery to the site of interest which results in a local therapeutic effect, while such application avoids or minimises systemic side effects. Problems related to extensive first pass effects, primarily due to metabolism by the liver but also to a lesser extent the by the gastrointestinal tract (GIT), encountered by most orally administered drugs, may be avoided. The main drawback of using the skin as an alternate route of drug delivery is that not all drugs are suitable candidates for topical administration (either local or systemic use) due to their physico-chemical properties amongst others (2). Ideally, a suitable drug candidate should have a low molecular mass (< 500 Da), high lipophilicity (log *P* in the range of 1-3), low melting point (< 200°C) and high potency (< 50 mg/day). Nevertheless, the relative ease of use and increased patient acceptance of topical formulations have made their use widespread (3,4) and many products for topical administration are currently available in a number of countries. Topical products available on the South African market include anti-bacterials, anti-fungals, anti-virals, anti-inflammatories, analgesics, corticosteroids, and also anaesthetics (5).

Topical formulations applied to the skin can be subdivided into 3 categories:

i. dermatological formulations (creams, ointments, gel, lotions) intended for the treatment of local skin disorders;
ii. dermatological formulations (creams, ointments, gels, lotions, sprays) intended for regional disorders;
iii. dermatological formulations and delivery systems (creams, ointments, gels and transdermal patches) intended for the treatment or prevention of systemic diseases (6,7).

While topical formulations to treat local and/or regional ailments have been in existence for centuries, those used to treat systemic ailments only started to appear on the market towards the end of the last century (8). Interestingly, the principles underlying the development and optimisation of topical formulations for systemic therapy appear to be better understood than those for local and/or regional therapy (3,4). Since absorption through the skin (percutaneous) is pivotal to the effectiveness of topical formulations for local, regional or systemic activity, there is a tendency amongst researchers to

misinterpret the intentions of topical delivery systems where a great deal of confusion currently reigns with respect to definitions and semantics. It is therefore extremely important to distinguish the actual objectives of topical formulations, i.e., whether intended for local, regional or systemic use, and the associated clinical outcomes.

1.2. Topical formulations intended for local and/or regional delivery

1.2.1. Objectives

Topical formulations for local activity are intended to treat cutaneous disorders (e.g., acne) or the cutaneous manifestations of a general disease (e.g., psoriasis) whereas topical formulations for regional activity are intended to treat diseases or alleviate disease symptoms in deep tissues (e.g., inflammation) beneath the site of application (9).

1.2.2. Principle

The intention of local delivery is to confine the pharmacological effect of the drug to the surface of the skin (i.e., at the site of application) or within the skin whereas the intention of regional delivery is to effect the pharmacological action of the drug within musculature, vasculature, joints and tissues beneath and around the site of application (9). Since the skin is the site of delivery for drugs intended for either local or regional activity, high dermal drug concentrations with low exposure to systemic circulation are desirable (3,4).

1.2.3. Types of formulation

Semisolid formulations dominate systems for local delivery but foams, sprays, medicated powders, solutions and even adhesive systems are also used. Regional delivery has been traditionally accomplished by administration of ointments and creams onto the skin as well as using large adhesive patches, plasters, poultices and cataplasms (9).

1.2.4. Formulation characteristics

Formulations are generally applied (rubbed) over diseased or inflamed skin with no visible mass left on the skin surface after repeated application. In most cases the formulation is left unoccluded, which permits the components of the formulation to be absorbed through the skin, evaporated from the skin or sloughed off the skin. This class of topical formulation usually contains several excipients which may partition into the skin in accordance with their physico-chemical properties. Excipients present in the formulation may change the integrity of the *stratum corneum* (SC) which alters the solubility of the active ingredient within the horny layer and/or facilitates the ease with which they diffuse through the affected tissue. The act of physically rubbing the formulation on the skin usually results in

changes in physico-chemical and thermodynamic conditions which may enhance the permeation of the drug through the skin following application (3,4).

1.3. Topical formulations intended for systemic delivery

1.3.1. Objective

Topical formulations intended for systemic effects also involve the application of the drug product to the skin (9) but are used to treat systemic diseases.

1.3.2. Principle

The intention is to achieve systemically active concentrations of drug with minimum or no drug retention in the skin although such accumulation is largely unavoidable (9). The drug diffuses through the relatively small diffusional window at the application site which is defined by the contact area of the delivery system, e.g., a patch.

1.3.3. Formulation types

Although traditional dosage forms such as ointments can be employed in this kind of therapy (e.g., nitroglycerin ointments used in the management of angina), transdermal adhesive systems of precisely defined size are commonly used (9).

1.3.4. Formulation characteristics

Topical formulations for systemic effects usually involve the use of prefabricated devices, commonly known as transdermal patches, in which the drug is incorporated into a reservoir or as a dispersion in an adhesive. The system is fastened adhesively to the skin surface and the drug, contained in a matrix, is rarely exposed to atmospheric conditions. Although such dosage forms may cause perspiration, the physico-chemical and thermodynamic conditions remain constant and therefore provide an approximately constant rate of drug delivery through the skin (3,4). Semisolid preparations may also be used for systemic effects, however such dosage forms are usually associated with unpredictable plasma drug concentrations due to the difficulty in controlling the amount applied which results in variability of drug concentration in the systemic circulation. Furthermore, variability in the method of application (involving the degree of rubbing), area of application and the need for frequent application are distinct disadvantages (2).

For the purposes of this research, only topical formulations intended for local and/or regional activity will be considered.

1.4. Bioavailability and bioequivalence

1.4.1. Definitions

- *Bioavailability* is defined by regulatory bodies as the *rate* and *extent* to which the active ingredient or active moiety is absorbed from the drug product and becomes available at the site of action (10-12).

- *Relative bioavailability* is defined as the *rate* and *extent* to which the active ingredient or therapeutic moiety becomes available in the organism from a dosage form, compared with a reference standard administered by the same route (9).

- *Absolute bioavailability* is the *extent* to which the active ingredient or therapeutic moiety becomes available in the organism from a dosage form in comparison with an intravenously administered reference standard, which is taken to be 100% bioavailable (9).

- *Pharmaceutical alternatives* are dosage forms that have the same chemical moiety but differ in chemical form, dosage form type or strength of the therapeutic moiety (13).

- *Bioequivalence* is the absence of a significant difference in bioavailability between pharmaceutical equivalents or pharmaceutical alternatives when administered at the same molar dose under similar conditions in an appropriately designed study (9,12,14).

- *Formulation performance* is defined as the release of the drug substance from the drug product leading to bioavailability of the drug substance and eventually leading to 1 or more pharmacologic effects, both desirable and undesirable (12,15).

- *Pharmaceutical equivalents* are defined as dosage forms that contain the same active ingredient(s), are of the same dosage form, route of administration and are identical in strength or concentration when compared with a reference product (13).

- *Multisource drug products* are pharmaceutically equivalent or pharmaceutically alternative products that may or may not be therapeutically equivalent. Multisource pharmaceutical products that are therapeutically equivalent are interchangeable (14,16).

- *Therapeutic equivalent products* are pharmaceutical products which when administered in the same molar dose, their effect with respect to both efficacy and safety are essentially the same

when administered to patients by the same route under the conditions specified in the labelling (16).

In the early 1960s, reports of physiological availability of vitamins were found to be erratic, which provided initial indications of the potential for bioavailability and bioequivalence problems with multisource drug products (9). The variations in absorption profiles of active ingredients from generic drug products when compared to the innovator or "Brand" product were soon recognised as a potential health hazard when episodes of drug toxicity were reported. Such variations were noted when excipients in pharmaceutical delivery systems or in manufacturing processes were changed or altered. Major advances in bioanalytical technology led to further investigations which permitted the determination of bioavailability of marketed drug products through the measurement of their concentration in biological fluids. Reports of bioinequivalence of a number of drugs such as digoxin, phenytoin and chloramphenicol, amongst others (9,15) were published in the scientific literature. The bioavailability of drugs and/or bioequivalence of drug products have therefore emerged as important national and international regulatory and scientific issues (9,17) and assessment of bioavailability has therefore being employed as a tool by regulatory authorities to monitor the quality, safety and efficacy of multisource drug products (generic), labelled as "test" versus the innovator or "Brand" product, labelled as "reference". For generic drug products to be interchangeable with the innovator drug product, a generic drug product must not only be pharmaceutically equivalent or a pharmaceutical alternative, but must be bioequivalent to an acceptable relevant reference product.

Innovator/"Brand" companies utilise bioavailability and/or bioequivalence in formulation development to assess formulation performance between 2 or more pharmaceutically equivalent drug products (15). E.g., based on the outcome of a clinical study that made use of the initial formulation, manufacturers effected minor changes to the formulation or altered the manufacturing protocol to improve the formulation. The revised formulation (test) is compared with the initial clinical trial product (reference). Therefore, bioequivalence not only plays an important role in assuring the therapeutic quality of multisource drug products but is also useful during formulation development.

1.4.2. Topical bioavailability and bioequivalence

There is an increased interest amongst pharmaceutical and medical scientists in the development of topical formulations intended for local and/or regional activity. In spite of confirmed data showing that significant concentrations of NSAIDs have been found in tissues underlying the application site of topical products (18-22) some medical practitioners and rheumatologists remain sceptical about how the non-steroidal anti-inflammatory drugs in particular, can penetrate the skin barrier in sufficient amounts to reach the joints and muscles and exert local and/or regional therapeutic effects. This

scepticism is based partly on the presumption that any clinical effect found after topical application of a drug product intended for local or regional effects was due to systemic absorption. However, concentrations attained in blood following topical applications intended for local and/or regional activity generally do not achieve drug levels associated with therapeutic activity and moreover, there is no clear explanation as to how such clinical effects can result from plasma drug concentrations that are otherwise considered sub-therapeutic following orally administration of the same drug (23).

Bioavailability relates to the "release/availability" of drug into (usually) the systemic circulation following extravascular administration or, when not intended for the systemic circulation, "availability" at the site of action. For orally administered drugs, it indicates "absorption" whereas for topically applied products intended for local and/or regional action, no "absorption" *per se* is intended so it is simply a measure of *in vivo* release or "availability *in vivo*". On the other hand, the use of bioavailability as a surrogate measure of safety or efficacy is premised on the assumption that there is a relationship between safety/efficacy and the concentration of drug in the systemic circulation.

The assessment of bioavailability of topical formulations intended for local and/or regional activity should therefore be performed by measuring drug concentrations within the skin (i.e., at the site of action) (9,24,25) and not in blood/plasma/serum or urine (26) which is the norm for topical formulations intended for systemic activity or for drugs administered orally. However, the determination of plasma drug concentrations after the application of a topical formulation intended for local and/or regional activity may be used as an indicator of toxicity since the principle of local and/or regional activity indicates high dermal drug concentrations and minimal or no drug in the plasma. E.g., with inhaled corticosteroids from aerosol delivery systems, the corticosteroid is intended for local action in the lung (alveoli etc.) and appearance in the systemic circulation has implications for possible adrenal suppression, especially if fairly high concentrations result. That can quite easily occur if after a dose, a large amount is swallowed rather than inhaled.

1.4.2.1. Bioavailability of drugs from dermatological formulations

The determination of the bioavailability of drugs from dermatological formulations requires a thorough understanding of the human skin. The structure of the human skin and its barrier functions have previously been published (27). In summary, the skin is the largest organ of the human body and it is composed of 3 layers namely the SC, which is the outermost part of the epidermis, the living epidermis and the dermis penetrated by a highly complex network of capillaries involved in the removal of drugs from the skin into the systemic circulation. In addition, several pilosebaceous and sweat glands are dispersed throughout the skin, in various numbers and size, depending on body site (28). A detailed illustration of the structure of the skin is shown in Figure 1.1.

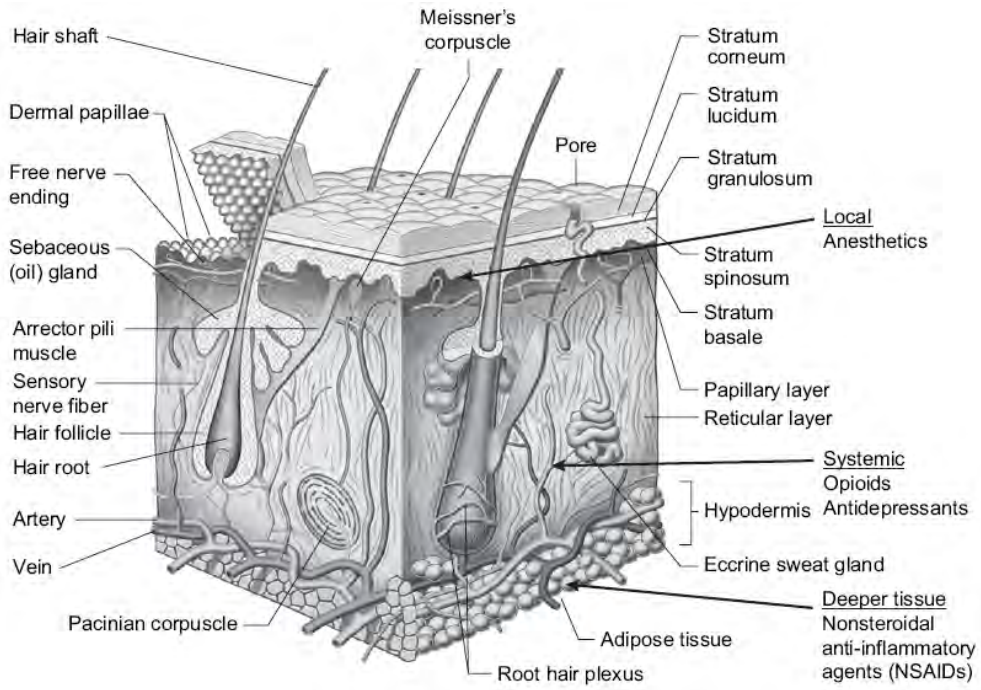


Figure 1.1. Anatomy and physiology of the skin with the potential target or sites of action of selected analgesics. Adapted from Reference (27).

The most important function of human skin is to act as a barrier by reducing water loss while barring the percutaneous absorption of harmful or unwanted molecules from the external environment. The SC contributes the rate-limiting step in the sequence of processes involved in percutaneous absorption, although the viable tissue can hinder the penetration of very hydrophobic compounds (29). Biophysical, morphological and biochemical data indicate that the SC forms a continuous sheet of protein enriched corneocytes embedded in an intercellular matrix enriched in non-polar lipids and organised as lamellar lipid layers (30). It has a thickness of about 10-20 µm and comprises about 20 cell layers (28,31). Penetration of drug substances through the skin involves complex processes but the major barrier to permeation resides within the SC. The reason for the impermeability of the skin is that a diffusing molecule has first to pass through a tortuous route around the dead corneocyte cells and through the intercellular lipids which comprise mainly ceramides, cholesterol and cholesterol derivatives (32). Generally, small non-polar, lipophilic molecules are most readily absorbed, while high water solubility confers poor percutaneous absorptive capacity through normal skin. The octanol/water partition coefficient (log *P*) of a drug is an indication of its lipophilicity and substances with a log *P* value of 2 are absorbed best across the skin (33).

However, despite its barrier properties, the skin is an important route of entry into the body for many topically applied drugs. Topical formulations for local use are assumed to exert their effect through the penetration of the active ingredient directly into deeper tissues although controversy exists over whether the dermal blood supply removes many drugs before they reach their site of action (34). The

sites of action of selected analgesics when applied topically from formulations intended for local and/or regional activity are illustrated in Figure 1.1. The occurrence of a response, its time of onset, duration and magnitude when a topical formulation is intended for local and/or regional activity, depends upon the relative efficiency of 3 sequential processes:

i. release of the drug from the vehicle,
ii. penetration of the drug through the skin barriers and
iii. activation by the drug of the desired pharmacological effect (7,35).

1.4.2.2. Bioequivalence of topical formulations

Bioequivalence assessment between 2 sources of a drug product as stipulated by the FDA (36) can be evaluated by 4 methods in order of preference: pharmacokinetic, pharmacodynamic, comparative clinical trials or *in vitro* studies.

Currently, in the USA, with the exception of topical corticosteroids, the only means by which a generic company can demonstrate bioequivalence of a topical formulation intended for local and/or regional activity to a formulation manufactured by an innovator/"Brand" company is through comparative clinical trials with a bioequivalence endpoint (37,38).

An innovator/"Brand" company that wishes to replace an approved post-1962 topical dermatological product with a new formulation that involved appreciable compositional changes, must demonstrate bioequivalence using data from clinical studies (39,40). For topical corticosteroid formulations, the demonstration of bioequivalence of 2 pharmaceutically equivalent (e.g., cream versus cream) products may be accomplished by performing a human skin blanching assay (HSBA) and following the appropriate protocol outlined in the FDA Guidance (41).

Clinical endpoint trials, generally considered as the "*gold standard*" in establishing bioequivalence of drug products, usually involve the use of large population sizes which makes these studies time consuming and expensive. A clinical endpoint study which involved the topical application of tretinoin gel formulations in the management of acne reported that, in order for bioequivalence to be established with significant statistical power, a study population size of between 275 and 300 is required (25). Therefore, there is increasing interest in developing and validating appropriate methodologies including a method permitting the measurement of drug concentrations within the skin to assess bioavailability. However, there is still some uncertainty as to which is the most appropriate layer of skin to measure drug concentrations for a given topical product (42).

1.4.2.3. Bioequivalence assessment

A 2 one-sided test procedure is the currently recommended statistical method for use in bioequivalence assessment (43,44). The area under the plasma concentration versus time curve (AUC) is used as an index of the *extent* of drug penetration (topical application) and maximum concentration (C_{max}) is used as an index of the *rate* of drug penetration (topical application). By convention, bioequivalence data are expressed as a ratio of the average response (AUC and C_{max}) for Test/Reference. The statistical criteria for acceptance of bioequivalence are that both the AUC and C_{max} confidence intervals (CIs) for the generic product (test) must fall within 80-125% of the innovator/"Brand" product (reference) based on log transformed data. These statistical tests are carried out using an analysis of variance (ANOVA) procedure and calculating the 90% CI for each pharmacokinetic parameter (AUC and C_{max}) (12).

Wide sample collection intervals and an insufficient number of enrolled human subjects are 2 main reasons why a clinical study may fail to establish bioequivalence between 2 pharmaceutically equivalent products unless the products are indeed inequivalent. The use of wide sampling intervals affects the C_{max} values due to the possibility that the true C_{max} value may be missed. Inherent intra-subject variability requires that a sufficient number of subjects are included. This is necessary to provide the requisite power to be able to detect a 20% difference in bioavailability. A pilot bioequivalence study using a relatively few subjects (8-12) can provide useful information relating to the choice of the appropriate sampling times and also provide data on intra-subject variability which can then be used to estimate the number of subjects to be enrolled (12).

1.4.2.4. Add-on study

In Canada, if an insufficient number of enrolled human subjects results in a clinical study failing to establish bioequivalence, an additional number of subjects may be studied so that when included to the initial study, the total number may be sufficient to pass the bioequivalence assessment (45). In addition to performing the bioequivalence assessment for the combined number of subjects, a second statistical test is required which seeks to establish homogeneity between the 2 sets of data. The residual variances between the 2 separate groups are compared using an *F* test and pooling of data can only be done when no statistically significant difference is reported, although it is not a requirement that each group meet the 90% CI for AUC and C_{max} (46). Suitable *F* tests used for the determination of homogeneity of variances are the Levene's test (47) and the Brown-Forsythe's test (48) which are described by Equations 1.1 and 1.2 respectively.

$$W = \frac{(N-k)}{k-1} \frac{\sum_{i=1}^{k} N_i (Z_{i.} - Z..)^2}{\sum_{i=1}^{k} \sum_{j=1}^{Ni} (Z_{ij} - Z_{i.})^2} \quad 1.1.$$

$$F = \frac{(N-p)}{(p-1)} \frac{\sum_{j=1}^{p} N_j (Z_{j.} - Z..)^2}{\sum_{j=1}^{p} \sum_{i=1}^{Nj} (Z_{ij} - Z_{j.})^2} \quad 1.2.$$

where:

- N is the total number of observations
- k and p represent the number of groups
- $Z_{ij} = \left| Y_{ij} - \overline{Y}_{i.} \right|$ with $\overline{Y}_{i.}$ either the mean (Levene's test) or median (Brown-Forsythe test) of group i.
- $Z.. = \frac{1}{N} \sum_{i=1}^{k} \sum_{j=1}^{Ni} Z_{ij}$ is either the mean (Levene's test) or median (Brown-Forsythe test) of all Z_{ij}.
- $Z_{i.} = \frac{1}{N_i} \sum_{j=1}^{Ni} Z_{ij}$ is either the mean (Levene's test) or median (Brown-Forsythe test) of the Z_{ij} for group i.

1.4.2.5. In vitro determination of dermal drug concentration

Considerable effort has been directed towards the development of *in vitro* methods for the determination of bioavailability of drugs or bioequivalence of topical formulations. However these methodologies have been associated with high variability when determining drug release from the topical dosage forms and therefore provided neither adequate nor consistent information. The high variability associated with these studies usually resulted from the source and treatment of skin tissues used as membranes to study drug diffusion *in vitro*. With excised human skin, the problem encountered was that the skin tissue was not only obtained from various body parts of cadavers but the absence of a standard protocol for the pre-treatment of such skin tissues had also been identified as the major cause of variability. This problem was also observed with animal tissue (35). Moreover the use of excised skin (i.e., either human or animal) tissue does not take into account the possible effects of skin flora and skin metabolism on drug release from topical formulations.

The use of commercially available synthetic membranes has been demonstrated to be useful in the development of topical formulations (1). Synthetic membranes function as an inert support which separates the formulation from the receptor phase (17). Several reports using various diffusion cells for the determination of drug release from topical formulations have been published (49-51).

Diffusion cells frequently used are the Franz diffusion cell (FDA approved) and the European Pharmacopeia diffusion cell (1,52,53).

While these *in vitro* experiments are generally conducted under controlled laboratory conditions, *in vitro* data bear little relation to delivery kinetics *in vivo* because vehicles also have an effect on the barrier properties of the skin and such vehicle effects cannot be assessed *in vitro* (54).

Whilst *in vitro* drug diffusion experiments are useful to evaluate quality, batch-to-batch uniformity and evaluation of changes in manufacturing process and drug release from the dosage form, the use of *in vitro* methods for the assessment of bioavailability/bioequivalence has not found acceptance by most regulatory agencies around the world.

1.4.2.6. In vivo determination of dermal drug concentration

Animal skins have been reported to be more permeable to drugs than human skin (6,55). This makes animal skin an inaccurate predictor for percutaneous penetration in man.

Although various *in vivo* techniques such as suction blister sampling or biopsies have been developed to study kinetic and dynamic parameters of topical drug applications, they are either too non-specific or too traumatic to be used readily and repeatedly in both normal and diseased skin in human subjects because of their invasive nature (9). Additionally, the cost, technical demands and ethical considerations limit the applicability of these techniques in human volunteers (30,56). None of these techniques permit the continuous monitoring of drug penetration and metabolism in the same individual on the same test area. Moreover, the determination of drug concentration in the skin requires that the skin be excised, homogenised, extracted and the extract analysed. This methodology defeats the purpose of monitoring drug concentration at the site of action, i.e., either the SC (local) or tissues (regional). Moreover, these *in vivo* techniques are quite restrictive with respect to the amount of information generated and also require a large number of subjects or a large number of sampling sites per subject, which increases invasiveness.

Currently, there are no sampling techniques which can demonstrate dermal bioavailability and bioequivalence of topical formulations intended for local and/or regional activity which are both minimally invasive and provide an indication of tissue concentration at the target site with time. Hence, recourse to clinical end-point studies remains the only route to assess bioequivalence of topical drug products. It is thus apparent that the development of a method to assess topical bioavailability of drugs or bioequivalence of topical drug products not intended for the systemic

circulation without the need to conduct expensive and time consuming clinical studies in humans should make a significant contribution to this particular area of endeavour.

Recently, the 2005 Bio-International Conference hosted by the Royal Pharmaceutical Society of Great Britain (London, UK) recognised dermal microdialysis (Chapter 4) and a revised tape stripping technique (Chapter 5) as having the potential for use in assessing the bioavailability of drugs from topical formulations intended for local and/or regional activity.

Hence the aim of this research was to develop and evaluate dermal microdialysis and tape stripping techniques for use in the assessment of the bioavailability/bioequivalence of ketoprofen from topical gel formulations.

CHAPTER TWO

REVIEW OF TOPICAL NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

2.1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are amongst the most commonly prescribed drugs worldwide (57-62). NSAIDs are central to the long-term pharmacologic treatment of patients with musculoskeletal and joint disorders, ankylosing spondylitis, osteoarthritis (OA) and rheumatoid arthritis (RA) (27). They are also used in peri-articular disorders such as tendonitis and in mild-to-moderate pain (58), muscle pain syndromes and various spine-related neck and lower back conditions (27). Approximately 30 million people consume NSAIDs on a regular basis (62) and with the availability of many different NSAID formulations as over-the-counter (OTC) products, this further significantly increases the number of people exposed to this class of drugs.

2.2. Disadvantages of oral NSAID therapy

Oral administration of NSAIDs in the management of acute conditions is generally not associated with significant issues of toxicity. However, chronic use of oral NSAIDs have been associated with a variety of adverse effects, which mainly involve the GIT, kidney, cardiovascular system, skin and respiratory tract (58,59,63,64). GIT damage includes small multiple lesions, gastric and duodenal ulcers, perforated ulcers and even severe bleeding of the upper GIT (58). As many as 15-30% of patients on chronic regimens of oral and/or systemic NSAIDs develop endoscopically detectable gastrointestinal ulcers (62,65). Moreover due to the fact that oral NSAIDs have to be administered in multiple daily doses to maintain therapeutic effects, patient non-compliance is a common therapeutic problem in the management of chronic inflammatory diseases (66).

The extensive use of prescription and OTC NSAIDs that is associated with significant adverse effects prompted efforts to find solutions to curtail this problem. Strategies which may reduce adverse effects include attempts to minimise oral NSAID use by education or legislation, co-administration of gastroprotective agents, development of potentially better tolerated drugs such as selective cyclo-oxygenase-2 (COX-2) inhibitors and modification of the delivery system (58,60,63,67).

2.3. Topical NSAID formulations

Humans have used salves, lotions, potions and the like, on their skin from ancient times and the concept of delivering drugs through the skin is a practice which dates as far back as the 16th century (8). Phenylbutazone cream was utilised over 30 years ago to treat superficial thrombophlebitis (19)

and the renewed interest in the development of topical formulations intended for local and/or regional activity stems from the potential advantages of topical formulations intended for systemic activity. There has been a dramatic increase in the development and marketing of topical NSAIDs. In 1980, benzydamine was the first topical NSAID to be licensed in the UK and since then several other topical NSAIDs have been licensed in Europe, Japan and South Africa (19,57). Recently, the first topical diclofenac gel and patch products were approved in the USA (68-70) further reflecting the increasing demand for topical NSAIDs by both physicians and patients which may be attributable primarily to their good safety record (59). Topical NSAIDs are also readily available without prescription in many countries (58,63,67). A list of commercially available topical NSAIDs intended for local, regional or systemic activity registered in South Africa is shown in Table 2.1 (5). Topical NSAID products applied for local and/or regional effects should achieve therapeutic concentrations in the tissues subjacent to the site of application while maintaining low plasma concentration which minimises possible systemic adverse effects (19,60).

2.3.1. Topical dosage forms available in South Africa

Various topical NSAID dosage forms available on the South African market are listed in Table 2.1.

Table 2.1. Commercially available preparations of topical NSAIDs in South Africa

Generic name	Trade name	Manufacturer	Strength	Dosage form
Aescin	Reparil	Altana Madaus	5 g/100 g	Gel
Diclofenac	Athru-Derm	Sandoz	1 g/100 g	Lotion
	DicloHexal	Hexal Pharma	1.16 g/g	Gel
	Panamor	Aspen Pharmacare	1292 g/100 g	Gel
	Voltaren	Novartis Consumer	1.16 g/100 g	Emulgel
Flurbiprofen	TransActLAT	Boots	40 mg	Patch
Ibuprofen	Nurofen	Boots	50 mg/g	Gel
Indomethacin	Adco-Indogel	Adcock-Ingram	10 mg/g	Gel
	Elmetacin	Pharmafrica	10 mg/g	Solution
Ketoprofen	Fastum	Adcock-Ingram	2.5 g/100 g	Gel
Methyl salicylate	Counterpain	Adcock-Ingram	102 mg/g	Cream
	Docrub	Aspen Pharmacare	1.77 ml/100 g	Ointment
	Thermo-Rub	Adcock-Ingram	10 g/100 g	Ointment
Piroxicam	Rheugesic	Cipla Medpro	5 mg/g	Gel

2.3.2. Benefits and limitations

Topical administration of NSAIDs intended for local and/or regional activity offer the advantage of local and/or regional enhanced drug delivery to affected tissues, with reduced incidence of systemic adverse effects, such as peptic ulcer disease and gastrointestinal haemorrhage. The short distance of transmission from the site of application to the target site and the avoidance of the enterohepatic cycle are attractive benefits (60). A summary of the benefits and limitations of topical formulations intended for local/regional activity (27) are listed below in Table 2.2.

Use of topical NSAID dosage forms lessens the risk of potentially serious systemic adverse effects secondary to NSAID-induced prostaglandin inhibition such as acute renal insufficiency, nephritic syndrome, gastropathy, prolonged bleeding time and fluid retention. Topical application also minimises the risk of drug-drug interactions, such as NSAID displacement of protein bound warfarin (19). This is particularly profound in patients on chronic medication and in the elderly who have been reported (60) to be the largest consumers of topical NSAIDs as they tend to be more sensitive to adverse effects of oral NSAIDs than younger individuals.

Table 2.2. Benefits and limitations of topical NSAID formulations (27)**.**

Benefits	Limitations
• First pass effect and other variables associated with the GIT (such as pH and gastric emptying time) are avoided. • Reduced side effects and the minimisation of systemic availability • Ease of dose termination in the event of untoward side effects. • Delivery can be sustained and controlled over a prolonged period. • Direct access to the target site. • Convenient and painless administration. • Improved patient acceptance and adherence to therapy. • Ease of use may reduce overall health treatment costs. • Provide a viable solution for treatment when oral dosing is not feasible (i.e., in unconscious or nauseated patients).	• Diffusion across the SC only occurs for molecules less than 500 Da. • Topical agents must have both aqueous and lipid solubility. • Both intra- and inter-individual variability in the permeability of skin, as well as differences between healthy and diseased skin, cause variable efficacy. • Skin enzymes can cause metabolism before cutaneous absorption, reducing the potency of the drug. • Localised skin irritation, such as erythema, can occur.

2.4. Clinical indications

Topical NSAIDs are primarily indicated for well-localised musculoskeletal conditions where pain or inflammation is likely to respond to inhibition of prostaglandin and leukotriene production from arachidonic acid (57). Common conditions for which topical NSAID formulations intended for local and/or regional activity are recommended include OA, RA, lower back pain (LBP), sport injuries and inflammatory conditions in the eye and breast.

Arthritis is a term that describes over 100 conditions affecting joint and connective tissue. The most common of these conditions are OA and RA. In the USA an estimated 7 million people are coping with this disease (65). The pain and decreased mobility associated with arthritis have a significant impact on the quality of life. Patients with this disease have to rely on the assistance of others for their routine daily activities. This disability often contributes to feelings of helplessness, anxiety, depression, stress and anger. Social isolation and decreased involvement in the community are not uncommon (65). First-line treatment of localised arthritic pain and joint stiffness normally consists of oral paracetamol (acetaminophen). Oral and/or systemic NSAIDs are effective (71-73) but are

generally considered second-line therapy because of their adverse effect profile. In the UK, topical NSAIDs and counter-irritants such as capsaicin have been found to be useful (19,65,74).

LBP is another common musculoskeletal disorder that creates a significant burden on society due to its high prevalence and consequent economic impact. LBP is the most reported musculoskeletal pain and as many as 84% of adults will experience LBP in their lifetime. In chronic situations, the management of LBP has been found to be challenging as it involves both musculoskeletal and neuropathic components (27).

Pain and inflammation brought on by physical activity such as sports injuries are another indication for the use of NSAIDs which are the foundation treatment for athletic injuries. In acute injuries such as ankle sprain, studies have not been able to demonstrate that topical NSAIDs reduce the inflammatory response. Medical practitioners and rheumatologists believe that topical NSAIDs offer analgesic relief rather than an inflammatory relief to patients. The use of topical NSAIDs has a valuable application in this area where pain is usually limited to a distinct region (75).

NSAIDs can be used topically in the eye to reduce inflammation and pain (76,77). Over the past 2 decades, topical NSAID solutions have been investigated for ophthalmic use. Ketorolac tromethamine (0.5%, m/v) has been evaluated for cystoid macular oedema (78), allergic conjunctivitis (79), surgically induced miosis (80) and for the management of pain and inflammation associated after cataract surgery (81). Topical indomethacin (0.1%, m/v) (82) and diclofenac (0.1%, m/v) (83) solutions have also been evaluated for inflamed pterygia and pingueculae, and glaucoma.

Breast pain is an important clinical feature and a common problem in women of all ages. Occasionally, it can be a distressing symptom, which may or may not be associated with nodularity (73). Re-assurance following the exclusion of cancer is the keystone of management for the majority of patients. However, in spite of this, there remains a small group of patients with severe and persistent mastalgia who require therapy. Severe breast pain significantly impairs the quality of life of these women including work performance, sleep, personal and sexual relations, which are all indications for active treatment. Topical NSAIDs have been shown to be useful in the management of mastalgia (73,84).

Topical NSAIDs may also be of value in *erythema nodosum*, urticarial vasculitis, nodular prurigo, palmoplantar pustulosis or erthyromelalgia (57). Roles for topical NSAIDs are being investigated for postoperative pain, prevention of thrombophlebitis in peripheral cannulation, periodontal disease (85), herpetic neuralgia, human pain models and burn injuries (63).

2.5. Pharmacokinetic studies

The concept of local and/or regional delivery of topical formulations requires proof that the drug does indeed reach the dermis and its surrounding tissues in high and therapeutically effective concentrations. The determination of dermal concentrations of NSAIDs from topical formulations is essential to clarify the issue of whether topical formulations do indeed bring about relief through their anti-inflammatory action or whether they simply act as rubefacients. Clarification and confirmation of the *modus operandi* of topical NSAIDs are needed in order to dispel the current scepticism amongst medical practitioners and rheumatologists regarding the efficacy of topical NSAID formulations.

Pharmacokinetic data suggest that topically applied NSAID formulations intended for local and/regional activity result in significant dermal concentrations of the drug without significant toxic effects. In a two-way crossover design, 11 healthy volunteers received either oral ibuprofen (800 mg) or ibuprofen gel (5%, m/m) applied (16 g) onto the skin of the thigh of a defined area (17 x 19 cm). For determination of drug in the subcutaneous and muscle, concentric microdialysis probes were inserted 4-5 mm and 25-30 mm respectively (20). Mean values of dialysate after topical and oral administration were 731.2 ± 605.0 ngh/ml and 176.6 ± 122.9 ngh/ml for subcutaneous tissue and 63.5 ± 90.3 ngh/ml and 213.4 ± 117.2 ngh/ml for muscle respectively. The ibuprofen concentrations in the dermis were 22.5-fold greater when delivered topically than via the oral route (20). In another microdialysis study involving 20 human volunteers, Müller *et al*. (86) determined diclofenac concentrations at superficial and deep layers within the skin after topical administration of a single dose (~ 300 mg/100 cm^2). Higher concentrations were observed in the superficial layers (532 ± 197 µgmin/ml) in comparison to the deeper layer (438 ± 249 µgmin/ml). Rolf *et al*. (18) determined ketoprofen concentrations in the intra-articular tissues of 100 patients following either a single application of a 30 mg plaster, multiple applications daily for 5 days, or administration of a 50 mg oral dose. The median C_{max} values for topical and oral administration were 568.9 ng/g and 85.7 ng/g respectively in the cartilage. In contrast, the plasma values were 18.7 ng/ml for topically administered ketoprofen and 2 595.3 ng/ml for the oral route. Overall, when applied topically, the ketoprofen concentration was 30-fold greater in the cartilage than in the plasma (18). Similar results were also observed in a study conducted by Brunner *et al*. (87). These authors also noted that relative bioavailability of diclofenac in subcutaneous adipose and skeletal muscle tissue was substantially higher after topical application compared with oral dosing whereas the plasma bioavailability was 50-fold lower. Plasma C_{max} values were approximately 250-fold lower after topical treatment compared with oral administration (87).

In summary, these studies have demonstrated that following the application of topical NSAID formulations intended for local and/or regional activity, high concentrations of the drug were achieved

in underlying tissues and moreover, peak plasma drug concentrations were only 3-5% of total systemic absorption compared with oral administration and without any associated systemic toxicity.

2.6. Efficacy

The analgesic and anti-inflammatory properties of NSAIDs are assumed to be primarily related to their ability to inhibit prostaglandin synthesis at the damaged tissue, therefore topical administration of these medicines should offer the advantage of relieving the damaged tissue by suppressing the local inflammatory responses with reduced incidence of systemic adverse effects (59).

2.6.1. Topical NSAIDs versus placebo formulations

Although topical NSAID formulations intended for local and/or regional activity have been demonstrated to produce high concentrations in the dermis (18-22) and despite being licensed in a number of countries including more recently, the USA (68,70), scepticism still exists as to whether topical NSAID formulations have any action other than as rubefacients (22,88). This has been attributed to the relatively high placebo positive response that has been claimed to be associated with rubefacients (19,67). These findings suggest that physical rubbing of the formulation into the skin at the affected site contributes significantly to the reduction of inflammation and pain rather than through the pharmacological action of the active ingredients present in the formulation. Additionally, topical NSAID dosage forms intended for local and/or regional activity have been misrepresented as rubefacients which were classified as topical anti-rheumatic agents, despite the lack of efficacy data from clinical trials to show that rubefacients provide true clinical benefit (67).

Vaile and Davis (63) reported that the placebo response associated with topical treatment of soft tissue complaints can be as high as 60-80%. While the natural progression of healing may account for the high placebo effect in patients with acute musculoskeletal complaints, the rate of inflammatory suppression reported in chronic rheumatological diseases remains unclear.

Moore *et al*. (22) in their systematic review, evaluated the impact of topical NSAIDs. Based upon data from placebo-controlled trials, they reported that topical NSAIDs were effective and that the effect of topical NSAIDs was not simply due to rubbing.

Nevertheless the continued popularity of rubefacients (i.e., topical applications without NSAID) supports the idea that responses of patients to topical NSAIDs may also be partly due to rubbing the affected tissue (89). Studies, however, have reported (22,59,60,77) that patients who received topical NSAIDs achieved pain relief quicker than those who received placebo.

2.6.2. Duration of action

Trials conducted by Moore *et al*. (22) compared topical NSAIDs intended for local and/or regional activity with their systemic oral equivalent in both acute and chronic conditions. None of these trials found oral NSAIDs to be superior to the same agent applied topically. Similar outcomes were observed in another study reported by Lin *et al*. (90). These authors, in a meta-analysis that explored the use of topical NSAIDs in the treatment of OA, found that topical NSAIDs intended for local and/or regional activity were superior to placebo in the reduction of pain and improvement of function. Interestingly, this study indicated that topical NSAIDs only showed efficacy over a fortnight, but also reported that these effects were lost after 4 weeks suggesting the development of tolerance. The authors therefore concluded that little evidence existed to support the long term use of topical NSAIDs in OA. Similar observations were noted in studies conducted by Bjordal *et al*. (91) and Mason *et al*. (72). This observation was not immediately noted because most randomised controlled trials were of short duration (i.e., 2 weeks or less) and not a single study extended beyond a period of a month (61). In an attempt to conduct clinical studies for longer than 2 weeks, Baer *et al*. (92) and Bookman *et al*. (93) evaluated the efficacy of topical diclofenac solutions in the treatment of primary OA of the knee. Although efficacy was established within 2 weeks, skin irritation, typically dryness was reported leading to the discontinuation of some patients from the studies.

The early effective waning off of topical NSAIDs intended for local and/or regional activity, suggested that topical therapy was best used for short periods in acute conditions. Since topical NSAIDs have been found to be effective only over a relatively short period of time their use in acute conditions (22,59,72) may be justified whereas use for chronic inflammatory disease is questionable (19).

2.7. Safety

Topical NSAIDs are generally well tolerated. Skin reactions, which are reversible on discontinuation of therapy, are the most commonly observed side effects (94). The incidence of skin reactions is however low and also often observed with placebos (64,67), suggesting that the vehicle itself may be responsible for a significant proportion of the adverse effect (19,63). The vast majority of adverse effects occurring in approximately 10-15% of patients are localised pruritis, itching, burning and/or rash at the site of administration (59). Local reactions in the form of delayed hypersensitivity dermatitis may occur at the site of the application, but these are also relatively uncommon. Cross-sensitisation between topical ketoprofen and other propionic NSAIDs has been reported (60,73,95).

Whereas most NSAIDs administered topically could potentially induce phototoxic reactions, in particular, topical formulations of ketoprofen have been associated with the highest frequency of

photosensitivity reactions which include both phototoxic and photoallergic reactions (58,59,63). Since their introduction in France in 1989, ketoprofen gels have been responsible for various cutaneous adverse effects, essentially photosensitisation and photocontact dermatitis (58). These reactions have been linked to the carbonyl functionality within the chemical structure, possibly related to the benzophenone chromophore (74). Since these products have often been classified as OTC allowing patients to obtain these formulations without any medical supervision, a distinct possibility exists that patients may exceed their recommended use.

Topical application of copious amounts of NSAIDs intended for local and/or regional activity may result in systemic adverse effects including hypersensitivity and asthma (60). On the other hand, systemic reactions due to the use of topical NSAID formulations intended for local and/or regional treatment, such as dyspepsia, nausea and diarrhoea, have rarely been reported (95) following recommended use.

While extensive clinical experience has generally shown the use of topical ocular NSAIDs to be well-tolerated, side effects including impaired corneal sensation, persistent epithelial defects, superficial punctuate keratitis, stromal infiltrates and subepithelial infiltrates have been reported (76). Recently, more severe lesions including corneal stromal ulceration requiring treatment with tissue adhesive, patch grafting and penetrating keratoplasty have been described in association with topical ocular use of Falcon®, a generic version of Voltaren® (diclofenac 0.1%, m/m). Interestingly, however, the generic product has been withdrawn from the market because of adverse effects (76).

Patients should also be cautioned on the use of heating pads in conjunction with topical NSAIDs, as the threshold to induce a burn is reduced. In addition, patients with pre-existing salicylate allergy may experience allergic reactions when salicylates are topically applied (65).

2.8. Cost effectiveness

It has been reported that the cost of therapy involved in treating a patient on chronic oral NSAID therapy is higher than the cost to treat a patient on topical NSAIDs. Hosie and Bird (67) reported significant cost savings to hospital budgets when topical NSAIDs were prescribed instead of oral NSAIDs. The economic argument was based on a model produced by Knill-Jones (96) that showed the probability of how a patient would be treated as well as the treatment costs in the UK, if peptic ulceration were to develop. Using an oral NSAID ulcer rate of 10%, the total cost (the basic drug price of the NSAID plus the cost of treating any resulting GIT adverse effect) of treating 1 000 patients with a month's supply of oral NSAIDs ranged from £39 678 to £58 858, while the equivalent total cost of treating 1 000 patients with topical NSAIDs was just £7 319. If the ulcer rate was reduced

to 5%, this study showed that treatment with any oral NSAID would still be more expensive than with topical NSAIDs.

Generally, topical NSAID formulations (gels, sprays, and patches) are significantly more expensive than oral NSAID dosage forms. E.g., a 100 g tube of a topical NSAID gel was reported as being approximately 3 times the cost of the equivalent oral dosage form required for a week's therapy (95). This led to the concern by Bagheri *et al*. (58) regarding benefit/risk and cost/benefit of topical NSAIDs. These authors noted that £30 million is spent each year in the UK on topical NSAIDs. However it is only possible to assess the true cost/benefit by considering the total cost of oral NSAIDs, which includes side effects and/or adverse reactions. It could be argued that the total cost associated with treatment with oral NSAIDs could potentially be reduced by co-prescription of gastro-protective drugs, however, Knill-Jones (96) showed that topical NSAIDs are more cost-effective than a combination product containing the oral NSAID diclofenac plus the prostaglandin analogue, misoprostol. Although there are regional variations in drug and treatment costs, this study highlighted the importance of investigating the direct costs involved for any drug which in most circumstances is borne by the patient.

CHAPTER THREE

KETOPROFEN ANALYSIS

3.1. Introduction

The development of rapid and efficient procedures for quantitative analysis is considered as a high priority objective in the pharmaceutical industry (97,98) since it permits a large number of samples to be analysed by reducing sample run times. The amount of high purity and expensive solvents used in the preparation of mobile phases (MPs) and associated wash solutions are thereby significantly reduced (99).

3.1.1. High performance liquid chromatography (HPLC)

Liquid chromatography (LC) coupled with different universal and/or selective detectors still remains the preferred method of choice in pharmaceutical analysis for the determination of drug in various matrices (97). The commonly used HPLC is, however, often associated with relatively lengthy sample run times (97,98). A key objective over recent times has been to develop strategies which can be employed to significantly reduce sample run times to a few min and even to less than 1 min where possible, without compromising efficiency.

The main limitation of HPLC is the lack of high efficiency, especially when compared to gas chromatography (GC) and capillary electrophoresis (CE). The small diffusion coefficient of solute in MPs translates into slow diffusion speeds into the stationary phase and has been attributed to the lower efficiency experienced in HPLC in comparison to GC and CE (100).

The efficiency of separation is reflected by the column equivalent to a theoretical plate (HETP) which varies with linear velocity of the MP (101). Separation efficiencies can be improved by using smaller column particles to shorten the diffusion path of drugs. This is described by the simplified van Deemter equation below.

$$H = 2\lambda d_p + \frac{2\gamma D_m}{u} + f(k)\frac{d_p^2}{D_m}u = a + \frac{b}{u} + cu \qquad 3.1.$$

where, efficiency is expressed as HETP, (H), (u) is the linear velocity, (d_p) is particle size in which λ is the packing constant, γ is an obstruction factor for diffusion in a packed bed, D_m is the diffusion coefficient in the MP and $f(k)$ is a function of the retention factor (k). The symbol a

describes the labyrinthine nature of the packed bed, the molecular diffusion (in the direction of the axis) is represented by b and c represents the resistance to mass transfer in the MP.

Since increasing the MP flow rate is normally the simplest approach to reduce analysis time, as seen in Equation 3.1, an increase in optimal linear velocity without compromising efficiency is only possible using columns packed with small particles (99,102). However, the use of columns packed with smaller particles is limited by a rapid increase in pressure drop (101) according to Darcy's law as shown in Equation 3.2. This necessitates the use of extremely high operating pressures which are not possible using conventional HPLC pumps since these instruments generally have a maximum operating pressure of ~ 400 bar (~ 5800 psi).

$$\Delta P = \phi \frac{\eta L u}{d_p^2} \qquad 3.2.$$

where L is the column length, u is the average MP velocity, η is the MP viscosity, ϕ is the flow resistance and d^2 is the particle size. Since u increases while d^2 decreases, a back-pressure higher than ~ 400 bar (~ 5 800 psi) could be generated with column lengths longer than 30 mm (97,101).

Various methodologies have been proposed to bypass the inherent limitations of HPLC. The use of high temperature liquid chromatography (HTLC) is considered as a valuable tool to reduce sample run time (97,99). An increase in temperature (80-200°C) can induce a 5-10 fold reduction in MP viscosity, allowing the application of high flow rates with limited back-pressure (98) even with small particle columns. However, the availability of stationary phases that are stable at elevated temperature is limited. Moreover, the HPLC instrumentation may have to be modified to accommodate and maintain the temperature of the MP (97) and there is also a possible risk of thermal degradation of labile compounds (98,103).

Another strategy is the use of monoliths as the stationary phase in columns (97,104,105). While conventional HPLC columns are filled with small silica spherical particles, monolithic columns contain special silica, which are not formed by particles. They are made by sol-gel technology, which enables formation of highly porous material, containing macropores and mesopores. The macropores (i.e., large pores, ~ 2 µm) are responsible for a low flow resistance and therefore allow the application of high eluent flow rates, while the mesopores (i.e., small pores, ~ 12 nm) ensure sufficient surface area for separation efficiency. Columns fitted with monoliths as stationary phases require short column equilibration times and are used in HPLC. This is especially beneficial when gradient elution is used (105). Monoliths exhibit equivalent quantitative features compared with conventional

stationary phase column packing. However, their need for high eluent flow rates invariably results in consumption of large volumes of organic solvents and limits their compatibility with mass spectrometry (98).

More recently, columns packed with 1.7 µm particles have become commercially available and which generate a 27 times higher column pressure drop than with 5 µm particles (99). Hence, dedicated instrumentation with low extra-column volumes and high detection acquisition rates that can accommodate such high back-pressures generated from the use of columns packed with sub-2 µm particles (106) have been developed. Hence, ultra-high pressure liquid chromatography (UHPLC) associated with high separation efficiencies on columns packed with sub-2 µm particles have been designed.

3.1.2. Ultra performance liquid chromatography (UPLC)

The first commercially available system that addressed the challenge of using elevated pressures coupled with sub-2 µm column particle sizes was introduced by Waters® Corporation (Milford, Massachusetts, USA). Pressures of up to ~ 1034 bar (~ 15 000 psi) with the use of commercially available columns packed with 1.7 µm particles are attainable with this system (107,108). These systems required extensive modifications compared to conventional instrumentation. Not only was there a need to develop high pressure pumps, sample injector systems needed to be re-designed to handle fast injection cycles, low injection and overall system volumes, negligible carryover and temperature control (4-40°C) which together contribute to rapid sample analysis (102).

The Acquity™ UPLC system incorporates specially designed Acquity™ UPLC columns with 1.7 µm Ethylene Bridged Hybrid (BEH) particles. A newer particle technology, 1.8 µm High Strength Silica (HHS) has recently been introduced. Although both technologies ensure column stability under high pressure, BEH is applicable for use over a wider pH range (i.e., 1-12) whereas HSS is used over a narrower pH range (i.e., 1-8). BEH is available in 5 column chemistries namely C_{18}, C_8, Shield RP_{18}, phenyl and HILIC whereas HHS is currently available in 2 column chemistries T3 and C_{18}.

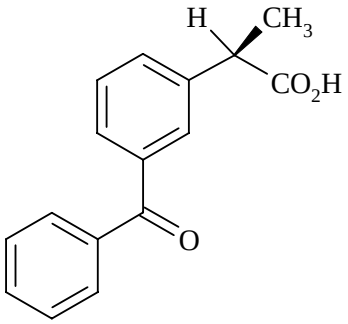
These UPLC systems may be equipped with a range of detectors such as a Photo Diode Array (PDA) UV detector and/or mass spectrometer (MS). It is therefore expected that the use of UPLC coupled to a MS should provide for fast, high resolution separations with significant advantages over conventional HPLC-MS/MS systems.

3.2. HPLC-UV method development and validation

3.2.1. Methods

3.2.1.1. Reagents and chemicals

Ketoprofen (99.4%) (Figure 3.1) was purchased from Sigma-Aldrich (Atlasville, South Africa). Acetonitrile 200 far UV ROMIL-SpS™ Super Purity Solvent and Methanol 215 ROMIL-SpS™ Super Purity Solvent were purchased from ROMIL Ltd. (Cambridge, UK). The following analytical grade chemicals and reagents were obtained from MERCK (Wadeville, South Africa); acetic acid glacial, sodium hydrogen pellets and potassium dihydrogen orthophosphate. Sterile normal saline solution (sodium chloride 0.9%, m/v) was purchased from Bodene (Pty) Ltd. (Port Elizabeth, South Africa). HPLC grade water was obtained from a Milli-Q® A10 water purification system (Millipore, Molsheim, France) that comprised a Quantum™ EX ultrapure organex cartridge and a Q-Gard™ purification pack. The water was filtered through a 0.22 µm Millipak stack filter prior to use.



$C_{16}H_{14}O_3$ MM 254.3 g/mol
pKa = 4.23

Figure 3.1. Structure of ketoprofen

3.2.1.2. Instrumentation

Sample analysis was performed on an Alliance 2695 HPLC system equipped with a 2996 PDA detector, autosampler and column heater (Waters® Corporation, Milford, Massachusetts, USA). Instrument control, data acquisition and evaluation were carried out with Empower Pro™ software (Waters® Corporation, Milford, Massachusetts, USA).

3.2.1a. HPLC analysis of microdialysis (MD) samples

3.2.1a.1. Equipment

A Model AG 135 analytical balance (Mettler Toledo, Greifensee, Zurich, Switzerland) and an ultrasonic bath Model 8845-30 (Cole-Parmer Instruments, Chicago, Illinois, USA) were used for sample preparation.

The method employed for the analysis of dialysis samples was adapted from a previously developed method used for the quantitative determination of ketoprofen obtained from diffusion experiments (1).

Samples (10 µl) were injected onto a Spherisorb® ODS2 C_{18} (250 x 4.1 mm i.d., 5 µm) stainless steel analytical column (Waters® Corporation, Milford, Massachusetts, USA) maintained at ambient temperature (22 ± 0.5°C) using a MP consisting of acetonitrile/20 mM phosphate buffer (pH 2.5): (60/40, v/v) at a flow rate of 1.0 ml/min. The eluate was monitored at a wavelength of 255 nm.

3.2.1a.2. Sample preparation

Relevant quantities of ketoprofen for the preparation of stock solutions were accurately weighed and made up to volume with normal saline solution. All weights were performed in 4 x 12 x 4 mm weighing boats (Sigma-Aldrich, Atlasville, South Africa). Calibration standards (0.2-15 µg/ml) and 3 quality control (QC) standard solutions (low, medium and high) were prepared by serial dilution of a stock solution (~ 100 µg/ml) and the solutions were sonicated for 10 min. A separate stock solution was used to prepare the QC solutions.

Since ketoprofen photodegradation under normal laboratory lighting conditions at room temperature (22 ± 0.5°C) has previously been established (1,109), all sample handling was performed under filtered fluorescent (deep golden amber) light (Lee Filters, Andover, Hampshire, England). This precaution prevents the transmission of light at wavelengths lower than 530 nm and protects ketoprofen photodegradation. Amber glassware was used as a further precaution to prevent the possibility of ketoprofen photolysis.

3.2.1a.3. Linearity

Plots of peak areas of ketoprofen versus concentrations were constructed following the analysis of relevant standard solutions (*n=3*) and the data was evaluated using linear regression analysis.

3.2.1a.4. Accuracy and precision

Intra- and inter-day precision and accuracy of the assay were assessed over a period of 3 days using 3 QC standards (low, medium and high). Five separate determinations of each concentration were analysed to assess accuracy using freshly prepared working standards. For the precision studies, a single working stock solution was used and stored at ambient temperature under filtered light and also served as an indicator of stability.

3.2.1a.5. Limits of quantification and detection

Standard stock solutions were diluted appropriately to obtain concentrations for the estimation of the limit of detection (LOD) and the lower limit of quantification (LLOQ) based on signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively.

3.2.2a. Results and discussion

A typical chromatogram showing the elution of ketoprofen (RT = 3.5 min) in normal saline is shown below (Figure 3.2). Samples in normal saline were directly injected unto the column without any pre-treatment or deleterious effects on separation efficiency, quantitation or significant increase of column back-pressure. Care was taken to ensure that all saline was flushed out daily from the column with HPLC-grade water (~ 10 column volumes) using a flow rate of 1 ml/min.

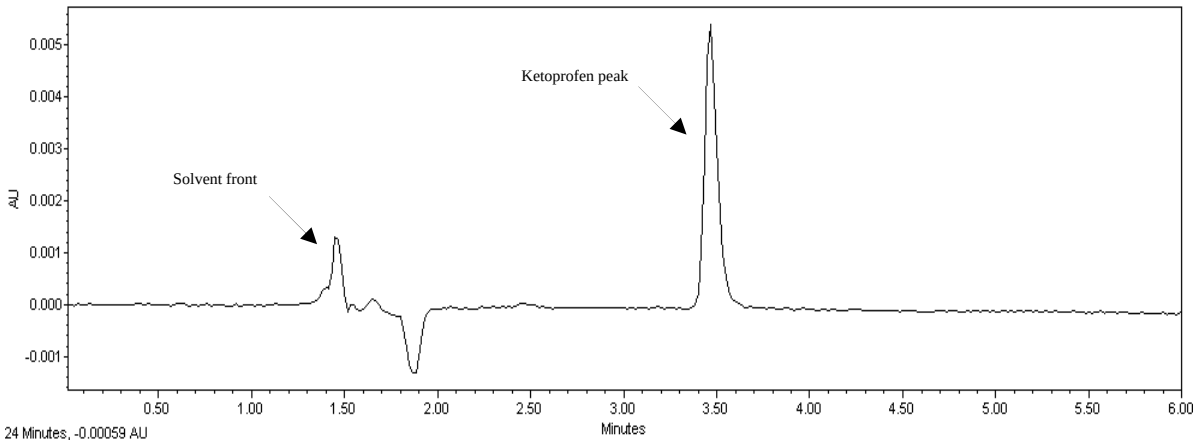


Figure 3.2. HPLC PDA chromatogram of ketoprofen (10 µg/ml) in normal saline solution

Conditions:- Elution chromatography: Isocratic; Column: Waters® Spherisorb® ODS2 C_{18} (250 x 4.1 mm i.d., 5 µm); Column temperature: 22 ± 0.5°C; MP: Acetonitrile/20 mM PBS (acetic acid glacial, pH 2.5) (60/40, v/v); Flow rate: 1.0 ml/min; Injection volume: 10 µl; UV detection wavelength: 255 nm.

3.2.2a.1. Linearity

The calibration plot for ketoprofen was linear with correlation coefficients better than 0.9999 ($y = 33799x + 2366$).

3.2.2a.2. Accuracy and precision

The accuracy of the method (Table 3.1) was found to be in the range 100.47-102.35% with RSDs less than 3.5%. Intra- and inter-day results (Table 3.2) showed good precision and also demonstrated that samples prepared under filtered fluorescent light were stable over a period of 3 days when kept at ambient temperature.

Table 3.1. Accuracy studies of ketoprofen MD QC samples (*n=5*)

MD samples	Day	Actual Conc (µg/ml)	Calculated Conc (µg/ml)	Accuracy (%)	Inter-day RSD (%)
	1	0.85	0.87	102.35	3.25
Low	**2**	0.88	0.90	102.27	2.59
	3	0.86	0.88	103.19	2.22
	1	6.15	6.26	101.78	2.15
Medium	**2**	6.37	6.40	100.47	1.56
	3	6.42	6.32	98.44	1.11
	1	13.15	13.25	100.76	2.01
High	**2**	13.62	13.78	101.17	0.87
	3	12.84	13.06	101.69	1.18

Table 3.2. Precision studies of MD QC samples

MD samples	Day 1 Mean Conc (µg/ml) ± SD (RSD %) (*n=5*)	Day 2 Mean Conc (µg/ml) ± SD (RSD %) (*n=5*)	Day 3 Mean Conc (µg/ml) ± SD (RSD %) (*n=5*)	Inter-day Mean Conc (µg/ml) ± SD (RSD %) (*n=3*)
Low	0.87 ± 0.01 (1.51)	0.85 ± 0.02 (0.02)	0.88 ± 0.01 (1.30)	0.87 ± 0.01 (1.29)
Medium	6.26 ± 0.04 (0.57)	6.26 ± 0.02 (0.38)	6.27 ± 0.02 (0.38)	6.26 ± 0.01 (0.15)
High	13.26 ± 0.02 (0.11)	13.25 ± 0.01 (0.06)	13.26 ± 0.01 (0.11)	13.26 ± 0.01 (0.02)

3.2.2a.3. Limits of quantification and detection

The LOD and the LLOQ of ketoprofen were found to be 0.2 and 0.5 µg/ml respectively and the ULOQ set at 15 µg/ml.

3.2.1b. HPLC analysis of tape strips

The method, equipment and chemicals were the same as described for the analysis of MD sample except for the following:

3.2.1b.1. Method

Initially, an un-spiked non-stripped tape strip was extracted as described below (section 2.3.1b.3) and injected into the chromatographic system using the same MP employed in the analysis of MD samples which involved isocratic elution. The MP was subsequently adjusted for use in gradient elution using Solvent A [Acetonitrile/20 mM phosphate buffer (pH 2.5) (60/40, v/v)] and Solvent B [Methanol].

3.2.1b.2. Equipment

A Model 5415 centrifuge (Eppendorf®, Hamburg, Germany) and a 100 µl Model 710SNR digital syringe (Hamilton, Reno, Nevada, USA) were used.

3.2.1b.3. Sample preparation and extraction

Calibration standards (5-200 µg/ml) and 3 QC solutions of ketoprofen in methanol were prepared. Pre-cut tape strips (2 x 2 cm) prepared from Transpore™ 1527 (24 mm x 5 m) dressing tapes (3M, Isando, South Africa) were spiked by applying 100 µl of each standard solution onto the tape strips which had previously been applied to human skin (stripped) and then removed to provide a layer of intact SC. The tape strips dried for 10 min, after which each tape strip was carefully rolled up, placed into a polyethylene (1.5 ml) centrifuge tube (Eppendorf®, Hamburg, Germany) and ketoprofen extracted with methanol (1 ml). Samples were vortexed for 10 s prior to centrifugation at 12 000 rpm (8050 × *g*) for 5 min and filtered through 0.45 µm hydrophilic low protein binding durapore (PVDF) filters (Millipore®, Molsheim, France). Approximately 200 µl of the supernatant was pipetted into glass sample inserts (300 µl) with pre-installed plastic springs and placed into 9 mm screw top glass amber sample (12 x 32 mm) vials (Waters® Corporation, Milford, Massachusetts, USA) and analysed. Sample preparation and extraction were conducted at ambient temperature (22 ± 0.5°C) under filtered light. All validation procedures were based on the extraction of tape strips spiked with relevant ketoprofen standards.

3.2.1b.4. Linearity

Same as previously described in section 3.2.1a.3.

3.2.1b.5. Accuracy and precision

Same as previously described in section 3.2.1a.4.

3.2.1b.6. Limits of quantification and detection

Same as previously described in section 3.2.1a.5.

3.2.1b.7. Recovery

Extraction recovery of ketoprofen was assessed by analysing spiked samples ($n=5$) of 3 different concentrations corresponding to the concentrations of the QC samples (low, medium and high) and compared with data from the analysis of methanolic solutions of ketoprofen at similar concentrations.

3.2.1b.8. Specificity

Specificity was evaluated by injecting an extract from a blank (non-stripped and un-spiked) tape strip as well as an extract from a blank (stripped but un-spiked) tape strip.

3.2.2b. Results and discussion

Injections of extracts of blank (non-stripped and un-spiked) tape strips following isocratic elution are shown in the chromatogram below (Figure 3.3).

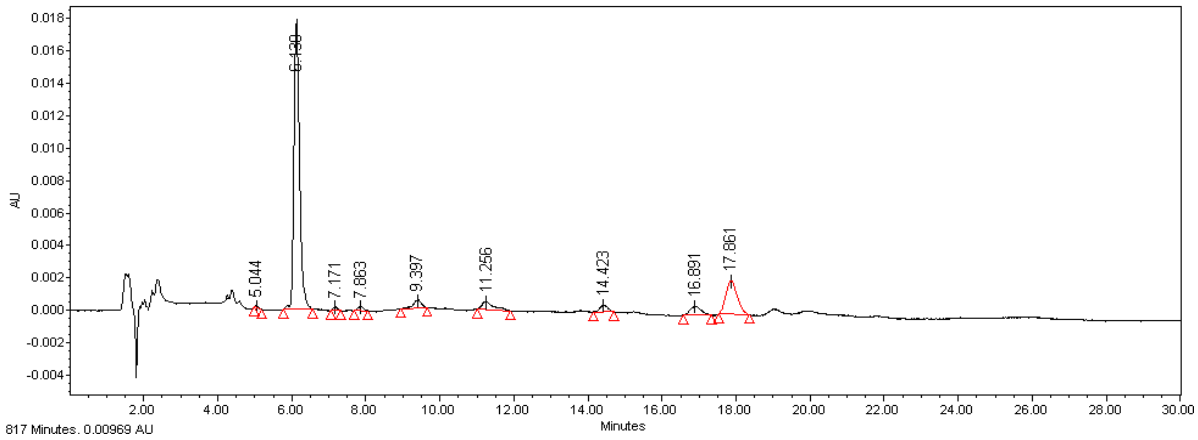


Figure 3.3. HPLC PDA chromatogram of a non-stripped un-spiked blank tape strip extract

Conditions:- Elution chromatography: Isocratic; Column: Waters® Spherisorb® ODS2 C_{18} (250 x 4.1 mm i.d., 5 µm); Column temperature: 22 ± 0.5°C; MP: Acetonitrile/20 mM PBS (acetic acid glacial, pH 2.5) (60/40, v/v); Flow rate: 1.0 ml/min; Injection volume: 10 µl; UV detection wavelength: 255 nm.

Injections of extracts of blank (un-spiked) tape strips including stripped tape strips following the step gradient elution are presented in Figures 3.4 and 3.5 respectively.

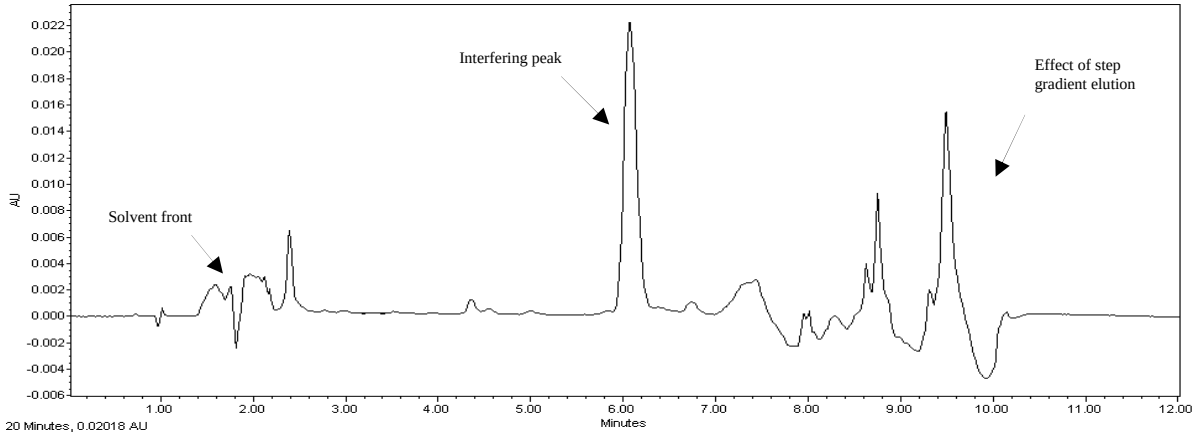


Figure 3.4. HPLC PDA chromatogram of a blank non-stripped tape strip extract

Conditions:- Elution chromatography: Step gradient; Column: Waters® Spherisorb® ODS2 C_{18} (250 x 4.1 mm i.d., 5 µm); Column temperature: 22 ± 0.5°C; MP: Solvent A [Acetonitrile/20 mM PBS at pH 2.5 (60/40) (v/v)]: Solvent B [Methanol]; Flow rate: 1.0 ml/min; Injection volume: 10 µl; UV detection wavelength: 255 nm.

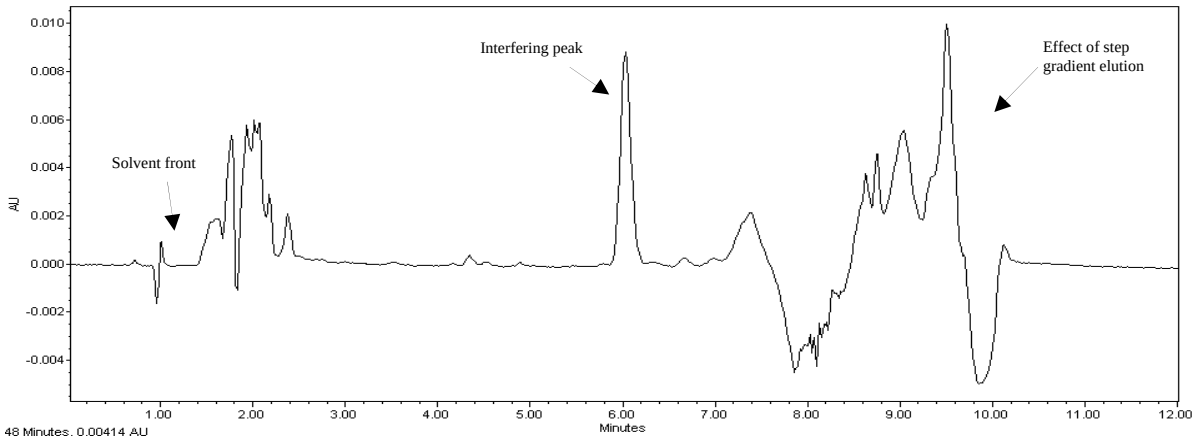


Figure 3.5. HPLC PDA chromatogram of a blank stripped tape strip extract

Conditions:- Elution chromatography: Step gradient; Column: Waters® Spherisorb® ODS2 C_{18} (250 x 4.1 mm i.d., 5 µm); Column temperature: 22 ± 0.5°C; MP: Solvent A [Acetonitrile/20 mM PBS at pH 2.5 (60/40, v/v)]: Solvent B [Methanol]; Flow rate: 1.0 ml/min; Injection volume: 10 µl; UV detection wavelength: 255 nm.

More interference was seen in the early portion (i.e., 1.5-2.5 min) of the chromatogram (Figure 3.5) following injection of a stripped tape strip extract using step gradient elution. With isocratic elution the interfering peaks eluted up to approximately 18 min (Figure 3.3) thus prolonging the run time. Hence a step gradient elution method was necessary to introduce methanol after the elution of the ketoprofen peak in order to flush out the interfering peaks to reduce the run time (~ 12 min) which also incorporated a 2 min re-equilibration (Figure 3.5).

3.2.2b.1. Linearity

The calibration plot for ketoprofen was linear with correlation coefficients better than 0.9999 ($y = 37948x - 6412.3$).

3.2.2b.2. Accuracy and precision

The accuracy of the method (Table 3.3) was found to be in the range 100.07-102.58% with RSDs less than 3%. Intra- and inter-day results (Table 3.4) showed good precision.

Table 3.3. Accuracy studies of ketoprofen from tape strips (*n=5*)

Tape strip	Day	Actual Conc (ng/ml)	Calculated Conc (ng/ml)	Accuracy (%)	Inter-day RSD (%)
	1	5.12	5.23	102.15	2.15
Low	**2**	5.31	5.42	102.07	2.14
	3	4.98	5.02	100.80	2.58
	1	50.34	50.41	100.14	1.02
Medium	**2**	52.21	52.33	100.23	2.01
	3	48.97	50.01	102.12	2.25
	1	150.41	150.54	100.09	0.52
High	**2**	155.99	160.02	102.58	1.15
	3	146.31	146.45	100.10	1.25

Table 3.4. Precision studies of tape strips

Tape strips	Day 1 Mean Conc (ng/ml) ± SD (RSD %) (*n=5*)	Day 2 Mean Conc (ng/ml) ± SD (RSD %) (*n=5*)	Day 3 Mean Conc (ng/ml) ± SD (RSD %) (*n=5*)	Inter-day Mean Conc (ng/ml) ± SD (RSD %) (*n=3*)
Low	5.24 ± 0.03 (0.57)	5.23 ± 0.02 (0.41)	5.25 ± 0.01 (0.19)	5.24 ± 0.01 (0.20)
Medium	50.45 ± 0.03 (0.06)	50.41 ± 0.02 (0.05)	50.47 ± 0.04 (0.07)	50.44 ± 0.03 (0.06)
High	150.58 ± 0.04 (0.03)	150.53 ± 0.02 (0.02)	150.45 ± 0.10 (0.07)	150.52 ± 0.07 (0.04)

3.2.2b.3. Limits of quantification and detection

The LOD and the LLOQ of ketoprofen were found to be 2 and 5 µg/ml respectively and the ULOQ was set at 200 µg/ml.

3.2.2b.4. Recovery

The recovery of ketoprofen ranged from 98.48-98.51% with RSDs less than 2.5% (Table 3.5).

Table 3.5. Ketoprofen recovery from tape strips

Tape strips	Actual Conc (ng/ml)	Calculated Conc (ng/ml)	% Recovery	% RSD (*n=5*)
Low	5.26	5.18	98.48	2.15
Medium	50.14	49.39	98.50	1.26
High	150.19	147.95	98.51	1.59

3.2.2b.5. Specificity

Although various compounds were co-extracted from the tape strips, none of those interfered with the elution of ketoprofen (Figures 3.4, 3.5 and 3.6).

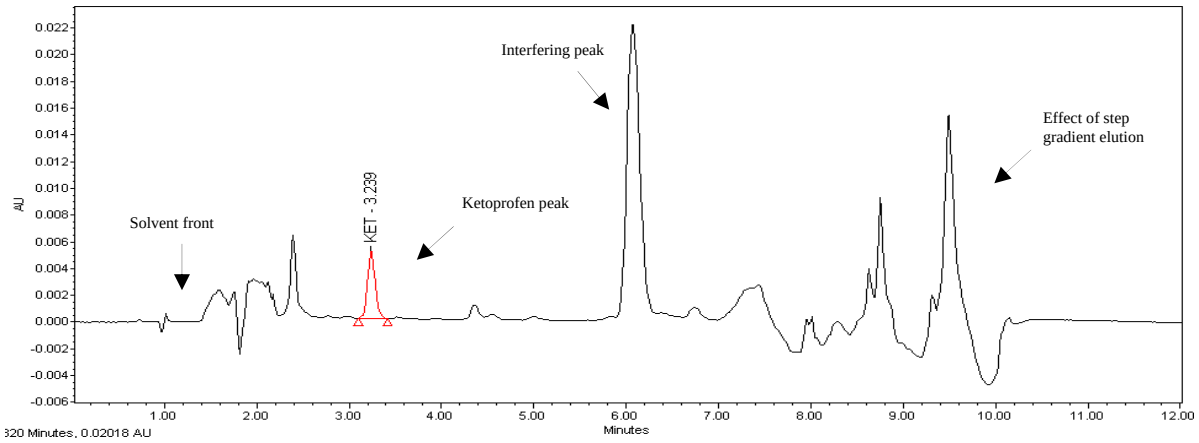


Figure 3.6. HPLC PDA chromatogram of a spiked (5 µg/ml) stripped tape strip extract

Conditions:- Elution chromatography: Step gradient; Column: Waters® Spherisorb® ODS2 C_{18} (250 x 4.1 mm i.d., 5 µm); Column temperature: 22 ± 0.5°C; MP: Solvent A [Acetonitrile/20 mM PBS at pH 2.5 (60/40, v/v)]: Solvent B [Methanol]; Flow rate: 1.0 ml/min; Injection volume: 10 µl; UV detection wavelength: 255 nm.

3.2.3. Conclusions

Analytical methods were developed and validated for the quantitative determination of ketoprofen in a) MD samples and b) tape strips. Normal saline solutions did not appear to affect the analysis nor cause any significant increase in column back-pressure. Using a gradient elution for tape strips resulted in a reduction in run time since tape strip extracts resulted in late eluting peaks which would have necessitated longer run times using the isocratic system applied to analyse MD samples. The concentration ranges for MD samples and tape strip extracts were from 0.5-15 µg/ml and from 5-200 µg/ml, respectively. Both methods were accurate (100.47-102.35% and 100.07-102.58% for MD and tape strips, respectively) and precise (with % RSDs less than 2% and 1% for MD and tape strips, respectively) and recovery of ketoprofen from the tape strips was approximately 98%.

3.3. UPLC-MS/MS analysis of dermal microdialysis (DMD) samples

The reagents, chemicals and equipment used were the same as described under 3.2 except for the following:

3.3.1. Reagents and chemicals

Naproxen (98%), flurbiprofen (99%) and ibuprofen (97%) were purchased from Sigma-Aldrich (Atlasville, South Africa). HPLC grade isopropyl alcohol was purchased from Burdick & Jackson, Inc. (Muskegon, Michigan, USA) and ethyl acetate, *n*-hexane, sodium dichromate dehydrate and sulphuric acid (98%, v/v) were of analytical grade and obtained from MERCK (Wadeville, South Africa).

3.3.2. Equipment

A Model MX5 analytical ultra-microbalance (Mettler Toledo, Greifensee, Zurich, Switzerland), a 200 µl pipette (Pipetman®, Gilson Medical Electronics, Villiers-le-Bel, France), a N-EVAP 24 place Model 112 nitrogen analytical evaporator (Organomation Associates Inc., South Berlin, Massachusetts, USA) coupled to a cylinder of high purity nitrogen gas (Afrox, Port Elizabeth, South Africa).

3.3.3. Instrumentation

Sample analysis was performed on an Acquity™ UPLC system (Waters® Corporation, Milford, Massachusetts, USA) which consisted of a binary pump solvent manager capable of generating pressures up to 1000 bar (~ 15 000 psi). Detection was carried out using an Acquity™ PDA UV-Vis detector coupled in series with an Acquity™ TQD tandem-quadrupole MS equipped with a Z-spray electrospray interface (Manchester, UK). The UV-Vis detector contained a 500 nl flow cell, the time constant was set at 25 ms and data sampled at 80 Hz. The system was equipped with strong and weak wash solution reservoirs. Instrument control, data acquisition and processing were carried out with MassLynx™ (version 4.1) and IntelliStart™ software (Waters® Corporation, Milford, Massachusetts, USA) was used to control the fluidics device to infuse solutions for tuning the MS.

The objective was to develop a rapid, accurate, precise and reproducible analytical method with the requisite sensitivity suitable for the quantitative determination of ketoprofen in DMD samples obtained from human subjects using UPLC-MS/MS. HPLC-MS/MS methods in negative-ion (NI) or positive-ion (PI) with electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) for ketoprofen analysis previously reported in the literature are shown in Table 3.6.

Table 3.6. Published methods/reports using HPLC-MS/MS for the analysis of ketoprofen

Stationary phase	MP and elution chromatography	Flow rate (ml/min)	RT (min)	LOD	Sample type analysed	Sample cleaning	Ionisation mode	Injection vol (µl)	Reference
Genesis C_{18} (150 x 2.1 mm, 4 µm)	Solvent A [Acetonitrile/methanol (40/60, v/v)]; Solvent B [20 mM ammonium acetate]-Gradient elution	0.20	8.51	20.00 pg/ml	Sewage samples	SPE	NI ESI	25	(110)
LiChrospher 100 RP-18	Acetonitrile/water (formic acid, pH 2)-Gradient elution	1.00	16.50	28.00 pg/ml	Surface and waste water samples	SPE	NI ESI	20	(111)
LiChrospher RP-18 (125 x 3.0 mm, 5 µm)	Acetonitrile/water (formic acid, pH 2)-Gradient elution	0.30	9.90	0.10 ng/ml	River sediment samples	SPE	NI APCI	50	(112)
Purospher Star RP-18 (125 x 2.0 mm, 5 µm)	Methanol/water-Gradient elution	0.20	17.13	26.00 pg/ml	Natural and treated water Samples	SPE	NI ESI	10	(113)
Symmetry C_{18} (150 x 2.1 mm, 3 µm)	Methanol/water-Gradient elution	0.20	4.80	0.10 pg/ml	Surface and waste water samples	SPE	NI ESI	20	(114)
Shimpack GLC-CN (150 x 4.0 mm, 5µm)	Acetonitrile/20 mM ammonium acetate (84/16, v/v)-Isocratic elution	1.00	3.63	100.00 ng/ml	Pharmaceutical formulations		NI APCI	10	(115)
OASIS HLB SPE (20 x 2.1 mm)	Acetonitrile/water (ammonium hydroxide 0.025%) (80/20, v/v)-Isocratic elution	0.50		25.00 ng/ml	Rat MD interstitial fluid samples	SPE	NI ESI	10	(116)
Agilent Zorbax Eclipse XDB C_{18} (150 x 4.6 mm, 5 µm)	Acetonitrile/water (acetic acid 0.1%)-Gradient elution	1.00	19.90	150.00 ng/ml	Homeopathic medical products	filter (0.22 µm)	NI ESI	5	(117)
Symmetry C_{18} (150 x 2.1 mm, 5µm)	Acetonitrile/water (formic acid 0.1%)-Gradient elution	0.25	10.67		Milk	filter (0.22 µm)	PI ESI	40	(118)
Purospher Star RP-18 (125 x 2.0 mm, 5 µm)	Methanol/water-Gradient elution	0.20	14.71	6.00 ng/ml	Wastewaters samples	SPE	NI ESI	10	(119)
Phenomenex® Luna™ C_8 (50 x 4.6 mm, 3 µm)	Acetonitrile/water (acetic acid 0.1%) (65/35, v/v)-Isocratic elution	1.00	1.10	0.47 ng/ml	Human MD normal saline samples	SPE	NI APCI	40	(21)

3.3.4. Method development and validation

3.3.4.1. UPLC conditions

An Acquity™ BEH C_{18} (100 x 2.1 mm i.d., 1.7 µm) stainless steel analytical column (Waters® Corporation, Milford, Massachusetts, USA) maintained at ambient temperature (22 ± 0.5°C) was used for the analysis. Methanol/water and acetonitrile/water mixtures were initially investigated for use as MP. A MP consisting of acetonitrile/methanol/water (60/20/20, v/v/v) was used at flow rate of 0.30 ml/min and the eluate monitored at a UV wavelength of 255 nm. Ibuprofen, naproxen and flurbiprofen were investigated as possible candidates for use as an internal standard (IS). The chemical structures of these compounds are shown below in Figure 3.7.

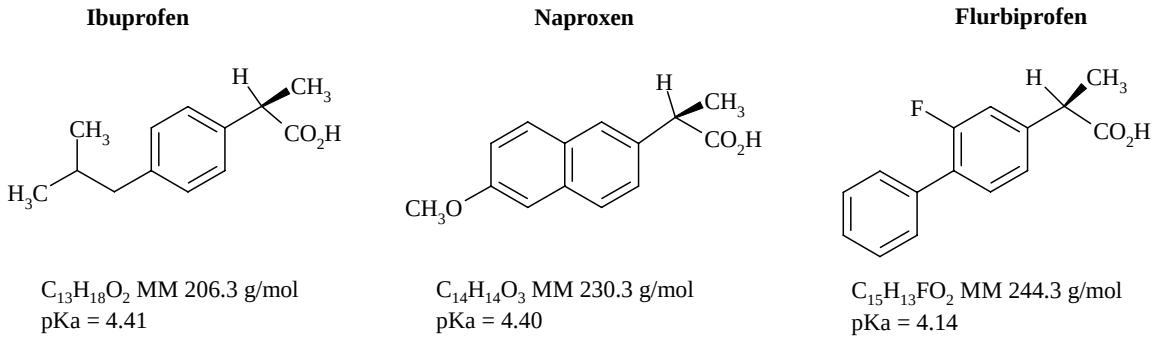


Figure 3.7. Chemical structures of ibuprofen, naproxen and flurbiprofen

3.3.4.2. MS tuning

The MS was tuned in NI ESI for the detection of precursor ions $[M-H]^-$ and the dissociation of these ions was induced. Methanolic solutions (100 ng/ml) of ketoprofen and IS were infused at 10 µl/min under software control, into the mass analyser. Nitrogen, used as a nebulising and desolvation gas, was provided by a high purity nitrogen generator NM 30LA 230VOC (Peak Scientific Instruments, Renfrewshire, Scotland, UK) and 2.55 x 10^{-4} mbar instrument grade (IG) argon (99.999%) (Afrox, Port Elizabeth, South Africa) was used as the collision gas.

3.3.4.3. MS optimisation

The most abundant ESI-MS/MS transition for each compound was monitored in the multiple reaction monitoring (MRM) mode to obtain the highest quantitative sensitivity. The choice of fragmentation products for each analyte based on the most intense signal and the optimisation of cone voltages, energy collisions and other instrument parameters were individually investigated for each compound in the combined flow-state mode through direct infusion of standard solutions in methanol (100 ng/ml). This was performed by UPLC where the MP was pumped directly into the MS via a switching valve. The optimised MS settings employed for both ketoprofen and IS are reported in Table 3.7.

Table 3.7. Optimised MS instrument parameters for NI ESI

Capillary voltage	3.50 kV
Extractor voltage	2.00 V
RF lens voltage	0.50 V
Source temperature	120°C
Desolvation temperature	500°C
Cone gas flow rate	109 l/h
Desolvation gas flow rate	509 l/h
Multiplier voltage	650 V
Gas cell Pirani pressure	$2.55e^{-3}$ mbar

3.3.4.4. Sample carryover

Sample carryover was evaluated by sequential serial injections of a blank methanol solution, followed by the injection of a solution that contained a relatively high concentration (1 µg/ml) of ketoprofen and IS and subsequently by the injection of further blank solutions. Sample inserts and sample vials were also assessed for possible carryover or contamination. Approximately 30 µl of methanol was pipetted into randomly selected sample inserts from 3 different lots (Lot number 4170672080), then placed in sample vials, capped and analysed.

3.3.4.5. Matrix effects

Matrix effect on MS response was evaluated by comparing responses obtained by injecting standard solutions of KET (200 ng/ml) in normal saline and methanol onto the column for on-line MS/MS analysis.

3.3.4.6. Sample preparation and extraction

Calibration standards (0.5-500 µg/ml), 3 QC solutions of ketoprofen and IS (20 ng/ml) in normal saline solution were prepared. Equal volumes (30 µl) of ketoprofen and IS were pipetted into centrifuge tubes making a total volume of 60 µl. Samples were extracted ethyl acetate (200 µl) and the tubes were vortexed for 10 s prior to centrifugation at 12 000 rpm (8050 × *g*) for 5 min. Approximately 180 µl of the supernatant from each centrifuge tube was pipetted into sample inserts which were placed into amber sample vials and evaporated to dryness in a nitrogen evaporator under a slow stream of high purity nitrogen gas. Samples were then reconstituted with methanol (30 µl), the sample vials capped and vortexed for a further 10 s before analysis. Sample preparation and extraction were conducted at ambient temperature (22 ± 0.5°C) under filtered light. All validation procedures were based on extracts of the relevant standard solutions of ketoprofen in normal saline.

3.3.4.7. Linearity

Same as previously described in section 3.2.1a.3.

3.3.4.8. Accuracy and precision

Same as previously described in section 3.2.1a.4.

3.3.4.9. Limits of quantification and detection

Same as previously described in section 3.2.1a.5.

3.3.4.10. Recovery

Same as previously described in section 3.2.1b.7.

3.3.5. Results and discussion

3.3.5.1. UPLC conditions

To my knowledge, to-date there has not been any publications or reports which have focused on the development and validation of an analytical method using UPLC for the quantitative determination of ketoprofen for use in human DMD studies. Most published HPLC-MS/MS methods have reported the use of buffers or inclusion of acids or bases in the MP (21,110-112,115-118) for the analysis of ketoprofen, although there have been reports that used MPs without incorporating such reagents (113,114,119). Special caution is however, necessary when using a UPLC system which involves columns packed with sub-2 µm particles and equipment which utilises very narrow tubing and fittings coupled with very high operation pressures.

Previously reported methods that used "non-buffer" MPs employed gradient as opposed to isocratic elution. Although gradient elution has been purported to provide an increase in sensitivity (106) by increasing peak capacity and decreasing peak widths (120), the use of gradient elution is more complex than isocratic procedures. Many more variables need to be controlled when using gradient elution compared to isocratic elution with gradient elution being a generally slower technique since the column has to be re-equilibrated after each run. Gradient elution is also associated with a higher incidence of the appearance "ghost" peaks and increased baseline noise (121).

During the initial method development, the influence on RT, peak symmetry and detection sensitivity was investigated by varying the volume of the organic modifiers (methanol and/or acetonitrile) in the MP. Tailing of the leading edge of the ketoprofen peak was observed using a MP consisting of varying proportions of methanol/water (Figure 3.8). Sensitivity increased with increasing methanol content to 70% but was associated with broadening and deteriorating peak shapes.

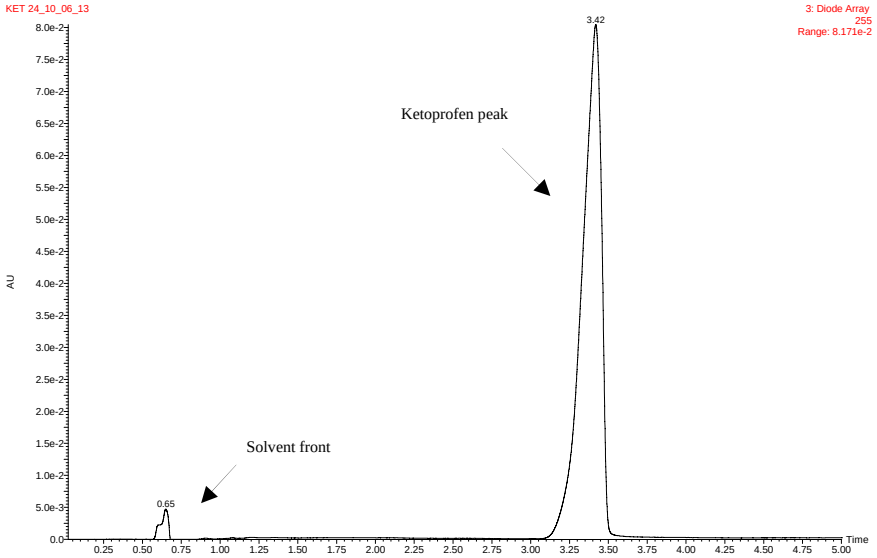


Figure 3.8. UPLC PDA chromatogram of a ketoprofen standard (200 ng/ml) employing a MP composition of methanol/water (50/50, v/v)

Conditions:- Column: Acquity™ BEH C_{18} (100 x 2.1 mm i.d., 1.7 µm); Column temperature: 22 ± 0.5°C; MP: (methanol/water) (50/50, v/v); Flow rate: 0.3 ml/min; Injection volume: 5 µl; Ionisation mode: NI ESI; MRM: 253.00 > 209.00; Capillary voltage: 3.5 kV; Source temperature: 120°C; Desolvation temperature: 500°C; Desolvation gas flow: 509 l/h; Cone gas flow: 109 l/h.

When methanol was replaced with acetonitrile, sharp symmetrical peaks but with reduced sensitivity, was observed. (Figure 3.9).

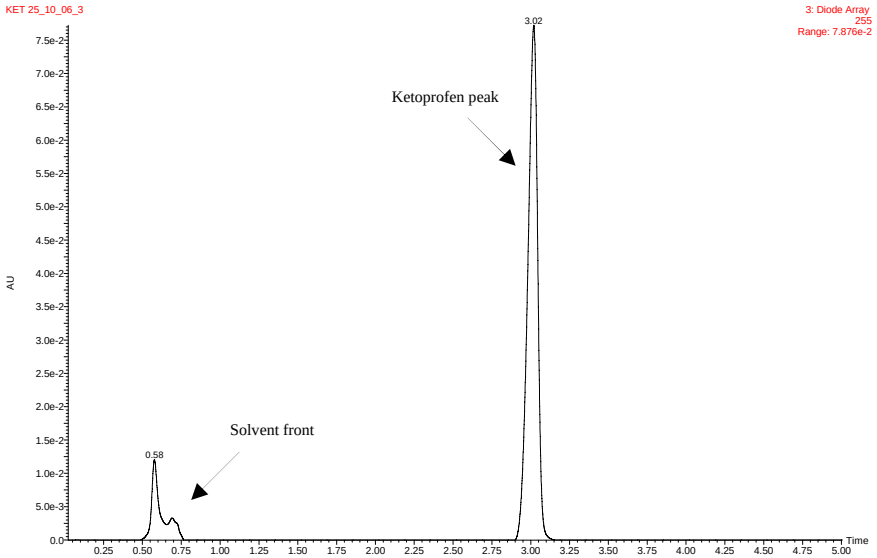


Figure 3.9. UPLC PDA chromatogram of a ketoprofen standard (200 ng/ml) employing a MP composition of acetonitrile/water (50/50, v/v)

Conditions:- Column: Acquity™ BEH C_{18} (100 x 2.1 mm i.d., 1.7 µm); Column temperature: 22 ± 0.5°C; MP: (acetonitrile/water) (50/50) (v/v); Flow rate: 0.3 ml/min; Injection volume: 5 µl; Ionisation mode: NI ESI; MRM: 253.00 > 209.00; Capillary voltage: 3.5 kV; Source temperature: 120°C; Desolvation temperature: 500°C; Desolvation gas flow: 509 l/h; Cone gas flow: 109 l/h.

Initially, an isocratic MP consisting of acetonitrile/methanol/water (30/35/35, v/v/v) resulted in the requisite sensitivity, but the peaks were somewhat asymmetrical with tailing on the leading edge. As expected, increasing the composition of acetonitrile, while concurrently decreasing the composition of methanol (although maintaining an equal ratio of methanol/water), produced an acceptable

symmetrical peak (Figure 3.10) with a composition of acetonitrile/methanol/water (60/20/20, v/v/v) without compromising sensitivity.

The associated increase or decrease in sensitivity with the use of methanol or acetonitrile respectively was in agreement with the published methods/reports presented in Table 3.6. The published methods that used an acetonitrile-based MP system incorporated either acid (formic acid or acetic acid) or base modifiers (ammonium hydroxide) to achieve high sensitivity (21,110-112,115-118), whereas reports that used methanol/water MPs were not only unmodified (i.e., no acids or bases), but produced the highest sensitivity (113,114,119), which suggested that methanol might have a significant role in the ionisation of ketoprofen in NI ESI.

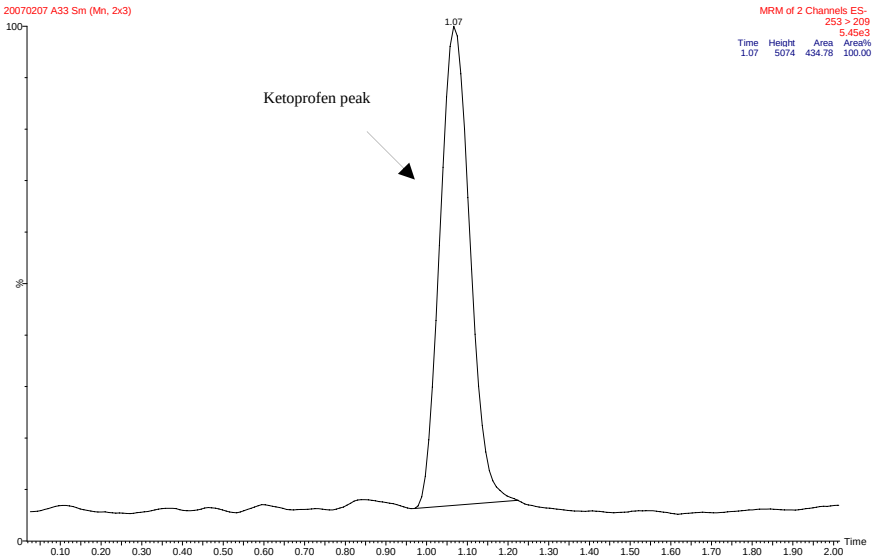


Figure 3.10. ESI-MS/MS chromatogram of a ketoprofen standard (10 ng/ml) employing a MP composition of acetonitrile/methanol/water (60/20/20, v/v/v)

Conditions:- Column: Acquity™ BEH C_{18} (100 x 2.1 mm i.d., 1.7 µm); Column temperature: 22 ± 0.5°C; MP: (acetonitrile/methanol/water) (60/20/20, v/v/v); Flow rate: 0.3 ml/min; Injection volume: 5 µl; Ionisation mode: NI ESI; MRM: 253.00 > 209.00; Capillary voltage: 3.5 kV; Source temperature: 120°C; Desolvation temperature: 500°C; Desolvation gas flow: 509 l/h; Cone gas flow: 109 l/h.

Another interesting observation reported in the published literature was the high sensitivity obtained with acid modifiers in NI ESI. It is commonly accepted that for weakly acidic analytes in NI mode, base modifiers facilitate analyte deprotonation resulting in increased sensitivity, whereas acid modifiers facilitate analyte protonation thereby resulting in decreased sensitivity. However, a number of weak carboxylic acids (e.g., acetic acid) have been reported to increase NI ESI response. The magnitude of this response however depends on the acid modifier, its concentration and the properties of the analyte (122). The decreased sensitivity with acetonitrile/water MPs has been related to the unavailability of protons from either water or analyte for chemical reduction, which is the principal electrochemical reaction that occurs in NI ESI at the MS spray tip. The protons are reduced to hydrogen gas and thus additional protons provided by an acid modifier will facilitate the reduction process by providing excess negative charges hence increasing sensitivity (122). Since methanol is a

weak acid and may also make protons available to facilitate the reduction process, the increased sensitivity with methanol/water MPs may then be explained by the reduction potential of methanol.

The increased sensitivity observed with the combination of acetonitrile/methanol/water MP compositions confirmed that methanol indeed acted as an acid modifier, although increasing volumes of methanol resulted in asymmetric peaks. The decreased sensitivity observed when the methanol content was reduced by 5% or more, suggested that the volume of the acid modifier (methanol) insufficiently provided protons to the ketoprofen for chemical reduction. An isocratic composition of acetonitrile/methanol/water (60/20/20, v/v/v) at a flow rate of 0.30 ml/min was therefore deemed optimum for use as the MP.

No significant improvement in peak sensitivity was observed with increased flow rates, although shorter elution times were observed. Moreover the higher flow rates resulted in undesirable higher column back-pressures.

Flurbiprofen, naproxen and ibuprofen compounds which are structurally and physico-chemically similar to ketoprofen, were investigated as an IS in order to compensate for possible errors in the extraction procedure. However, the former 2 compounds co-eluted with ketoprofen at 1.15 and 1.21 min respectively, whereas ibuprofen was well-resolved from ketoprofen and eluted at 1.49 min. The chosen flow rate of 0.3 ml/min was compatible with the use of ibuprofen as IS (Figure 3.11).

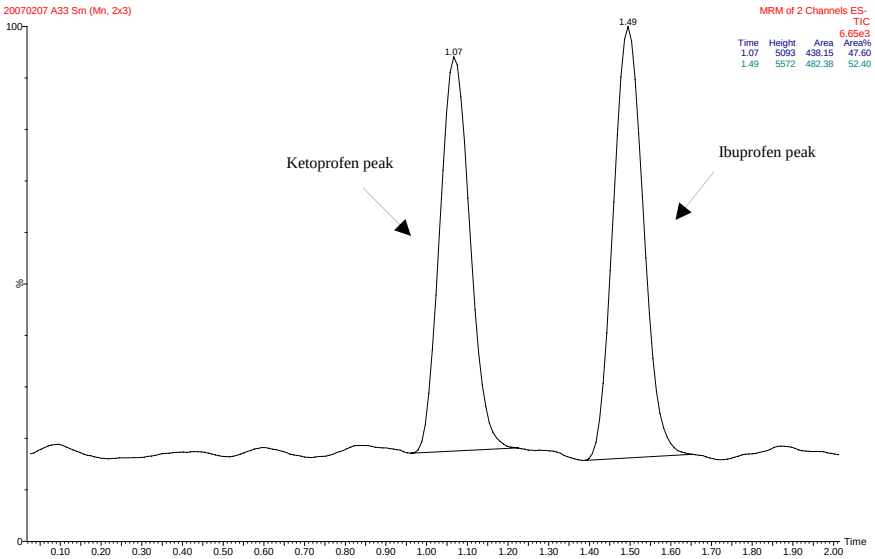


Figure 3.11. ESI-MS/MS TIC chromatogram of ketoprofen (50 ng/ml) and IS (50 ng/ml)

Conditions:- Column: Acquity™ BEH C_{18} (100 x 2.1 mm i.d., 1.7 µm); Column temperature: 22 ± 0.5°C; MP: (acetonitrile/methanol/water) (60/20/20, v/v/v); Flow rate: 0.3 ml/min; Injection volume: 5 µl; Ionisation mode: NI ESI; MRM: 253.00 > 209.00 and 205.00 > 161.00; Capillary voltage: 3.5 kV; Source temperature: 120°C; Desolvation temperature: 500°C; Desolvation gas flow: 509 l/h; Cone gas flow: 109 l/h.

3.3.5.2. MS tuning and optimisation

NI ESI (110,111,113,114,116,117,119) was the commonly used ionisation mode presented in Table 3.6. However, Daeseleire *et al*. (118) reported ketoprofen detection in PI ESI and conditions for NI APCI have also been reported (21,112,115). The most sensitive methods/reports were attained with the use of NI ESI, hence this mode was subsequently investigated. The TQD MS tuned spectra methanolic solutions (100 ng/ml) of ketoprofen (Figure 3.12) and IS (Figure 3.13) revealed 2 product ions in each spectrum. Under the tuned conditions, product ions were produced for ketoprofen (*m/z* 209.01 and 196.99) and ibuprofen (*m/z* 160.82 and 158.87) with the most abundant fragmentations observed were *m/z* 209.01 and *m/z* 160.82, respectively.

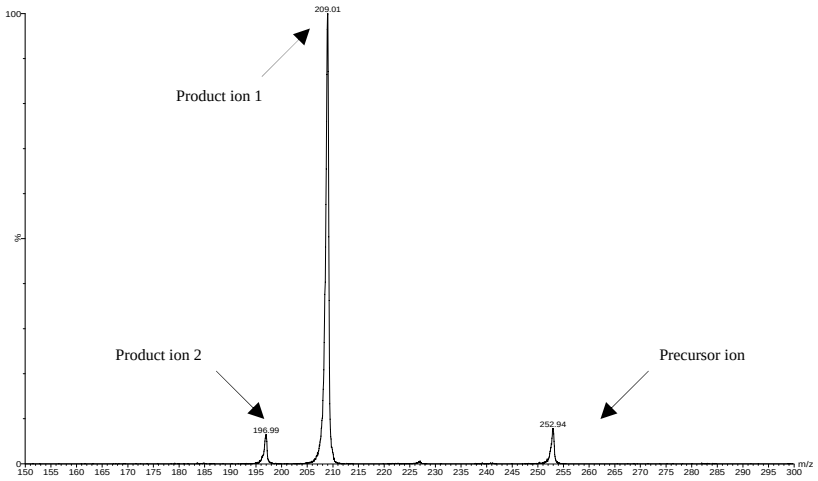


Figure 3.12. Spectrum of precursor (*m/z* 252.94) and product (*m/z* 196.99; 209.01) ions of ketoprofen

Conditions:-Ionisation mode: NI ESI; Flow state: Combined; Solvent: Methanol; Infusion flow rate: 10 µl/min; MP: (acetonitrile/methanol/water) (60/20/20, v/v/v); Flow rate: 0.3 ml/min; MRM: 253.00 > 209.00; Cone voltage: 15 V; Collision energy: 8 eV; Capillary voltage: 3.5 kV; Source temperature: 120°C; Desolvation temperature: 500°C; Desolvation gas flow: 509 l/h; Cone gas flow: 109 l/h.

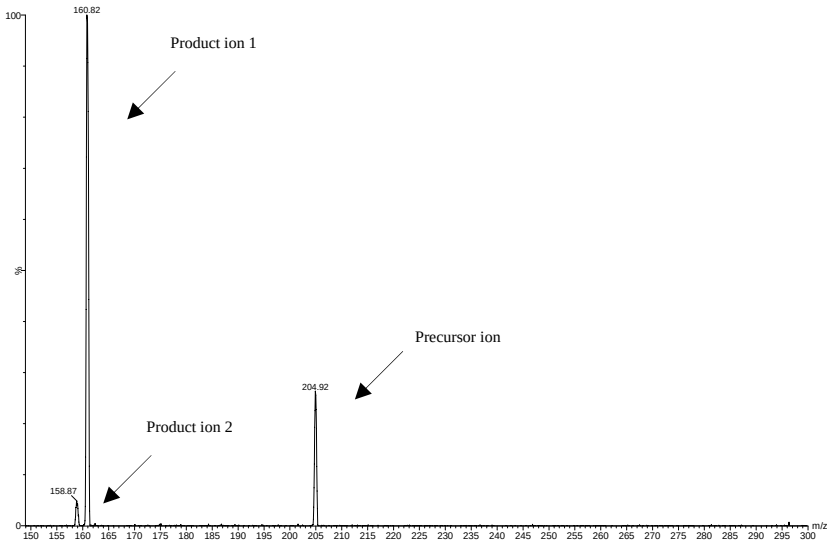


Figure 3.13. Spectrum of precursor (*m/z* 204.92) and product (*m/z* 158.87; 160.82) ions of IS

Conditions:-Ionisation mode: NI ESI; Flow state: Combined; Solvent: Methanol; Infusion flow rate: 10 µl/min; MP: (acetonitrile/methanol/water) (60/20/20, v/v/v); Flow rate: 0.3 ml/min; MRM: 205.00 > 161.00; Cone voltage: 21 V; Collision energy: 7 eV; Capillary voltage: 3.5 kV; Source temperature: 120°C; Desolvation temperature: 500°C; Desolvation gas flow: 509 l/h; Cone gas flow: 109 l/h.

As previously mentioned, since the principal electrochemical reaction in NI ESI is reduction or deprotonation of molecules [M], the detection of the precursor ions was achieved by the loss of the carboxylic acid proton $[M-H]^-$. For both analytes, the product ions formed by the expulsion of CO_2 $[M-H-CO_2]^-$ were the most intense ions and this was in agreement with published MS/MS methods (110-114). Secondary fragments $[M-H-C_2H_3O_2]^-$ and $[M-H-CO_2-H]^-$ corresponding to ketoprofen and IS respectively were too low to be used quantitatively. The MRM transitions used for the quantification of ketoprofen and IS are presented in Table 3.8.

Table 3.8. MRM NI ESI-MS/MS conditions for ketoprofen and IS

Analyte	Collision (eV)	Cone voltage (V)	MRM transition (*m/z*)	Precursor and product ions (*m/z*)	
Ketoprofen	8.0	15.0	253.00 > 209.00	252.94	$[M-H]^-$
				209.01	$[M-H-CO_2]^-$
				196.99	$[M-H-C_2H_3O_2]^-$
Ibuprofen	7.0	21.0	205.00 > 161.00	204.92	$[M-H]^-$
				160.94	$[M-H-CO_2]^-$
				158.99	$[M-H-CHO_2]^-$

3.3.5.3. Sample carryover

The UPLC system is equipped with 2 needle wash solution reservoirs, one containing a "weak" needle wash using 200 µl/run and the other a "strong" needle wash using 600 µl/run which clean the needle and wash station before, during and after each injection cycle. This is an added measure to avoid carryover and trace contamination when using highly sensitive systems. During the initial stages of method development, small peaks were seen at the RTs of ketoprofen and IS following blank methanol injections. The composition of the strong wash solution was initially MP but subsequently replaced with (1500 µl/run) isopropanol alcohol/methanol/acetonitrile (40/35/35, v/v/v) while the weak wash solution (500 µl/run) was maintained with MP. This modification in the strong wash solution and change in the volumes of the washes resulted in the elimination of those peaks.

Different ESI-MS/MS responses from 3 batches of sample inserts using the same blank methanol solution are presented in Figures 3.14-3.16 below. The analysis of Lot 1 sample inserts showed no interference in both ketoprofen and ibuprofen MRM transitions whereas blank injections from Lot 2 inserts produced a response which interfered with the ibuprofen MRM transition and blank injections from Lot 3 inserts resulted in interference with both ketoprofen and ibuprofen MRM transitions. Although the sample vials were only used as supports for the sample inserts, methanol was added to those vials and injected into the system but no response was observed indicating contamination only from Lot 2 & 3 sample inserts.

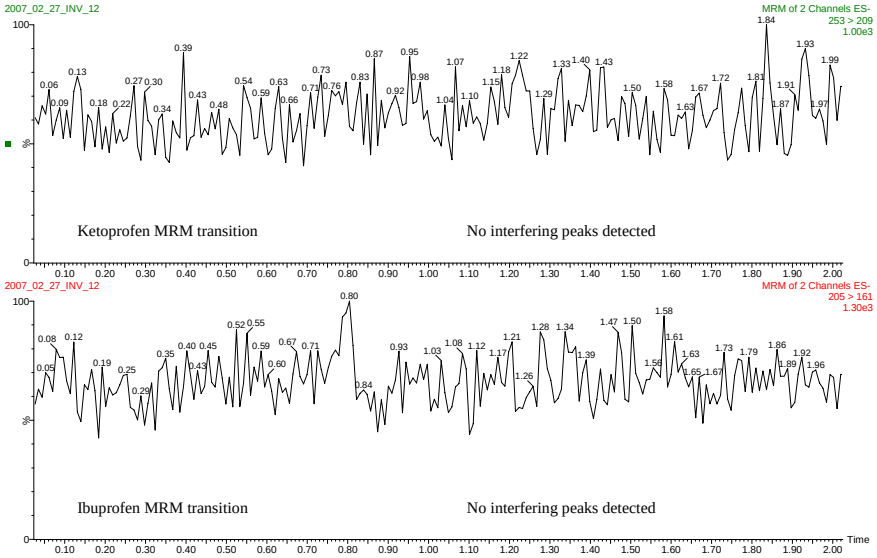


Figure 3.14. ESI-MS/MS chromatogram illustrating analysis of blank methanol solution from sample inserts (Lot 1)

Conditions:- Column: Acquity™ BEH C_{18} (100 x 2.1 mm i.d., 1.7 µm); Column temperature: 22 ± 0.5°C; MP: (acetonitrile/methanol/water) (60/20/20, v/v/v); Flow rate: 0.3 ml/min; Injection volume: 5 µl; Ionisation mode: NI ESI; MRM: 253.00 > 209.00 and 205.00 > 161.00; Capillary voltage: 3.5 kV; Source temperature: 120°C; Desolvation temperature: 500°C; Desolvation gas flow: 509 l/h; Cone gas flow: 109 l/h.

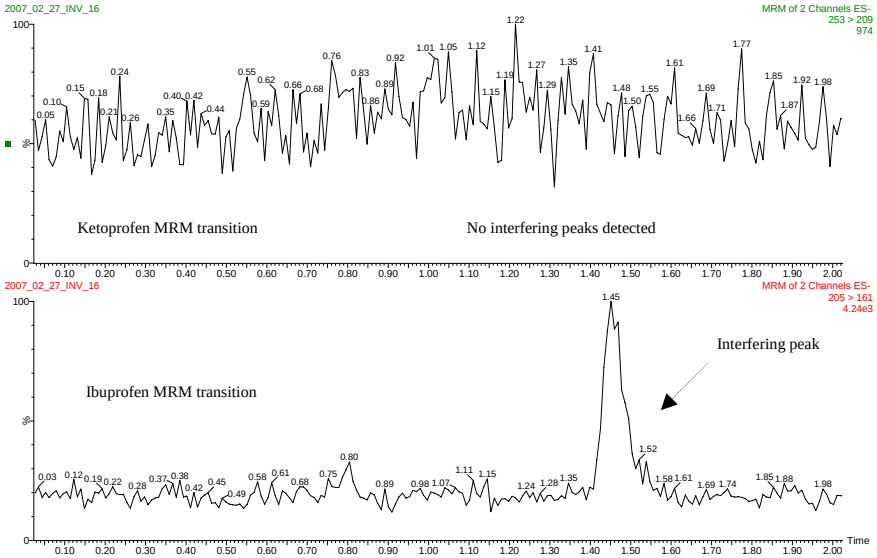


Figure 3.15. ESI-MS/MS chromatogram illustrating analysis of blank methanol solution from sample inserts (Lot 2)

Conditions:- Column: Acquity™ BEH C_{18} (100 x 2.1 mm i.d., 1.7 µm); Column temperature: 22 ± 0.5°C; MP: (acetonitrile/methanol/water) (60/20/20, v/v/v); Flow rate: 0.3 ml/min; Injection volume: 5 µl; Ionisation mode: NI ESI; MRM: 253.00 > 209.00 and 205.00 > 161.00; Capillary voltage: 3.5 kV; Source temperature: 120°C; Desolvation temperature: 500°C; Desolvation gas flow: 509 l/h; Cone gas flow: 109 l/h.

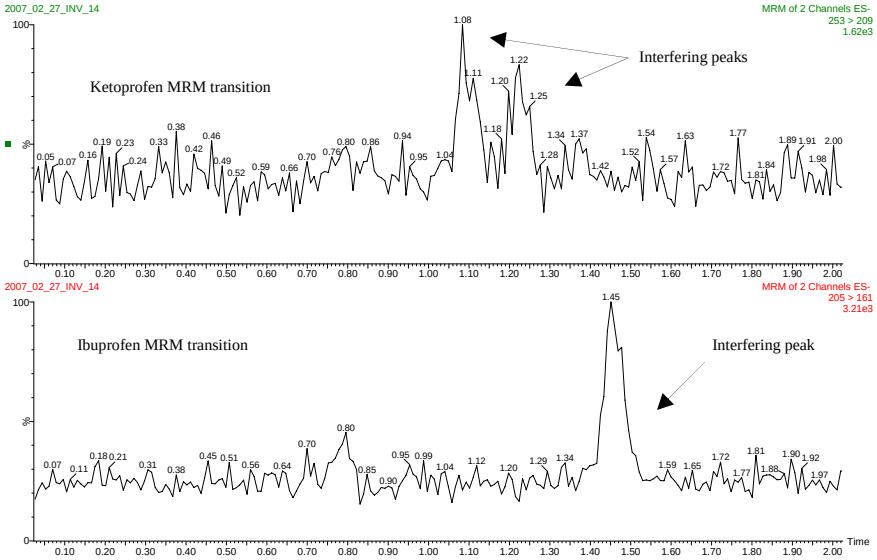


Figure 3.16. ESI-MS/MS chromatogram illustrating analysis of blank methanol solution from sample inserts (Lot 3)

Conditions:- Column: Acquity™ BEH C_{18} (100 x 2.1 mm i.d., 1.7 µm); Column temperature: 22 ± 0.5°C; MP: (acetonitrile/methanol/water) (60/20/20, v/v/v); Flow rate: 0.3 ml/min; Injection volume: 5 µl; Ionisation mode: NI ESI; MRM: 253.00 > 209.00 and 205.00 > 161.00; Capillary voltage: 3.5 kV; Source temperature: 120°C; Desolvation temperature: 500°C; Desolvation gas flow: 509 l/h; Cone gas flow: 109 l/h.

Examination of the sample vials and inserts revealed that LCMS grade sample vials were supplied (Microsep, Sandton, South Africa) with HPLC grade sample inserts from the same supplier. Attempts were made to clean the sample inserts by individually flushing with methanol but without success. Further attempts were made to remove the contamination by immersing the inserts in a solution of chromic acid for 12 h followed by removal from the acid and thorough flushing, first with double distilled deionised water and then with HPLC grade water and finally sonicated in methanol for 30 min. This treatment was successful and all sample inserts were subsequently treated in this way prior to use.

3.3.5.4. Matrix effects

Signal suppression was observed when analytes in normal saline were introduced into the MS (Figure 3.17) and, moreover, the column back-pressure increased with increasing number of normal saline sample injections.

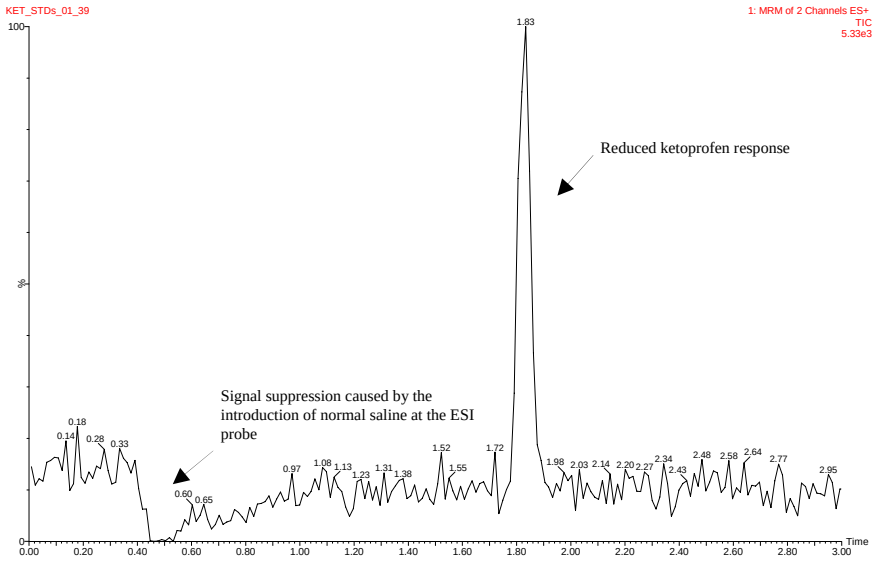


Figure 3.17. ESI-MS/MS TIC chromatogram of normal saline solutions ketoprofen (200 ng/ml)

Conditions:- Column: Acquity™ BEH C_{18} (100 x 2.1 mm i.d., 1.7 µm); Column temperature: 22 ± 0.5°C; MP: (acetonitrile/methanol/water) (50/25/25, v/v/v); Flow rate: 0.3 ml/min; Injection volume: 5 µl; Ionisation mode: NI ESI; MRM: 253.00 > 209.00 and 205.00 > 161.00; Capillary voltage: 3.5 kV; Source temperature: 120°C; Desolvation temperature: 500°C; Desolvation gas flow: 509 l/h; Cone gas flow: 109 l/h.

DMD samples generally contain various salts and other components since the perfusates are often normal saline. Such solutions are not compatible for use with MS as these samples clog the ionisation source which creates high background noise (123). In an attempt to circumvent signal suppression and high column back-pressure, a gradient elution method was developed using MP [Solvent A] and water [Solvent B] to introduce a water wash before and after the elution of the ketoprofen and IS. If the increased column back-pressure was attributed to precipitation of normal saline, water was expected to dissolve and flush out the precipitate while the eluent was simultaneously diverted away from the ESI probe and the eluent later diverted towards the ESI probe for analyte detection. Although signal suppression was reduced, the column back-pressure still increased exponentially until the system maximum of 15 000 psi was reached following approximately 50 sample injections. This unexplained phenomenon re-occurred when the column was replaced on 2 further occasions and the problem was not resolved even when the columns were flushed with aqueous MP (0.3 ml/min) at elevated temperatures (50°C) over 2 days.

Subsequently, sample clean-up techniques were considered and among the various available techniques such as SPE, silica filtration and attempts at "salting out" the analyte, a sample extraction method was developed. Various solvents such as *n*-hexane, diethyl ether and ethyl acetate were initially investigated as extraction solvents due their low polarity indices. Ethyl acetate and diethyl ether both gave similar extraction efficiencies but faster evaporation times were obtained with diethyl ether. Diethyl ether was however problematic for use as a routine extraction solvent due to its high volatility and low viscosity making it difficult to accurately dispense. Use of *n*-hexane resulted in low recoveries of both ketoprofen and IS. Re-extraction with *n*-hexane to improve the recovery was not

considered since a single step extraction was preferred, hence ethyl acetate was selected. Methanol was used to re-constitute samples due to its ability to enhance the sensitivity in NI ESI and moreover, both ketoprofen and IS are highly soluble in methanol.

3.3.5.5. Linearity

The calibration plot for ketoprofen was linear with correlation coefficients better than 0.9999 ($y = 0.0427x + 0.0079$).

3.3.5.6. Accuracy and precision

The accuracy of the method (Table 3.9) was found to be in the range 99.97-104.67% with RSDs less than 2%. Data from the precision studies are presented in Table 3.10.

Table 3.9. Accuracy studies of ketoprofen DMD samples (*n=5*)

DMD samples	Day	Actual Conc (ng/ml)	Calculated Conc (ng/ml)	Accuracy (%)	Inter-day RSD (%)
	1	1.46	1.52	104.11	3.22
Low	**2**	1.42	1.44	101.41	2.15
	3	1.48	1.49	100.67	2.51
	1	152.32	152.61	100.19	1.52
Medium	**2**	148.15	148.32	100.11	1.15
	3	154.41	154.43	100.01	1.05
	1	453.84	453.71	99.97	0.57
High	**2**	441.42	441.44	100.01	0.82
	3	460.07	460.10	100.01	0.25

Table 3.10. Precision studies of DMD samples

DMD samples	Day 1 Mean Conc (ng/ml) ± SD (RSD%) (*n=5*)	Day 2 Mean Conc (ng/ml) ± SD (RSD %) (*n=5*)	Day 3 Mean Conc (ng/ml) ± SD (RSD %) (*n=5*)	Inter-day Mean Conc (ng/ml) ± SD (RSD %) (*n=3*)
Low	1.50 ± 0.02 (1.38)	1.52 ± 0.03 (1.96)	1.50 ± 0.02 (1.61)	1.51 ± 0.01 (0.67)
Medium	152.50 ± 0.28 (0.18)	152.62 ± 0.06 (0.04)	152.68 ± 0.03 (0.02)	152.60 ± 0.09 (0.06)
High	453.71 ± 0.01 (0.00)	453.71 ± 0.01 (0.00)	453.72 ± 0.01 (0.00)	453.71 ± 0.01 (0.00)

3.3.5.7. Limits of quantification and detection

The LOD and the LLOQ of ketoprofen were found to be 0.1 and 0.5 ng/ml respectively and the ULOQ set at 500 ng/ml.

3.3.5.8. Recovery

The recoveries of ketoprofen and ibuprofen are depicted in Table 3.11 below.

Table 3.11. Recovery studies of ketoprofen and ibuprofen in DMD samples

DMD samples	Actual Conc (ng/ml)	Calculated Conc (ng/ml)	% Recovery	% RSD (*n=5*)
Low	1.44	1.27	88.25	2.16
Medium	150.23	131.72	87.68	1.68
High	447.61	394.57	88.15	0.53
Ibuprofen	20.23	19.25	95.16	0.86

3.3.6. Conclusions

An analytical method was developed and validated for the quantitative determination of ketoprofen in DMD samples. This method demonstrated that small injection sample volumes (5 µl) not only achieved rapid, accurate and reproducible analysis but attained the requisite sensitivity. Methanol acted as a weak acid which provided protons that promoted chemical reduction at the ESI spray tip thereby enhancing sensitivity. Optimisation of wash solution volumes was necessary which eliminated sample carryover and system contamination and sample inserts were identified as the source of interfering peaks which were subsequently cleaned with chromic acid. Ibuprofen was identified for use as IS which eluted at 1.49 min and the most abundant fragments in NI ESI for ketoprofen and ibuprofen were 209.01 *m/z* and 160.82 *m/z* respectively. Normal saline solutions caused considerable signal suppression hence sample extraction was necessary. Calibration plots were linear over the range, 0.5-500 ng/ml and the method was accurate (99.97-104.67%) and precise with % RSDs less than 2% and recovery of ketoprofen and ibuprofen from DMD samples were approximately 88 and 95% respectively.

CHAPTER FOUR

MICRODIALYSIS

4.1. Introduction

MD is an *in vivo* sampling technique used to measure endogenous and/or exogenous compounds in extracellular spaces (124-126). The technique involves the implantation of a semi-permeable membrane into a specific region of a tissue or fluid-filled space (127). The technique was originally developed for use in neuroscience research which monitored rodent behaviour; subsequently the procedure was adapted for use in humans from which studies in many tissues have been reported (125,128). Although the MD technique was introduced for pharmacokinetic studies in animals in 1972, it was not until 1987 that the first pharmacokinetic study in humans was published (129). Preliminary clinical studies that employed MD demonstrated the potential of this technique for the determination of drug concentrations in the *interstitium* of target tissues. This allowed relative changes of concentration-time profiles of drugs to be described. While MD is used in neuroscience and metabolic studies to detect metabolic disorders, its application in clinical pharmacokinetics provides information on concentration-time profiles of drugs in the interstitial fluid (ISF) (129). The development and refinement of this technique over the past 2 decades has led to its increased acceptance in studies of drug distribution, metabolism and pharmacodynamics (127,130). This technique has been successfully used for the continuous sampling of low molecular weight compounds including glucose, lactose, pyruvate, glycerol, glutamate and urea as well as pharmacologically active agents in extracellular fluid (ECF) (131). MD has also been used to investigate basic physiology and endogenous substances, as well as the pathophysiology of inflammation and allergic responses, pharmacokinetics and pharmacodynamics of topical and systemic drugs, skin barrier function and drug penetration into the skin (30,125,132,133).

4.2. Theoretical principles

The principle of the MD technique is based on the passive diffusion of compounds down a concentration gradient across the semi-permeable membrane of a dialysis fibre (134). This technique mimics the functions of a capillary blood vessel which permits the exchange of solutes in and out of the ECF (55,125,135,136). Movement of drug across the membrane is based on Fick's Law of diffusion (137). Facilitated diffusion has also been reported with the use of certain types of perfusates (138).

4.2.1. Dermal microdialysis

DMD is a relatively new application of MD which allows continuous monitoring of endogenous and/or exogenous solutes in the interstitial fluid (ISF) of dermal tissue with minimal tissue trauma (127). The technique involves the placement of small perfused membrane systems at given depths within the dermis (Figure 4.1).

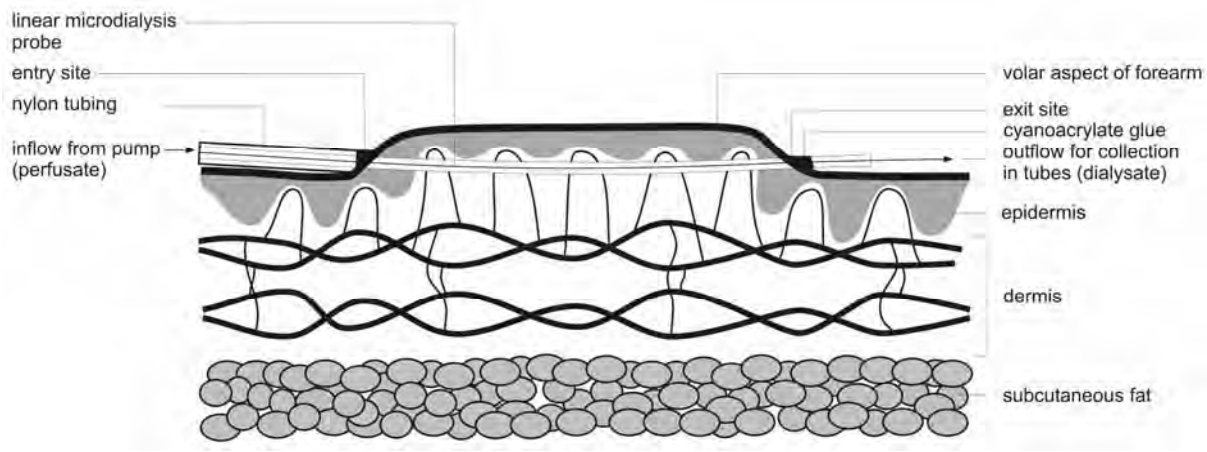


Figure 4.1. Membrane system implanted into the dermis. Modified from Benfeldt (55)

When a topical formulation is applied onto the skin and perfusate is pumped through the implanted membrane system, drug molecules from the topical formulation present in the dermal ISF diffuse (driven by the concentration gradient) into the lumen of the membrane resulting in the presence of net gain of drug in the perfusion medium collected (dialysate). The dialysate is collected at timed intervals and the drug concentration in the dialysate can be determined quantitatively (127).

Since the first report of DMD, this technique has been used successfully in human volunteers to:

- study the endogenous cutaneous release of histamine in response to various topical stimuli and the penetration of a number of topically applied organic solvents (e.g., ethanol, isopropanol) (34)
- measure inflammatory mediators in the dermis
- study skin metabolism
- determine the absorption of drugs or other agents in the skin.

DMD has also been utilised as an alternate route of drug administration (139).

Dermal tissue is an attractive sampling site since the tissue is relatively uniform with the ECF in constant equilibrium with the systemic circulation. Moreover, the implantation of the membrane system in the dermis involves a relatively simple procedure although training is imperative (134). DMD has been considered as a promising technique for the assessment of bioavailability and

bioequivalence of topical formulations and has garnered a lot of interest amongst research scientists, dermatologists and the pharmaceutical industry (39,40,125,140). The technique is minimally invasive and capable of producing concentration-time profiles sampled directly in the dermis, the target tissue, and is therefore suited to study the local and/or regional delivery of drugs following topical administration. Table 4.1 depicts the various applications of MD to study drug diffusion through human and animal skin.

4.2.2. Membrane system design

Membrane systems used for MD differ extensively in shape and type of material, depending on the tissue being sampled (126). Two types of membrane systems commonly used in skin studies are either linear or concentric. Linear systems are normally employed in DMD studies (55,140-142) whereas concentric systems are normally employed in deeper tissue studies involving the cutaneous and subcutaneous regions (20,21,86). The application of the different types of membrane systems employed in dermal, cutaneous and subcutaneous MD studies are reported in Table 4.1.

Linear membrane systems are fabricated from hollow fibres (heamodialysis cylinders) often taken from artificial kidneys. These membrane system types are not generally commercially available but are simple to manufacture in the laboratory and are therefore inexpensive. Their use requires entry and exit punctures by means of a guide cannula through the skin when placed in the tissue. In contrast, commercially available concentric membrane systems require 1 entrance puncture (125).

MD membrane systems are commonly referred to as “probes” which comprise the permeable membranes attached at either one or both ends to impermeable nylon or other inert materials such as Peek® tubing. Henceforth MD membrane systems will be referred to as MD probes.

The membrane materials used in MD probes are available in different types and pore sizes (30). Common substances used as membrane materials are cellulose acetate, polyacrylonitrile, polycarbonate, AN-69 copolymer (which consists of polyacrylonitrile and methylsulphonate), polyethersulphone and cuprophan (regenerated cellulose membrane) (126,136,143-147). MD membranes are porous in nature which permits diffusion to occur through water spaces within the pores of the membrane (146).

The physico-chemical properties of these membranes have been reported (148). Different membrane materials have different chemistries and will have an effect on recovery and/or delivery of drugs. The molecular weight cut-off (MWCO), a physical property commonly used by manufacturers of these membranes, describes the ability of a membrane to reject 90% of molecules with a specific molecular

Table 4.1. Dermal, cutaneous and subcutaneous MD studies

Drug	Probe specification	Perfusate	Analytical method	Species	References
17-β-Oestradiol	Concentric, 20 kDa	Ringer's solution	Radioimmuno assay	Healthy human volunteers	(149)
5-Fluorouracil	Linear, 9 kDa		HPLC-UV	Fuzzy rats	(150)
Acyclovir	Linear, 2 kDa	Ringer's solution	HPLC-UV	Healthy human volunteers	(33)
Betamethasone-17-valerate	Linear, 2 kDa	Ringer's solution or sterile PBS	HPLC-UV	Healthy human volunteers and Hairless rats	(142)
Diclofenac	Concentric, 20 kDa	Ringer's solution	HPLC-UV	Healthy human volunteers	(86)
Ethanol	Concentric, 20 kDa	Ringer's solution	GC-Flame ionisation	Healthy human volunteers	(143)
Fluconazole	Linear, 5 kDa	0.01 M Na_2HPO_4/NaCl 8.288 g/l	HPLC-UV	Hairless rats	(151,152)
Fluconazole	Concentric, 20 kDa	Normal saline	HPLC-UV	Healthy human volunteers	(56)
Flurbiprofen	Linear, 5 kDa	Isotonic PBS (pH 7.4)	HPLC-Fluorescence	Hairless rats	(153)
Fucidic acid	Linear, 2 kDa	Ringer's solution	HPLC-UV	Healthy human volunteers and Hairless rats	(142)
Ibuprofen	Concentric, 20 kDa	Normal saline	HPLC-MS/MS	Healthy human volunteers	(20)
Ketoprofen	Concentric, 20 kDa	Normal saline	HPLC-MS/MS	Healthy human volunteers	(21)
Lidocaine	Linear, 2 kDa		HPLC-MS/MS	Healthy human volunteers	(140,154)
Nicotine	Concentric, 20 kDa	Ringer's solution	HPLC-UV	Healthy human volunteers	(149,155)
Pencyclovir	Linear, 2 kDa	Ringer's solution	HPLC-UV	Healthy human volunteers	(33)
Salicylic acid	Linear, 2 kDa	Sterile PBS (pH 7.4)	HPLC-UV	Healthy human volunteers (perturbed skin)	(141)
Salicylic compounds	Linear, 5 kDa	0.05 M PBS (pH 7.4)	HPLC-UV, Liquid scintillation	Hairless rats	(156,157)
Toluene	Linear, 3000 kDa	Albumin solution (5%)	GC-Electrochemical detector	Hairless rats	(158)

weight. The MWCO value however gives little insight into whether or not a particular membrane will result in a higher recovery for a particular drug except for larger drugs such as neuropeptides and hormones where their size is the major constraining factor. It is important to note that the MWCO value does not describe the porosity of the membrane, i.e., how many pores per unit area exist for drug diffusion to occur, nor does it predict possible drug interactions with the material of the membrane (146). However, even if the drug molar weight falls below the MWCO value, an acceptable extraction efficiency (*EE*) will only be attained with substances having a molar weight lower than approximately one-fourth of the value.

Although most membrane pore sizes range from 6 to 3000 kDa, the majority of MD experiments have been conducted using membranes with MWCO of 20 kDa (30). Researchers make do with this MWCO size because it is small enough to permit diffusion of a large number of drugs, whilst restricting the entry of large endogenous compounds such as proteins and other macromolecules (123). A study of the effect of diffusion of acetaminophen, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid and homovanillic acid by 3 different semi-permeable membranes has been reported (159). This study indicated that although *in vitro* differences were observed between membranes, no significant difference was observed *in vivo*.

The choice of the membrane type is an essential element in searching for the optimal probe for a particular application. It is important that the membrane as well as any other component of the MD system does not interact with the drug since this would reduce the drug concentration in the dialysate (147). The membranes incorporated in linear probes are usually reinforced with stainless steel guide wire during manufacture for mechanical strength. Klimowicz *et al*. (160) reported no significant effect on the presence of an intra-luminal guide wire in linear MD probes during sampling. When manufacturing MD probes, the length and inner diameter of the outlet tubing should be considered to minimise mixing of the dialysate and to prevent hydrostatic pressure build-up across the probe membrane (147).

4.2.3. Probe calibration

MD probes should be checked to ensure reproducible recovery of the study drug to ensure minimisation of probe-to-probe differences. This is especially important for in-house fabricated MD probes (146,148). Calibration of probes may be performed *in vitro* and *in vivo*. For *in vitro* studies the surrounding medium is referred to as the periprobe whereas the surrounding medium for *in vivo* studies is the tissue ISF.

Since MD is a dynamic technique with the perfusate continuously being pumped through the probe, equilibrium is not established and dialysate concentrations represent only a fraction of actual concentrations in the tissue ISF or the periprobe (161,162). The fraction obtained is referred to as the extraction efficiency EE which has to be determined in order to quantitatively relate drug dialysate concentrations in either the tissue or in the periprobe. However if the desired information from a MD experiment is the relative change in drug concentration, knowing the *in vivo* EE is not absolutely necessary. However, knowledge of the *in vitro* EE provides information on the reproducibility and patency of the MD probe being used (162).

The general working definition of the EE is shown in Equation 4.1.

$$EE = \frac{C_p - C_d}{C_p - C_s} \qquad 4.1.$$

where C_d is the drug dialysate concentration, C_s is the known concentration in the external medium and C_p is the drug concentration in the perfusate. If the C_p equals zero, EE is also referred to as the relative recovery (RR). The EE describes the overall mass transport of drugs to and from the MD probe and is commonly used as a means to calibrate the device (145,163).

Several approaches have been reported to determine the EE. The most frequently used calibration methods are:

- the low-flow rate method
- the no-net-flux (or zero-net-flux) method
- the dynamic (or extended) no-net flux method
- retrodialysis by drug or by calibrator methods (127).

The assumption employed with the use of most calibration techniques such as no-net-flux, extrapolation to zero flow rate and the use of very slow perfusion rates, is that at very slow flow rates equilibrium is established across the dialysis membrane (162,164). These approaches are however time consuming and require long steady-state periods before any kinetic information is obtainable (144).

The simplest approach to calibrate a MD probe is by using a standard solution. For *in vitro* calibration studies, since the drug concentration in the periprobe is known and the perfusate contains no drug (i.e., $C_p = 0$), diffusion of the drug occurs from the periprobe into and through the membrane and is

collected as dialysate. The EE obtained from a measure of drug concentration recovered in the dialysate (EE_r) is described by Equation 4.2. EE_r is also referred to as recovery by gain.

$$EE_r = \frac{C_d}{C_s} \qquad 4.2.$$

For *in vivo* and/or *in vitro* calibration studies, EE may be determined by using a standard solution as perfusate with no drug in the tissue or periprobe ($C_s = 0$). Diffusion occurs from the perfusate into the tissue or periprobe. The EE in this instance, referred to as retrodialysis, recovery by delivery or recovery by loss (EE_d), is defined as the ratio of loss of drug from the perfusate relative to the perfusate drug concentration as described in Equation 4.3.

$$EE_d = \frac{C_p - C_d}{C_p} \qquad 4.3.$$

Retrodialysis experiments can either be performed using the same drug as the calibrant (i.e., retrodialysis by drug; RD_D) or using a calibrant with physico-chemical properties identical to the drug and which does not interfere with the experiment (i.e., retrodialysis by calibrant; RD_C). It is worth noting that if RD_C is the preferred option, the development of an analytical method to ensure that no peak interference between the drug under study and the calibrant exists which is very likely due to their similar physico-chemical properties. Although the advantage of performing RD_D involves the use of the actual study drug for calibration, the RD_D cannot be performed during the MD experiment due to possible tissue contamination. It must be performed before and/or after the actual experiment. It is however assumed that the EE does not change (162).

Initially the EE_r determined *in vitro* was used as the EE for experiments performed *in vivo*. This was mostly because the EE_r was easily determined *in vitro* while calibration *in vivo* was cumbersome. However, considerable evidence has shown that it is not reliable to use an EE determined *in vitro* for the EE *in vivo*. For most *in vivo* systems, transport through the tissue is the rate limiting step determining the EE rather than transport through the dialysis membrane as is the case for most systems *in vitro*. It is advisable to perform *in vitro* experiments before human use, to check for *in vitro* adsorption to tubing, time delays in drug movement and to compare drug gain and loss (125,127).

4.2.3.1. Effect of temperature on EE

The diffusion process is directly proportional to temperature and therefore the MD study should be conducted at a constant, preferably body temperature environment. The relationship with temperature is depicted in Equation 4.4 which describes the Stokes-Einstein equation:

$$D = \frac{k_b T}{6\pi\eta\sigma} \qquad 4.4.$$

where k_b is the Boltzmann constant (1.38 x 10^{-23} J/K), T is the absolute temperature, η is the viscosity of the suspending fluid and σ is the particle radius (126).

4.2.3.2. Effect of perfusion flow rate on EE

An influential factor on EE is the flow rate. In general, low flow rates result in higher recoveries and high flow rates in lower recoveries, according to Equation 4.5 below.

$$EE = (1 - e^{-rA/F}) \times 100 \qquad 4.5.$$

where r is the mass transport coefficient, A is the surface area of the MD membrane and F is the flow rate. Low perfusion rates are often limited by the small sample volumes and the quantification limit of the analytical method. Therefore, it is not advisable to choose minimal flow rates as this would increase the sample collection interval and consequently result in worse temporal resolution (126,165). On the other hand, it is also not advisable to choose a flow rate of 10 µl/min or higher as this would significantly result in reduced EE. Increasing the flow rate might also be conducive to the process of ultrafiltration due to the built-up pressure in the dialysis tubing, resulting in a net flow out of the probe (126).

EE is also influenced by several solute and tissue-related factors. Among these factors are the physico-chemical properties of the solute of interest and its diffusion coefficient in the tissue, the ECF volume fraction and the processes for elimination from the tissue, including active transport mechanism (30,127).

4.2.4. Assessment of probe depth in DMD

The effect of probe depth on drug concentration has been a subject of debate. Whereas Benfeldt and Serup (141) found increased drug concentrations in the dialysate with superficial probe insertions, in contrast, Benfeldt *et al*. (166), Hegemann *et al.* (155), Müller *et al.* (86) and Simonsen *et al*. (156)

reported no such correlation. A probe depth of 0.6-1.0 mm is considered acceptable for DMD studies (125). The depth of the probe insertion, i.e., the distance of the dialysis membrane within the skin to the skin surface can be measured by ultrasound imaging using a frequency of 20 MHz.

4.2.5. Composition of perfusates

Perfusates used in MD experiments vary widely in composition and pH. Ideally the composition, ion strength, osmotic value and pH of the perfusate should be identical to those of the ECF of the dialysed tissue (147) i.e., the perfusate chosen should be physiologically compatible with the dermis environment (123). This prevents the excessive migration of molecules into or out of the periprobe fluid due to osmotic differences. The perfusate is normally perfused at low flow rates of 1-10 µl/min (161). Although isotonic perfusates have been employed during MD experiments, fluid losses in the dialysates have been reported (146). These losses have been attributed to sample evaporation during the experiment at ambient conditions. Table 4.1 shows a list of commonly used perfusates (Ringer's solution, isotonic phosphate buffers (PBS) and normal saline) in skin MD, although a comprehensive list of different perfusates have been reported (147). Perfusates should be sterile when used in human and animal experiments.

The choice of perfusate used in MD studies affects drug recovery. Studies have demonstrated that the inclusion of β-cyclodextrin in the perfusate as a complexing agent enhanced the relative recovery (RR) of ibuprofen in an *in vitro* experiment by a factor of 1.5-2.0 (167). Cyclodextrins have also been used to prevent adsorption of hydrophobic materials onto plastics (145). Trickler and Miller (168) studied the inclusion of an osmotic agent, bovine serum albumin (BSA), to the perfusate which increased the recovery of macromolecules such as tumor necrosis factor and interleukin-1. *In vivo* studies have also demonstrated the use of Intralipid®, a fat emulsion used for nutritional disorders, and 2-hydroxypropyl-β-cyclodextrin as perfusate for recovery enhancement of lipophilic, highly protein-bound compounds (138).

4.2.6. Invasiveness and trauma

Although DMD has been described by authors as a minimally invasive technique (30,56,137,161,169), human subjects under study do experience some degree of reversible trauma caused by the insertion of probes (126). The trauma thus experienced is a result of inflammatory reactions that occur due to the implantation of the probes but the inflammation is reversible with little bleeding and oedema (123).

Insertion of MD probes has been reported (143) to increase local blood flow which has been confirmed with laser Doppler perfusion imaging. The skin blood flow has been observed to return to

normal by 60 min after insertion. Probe implantation also causes histamine release into the skin which returns to baseline after 40 min (143).

Histological examinations have shown no cellular infiltration or tissue disruption around dialysis probes in the skin within the first 6-10 h (150). However to my knowledge, no DMD studies in human volunteers have been published that extended this time interval (30). Apart from the inflammatory response, the probe might also introduce bacteria therefore causing infection and purulent response. It is important to sterilise the probes before they are implanted in the dermis. Ethanol has been reported not to damage the dialysis membrane and ethanol solution (70%, v/v) has been used as sterilisation medium before implantation in human tissue (143).

4.2.7. Analytical challenges

A major limitation with the use of MD is the production of extremely small volumes (1-30 µl) of dialysate. Due to the low perfusion flow rates normally employed in MD, long sampling collection times may be necessary in order to collect sufficient volumes of dialysate for reliable quantification (170). Volumes deemed as sufficient will be dictated by the minimum volume required by the analytical instrumentation required for reproducible analysis. The generating of more sample volume by increasing the perfusion rate, thereby decreasing the sample collection interval, results in sample dilution. Therefore, an analytical method that can either make use of small sample volumes collected during the MD process, or be of sufficient sensitivity to measure the drug concentration in the dialysate i.e., the lower quantification limit (LLOQ) of the analytical method, is essential (126). Moreover, the temporal resolution is determined by a combination of perfusion rate through the MD probe and sample volume requirement of the analytical technique (151,170). Commonly used analytical techniques employed in DMD studies are reported in Table 4.1 above.

4.2.8. Advantages and limitations

DMD has several advantages for *in vivo* sampling of drugs. No endogenous fluid is removed, so continuous sampling can be performed with minimal disruption of the physiological system (123). This technique is also valuable in patients with minimal blood supply such as children or neonates (126). Although minimally invasive, the procedure is well tolerated by subjects and dermal implantation of the probes may be achieved without the use of drug anaesthesia (171). The probe can be implanted directly into the tissue of interest and since the probe collects only free fraction of the drug, the therapeutically active portion of the dose can be monitored (165).

Provided that the perfusate used is simple and the type of the analytical method employed for the analysis of samples, the MD technique eliminates the need for complex, elaborate and time-

consuming sample preparation which is normally the case with plasma samples (126,147,161). The membrane can also act as an effective enzyme inhibitor because it excludes enzymes that could cause degradation of the drug especially during sample storage (123).

DMD may also address the issue of bioequivalence of topical formulations intended for local and/or regional delivery as acknowledged at a FDA workshop (39,40). Since more than 1 probe can be inserted in a subject, this reduces the number of subjects needed for pharmacokinetic investigations (130,147). Drug metabolism in tissues can be studied locally (171). The method can be used to evaluate drug permeation across both normal and diseased skin. MD may also have the potential to quantify a biomarker, or concentration of another surrogate measure, for therapeutic activity. Concomitant pharmacokinetic and pharmacodynamic evaluations are therefore possible (28). Finally MD can be performed in almost any organ or tissue of the body (i.e., brain, blood, liver, muscle, heart, subcutaneous tissue, etc.) (168).

The MD technique has a few main limitations. As previously noted, the small sample volumes coupled with the extremely low drug concentrations, result in the necessity for very sensitive analytical methods (138). Lipophilic compounds adsorb onto the polymeric materials that are used in the manufacture of MD probes as well as the inlet and outlet tubing (145,164). Müller *et al*. (149) observed that 17-β-Oestradiol, even with the addition of albumin to the perfusion medium, was shown to be dialysable only in small amounts *in vitro*, whereas no detectable 17-β-Oestradiol concentration was obtained *in vivo*. Benfeldt and Groth (142) also reported a similar observation when an attempt to measure dermal concentrations of betametasone-17-valerate after topical drug administration resulted in no *in vivo* concentrations.

Many pharmaceutically active compounds demonstrate substantial *in vivo* dermal protein binding. Therefore the actual recovery of total drug concentration at the site of interest within the skin in an *in vivo* MD study can be well below 1%. This limitation may be overcome with high doses or particularly sensitive analytical techniques. However, this overall poor recovery at therapeutic concentrations has limited the routine application of MD in some areas of research (138).

Another limitation seems to be the high inter- and intra-individual variability associated with the recovery of exogenous substances. The variability has been reported (30) not to be caused by the MD technique itself, but due to variations in the dermal concentration after the penetration of an exogenous compound. However some aspects of the MD clinical methodology may contribute towards the variability. One of such aspects is the difficulty in the standardisation of a dose application procedure.

Finally, the MD technique requires training of the clinical investigators particularly with respect to the fabrication of the MD probes and the probe implantation procedure.

A summary of the advantages and limitation of DMD sampling is presented in Table 4.2.

Table 4.2. Advantages and limitations of DMD sampling (127)

Advantages	Limitations
Highly dynamic continuous sampling	Training of skills required
High-resolution real-time sampling	• Insertion of probes
Both drug and metabolites in 1 sample	• Probe manufacture
Minimally invasive	Sensitive analysis needed
Multiple sites in 1 subject	• Drug-specific problems
Sampling and/or delivery via the probe	• Lipophilic drugs
Purified samples (protein free)	• Highly protein-bound drugs
Highly reproducible	Absolute tissue levels more difficult to estimate
Simultaneous use of auxiliary techniques	Recovery dependent on tissue and time

4.2.9. Dermatological application

The application of topical formulations intended for local and/or regional activity is an attractive way of attaining high dermal concentrations of drug without significant systemic activity (Chapter 1; Section 1.2) but as previously mentioned it is often not clear whether adequate drug concentrations are reached at the site of action within the skin. Moreover there are currently no *in vivo* methods available for the direct characterisation of drugs applied topically for a local and/or regional effect. DMD provides the opportunity to address this problem due to its minimally invasive nature and its ability to generate concentration-time profiles at a target site with good time resolution provided a sufficiently sensitive analytical method is available (172). DMD may also be used to establish *in vivo* formulation performance with the possibility of optimising doses of topical formulations to produce minimum effective concentrations at the site of interest within the skin (127,148).

4.3. Validation of DMD probes

4.3.1. Methods

4.3.1.1. Reagents, chemicals and materials

Ketoprofen (K1751) was purchased from Sigma-Aldrich (Atlasville, South Africa).

Haemophan® fibre dialyser Alwall GFS Plus 12 (208 µm i.d., 216 µm o.d., 2 kDa MWCO) (Gambro, Hechingen GmBH, Germany), acetone UL (Ultrafine Ltd., Finchley, UK), stainless steel (0.10 mm o.d.) guide wire (At Sandvik Benelux Steel, Leuven, Belgium), Portex® non sterile autoclavable (0.50 mm i.d., 0.63 mm o.d.) nylon tubing and Loctite® Super glue gel (Scientific Laboratories Supplies Ltd., Nottingham, UK) were used for manufacture of linear MD probes.

Sterile normal saline (sodium chloride 0.9%, m/v) (Bodene (Pty) Ltd., Port Elizabeth, South Africa), ethanol (96%, v/v) solution (MERCK, Wadeville, South Africa), stainless steel (3 mm o.d., 0.0006 sphericity) 440C metal balls (Small Parts, Inc., Miramar, Florida, USA), an adhesive sealant SewSimple™ (Bostik Findley Ltd., Swords, Dublin, Ireland), sample inserts (300 µl) with pre-installed plastic springs and amber (9 mm, screw top, 12 x 32 mm) sample vials (Waters® Corporation, Milford, Massachusetts, USA) and Terumo® 10 ml plastic syringes (Terumo Europe, Leuven, Belgium) were used during the MD study.

4.3.1.2. Equipment

Perfusion was performed with MD CMA Model 400 syringe pump systems (Chromatography Sciences Company, Quebec, Canada). Exmire microsyringes (2.5 ml) were purchased from Aurora Borealis Control BV (Schoonebeek, The Netherlands). Blue tubing adapters were purchased from CMA MD AB (Stockholm, Sweden). A shaker was purchased from The Chemical Rubber Company (Cleveland, Ohio, USA).

A closed chamber MD cell (Figure 4.2) fabricated from a slab of perspex (7 x 4 x 4 cm) was used. The MD cell comprised 3 components, 2 identical inlet and outlet blocks (1 x 4 x 4 cm) and the main block (5 x 4 x 4 cm) which accommodates an enclosed circular chamber (5 x 1 cm i.d., 5 ml capacity) into which the periprobe solution was filled. Both inner and outer blocks had a minute opening in the centre through which the linear MD probe was introduced. Inlet and outlet tubes were connected to the periprobe chamber to permit filling and ultimately facilitate cleaning. Four holes were bored into each block which accommodated 4 bolts that secured the integrity of the cell. Leakages were prevented from the chamber by incorporating o-rings at each end.

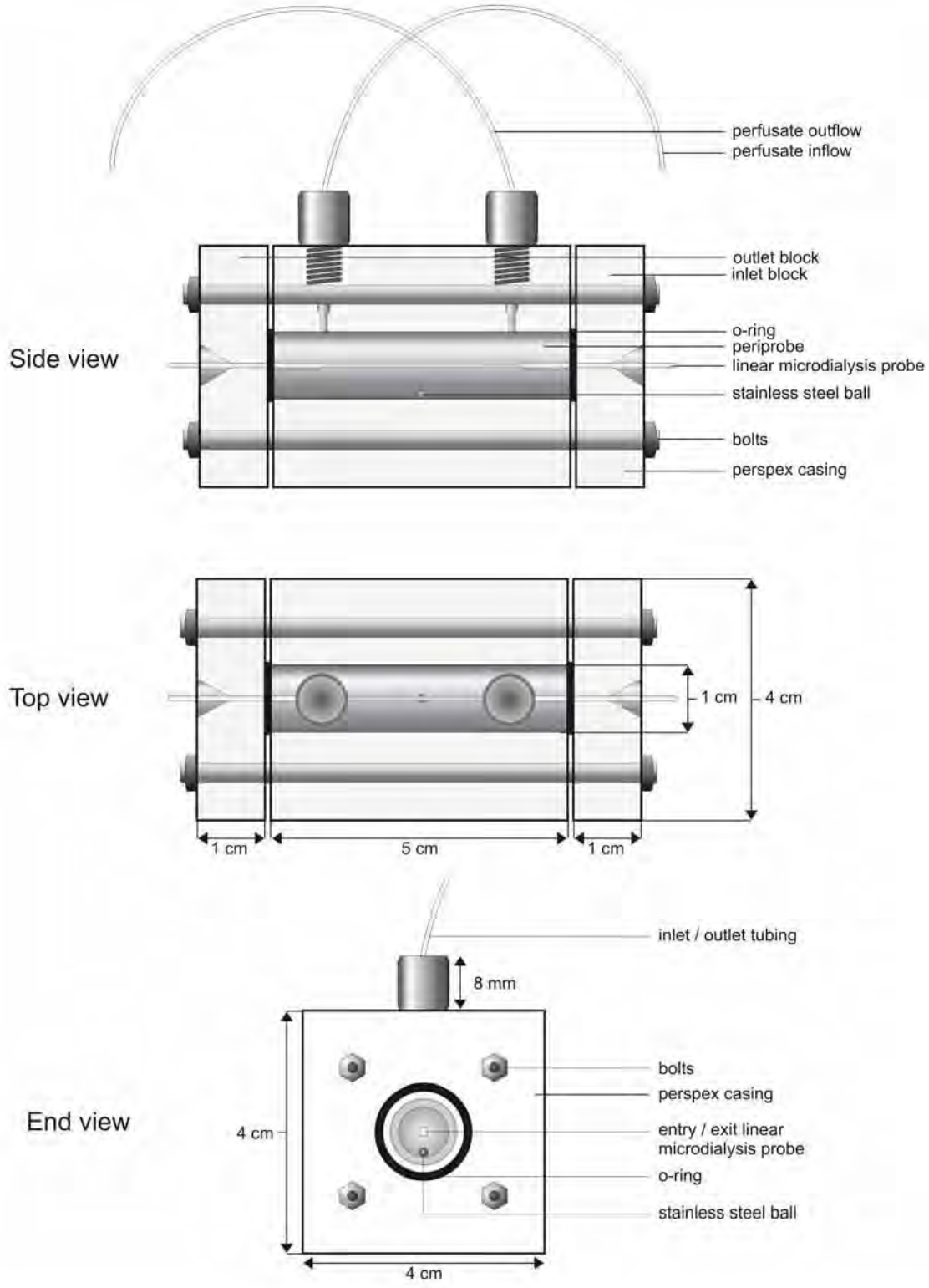


Figure 4.2. MD cell for *in vitro* applications

4.3.1.3. Preparation of linear MD probes

Two lengths (30 cm each) of Portex® nylon tubes were glued, one to each end of a pre-cut length of a dialysis membrane fibre and left to dry for a minimum of 2 h. Normally, for a 3 cm active membrane

window, a 5 cm length of membrane fibre is used. Lengths (7 cm) of stainless steel wire for use as a guide were degreased by treatment with acetone and each fibre was carefully threaded with the guide wire. The Portex® tubing, membrane and guide wire thus constitute the probe. Preliminary leak tests were performed with double-distilled water and a blue CMA tubing connector, previously soaked in ethanol was attached to the inlet nylon tubing of the probe. All probes were prepared within 24 h prior to use. A schematic illustration with the specifications as per protocol is presented in Figure 4.3 below.

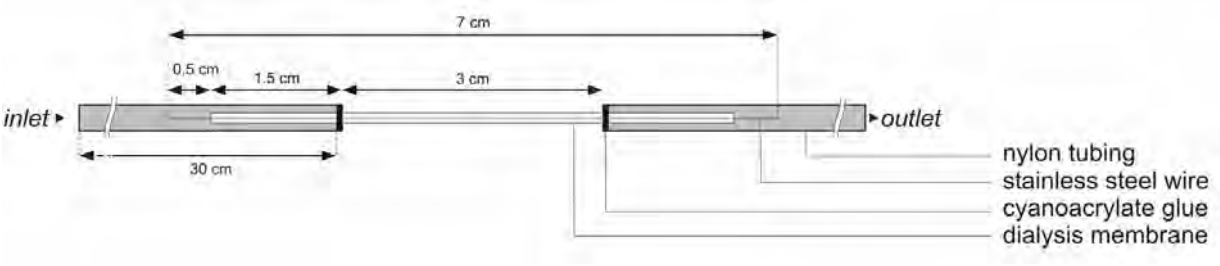


Figure 4.3. Linear MD probe for *in vitro* applications

4.3.1.4. MD setup

The probe (Figure 4.3) was inserted into the MD cell (Figure 4.2) as previously described. A stainless steel ball (3 mm o.d.) was placed in the periprobe chamber and the cell securely assembled. An adhesive sealant, SewSimple™, was used to seal both ends (probe entry and exit holes) of the MD cell. The periprobe chamber was completely filled with a relevant solution through the inlet tube using a plastic syringe and the cell assembly was then placed on a shaker. The blue tubing adapter at the end of the inlet probe tubing was attached to a microsyringe which was housed in the MD precision pump. The outlet end of the probe was placed into a pre-weighed sample insert-vial setup which served as the dialysate collection vessel. The system was equilibrated by pumping the relevant perfusate for 30 min prior to sample collection. Calibration was performed using 3 separate probes and 6 samples were collected from each probe using a pump flow rate to result in the collection of approximately 30 µl equivalent to 30 µg of dialysate per sample based on the specific gravity of water.

4.3.1.5. Determination of extraction efficiency

For recovery studies (EE_r), the periprobe chamber was filled with a standard solution of ketoprofen and the linear MD probes were perfused with normal saline solution whereas for delivery studies (EE_d), the periprobe was filled with normal saline solution and the linear MD probes perfused with a standard solution of ketoprofen.

Percent recovery of ketoprofen was calculated as the ratio of the dialysate concentration (C_d) to the concentration of the standard solution (C_s) in the periprobe, (Equation 4.6) i.e.,

$$EE_r(\%) = \frac{C_d}{C_s} \times 100 \qquad 4.6.$$

Percent delivery of ketoprofen was calculated as the ratio of the decrease in perfusate concentration $(C_p - C_d)$ to the initial perfusate concentration (C_p), (Equation 4.7) i.e.,

$$EE_d(\%) = \frac{C_p - C_d}{C_p} \times 100 \qquad 4.7.$$

4.3.1.6. Investigation of parameters affecting EE_r and EE_d

4.3.1.6.1. Sampling time

The recovery of ketoprofen from a standard solution (1 µg/ml) was monitored every 10 min from flow initiation at zero time to 240 min, hence no equilibration time was permitted.

4.3.1.6.2. Dialysis membrane length

The recovery of ketoprofen from a standard solution (1 µg/ml) using 3 different lengths of dialysis membrane (1, 3 and 4 cm) was determined for 3 probes. Dialysis membrane length dependency was investigated for ketoprofen with a pump perfusion rate maintained at 3 µl/min, sampling every 10 min.

4.3.1.6.3. Perfusion rate

The recovery of ketoprofen from a standard solution (1 µg/ml) was investigated using different perfusion rates and 4 cm lengths of dialysis membrane. Perfusion flow rates of 1, 2, 3 and 5 µl/min necessitated sampling collection intervals of 30, 15, 10 and 6 min, respectively, in order to provide 30 µl of dialysate which was measured gravimetrically. The system was equilibrated for 30 min prior to sample collection for each perfusion flow rate investigation.

4.3.1.6.4. Drug binding

To evaluate possible binding effects of ketoprofen onto any portion of the probe, the recovery of ketoprofen from a standard solution (1 µg/ml) was investigated. The periprobe chamber was left unfilled (air filled).

4.3.1.6.5. Drug concentration

Recovery and delivery of ketoprofen from 3 standard solutions (1, 2 and 5 µg/ml) were investigated. Equations 4.6 (EE_r) and 4.7 (EE_d) were used to calculate recovery and delivery, respectively and the results from each determination were then reconciled.

4.3.1.6.6. Ethanol sterilisation

To evaluate the effect of ethanol solution (70%, v/v), used as sterilisation fluid, on the recovery of ketoprofen, a standard solution (1 µg/ml) of ketoprofen was investigated using probes that had been treated with ethanol for 20 min and compared to untreated probes.

The above studies (4.3.1.6.1, 4.3.1.6.4-4.3.1.6.6) were conducted with 4 cm lengths of dialysis membranes using a pump perfusion rate at 3 µl/min with sampling every 10 min. All experiments were conducted in triplicates with 6 samples collected from each probe with the exception of 10 samples collected from the sampling time study (4.3.1.6.1).

4.3.1.7. Sample analysis

Ketoprofen concentrations in MD samples were determined by a validated HPLC-UV method described in Chapter 3; Section 3.2.1a.

4.3.2. Results

4.3.2.1. Sampling time

The mean recovery of ketoprofen was only 7.62% after the first sample was collected and increased to 34.58% in the next sample after which the recovery of ketoprofen was seen to stabilise to a value of approximately 64% (Table 4.3).

Table 4.3. Effect of sampling (*n=3*)

Sample No	Sampling time (min)	Mean recovery (%)
1	10	07.62
2	20	34.58
3	30	63.74
4	40	63.69
5	50	63.64
6	60	63.56
7	70	63.83
8	80	64.41
9	90	64.40
10	100	64.87

4.3.2.2. Dialysis membrane length

The mean recovery of ketoprofen increased with increasing membrane length (Table 4.4).

Table 4.4. Effect of dialysis membrane length (*n=3*)

Sample No	Mean recovery (%)		
	1 cm	3 cm	4 cm
1	19.75	36.12	64.08
2	19.48	36.48	64.25
3	20.02	38.77	64.51
4	19.75	38.06	63.99
5	20.19	39.47	64.16
6	19.75	39.29	63.56

4.3.2.3. Perfusion rate

Ketoprofen recovery decreased linearly with increasing perfusion rates (Table 4.5).

Table 4.5. Effect of perfusion rate on recovery of ketoprofen (*n=3*)

Sample No	Mean recovery (%)			
	1 µl/min	2 µl/min	3 µl/min	5 µl/min
1	93.40	84.70	65.80	43.33
2	93.45	84.90	65.43	43.41
3	93.59	84.84	65.97	43.04
4	93.82	84.79	66.67	43.33
5	94.78	84.86	66.45	43.46
6	93.31	84.67	66.29	43.41

4.3.2.4. Drug binding

The delivery of ketoprofen perfused through linear MD probes with air in the periprobe is presented in Table 4.6.

Table 4.6. Delivery in air (*n=3*)

Sample No	Mean delivery (%)
1	94.62
2	93.68
3	95.03
4	96.04
5	93.78
6	92.76
Mean ± SD	94.32 ± 1.16

4.3.2.5. Drug concentration

The recovery of ketoprofen from 3 different concentrations was independent of concentration and the data are presented in Table 4.7.

Table 4.7. Recovery and delivery at different concentrations (*n=3*)

Concentration (µg/ml)	Recovery and delivery (%)
1	67.50 ± 1.84
2	65.75 ± 1.63
5	69.20 ± 0.43
Mean ± SD	67.48 ± 1.72
RSD (%)	2.55

4.3.2.6. Ethanol sterilisation

No difference was obtained between ketoprofen recovery from probes pre-treated with the ethanol (70%, v/v) solution (66.21 ± 0.52%) and probes without the sterilisation procedure (65.32 ± 0.25%).

4.3.3. Discussion

Various factors which could affect the recovery of ketoprofen during DMD were investigated including effects of experimental parameters/operation and conditions. In addition, it is important to emphasise that some precautions are necessary when fabricating the probes and preparing the membrane systems for DMD. The choice of glue for use to connect the Portex® tubing to the membrane fibre is important in order to avoid the development of leaks; cyanoacrylate glues proved successful in this respect. Insertion of the stainless steel guide wires must ensure that tips of the wires do not pierce the membranes during the threading procedure. Furthermore, care must be taken to prevent sharp tips being formed on the wires during cutting, which can be avoided by cutting the wire at a 90° angle.

Investigations of the effect of sampling times on ketoprofen recovery established the minimum time required to equilibrate the probes. Several reports have indicated that different equilibration times are required for different drug candidates (131,136,160). Although a gradual increase in recovery with increasing time was initially observed where the rate of recovery of ketoprofen was extremely low after pumping for 10 min at a constant rate, continuation of the perfusion resulted in the recovery reaching a stable value after 30 min. The initial diffusion rate of ketoprofen was low and then increased after the first sample (10 min) until the second sample (20 min) and then decreased with time resulting in a relatively constant recovery after 30 min. The initial low diffusion rate was attributed to the initial state of the DMD probes before the inception of the experiment. After the probes were fabricated, they were checked for leaks by forcing distilled water (~ 1 ml) via a manually operated syringe (10 ml) and the wet probes were subsequently stored in a sealed container prior to use. The presence of the residual water likely resulted in some dilution of the ketoprofen concentration, thereby influencing the initial recovery data from 10-30 min. Hence, using a perfusion rate of 3 µl/min, an equilibration time of 30 min was established for use in DMD studies at that particular flow rate. The use of lower perfusion rates thus will require longer equilibration times and

vice versa. Although equilibrium was perceived to have occurred under the abovementioned experimental conditions, it is important to note that true equilibration probably never occurs since recovery never reaches 100%, although the use of certain types of β-cyclodextrins as perfusate have been reported to generate recovery greater than 100% (164).

Increasing the length of the dialysis membrane (i.e. from 1 to 4 cm) resulted in an increase in ketoprofen recovery. This is in accordance with Fick's law of diffusion where the rate of perfusion across a membrane is proportional to its area. Hence, the increased surface area associated with the use of longer dialysis membrane lengths would account for the higher recovery of ketoprofen. This trend has also been reported in published studies (136,173). The use of a 1 cm membrane length in DMD studies is thus not recommended since it will generate a low recovery of drug. Although a 4 cm membrane provided a relatively higher ketoprofen recovery, its use in DMD may be impractical since the volar aspect of the forearms of most volunteers, where probe implantation occurs, will generally not accommodate the use of membranes longer than 3 cm.

The lower recovery obtained with increased perfusion rate was attributed to less contact time of ketoprofen in the periprobe solution with the perfusate. However, the use of lower perfusion rates will necessitate sample collection over longer time intervals in order to provide sufficient sample volumes required for analysis.

The relatively high recovery of ketoprofen (94.32%) confirmed the lack of significant binding of ketoprofen to the probe by performing a recovery study in air within the periprobe chamber in the MD cell. Although ketoprofen is a hydrophobic drug (174), high recovery has been obtained *in vitro* which suggested that measurable recovery would be expected *in vivo*.

Recovery of ketoprofen was found to be independent of drug concentration in the range of ketoprofen concentrations studied. Ethanol solution (70%, v/v) used as the sterilisation medium did not have any effect on the recovery of ketoprofen from the linear MD probes. Anderson *et al*. (143) also reported that ethanol does not damage the membrane and therefore it can be safely used as a sterilisation medium prior to *in vivo* use.

4.3.4. Conclusions

An equilibration period after probe fabrication was essential to get reproducible recovery. These studies also provided useful data to facilitate the construction of appropriate probes for use in DMD studies to be conducted in human subjects. The effect of membrane length indicated that although a longer membrane length would provide a higher ketoprofen recovery, a membrane length of 3 cm as

opposed to 4 cm will be more practical in order to be accommodated on the volar aspect of human forearms. Data were also generated to establish the optimum perfusion rate which will be appropriate for use in DMD studies in human subjects. The decision is dependent on the sample collection volume and concentration of ketoprofen expected in the dialysate. If the perfusion rate is set too low, longer sampling intervals will be necessary to provide sufficient volume for analysis. However, on the other hand, if the pump flow rate is set too high, drug concentrations in the dialysate samples may be too low for quantitative analysis and the drug may even be undetectable. These preliminary investigations provided valuable information prior to undertaking DMD studies intended to be performed in human subjects.

4.4a. Human study I (HS I): Preliminary investigations

4.4a.1. Methods

The reagents, chemicals, materials and equipment used were the same as described under 4.3.1.1 except for the following:

4.4a.1.1. Reagents, chemical and materials

Medisan® mild liquid soap (Designer Group, Edenvale, South Africa), polyethylene (1.5 ml) centrifuge tubes (Eppendorf®, Hamburg, Germany), Leukoband® S (BSN medical (Pty) Ltd., Pinetown, South Africa), Medac ice packs (Medac (Pty) Ltd., Cape Town, South Africa), sterile latex surgical gloves (Neogloves, Middlesex, UK), Biocort® (hydrocortisone acetate 0.01%, m/m) cream (Adcock Ingram Ltd., Bryanston, South Africa), disposable (21G x 1½") needles (Korea Vaccine Ltd., Seoul, Korea), adhesive labels (Redfern Labels, Cape Town, South Africa), Micropore™ 1530 (12 mm x 10 m) dressing tape (3M, Isando, South Africa) and isopropyl alcohol (70%, v/v) swabs (Tyco Healthcare (Pty) Ltd., Midrand, South Africa) were used.

4.4a.1.2. Equipment

A Dermascan C® (Cortex Technologies, Hadsund, Denmark) ultrasound scanning device was used to measure probe depth in the skin. The instrument comprised 3 main parts: the C-probe, the elaboration and visualisation system and the memorising and data-storing system. The C-probe was surrounded with water and sealed at the point of contact with an ultra-thin plastic diaphragm.

4.4a.1.3. Formulations

Fastum® Gel (reference formulation; R) containing ketoprofen 2.5%, m/m (Adcock Ingram Ltd., Bryanston, South Africa) and Ketum® Gel (test formulation; T) containing ketoprofen 2.5%, m/m

(Menarini (Pty) Ltd., Rungis Cedex, France) were purchased from local pharmacies in Grahamstown, South Africa and Montpellier, France respectively.

4.4a.1.4. Ethics

The study protocol (Appendix I) was approved by the Rhodes University Ethical Standards Committee (RUESC, Grahamstown, South Africa) and performed in accordance with the recommendation of the guidelines as set out in the Declaration of Helsinki (1964) and its amendments (175), and carried out in compliance with the guidelines on the conduct of clinical trials in South Africa as set out by the Medical Control Council (MCC) South Africa in concurrence with the South African Department of Health (176).

4.4a.1.5. Subjects

The study population comprised 10 (5 females and 5 males) healthy black subjects with ages ranging from 19-26 yr and body weights ranging from 52.4-91.6 kg. Subjects who met the inclusion and exclusion criteria (Table 4.8) and successfully completed the pre-experimental medical assessment were enrolled in the study. All subjects gave written informed consent.

Table 4.8. Inclusion/exclusion criteria used in recruiting subjects

Inclusion criteria	Exclusion criteria
Female subjects who were using reliable contraception or abstaining	Female subjects who were breast feeding
Subjects who were aged between 18 and 50 years	Female subjects who were contemplating becoming pregnant in the time immediately following the study
Subjects who were in general good health	Female subjects who were pregnant
Subjects who were available for the entire study period	Subjects who had a known allergy/hypersensitivity to ketoprofen or any NSAID including aspirin
	Subjects who had any history of drug or alcohol abuse
	Subjects who had any mental deficiency or handicap
	Subjects who had hairy ventral forearm surfaces and/or abrasions on the underside of their forearms
	Subjects who had engaged in any sun-tanning or taken any sunny vacations within the last month
	Subjects who had participated in another NSAID DMD or tape stripping study within 2 months of the study date
	Subjects who had used any NSAIDs within the last 3 months
	Subjects who suffered from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema)
	Subjects who suffered from any skin disorder such as psoriasis, eczema or other relevant skin disorder
	Subjects who took regular medicine or tablets or used any creams within the last week (contraceptive pills excluded)
	Subjects who tested positive for HIV and Hepatitis B
	Subjects with a history of any neurological, kidney or liver disorders

4.4a.1.6. Preparation of linear DMD probes

The DMD probes were prepared as previously described in section 4.3.1.3 except that an 8 cm length of fibre was used with Portex® tubing glued at only at 1 end (Figure 4.4).

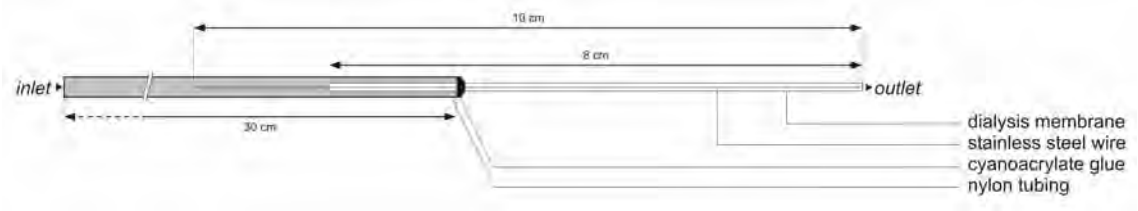


Figure 4.4. Linear DMD probe for *in vivo* applications

4.4a.1.7. Study design

Subjects were admitted into the clinic (Faculty of Pharmacy, Rhodes University, Grahamstown, South Africa) and remained there for the duration of the study. The forearms of each subject were washed with mild liquid soap and the subjects assumed a supine position with their arms placed on an armrest.

The wrists of the subjects were loosely restrained, to facilitate implantation of 4 probes into the dermis on the volar aspect of the forearm in each subject under the specific gel application sites (Figure 4.5) as indicated by the shaded areas.

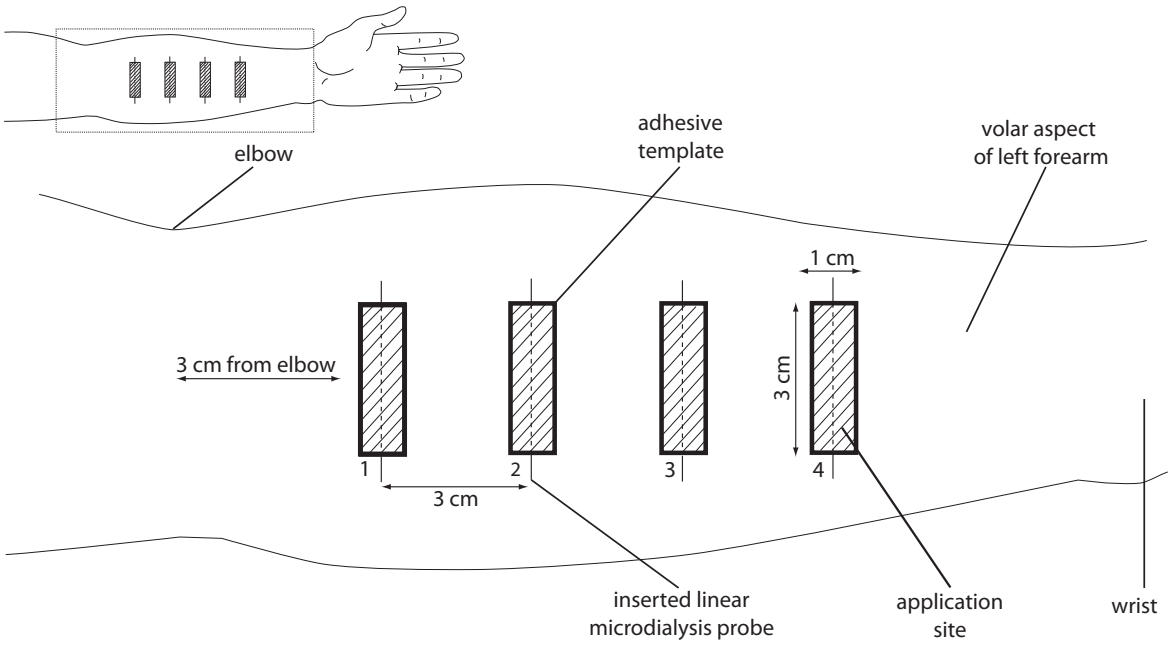


Figure 4.5. DMD study design (HS I)

Experimental conditions: 4 probe insertions, 4 application sites, 1 probe per site, probes were 3 cm apart, probes covered approximately 2 quarters of the volar aspect of the forearm of each subject

Ice packs were placed directly over the area demarcated for the probes for approximately 5 min prior to the insertion of the cannulae in order to induce a local anaesthetic effect. The DMD probes, which had previously been sterilised in ethanol (70%, v/v), were introduced through guide cannulae which were subsequently withdrawn, leaving the DMD probes implanted within the dermis (Figure 4.6). The insertions were guided by entry and exit sites marked on the forearms, ensuring that a length of 30 mm of the membrane portion of the MD probe was intra-dermally placed in each case.

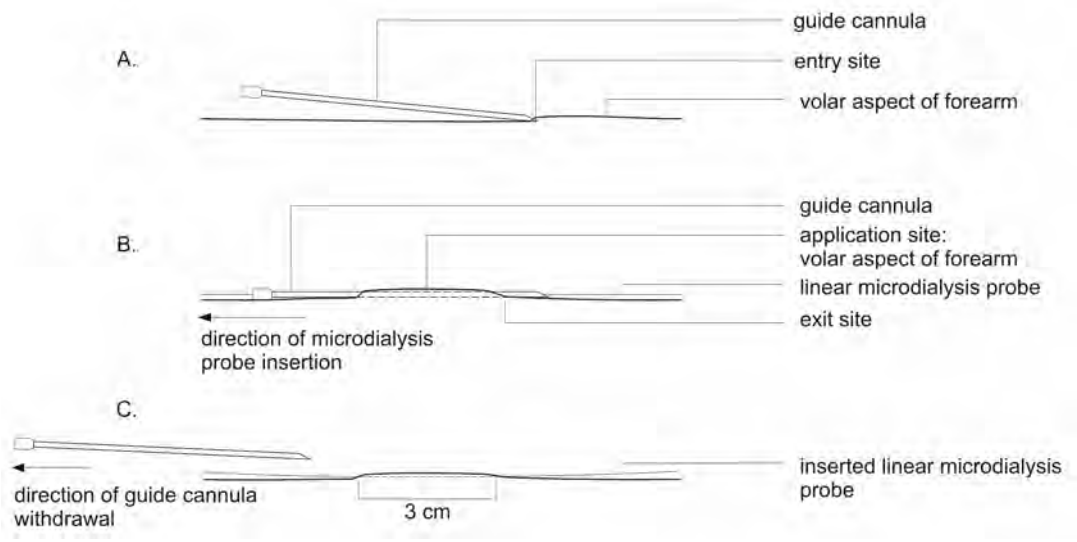


Figure 4.6. Implantation of linear DMD probes in the skin

A: Guide cannula insertion at the entry point marked on the skin. B: Guide cannula pierced through the exit point and MD probes inserted into the guide cannula. C: Guide cannula withdrawal leaving the MD probe within the dermis.

Pre-cut templates (25 x 45 mm with a fenestration measuring 10 x 30 mm in the centre) prepared from adhesive labels (Redfern Labels, Cape Town, South Africa), were positioned over the probes in the skin, covering the entry and exit sites as seen in Figure 4.5.

Following a successful probe leak test, both entry and exit sites were sealed and perfusion of the probes was initiated at 0.5 µl/min (15 min) and subsequently increased to 1.25 µl/min (45 min) to ensure sufficient baseline equilibration (60 min) in order for the insertion trauma to subside (125). The inlet tubes were secured with Micropore™ 1530 dressing tape and blank samples were collected prior to product application. The ketoprofen formulation (30 mg) was dispensed in a randomised sequence according to Table 4.9 from pre-loaded Combitips® (0.5 ml) in a HandyStep® dispenser (Eppendorf®, Hamburg, Germany) and a glass rod was used to spread the formulation within the application area. In order to monitor any possible loss in the dose as a result of the spreading pre-weighed glass rods were used to determine the amount removed by the glass rods.

Table 4.9. Randomisation sequence of applied formulations

	Randomisation sequence			
Subject	**Probe 1**	**Probe 2**	**Probe 3**	**Probe 4**
1	T	T	R	R
2	T	R	T	R
3	T	R	R	T
4	R	T	T	R
5	R	T	R	T
6	R	R	T	T
7	T	T	R	R
8	T	R	T	R
9	T	R	R	T
10	R	T	T	R

4.4a.1.8. Sample collection and preparation

The samples were collected every 0.5 h for 5 h into centrifuge tubes. A total of 44 samples per subject were collected (4 probes x 10 samples & 4 blanks) and the samples were stored in the dark at 4°C and analysed within 24 h.

4.4a.1.9. Probe depth measurement

At the end of the sampling period, the probes were disconnected from the pump and the templates removed from the application sites. Excess formulation was removed using isopropyl alcohol swabs and the depth of the probes measured in triplicate by ultrasound scanning at 20 MHz. The probe depth was measured, in the A-mode scan, as the vertical distance between the epidermis entrance echo and the echo of the DMD membrane (internal stabilising steel wire). At the end of the study, the probes were withdrawn from the skin, the sites dressed with alcohol swabs, and each subject was provided with a tube of hydrocortisone cream to apply twice daily for 2 weeks as a prophylactic measure against post-traumatic skin inflammation. Post-experimental medical follow-up examination of the skin sites were performed weekly for 4 weeks.

4.4a.1.10. Dialysate analysis

Ketoprofen concentrations in the DMD samples were determined with a validated UPLC-ESI-MS/MS analytical method described in Chapter 3; Section 3.3.

4.4a.1.11. Pharmacokinetic evaluation

The concentration-time profiles were generated by plotting ketoprofen concentrations at the mid-point between sample collections times and calculating the AUC from 0-5 h.

4.4a.1.12. Bioequivalence assessment

Pharmacokinetic and statistical parameters to determine bioequivalence were calculated with a statistical package SAS® (SAS Institute Inc., Cary, North Carolina, USA). AUC_{0-5} was tested for comparative bioavailability using a 2 one-sided test procedure and bioequivalence was concluded if the 90% CI of the log transformed AUC_{0-5} data for pairs of test/reference application areas were within the acceptance range of 80-125%.

4.4a.2. Results

4.4a.2.1. Clinical observations

The insertion of the guide cannulae with the subsequent implantation of the probes was acceptable for all subjects. Ice was well tolerated and efficient as an anaesthetic with no subjects finding the insertion procedure painful as such. All subjects completed the study and no disruptions to the study were recorded. No local adverse reactions from the formulations were reported during the study and the weekly post-trial medical examination showed no signs of residual inflammation, scarring or keloid formation at the sites of probe implantation. Six incidences of failed probes out of 44 probes were reported, however these probes were replaced before sampling occurred and did not affect the results obtained.

4.4a.2.2. Dose assessment

The mean actual dose on both the test and reference application sites for all subjects were 16.55 ± 5.37 mg (ranged from 3.11-26.98 mg; %RSD = 32%) (*n=20*) and 17.39 ± 5.35 mg (ranged from 2.67-28.68 mg; %RSD = 33%) (*n=20*) respectively (Table 4.10), calculated by re-weighing the glass rods after spreading. These results indicate that approximately 60% of the dose was removed by the spreading procedure.

Table 4.10. Actual mean doses remaining on test and reference sites (*n=20*)

	Dose rubbed (mg) (mean ± SD)	
Subject	**Test sites**	**Reference sites**
1	14.10 ± 5.23	12.71 ± 2.28
2	17.55 ± 2.62	14.58 ± 1.66
3	19.40 ± 2.12	17.16 ± 0.34
4	18.90 ± 1.27	18.79 ± 0.55
5	21.95 ± 0.49	19.93 ± 0.32
6	15.20 ± 3.68	17.06 ± 0.19
7	16.95 ± 1.77	10.69 ± 3.94
8	22.70 ± 1.98	21.59 ± 0.55
9	13.40 ± 6.08	17.53 ± 3.08
10	22.25 ± 0.78	21.85 ± 0.64
Mean	16.55 ± 5.37	17.39 ± 5.35
RSD (%)	32	33

4.4a.2.3. Probe depth measurements

A white hyper-reflecting dot confirming the presence of the stainless steel guide wire within the membrane was visualised *in situ* during the ultrasound scanning. The mean probe depths at the test and reference application sites for all subjects were 0.75 ± 0.076 mm (ranged from 0.62-0.88 mm; %RSD = 10%) (*n=20*) and 0.75 ± 0.088 mm (ranged from 0.62-0.89 mm; %RSD = 12%) (*n=20*) respectively (Table 4.11). The precision of probe insertions was extremely consistent and no correlation was observed between AUC_{0-5} and probe depth for all subjects.

Table 4.11. Mean probe depth measurements (HS I) (*n=4*)

	Probe depth (mm) (mean ± SD)	
Subject	**Test sites**	**Reference sites**
1	0.81 ± 0.10	0.78 ± 0.01
2	0.69 ± 0.08	0.70 ± 0.03
3	0.80 ± 0.07	0.86 ± 0.06
4	0.73 ± 0.04	0.63 ± 0.01
5	0.84 ± 0.03	0.86 ± 0.01
6	0.72 ± 0.05	0.65 ± 0.01
7	0.84 ± 0.04	0.86 ± 0.02
8	0.72 ± 0.07	0.77 ± 0.02
9	0.67 ± 0.04	0.67 ± 0.03
10	0.69 ± 0.02	0.75 ± 0.02
Mean	0.75 ± 0.09	0.75 ± 0.08
RSD (%)	12	10

4.4a.2.4. Pharmacokinetic evaluation

Mean dialysate concentration-time (a) and semi log-time (b) profiles (± SD) of both the test and reference formulations are illustrated in Figure 4.7.

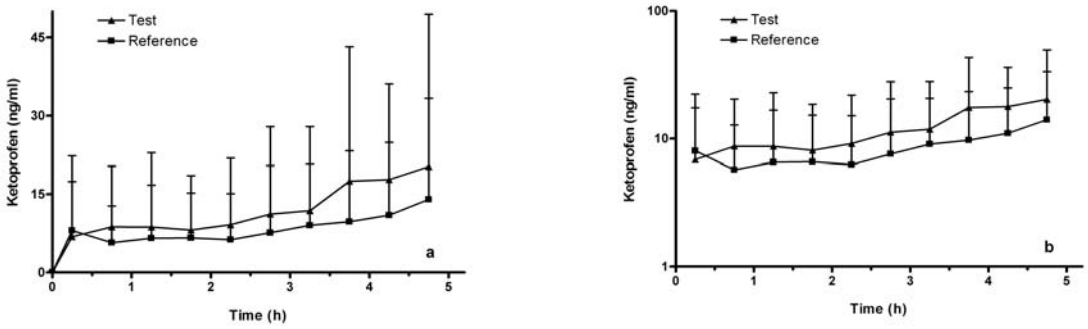


Figure 4.7. Mean dialysate concentration (a) and semi-log (b) time profiles (± SD) HS I (*n=10*)
Experimental: 4 probe insertions, 4 application sites, 1 probe per site, probes were 3 cm apart, probes covered approximately 2 quarters of the volar aspect of the forearm of each subject, 10 subjects, Test: Ketum® gel, Reference: Fastum® gel

The raw data and individual subject concentration-time profiles are located in Appendix II (DMD: HS I). Although most of the concentrations obtained were measurable, some concentrations were between the LOD and LLOQ. The AUC_{0-5} obtained for both the test and reference formulations were 54.99 ±

52.31 ngh/ml (ranged from 1.76-139.56 ngh/ml; RSD = 105.12%) and 38.79 ± 51.32 ngh/ml (ranged from 1.52-174.57 ngh/ml; RSD = 75.57%) respectively.

4.4a.2.5. Bioequivalence assessment

The bioequivalence assessment of the test and reference formulations are summarised in Table 4.12. The 90% CI for AUC_{0-5} and the point estimates were outside the acceptance range of 80-125%.

Table 4.12. Bioavailability comparison of test and reference formulations (*n=10*)

PK parameter	Units	Arithmetic means (mean ± SD)		%Ratio (T/R)	90% CI (lower limit, upper limit)	Power of ANOVA (%)	ANOVA CV (%)
		Test	Reference				
AUC_{05}	ngh/ml	54.99 ± 52.31	38.78 ± 51.32	149.77	(117.21, 191.37)	98.17	29.90

4.4a.3. Discussion

In general, the DMD technique was well tolerated with no residual scaring and inflammation observed after 4 weeks. Ice was effective as an anaesthetic which made probe implantation simple and virtually pain free. Probe failures and variable ketoprofen dialysate concentrations during the DMD studies appeared to be caused through attempts by some subjects to alleviate discomfort with resulting probe disturbance. In particular the posture of the relevant arm in relation to the body position was identified as the source of the problem. Normally, when a subject assumes the supine position with the arm stretched out, the level of the heart should be higher than that of the arm. This posture ensures sufficient blood circulation is available to the arm. When the converse occurs, i.e., the level of the heart is the same or below the level of the arm, poor blood circulation results inducing pain, with some subjects complaining of numbness and coldness. This discomfort causes subjects to momentarily flex their arms to improve blood circulation. Since, flexing resulted in the movement of the muscles underneath the probes, this reflex action may have mechanically disrupted the diffusion process by forcing more ketoprofen into the dermis thereby resulting in higher concentrations leading to variable results. Moreover, the cold arms may have resulted in poor ketoprofen diffusion since a decrease in temperature decreases diffusion. It was therefore important to ensure that the subjects were comfortable to minimise the effect any form of discomfort may have the outcome.

Five issues were identified as shortcomings in the study design and also as possible sources of variability associated with HS I. Based on these issues, the study design was amended to take these issues into account and implemented in subsequent studies in an attempt to reduce variability and validate the model for the determination of bioequivalence of topical products using ketoprofen as the model drug.

The sources of the formulations were investigated. Fastum® gel is a registered product in South Africa whereas Ketum® gel is a registered product in France. Although *in vitro* information for both products were found to be similar (1) the *in vitro* data for both products indicated similar pharmaceutical availability of ketoprofen. However, in the absence of an established *in vitro-in vivo* correlation, such data do not provide adequate information to suggest that the products are bioequivalent. Moreover, since Fastum® gel is the only topical ketoprofen gel product on the South African market, no comparative product was available for use as a reference in order to assess bioequivalence of Fastum® gel. In order to employ DMD routinely to assess bioequivalence of topical formulations intended for local and/or regional delivery, a validated model is necessary to show proof-of-concept. Such a model would need to confirm bioequivalence between bioequivalent products. In the absence of a reference product, validation could not be performed under the circumstances of this particular study design. Hence, a future study was considered to use the same topical gel formulation as both the test and reference formulation. In this way, since bioequivalence is expected when paired sites designated as test and reference are compared, the ratios of $AUC_{0\text{-}5}$ (T)/$AUC_{0\text{-}5}$ (R) should provide 90% CIs within the acceptance range of 80-125% in order to validate the model.

Although the probes were inserted within the region of the forearm between 3 cm from the elbow and 3 cm from the wrist, the probes were inserted over a wide area spanning across a greater length of the forearm of each subject (Figure 4.5). The vasculature across the forearm is variable with the most dense areas towards the wrists and elbows (39,40,55) and these differences in vasculature along the forearm may affect drug diffusion. An area with increased vasculature may produce low dialysate concentrations whereas an area with decreased vasculature may produce increased dialysate concentrations. The distance between probes was subsequently decreased from 3 to 1.5 cm in order to reduce variability resulting from the differences in vasculature across the volar aspect of the forearm.

The spreading of the formulation within the surface area of each template was another source of possible variability. Although an accurate amount of formulation from a calibrated formulation dispenser was dispensed at each application site, the use of a pre-weighed glass rod to spread the formulation across the surface area resulted in different amounts of the formulation remaining on each application site. On some sites, as much as 60% of the intended dose was lost to the glass rod during spreading. Differing amounts of ketoprofen were therefore available for diffusion through the SC into the dermis. Furthermore, the spreading procedure invariably contributed to the possibility of exerting variable pressures at the different application sites. Inconsistent pressures applied at different sites may have resulted in forcibly introducing more ketoprofen into the dermis. To avoid using a glass rod to spread the dispensed formulation across the surface area of the application site demarcated by the template, a sufficient amount of formulation was directly dispensed to cover the entire surface area of the application site. This ensured that each area received the same application dose.

The study was not conducted in a controlled environment. The clinic was not temperature or humidity controlled resulting in some fluctuation of both these conditions. During some study days, the clinic temperature was as low as 16°C and as high as 28°C on other days. The study was not conducted under filtered light to exclude UV rays from causing photodegradation of ketoprofen. Hence, subsequent studies should be conducted in a facility under controlled/regulated temperature and humidity conditions and performed under filtered lighting to exclude UV rays.

Based on these recommendations, an amendment to the research protocol was submitted to the RUESC for approval (Appendix I: RP Ammendments).

4.4a.4. Conclusions

The probe implantations and the overall study design were well tolerated by all subjects with no residual inflammation or scarring after 4 weeks, facilitated by the application of an anti-inflammatory cream immediately post probe removal, and moreover, ice was an effective anaesthetic. The lack of bioequivalence between the 2 products may have been due to either the test and reference products indeed being bioinequivalent since no data were available to show the basis of marketing approval for either of the products or a poorly designed study protocol. Subject discomfort during the study, the spreading of the formulation across the surface area of the application sites, the widely distributed probes across the volar aspect of the forearm and the absence of a controlled environment were also identified as issues requiring attention.

4.4b. Human study II (HS II): Bioequivalence model development and optimisation

4.4b.1. Methods

The methods were the same as described in section 4.4a.1 except for the following:

4.4b.1.1. Formulation

Fastum® Gel containing ketoprofen 2.5%, m/m (Adcock Ingram Ltd., Bryanston, South Africa) was used as the test and reference products.

4.4b.1.2. Subjects (1-10) (HS II)

The study population comprised 10 (5 females and 5 males) healthy black subjects with ages ranging from 20-45 yr and body weights ranging from 55.3-95.6 kg.

4.4b.1.3. Study design

The study was conducted in the Biopharmaceutics Research Institute (BRI, Rhodes University, Grahamstown, South Africa). The probes were implanted 1.5 cm apart (Figure 4.8) and 50 mg of Fastum® gel formulation was dispensed using 1.0 ml preloaded Combitips®. No spreading of formulation was performed and the entire surface area of the application site was covered as previously discussed. The entire study was conducted under filtered fluorescent (deep golden amber) light (Lee Filters, Andover, Hampshire, England) which prevented the transmission of light at wavelengths lower than 530 nm and protected against ketoprofen photodegradation. Amber glassware was used as a further precaution to prevent the possibility of ketoprofen photolysis.

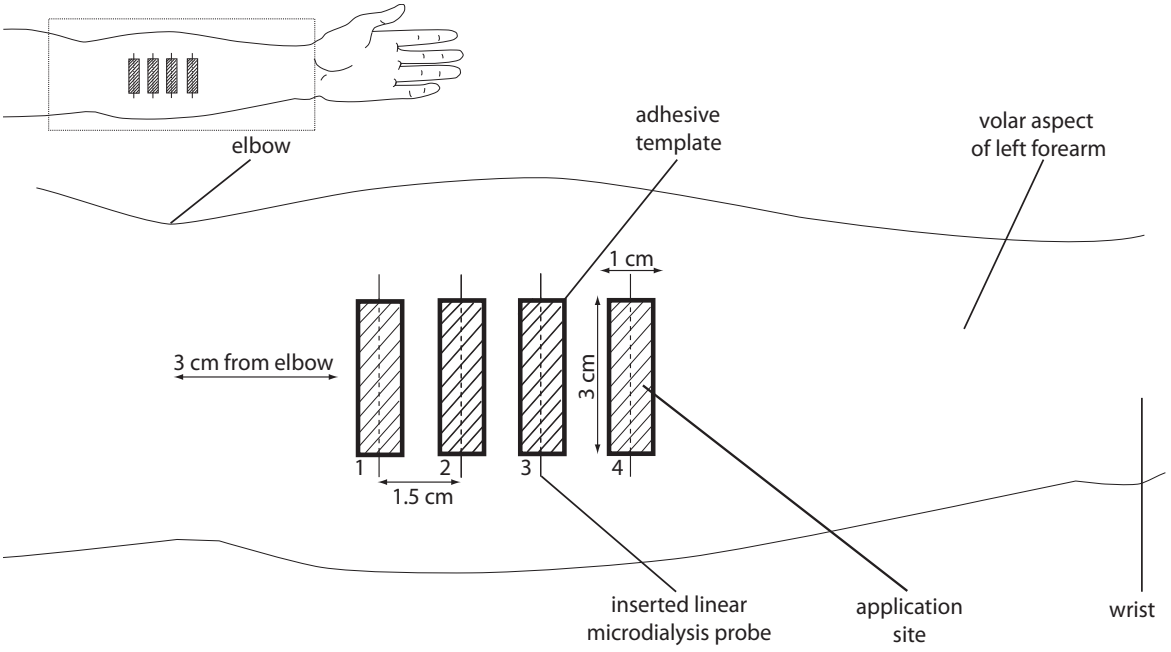


Figure 4.8. DMD study design (HS II)

Experimental conditions: 4 probe insertions, 4 application sites, 1 probe per site, probes were 1.5 cm apart, probes covered approximately 2nd quarter of the volar aspect of the forearm of each volunteer

4.4b.1.4. Bioequivalence assessment

In order to assess the resulting data for bioequivalence, 2 sites on each subject were designated as test sites (T) and the other 2 sites as reference sites (R). The designation of the sites was performed according to the following randomisation sequence: A (TTRR/RRTT), B (TRTR/RTRT) and C (TRRT/RTTR). The means of 2 pairs of sites for each subject were used in the bioequivalence assessment. The means of probes 1 and 2 versus 3 and 4 were designated as sequence A. The means of probes 1 and 3 versus 2 and 4 were designated as sequence B. The means of probes 1 and 4 versus 2 and 3 were designated as sequence C. Sequence D was a randomisation of sequences A, B and C.

4.4b.2. Results

4.4b.2.1. Clinical observations

Similar insignificant clinical observations as in the previous study were noticed however, no failed probes were observed in this study.

4.4b.2.2. Dose assessment

The dose applied at the sites sufficiently covered the entire surface area of the application sites demarcated by the templates and no spreading of the formulation was conducted.

4.4b.2.3. Probe depth measurements

The mean probe depths per subject (*n=4*) for all subjects ranged from 0.64-0.87 mm and the %RSDs ranged from 2.95-11.09% (Table 4.13).

Table 4.13. Mean probe depth measurements (HS II) (*n=4*)

Subject	Probe depth (mm) (mean ± SD)	RSD (%)
1	0.76 ± 0.08	11.09
2	0.65 ± 0.03	5.22
3	0.87 ± 0.05	6.23
4	0.66 ± 0.07	10.47
5	0.84 ± 0.08	9.00
6	0.64 ± 0.05	7.95
7	0.87 ± 0.03	3.15
8	0.77 ± 0.02	2.95
9	0.64 ± 0.06	9.27
10	0.72 ± 0.08	10.81

4.4b.2.4. Pharmacokinetic evaluation

Mean dialysate concentration-time (a) and semi log-time (b) profiles (± SD) from 4 probes are illustrated in Figure 4.9.

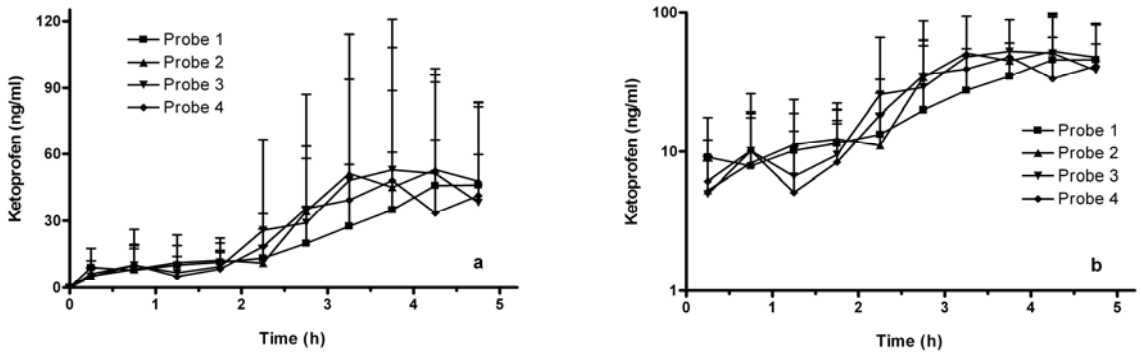


Figure 4.9. Mean dialysate concentration (a) and semi-log (b) time profiles (± SD) HS II (*n=10*)

Experimental: 4 probe insertions, 4 application sites, 1 probe per site, probes were 1.5 cm apart, probes covered approximately 2 quarters of the volar aspect of the forearm of each volunteer, 10 subjects, Formulation: Fastum® gel

The raw data and individual subject concentration-time profiles are located in Appendix II (DMD: HS II). All the concentrations obtained were measureable and above the LLOQ. The AUC_{0-5} values obtained from probes 1-4 were 101.60 ± 56.65 ngh/ml (ranged from 17.12-180.37; RSD = 55.76%), 127.89 ± 93.49 ngh/ml (ranged from 20.62-346.03 ngh/ml; RSD = 73.10%), 128 ± 122.96 ngh/ml (ranged from 18.52-460.16 ngh/ml; RSD = 95.47%) and 112.63 ± 98.57 ngh/ml (ranged from 18.21-351.20 ngh/ml; RSD = 87.52%), respectively. Although inter-subject variability (%RSD) for each probe was approximately 77%, the intra-subject variability (%RSD) between probes was 11.04%.

4.4b.2.5. Bioequivalence assessment

The bioequivalence assessment of sequence A, B, C and D are presented in Table 4.14. The AUC_{0-5} 90% CI for sequence A (80.34, 108.53) and B (80.21, 112.81) were within the acceptance limits of 80-125% but the AUC_{0-5} CIs for sequence C (75.17, 98.92) and D (70.79, 117.76) were just outside the lower limits. The power of the study for both sequence A (44.55%), B (34.44%) and D (45.69%) were rather low but high for sequence C (78.48%). The ANOVA CV% for sequences A, B, C and D were low.

Table 4.14. Bioavailability comparison of sequences (*n=10*)

Sequence	PK Parameter	Units	Arithmetic means (mean ± SD)		%Ratio (S1/S2)	90% CI (lower limit, upper limit)	Power of ANOVA (%)	ANOVA CV (%)
			Test	Reference				
A	AUC_{05}	ngh/ml	114.95 ± 88.29	120.51 ± 90.01	93.37	(80.34, 108.53)	44.55	18.34
B	AUC_{05}	ngh/ml	115.20 ± 84.20	120.26 ± 93.05	95.13	(80.21, 112.81)	34.44	20.80
C	AUC_{05}	ngh/ml	107.12 ± 73.22	128.34 ± 106.04	86.23	(75.17, 98.92)	78.48	16.75
D	AUC_{05}	ngh/ml	107.36 ± 91.86	120.54 ± 96.32	91.30	(70.79, 117.76)	45.69	19.67

4.4b.3. Discussion

The outcome of the study based on the recommendations implemented from HS I demonstrated the possibility of developing a DMD model to determine the bioequivalence of ketoprofen topical gel formulations in human subjects. The bioavailability comparisons showed that 2 out of 3 paired site combinations (T & R) met the AUC_{0-5} criteria for bioequivalence and moreover the improved ANOVA CV% was indicative of an improved study design.

The exclusion of UV light, the use of sufficient amount of formulation and the reduction of probe-to-probe distances minimised the variability associated with the study. A preliminary 2-subject study conducted under filtered light with the HS I dose (10 mg/cm^2) confirmed that the higher concentrations obtained in HS II were due to the exclusion of UV rays and not to the increased amount of formulation dispensed at the application areas. Although similar dialysate concentrations were observed with either dose from HS I (10 mg/cm^2) or HS II (16.67 mg/cm^2) the use of the latter dose was preferred due to the ability to dispense reproducible amounts of the topical formulation onto

each application area. The failure to show bioequivalence for sequence C coupled with the low power of the study indicated that the increased dose did not affect the discriminatory power of the study as a result of possible ketoprofen site saturation. Reducing the distance between the inserted linear DMD probes (i.e., from 3 to 1 cm) appeared to have resulted in reduced variability and contributed towards an improved ANOVA CV% for all sequences.

The probe depth measurements showed that very consistent implantation depths were obtained within the dermis of all subjects and was unlikely to contribute towards variability. No correlation between probe depth measurements and AUCs was observed.

The data indicated dermal penetration of ketoprofen to varying degrees between subjects although a clear maximum concentration of ketoprofen could not be unequivocally established within the 5 h study duration. Hence C_{max} could not be used as a bioavailability parameter in the bioequivalence assessment. Although the concentration-time profiles showed high inter-subject variability (~ 77%), more pronounced between 2-5 h, the study was associated with low intra-subject variability (11.04%). The high inter-subject variability is presumably associated with individual differences of the SC of the subjects and is not entirely unexpected (140,172). Whereas C_{max} is normally included in the assessment of bioequivalence for drugs intended for systemic absorption, it remains questionable whether such a parameter is appropriate for products not intended for the systemic circulation. In this respect, a well-established precedent to waive such a requirement has long been used in the assessment of topical corticosteroid formulations where only a single criterion, the area under the effect curve (AUEC) is used to determine bioequivalence between such topical preparations (41).

Although the 90% CI for AUC_{0-5} for sequences A and B were within the bioequivalence acceptance limits of 80-125%, the power of those studies was relatively low, 44.55% and 34.44%, respectively, indicating that more subjects would be required to show bioequivalence. The failure of sequence C to meet the bioequivalence assessment for AUC_{0-5} may be due to location of the application sites being compared which involved comparing data from sites 1 and 4 (outside sites) versus 2 and 3 (inner sites). Possible differences in vasculature may account for the variable diffusion patterns between the inner and the outer sites. Since sequence D involved the randomisation of sequences A, B and C, the failure of sequence D was attributed to sequence C not meeting the bioequivalence assessment.

4.4b.4. Conclusions

The recommendations implemented from the previous study (HS I) reduced the variability seen in this study (HS II). The use of sufficient amount of formulation to avoid the need to spread the formulation, reduction of the probe-to-probe distance as well as conducting the study in a controlled environment

under filtered lights reduced variability. A study population of 10 subjects was however insufficient to demonstrate bioequivalence and therefore and add-on study was necessary.

4.4c. Human study III (HS III): Add-on study

4.4c.1. Methods

An add-on study involving an additional 8 (Subjects 11-18) healthy subjects (4 females and 4 males) with ages ranging from 18-23 yr and body weights ranging from 61.5-85.69 kg was conducted under the same conditions as previously described.

4.4c.1.1. Homogeneity test

The Levene's test and Brown-Forsythe test for homogeneity set at a 5% level of significance for the 2 groups (HS II & HS III) of data were performed using SAS® (SAS Institute Inc., Cary, North Carolina, USA). The null hypothesis (H_o: $\sigma^2_{HS\ II} = \sigma^2_{HS\ III}$) described equal variances of data obtained from HS II and HS III whereas the alternate hypothesis (H_a: $\sigma^2_{HS\ II} \neq \sigma^2_{HS\ III}$) described unequal variances.

4.4c.2. Results

Once again there were neither no failed probes nor any significant untoward clinical observations. The mean probe depths ranged from 0.67-0.87 mm and the %RSDs ranged from 5.38-16.70% (Table 4.15).

Table 4.15. Mean probe depth measurements (HS III) (*n=4*)

Subject	Probe depth (mm) (mean ± SD)	RSD (%)
11	0.84 ± 0.13	15.57
12	0.70 ± 0.07	10.27
13	0.80 ± 0.07	9.23
14	0.67 ± 0.07	11.07
15	0.87 ± 0.05	5.38
16	0.79 ± 0.11	13.48
17	0.84 ± 0.06	7.29
18	0.75 ± 0.13	16.70

4.4c.2.1. Pharmacokinetic evaluation

4.4c.2.1.1. Subjects 11-18 (HS III)

Mean dialysate concentration-time (a) and semi log-time (b) profiles (± SD) from 4 probes assessed for subjects 11-18 are illustrated in Figure 4.10. The raw data and individual subject concentration-time profiles are located in Appendix II (DMD: HS III). All the ketoprofen concentrations obtained for subjects 11-18 were measureable and above the LLOQ. The AUC_{0-5} obtained from probes 1-4

were 192.38 ± 93.17 ngh/ml (ranged from 84.31-342.12; RSD = 48.43%), 220.55 ± 125.26 ngh/ml (ranged from 74.23-415.14 ngh/ml; RSD = 56.79%), 203.80 ± 114.42 ngh/ml (ranged from 68.53-406.53 ngh/ml; RSD = 56.14%) and 169.33 ± 90.60 ngh/ml (ranged from 56.16-302.27 ngh/ml; RSD = 53.51%), respectively. Inter-subject variability (%RSD) for each probe was approximately 78% and the intra-subject variability (%RSD) between probes was approximately 13%.

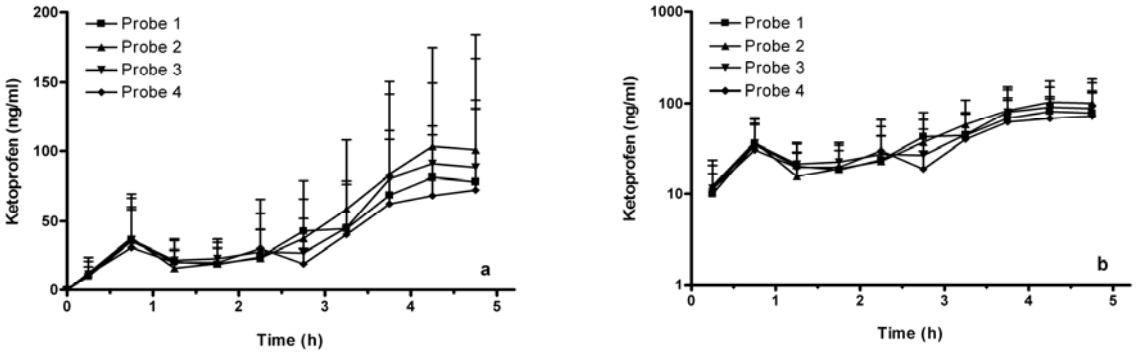


Figure 4.10. Mean dialysate concentration (a) and semi-log (b) time profiles (± SD) HS III (*n=8*)

Experimental: 4 probe insertions, 4 application sites, 1 probe per site, probes were 1.5 cm apart, probes covered approximately 2 quarters of the volar aspect of the forearm of each volunteer, 8 subjects, Formulation: Fastum® gel

4.4c.2.1.2. Homogeneity test

The results of the statistical procedure are provided in the Table 4.16 below.

Table 4.16. Homogeneity testing of data generated from HS II and HS III

Statistical test	F value	Pr > F
Levene's test	0.66	0.4201
Brown-Forsythe's test	0.07	0.7930

Since both p-values > 0.05, the null hypothesis was not rejected, hence there is sufficient evidence of homogeneity of data between HS II and HS III.

4.4c.2.1.3. Subjects 1-18 (HS II & III; Add-on)

Mean dialysate concentration-time (a) and semi log-time (b) profiles (± SD) from 4 probes assessed for subjects 1-18 are illustrated in Figure 4.11. The raw data and individual subject concentration-time profiles are located in Appendix II (HS II and III). The AUC_{0-5} obtained from probes 1-4 were 141.95 ± 86.18 ngh/ml (ranged from 17.12-342.12; RSD = 60.72%), 169.07 ± 115.47 ngh/ml (ranged from 20.61-415.14 ngh/ml; RSD = 68.29%), 162.13 ± 121.93 ngh/ml (ranged from 18.52-460.16 ngh/ml; RSD = 75.20%) and 137.83 ± 96.77 ngh/ml (ranged from 18.21-351.20 ngh/ml; RSD = 70.21%) respectively. Inter-subject variability (%RSD) for each probe was approximately 68% and the intra-subject variability (%RSD) between probes was approximately 10% when the data obtained from the total number of subjects were pooled.

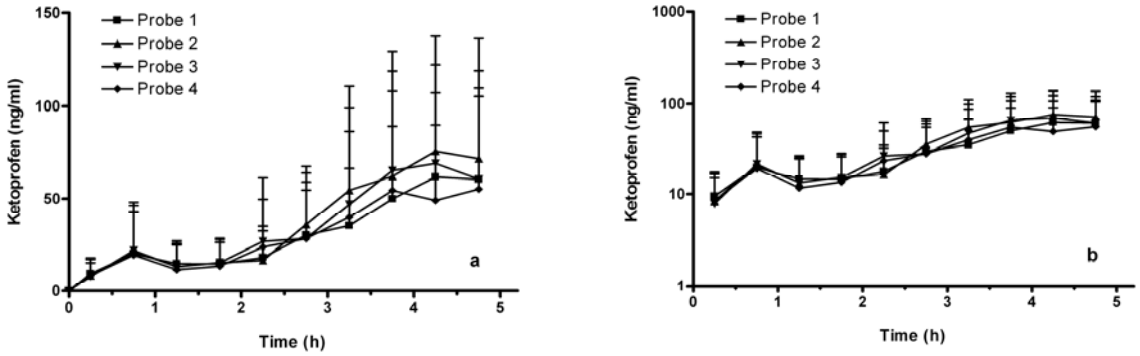


Figure 4.11. Mean dialysate concentration (a) and semi-log (b) time profiles (± SD) HS II and III (*n=18*)

Experimental: 4 probe insertions, 4 application sites, 1 probe per site, probes were 1.5 cm apart, probes covered approximately 2 quarters of the volar aspect of the forearm of each volunteer, 18 subjects, Formulation: Fastum® gel

4.4c.2.2. Bioequivalence assessment

The bioequivalence assessments of sequences A, B, C and D for 8 subjects are presented in Table 4.17. The AUC_{0-5} CI for sequences A (102.00, 121.88), B (94.81, 114.26) and C (107.02, 122.71) were found to be within the acceptance limits and sequence D (79.89, 119.92) was only slightly outside the lower limit and the point estimates for all sequences were within the limits. However, the power of the various sequences was low, particularly for sequences B (24.23%) and D (20.54%).

Table 4.17. Bioavailability comparison of sequences HS III (*n=8*)

Sequence	PK Parameter	Units	Arithmetic means (mean ± SD)		%Ratio (S1/S2)	90% CI (lower limit, upper limit)	Power of ANOVA (%)	ANOVA CV (%)
			Test	Reference				
A	AUC_{05}	ngh/ml	206.46 ± 108.29	186.57 ± 101.06	111.50	(102.00, 121.88)	51.11	22.23
B	AUC_{05}	ngh/ml	198.09 ± 102.16	194.94 ± 107.51	104.08	(94.81, 114.26)	24.61	24.23
C	AUC_{05}	ngh/ml	212.18 ± 118.90	180.86 ± 90.31	114.60	(107.02, 122.71)	45.67	21.25
D	AUC_{05}	ngh/ml	197.44 ± 120.57	188.35 ± 89.54	101.60	(79.89, 119.92)	20.54	29.12

The bioequivalence assessments of sequences A, B, C and D for all 18 subjects are presented in Table 4.18. The AUC_{0-5} CIs and point estimates for all sequences were within the acceptance limits with low ANOVA CV%s, although the power obtained for sequences C (53.99%) and D (51.04%) were still low.

Table 4.18. Bioavailability comparison of sequences (*n=18*)

Sequence	PK Parameter	Units	Arithmetic means (mean ± SD)		%Ratio (S1/S2)	90% CI (lower limit, upper limit)	Power of ANOVA (%)	ANOVA CV (%)
			Test	Reference				
A	AUC_{05}	ngh/ml	155.51 ± 98.89	149.98 ± 107.27	106.16	(97.39, 115.72)	92.88	14.88
B	AUC_{05}	ngh/ml	152.04 ± 99.23	153.45 ± 103.93	99.01	(89.86, 109.09)	95.95	16.72
C	AUC_{05}	ngh/ml	139.89 ± 87.28	165.60 ± 116.67	86.69	(80.37, 93.50)	53.99	13.04
D	AUC_{05}	ngh/ml	149.39 ± 112.14	150.68 ± 97.03	95.74	(82.20, 111.52)	51.04	26.30

4.4c.3. Discussion

In order to increase the power of the studies, an additional 8 subjects were enrolled into the study as determined from a power analysis based on the T/R ratio of %CV of the AUC_{0-5} data from the initial 10 subjects to provide a probability of 90%. When the data from all subjects were pooled, bioequivalence was met for all 3 sequences. However, although sequences C and D met the criteria for bioequivalence, they were associated with relatively low power which indicated that the factors previously discussed for these sites were probably involved. Justification of pooling the data was confirmed with both the Levene's and Brown-Forsythe's tests.

Although it is possible to improve the power of study sequences C and D with the use of even more subjects, modification of the study design may also be an important consideration. Since the least variable and thus strongest results were achieved with application sites 2 and 3, implantation of 2 probes in each of those sites instead of a single probe at the 2 outer sites (1 and 4) may be more appropriate (Figure 4.12).

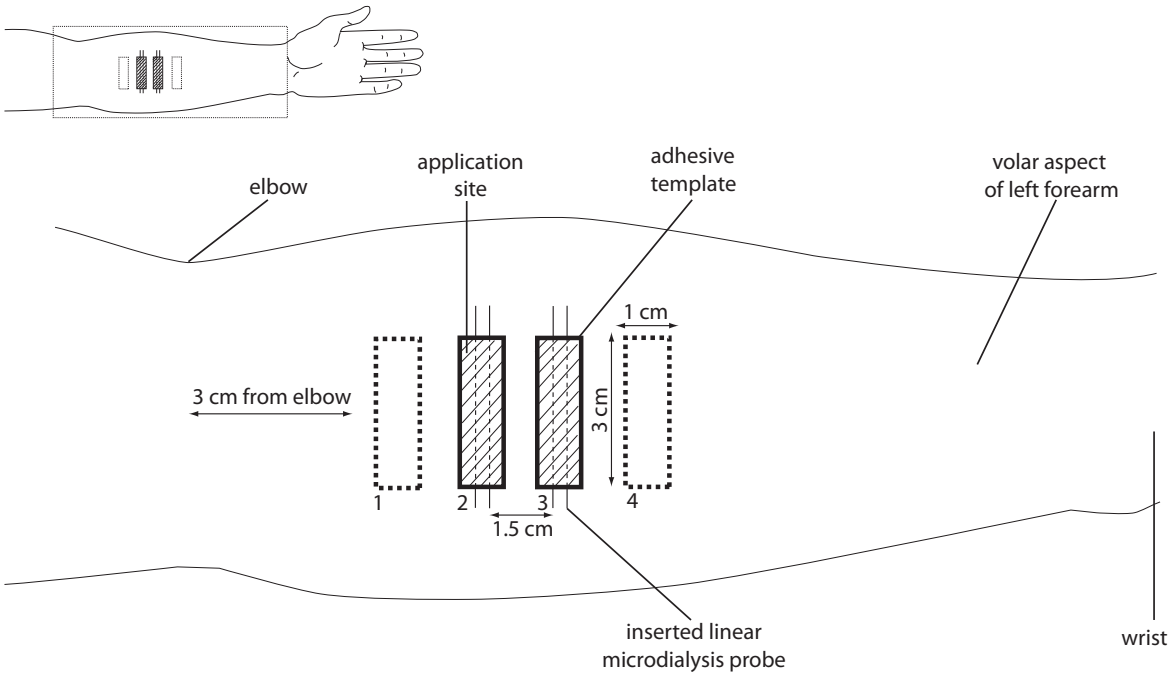


Figure 4.12. Proposed DMD study design

Experimental conditions: 4 probe insertions, 2 application sites, 2 probe per site, probes were 1.5 cm apart, probes covered approximately 2nd quarter of the volar aspect of the forearm of each volunteer

4.4c.4. Conclusions

The data from 10 initial subjects indicated that the study was under-powered to unequivocally show bioequivalence, hence the data from an add-on study of 8 additional subjects were pooled. As a result, the power of the study was increased to > 90% for 2 out of the 3 study sequences although all 3 of the

sequences indicated bioequivalence for those gel applications, with 1 of the 3 sequences being associated with a somewhat reduced power. The location of the application sites was seen to be an important consideration and a study modification to incorporate the implantation of 2 probes at sites 2 and 3 is proposed to minimise variability. A randomisation sequence of site (sequence D) may be performed with 4 application sites but more subjects will be needed to attain significant power. Since paired applications of ketoprofen gel used in this study consisted of the same preparation, bioequivalence was expected and subsequently proven. AUC_{0-5} was used as the main bioequivalence parameter since C_{max} was considered as an inappropriate parameter for products not intended to be absorbed into the systemic circulation in the same way as this parameter is not used for the assessment of the bioequivalence of topical corticosteroid preparations. The data from the study of the ketoprofen gel formulation provide the basis for and validates the use of DMD to assess bioequivalence of topical formulations applied to the skin and subsequently sampled in the target organ, the dermis. However, care must be taken to optimise the study design and application of the topical products to sites on the skin which have been appropriately identified. In order to justify the pooling of subjects, homogeneity testing is necessary.

CHAPTER FIVE

TAPE STRIPPING

5.1. Introduction

Tape Stripping (TS) is a method used to measure drug concentrations within the SC (35) and is based on the reservoir principle of the SC (177). The SC has the property to store drugs applied to the skin depending on the drug, the formulation, the application procedure and the state of the skin (24). If a formulation is applied to the skin for a limited period of time and removed, the amount of drug in the upper layers of the SC will be predictive of the drug bioavailability in the skin (178-180) and hence this technique may also be employed for local bioavailability and/or bioequivalence of topical formulations intended for local and/or systemic effect.

TS involves sequentially removing microscopic layers (~ 0.5-1.0 μm thick) of SC by placing an adhesive tape strip onto the skin surface, followed by gentle pressure to ensure good contact and subsequent removal by a sharp upward movement (28) which may be repeated 10 to more than 100 times (9,181). This technique although painless and non-invasive (28), disrupts the integrity of the water barrier properties of the SC (182) which is rapidly repaired by a homeostatic response in the dermis.

TS has been employed in the dermatological and pharmaceutical fields to evaluate the percutaneous penetration of drugs (24,140,183-192), barrier function (193-196), the possibility of animal skin as a surrogate for human skin, chemical toxicity (197), SC mass and thickness (198,199), wound healing processes and pharmacodynamic and clinical parameters (181). TS may also be useful for selecting or comparing vehicles for drugs to be applied topically.

The initial TS methodology outlining the bioavailability/bioequivalence protocol for topical formulations intended for local and/or regional activity, published in a draft guideline (200) was subject to criticism and resulted in its withdrawal. A number of limitations associated with the draft guidance are listed below but many of them still remain unresolved.

i. Dosing details such as size and duration of application as well as frequency of sampling were not clearly delineated.
ii. The number of tape strips needed is considerable which renders a dermatopharmacokinetic (DPK) evaluation time-consuming. For example, 10 applications per arm require 10 strips per site.
iii. Discarding of the first 2 tape strips was not substantiated.

iv. The same number of tape strips do not remove the SC from all subjects or even potentially within the same subject at different sites (28).

Moreover, there are several sources of variability (177) associated with this technique namely:

i. drug application procedure
ii. type of tape
iii. size of tape
iv. pressure applied by investigator
v. duration of application of pressure
vi. drug removal procedure
vii. drug extraction procedure
viii. analytical methods
ix. temperature
x. relative humidity
xi. skin type
xii. skin surface uniformity

Although most of these variables (i, ii, iii, vii, viii, ix and x) can be controlled, the degree of variability contributed by the others (iv, v, vi, xi and xii) is still significant (201-204). Löffler *et al*. (182) demonstrated significant influences of anatomical site, application pressure, application duration and TS removal from the application site during the skin stripping technique whereas the properties of adhesive tapes have been studied by Tsai *et al*. (193).

Among the list of variables, the number of tape strips needed to remove the SC has received much attention lately. As mentioned previously, the same number of strips do not remove the SC in all subjects due to age, gender and possibly ethnicity (205) and therefore determination of the SC removed from each strip would provide objective data, which are comparable between different subjects.

Sequential skin stripping permits fractions of the SC to be obtained after which the tape strips are extracted to allow the quantification of the absorbed drug which may be obtained from either combined or individual tape strips. Various analytical techniques have been employed for the quantification of drugs in tape stripped skin ranging from HPLC-UV, HPLC-florescence, HPLC-MS/MS, GC and infra-red (IR) spectrophotometry amongst others (28). Besides tape strip weighing before and after SC harvesting which is often time consuming and prone to error because of the SC moisture content (206), spectroscopic measurements determining the protein absorption in the UV range, measurements in the visible spectral range after staining of the corneocytes (207), spectral

signal connected with the SC or the microscopic determination of the covering density of the corneocytes can also be employed (182,195,201,206,208).

Transepidermal water loss (TEWL) is a non-invasive bioengineering technique that describes the outward diffusion of water through the skin (209). TEWL monitors the integrity of the SC water barrier function and is an indicator of skin water barrier alteration (182,205), with increased readings often indicating impairment of skin barrier function (209). Healthy SC typically has a water content of 10-20% and TEWL can be dramatically altered if barrier function is perturbed by physical, chemical, therapeutic and/or pathological factors (205). Typical basal values of TEWL in adults with healthy skin are between 5-10 $gm^{-2}h^{-1}$ (31).

Kalia *et al*. (31) reported the use of TEWL in TS experiments to determine the thickness of SC in an attempt to normalise the data by incorporating the SC thickness from each subject. This involves weighing each tape strip before and after SC harvesting to determine the amount (m) of tissue removed. Each stripped amount of SC can be converted to a distance (x) which reflects thickness of the removed skin strip, using Equation 5.1 below where (A) is the area of the application site, (ρ) is the density of the SC , reported as ~ 0.88-1.42 gm/cm^3 (210).

$$x = \frac{m}{(A \cdot \rho)} \qquad 5.1.$$

The additional standardisation to determine SC thickness by incorporating TEWL measurements was based on the SC functioning as a homogenous barrier to water transport *in vivo*, with the diffusional resistance equally distributed and not restricted to a particular tissue layer (31). Baseline TEWL $(TEWL_0)$ across non-stripped SC of thickness L (μm) is given by Fick's first law of diffusion expressed in Equation 5.2:

$$TEWL_0 = \frac{D \cdot K}{L} \Delta C \qquad 5.2.$$

where D and K are the diffusion coefficient of water in the SC and the SC viable tissue partition coefficient of water, respectively and ΔC is the water concentration difference (55 M ≡ 1 gcm^{-3}) across the membrane. After the TS procedure has removed a depth x of SC, the TEWL increases to a new value by:

$$TEWL_x = \frac{D \cdot K}{(L - x)} \Delta C \qquad 5.3.$$

Inversion of Equation 5.3 yields a linear relationship between TEWL and x (Equation 5.4) where the intercept on the x-axis equals the SC thickness, L (μm).

$$\frac{1}{TEWL_x} = \frac{L}{K\Delta C \cdot D} - \frac{x}{K\Delta C \cdot D} \qquad 5.4.$$

This equation allows the TS data to be expressed as an amount per normalised fraction of SC removed (x / L), a strategy which allows results from disparate subjects of different SC thickness to be normalised and compared.

The TS procedure coupled to TEWL measurements is time consuming and may pose a problem for fast diffusing drugs. Static electricity on tapes and misleading weights due to the presence of formulation excipients complicates gravimetric measurements.

This approach however demonstrates that the number of tape strips (10), as suggested in the FDA Draft Guidance (200) is a poor indicator of the actual amount of SC tissue removed since no information on the relative position within the SC is known and moreover 10 tape strips fail to permit meaningful comparisons between individuals (31).

The procedure is simple, inexpensive, relatively painless and non-invasive, given that only dead cells (corneocytes) are removed (182,204) and is the commonly used technique in DPK for the assessment of drug amounts in SC. TS is applicable to all drugs that are topically applied (211). Although the TS technique is a single point determination, it is possible to derive pharmacokinetic parameters such as AUC, C_{max} and T_{max} by sampling different sites progressively with application time, thereby providing a means to assess topical bioavailability of dermatological formulations (25).

Since the TS technique is accessible only to the SC but not the deeper tissues, e.g., the viable epidermis and dermis, this technique may not be applicable to drugs that have their activity in deeper tissues. Vehicle components of products influence both the adhesive properties of the tape as well as the cohesion of the corneocytes and even though TS is considered to be essentially non-invasive, stripped sites in certain dark-skinned individuals may remain pigmented for several months after healing (181).

5.2. Human study IV (HS IV): Preliminary investigations

5.2.1. Methods

The experimental protocols, ethical approval and subjects participating in this study were the same as previously described in section 4.4b.1 and 4.4c.1 except for the following:

5.2.1.1. Reagents, chemical and materials

Transpore™ 1527 (24 mm x 5 m) dressing tape (3M, Isando, South Africa) and Pelikan® clear film sheets (Hannover, Germany) were used.

5.2.1.2. Equipment

TEWL measurements were performed with a Vapometer Model SWL3N (Delfin Technologies, Oy, Kuopio, Finland). A Model AG 135 analytical balance (Mettler Toledo, Greifensee, Zurich, Switzerland) and a ZEROSTAT 3 anti-static gun (Sigma-Aldrich, Atlasville, South Africa) were used to weigh tape strips.

5.2.1.3. Study design

Three pre-cut templates (2 x 2 cm) prepared from a thin sheet of clear film were securely attached on the volar aspect of the arm. Two sites (Figure 5.1) were designated for formulation application whereas the third site was served as a blank for TEWL measurements. Forty milligrams (40 mg) of the ketoprofen gel formulation was dispensed onto application sites 1 and 2 and with the use of a pre-weighed glass rod, the dispensed formulations were evenly distributed.

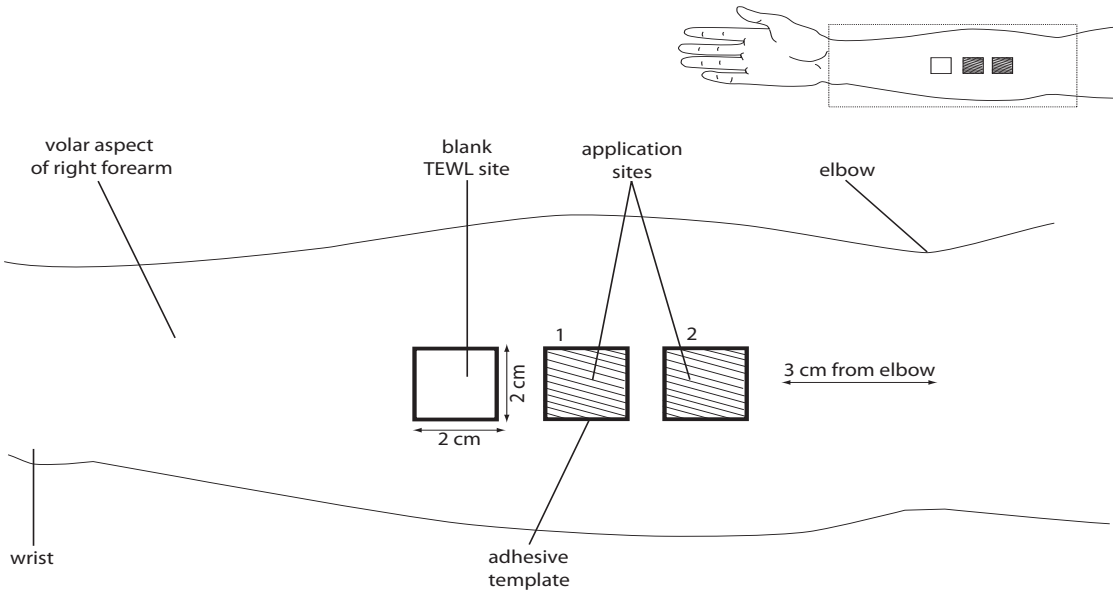


Figure 5.1. TS study design

Experimental: 2 application sites, 1 blank TEWL site covering approximately 2 quarters of the volar aspect of the forearm of each subject

Each site, including the blank, was subsequently stripped with 15 pre-cut (2 x 2 cm) tape strips which were previously weighed. Stripping was performed by alternating removal directions (N, E, S and W) and TEWL measurements were immediately recorded over a 10 s period after each strip from the blank application site. Thirty min (30 min) after the dose, the first 3 strips from each application site (i.e., sites 1 & 2) were discarded.

5.2.1.4. Sample collection

Each strip was subsequently re-weighed and individually placed in a centrifuge tube and samples were stored in the dark at 4°C prior to sample extraction (Chapter 3; Section 3.2.1b.3).

5.2.1.5. Sample analysis

Ketoprofen concentration in tape strips were determined with a validated HPLC-UV analytical method described in Chapter 3; Section 3.2.1b.

5.2.1.6. Data evaluation

The concentration-skin depth profiles were generated by plotting ketoprofen concentration against the cumulative stripped thickness which were normalised with the SC thickness of each subject and AUCs calculated. The determination of individual SC thickness with TEWL measurements was based on the model developed by Kalia *et al*. (31).

5.2.1.7. Bioequivalence assessment

In order to assess bioequivalence, a site was designated as the test and the other the reference site.

5.2.2. Results

5.2.2.1. TEWL measurements

The mean TEWL measurements for all 18 subjects are presented in Table 5.1. Although measurements were observed to be increasing with increasing number of stripped tapes, high RSDs were noted typically after the tenth strip. A linear relationship between $1/TEWL$ and the cumulative SC thickness was observed for all subjects and the x-intercept which represents the SC thickness of each subject are reported in Table 5.2.

Table 5.1. Mean TEWL measurements of subjects per strip (*n=18*)

Tape strip number	TEWL ± SD ($gm^{-2}h^{-1}$)	RSD (%)
1	10.32 ± 2.73	26.47
2	10.88 ± 3.71	34.11
3	12.13 ± 3.35	27.58
4	13.15 ± 1.58	11.98
5	14.31 ± 1.76	12.29
6	15.54 ± 2.51	16.17
7	16.25 ± 2.37	14.57
8	17.46 ± 3.37	19.32
9	19.58 ± 5.87	29.97
10	23.43 ± 17.05	72.76
11	25.77 ± 17.37	67.41
12	32.80 ± 34.31	104.60
13	47.82 ± 65.10	136.14
14	55.22 ± 71.47	129.42
15	59.52 ± 75.23	126.40

Table 5.2. SC thickness determined from TEWL measurements

Subject	SC thickness (µm)
1	7.89
2	9.38
3	13.91
4	10.17
5	14.08
6	22.29
7	13.09
8	15.16
9	27.16
10	23.40
11	15.14
12	18.21
13	25.15
14	27.18
15	10.11
16	12.25
17	14.14
18	18.15
Mean ± SD	**16.45 ± 6.17**
RSD (%)	**37.40**

5.2.2.2. Tape strips weights

The average weights of tape strips obtained from strips 4-15 from all subjects at both applications sites are presented in Table 5.3. Although reproducible weights were recorded at both application sites, high RSDs were observed (53.3-77.3%).

Table 5.3. Mean weights of tape strips from application sites

	Site 1		Site 2	
Tape strip number	**Mean ± SD (mg)**	**RSD (%)**	**Mean ± SD (mg)**	**RSD (%)**
4	0.3 ± 0.2	55.2	0.3 ± 0.2	53.3
5	0.3 ± 0.1	60.2	0.3 ± 0.2	62.3
6	0.3 ± 0.2	57.7	0.3 ± 0.2	60.2
7	0.3 ± 0.2	58.8	0.3 ± 0.2	58.8
8	0.3 ± 0.2	66.5	0.3 ± 0.2	64.0
9	0.3 ± 0.2	63.5	0.3 ± 0.2	65.3
10	0.3 ± 0.2	57.7	0.3 ± 0.2	57.9
11	0.3 ± 0.2	59.5	0.3 ± 0.2	61.1
12	0.3 ± 0.2	74.1	0.3 ± 0.2	68.9
13	0.2 ± 0.2	68.3	0.3 ± 0.2	65.4
14	0.2 ± 0.1	65.2	0.2 ± 0.2	77.3
15	0.2 ± 0.1	66.6	0.2 ± 0.1	59.4

5.2.2.3. Pharmacokinetics evaluation

The mean concentration cumulative SC depth profiles (± SD) of both application sites (i.e., site 1 and site 2) are illustrated in Figure 5.2. The raw data and individual subject concentration-SC depth profiles are located in Appendix III (TS: HS IV). The $AUC_{x/L}$ obtained for both site 1 and site 2 were 128.93 ± 82.74 (ranged from 33.86-367.01 ngm/l; RSD = 64.17%) and 105.93 ± 69.56 (ranged from 24.45-321.07 ngm/l; RSD = 65.67%) respectively. Inter-subject variability (%RSD) for each probe was approximately 70% and the intra-subject variability (%RSD) between probes was approximately 25%.

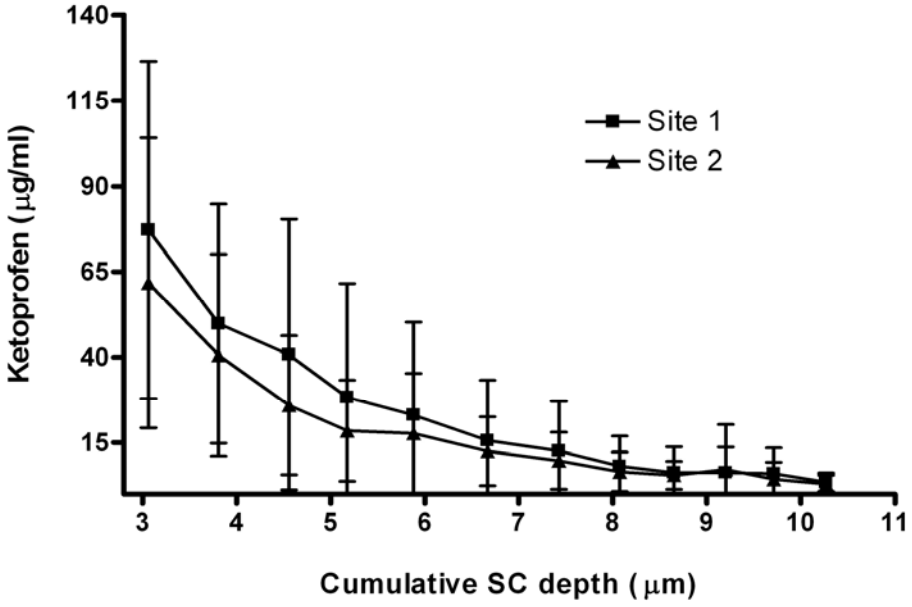


Figure 5.2. Mean concentration-cumulative SC depth profiles (± SD) HS IV (*n=18*)
Experimental: 2 application sites, 1 blank TEWL site, 15 strips, 12 strips harvested for analysis, 18 subjects, Formulation: Fastum® gel (Site 1 and 2)

5.2.2.4. Bioequivalence assessment

The bioequivalence assessments of site 1 and site 2 in 18 subjects are summarised in Table 5.4 below. The 90% CI for $AUC_{x/L}$ and point estimate was outside the acceptance range of 80-125%.

Table 5.4. Bioavailability comparison of application sites (*n=18*)

PK parameter	Units	Arithmetic means (mean ± SD)		%Ratio (T/R)	90% CI (lower limit, upper limit)	Power of ANOVA (%)	ANOVA CV (%)
		Site 1	Site 2				
$AUC_{x/L}$	ng.m/l	176.67 ± 228.15	134.58 ± 144.94	131.27	(100.71, 155.40)	99.97	41.15

5.2.3. Discussion

High variability associated with TS were observed with TEWL measurements (RSDs < 136.14%), tape strip weights (RSDs < 77.3%), the pharmacokinetic data (RSDs < 65.67%) and the bioequivalence assessment (ANOVA CV = 41.15%). The sources of variability have been indentified to be related to tape strips weight measurements, pressure application at sites, application procedure and the absence of formulation excipients effect with TEWL.

The use of TEWL demonstrated the ability to normalise TS data, which was confirmed by the linear relationship obtained from all subjects. The determination of the SC thickness not only appeared to normalise the data but also provided insight into the exact position within the SC (Figure 5.2). This is an improvement over previously reported approaches where the sum of the AUC was used as an indicator of bioavailability (140). However, formulation excipients have been known to alter the integrity of the SC (212) and the TEWL as currently incorporated does not take formulation excipients into account since measurements are performed on the blank site and not on the application sites. This shortcoming may become apparent when formulations with different excipients are compared for bioequivalence assessments.

Although a precision balance coupled with an electro-static source was used to weigh the tape strips, the time taken to achieve consistent weight measurements for each tape was relatively long with most determinations producing inconsistent readings after 1 min. The long duration allowed for possible desiccation of stripped SC on the tape strips as well as loss of volatile formulation excipients. The tapes were equally prone to adhesion of static matter from the ambient surroundings (i.e., within the clinic) which may have altered the weight of the strips.

The pressure applied as well as the duration of the applied pressure at each application site prior to stripping was subject to variability between sites but more importantly between subjects. Although sufficient pressure duration (10 s) was employed during the study, it was difficult to qualify the

applied pressure prior to each strip and this may have also contributed to increased variability of the data. The pressure used at the blank sites may vary from that used at the application sites due to the presence of the formulation. Ideally, the pressure used for the determination of the SC thickness should be used during the stripping procedure.

The use of the glass rod to spread the formulation within the application site may contribute to the variability by inconsistent removal of some of the dose. The use of a dose that would cover the application site similar to the DMD study to circumvent the use of the glass rod was not practical since excess formulation would remain even after several tape strips. Analysis of the first 3 tape strips showed high concentrations of ketoprofen which represented unabsorbed drug and hence discarding those strips served as a cleaning procedure. This cleaning procedure was chosen over previous methods which indicated the use of solvents or cotton swabs to remove excess formulation because the use of solvents are known to either increase of retard drug penetration and moreover, the physical rubbing with cotton swabs increases the possibility of mechanically pushing the drug into the SC hence producing higher concentrations than usual (24,213).

The incorporation of TEWL with TS as currently used may be sufficient to provide adequate information for bioavailability studies for the *in vivo* differentiation of formulations. However, the high variability still associated with this technique is still a challenge for bioequivalence assessments. Increasing the number of stripping sites and the average of test and reference $AUC_{x/L}$ values used in the bioequivalence assessment may circumvent this issue. However, a possible limitation would be the need to use smaller tape strips sizes to accommodate a sufficient number of application sites on the volar aspect of the forearm of subjects. This further complicates tape strip weighing measurements since smaller tape strips may require a more sensitive weighing balance fitted with an anti-static component thus making a this procedure more time-consuming and laborious.

5.2.4. Conclusions

Although normalisation of data between subjects using TEWL has been proposed as a measure to reduce the variability of the TS technique, TS still remains a highly variable technique due to the number of variables, such as pressure application, removal of strips, weighing of tape strips and formulation applications that are yet to be standardised and validated. Tape strips are sensitive to desiccation and static matter. The incorporation of TEWL which determines the SC thickness of each subject negates the effect of formulation excipients which may alter the thickness of the SC. More sites are therefore necessary for bioequivalence studies however a more sensitive analytical balance fitted with an anti-static component may be required.

CHAPTER SIX

CONCLUDING REMARKS

Analytical methods were developed and validated for MD, TS and DMD samples in full compliance with all the required analytical method validation parameters. HPLC-UV methods were developed for MD and TS samples whereas a rapid UPLC-ESI-MS/MS method was developed for DMD samples. Normal saline did not affect HPLC-UV analysis however due to signal suppression with UPLC-ESI-MS/MS analysis, DMD samples had to be extracted from normal saline into methanol to maintain sensitivity and column preservation. A step gradient elution was necessary for TS samples to reduce sample run time and flush out late eluting interfering peaks. Inclusion of methanol in both the mobile phase as well as in the sample reconstitution step following extraction was found to enhance sensitivity in NI ESI applications. Furthermore, system carryover and contamination from sample inserts which were detected were eliminated.

In vitro MD provided valuable information for the development and optimisation of DMD studies. An equilibration period was necessary to obtain reproducible recovery, a membrane length of 3 cm was identified as practical and an appropriate perfusion rate was determined which generated sufficient dialysate volumes for analysis.

The DMD studies demonstrated excellent potential for the determination of dermal bioavailability of topical formulations intended for local and/or regional activity although care must be taken to optimise the study design. Probe insertions were well tolerated by subjects with no residual inflammation or scarring after 4 weeks of post-experimental medical assessment. Limitations identified in the study design of the preliminary investigation were used to develop and optimise subsequent DMD investigations. Several necessary precautions were implemented which included the avoidance of any subject discomfort, care in the spreading of formulation, avoiding the widespread use of the volar aspect of the forearm by constraining product applications to specific sites and providing a controlled environment to result in an optimised study design with reduced variability. Although the 90% CI for AUC_{0-5} for a 10 subject study population was within the acceptable limits for bioequivalence for sequences A and B, the statistical power proved to be relatively low to conclude bioequivalence. The failure to show bioequivalence of sequence C was related to possible differences in vasculature at the sites being compared (i.e., 2 outer sites). An addition of 8 subjects resulted in the 90% CI for AUC_{0-5} for all sequences falling within the permissible limits describing bioequivalence. The statistical power for sequence C was however was still low whereas the statistical power of both sequences A and B were found to be excellent (> 90%) to conclude the declaration of bioequivalence. A study design modification to accommodate the low statistical power of sequence C

was proposed involving the use of 2 probes at sites 2 and 3 instead of the use of single probes within sites. Since C_{max} was not obtained in most of the subjects, it was considered as an inappropriate parameter, hence AUC_{0-5} was used as the main bioequivalence parameter similar to the situation which has been found acceptable in the assessment of the bioequivalence of topical corticosteroid formulations.

The relatively high variability obtained from TS studies is attributed to a number of variables and the optimisation and standardisation of these variables is essential to ensure that the method is optimised.

Optimisation of the dose duration, i.e., the time for which the formulation is left on the application site, is essential to allow for sufficient drug diffusion into the SC prior to the stripping procedure. If an insufficient duration is used, possible analytical challenges may be encountered regarding the LOQ. The converse may show site saturation which may not provide a model capable of discriminating different formulations.

The stripping technique has also been identified as a source of variability. Although the tape strips were removed in a sequential order (i.e., N, E, S and W) in order to ensure that approximately the same thickness of SC was consistently stripped, the pressure applied prior to the stripping procedure needs to be standardised and validated. An increased pressure would certainly increase the risk further by mechanically pushing the drug into the skin and beyond the SC.

The careful removal of residual application prior to skin stripping allows for the exclusion of excess formulation being stripped thus generating extremely high concentration/amounts. Although tape strips were used in this study to remove residual application during the first 3 strippings where these 3 tape strips were discarded, other “cleaning/removal methods” need to be investigated to ensure that the stripped skin contains drug that is present within the SC and not on the surface of the skin. For purposes of mass balance it will be useful to analyse the first 3 strips prior to discarding, for mass balance purposes.

Careful control of the dose and application of doses to demarcated skin sites need to be addressed. The use of a finite amount of dose requires the need to spread the formulation over the application sites which could mechanically alter drug diffusion patterns throughout the adjacent demarcated site thereby contributing to increased variability. The use of infinite dose obviates the need to spread the formulation over the site and is thus a preferable approach which can be readily standardised and validated. A dose-response model should also be used in order to determine the number of subjects required to provide the necessary discriminatory power to distinguish between different formulations, if differences do in fact exist.

The control of temperature and humidity is essential to avoid the possibility of increased drug diffusion during warm days and vice versa during cold days and also to prevent possible tape strip desiccation due to variable humidity. The desiccation of tape strips generates inconsistent mass readings thus affecting the determination of subject SC thickness.

The foregoing describes various issues and suggestions which could be pursued in order to further optimise the various methodologies. Resolution of these issues and implementation of these suggestions should allow the reduction of variability and provide better standardisation and facilitate the development of validation requirements to ensure consistent generation of reproducible data together with necessary quality control procedures.

APPENDIX I

Application Letter

Research Protocol

Amendments to Protocol

Advertisement application Letter

Copy of Advert

28 June 2007

Professor Roy Jobson
Chairman
Rhodes University Ethical Standards Committee
RHODES UNIVERSITY
GRAHAMSTOWN

Dear Professor Roy Jobson,

APPLICATION TO UNDERTAKE A DERMAL MICRODIALYSIS AND TAPE STRIPPING STUDY

The Biopharmaceutics Research Group (BRG) wishes to conduct a dermal microdialysis and tape stripping clinical study on healthy human volunteers for the assessment of the bioavailability of ketoprofen within the next 6 months.

Microdialysis is a sampling technique used to study the concentration of endogenous and exogenous compounds in extracellular space. Tape stripping involves the sequential removal of microscopic layers of the *stratum corneum*. Both techniques will be applied within the dermal region on the volar aspect of the forearms of subjects.

Healthy human volunteers will be drawn from Rhodes University for this study and as such the BRG requires approval from the Rhodes University Ethical Standards Committee. A research protocol outlining the study has been included in the application.

Thank you.

Sincerely,

……..
Ralph Nii Okai Tettey-Amlalo
BPharm, MSc, PhD Candidate (Rhodes)
Principal Investigator

Rhodes University
Faculty of Pharmacy
Biopharmaceutics Research Group
PO Box 94
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RESEARCH PROTOCOL

APPLICATION OF DERMAL MICRODIALYSIS AND TAPE STRIPPING FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF KETOPROFEN IN HEALTHY VOLUNTEERS

STUDY NUMBER: RNO_01_2007

PROTOCOL VERSION 1

JULY 2007

Test product **Fastum® Gel**

(Ketoprofen 2.5%, m/m)

Adcock Ingram Limited

South Africa

Principal Investigator **Ralph Nii Okai Tettey-Amlalo**

BPharm, MSc, PhD Candidate (Rhodes)

Biopharmaceutics Research Group (BRG)

Faculty of Pharmacy

Rhodes University

Grahamstown 6140

South Africa

Supervisor **Professor Isadore Kanfer**

BSc (Pharm), BSc (Hons), PhD (Rhodes)

Rhodes University

Grahamstown, 6140

South Africa

FINAL APPROVAL OF PROTOCOL

Study number	RNO_01_2007
Study title	Application of dermal microdialysis and tape stripping for the assessment of the bioavailability of ketoprofen in healthy volunteers
Test product	**Fastum® Gel** (Ketoprofen 2.5%, m/m) Adcock Ingram, South Africa

_____ _____

Principal Investigator **Date (yyyy-mm-dd)**

Ralph Nii Okai Tettey-Amlalo

BPharm, MSc, PhD Candidate (Rhodes)

_____ _____

Supervisor **Date (yyyy-mm-dd)**

Professor Isadore Kanfer

BSc (Pharm), BSc (Hons), PhD (Rhodes)

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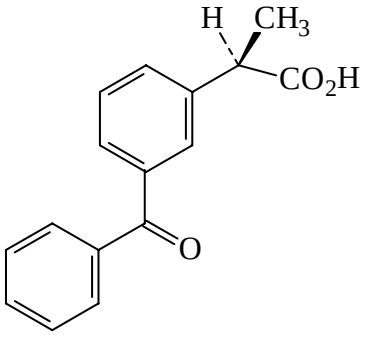
1. BACKGROUND INFORMATION

1.1. Ketoprofen

1.1.1. Molecular structure

Ketoprofen is an anionic non-steroidal anti-inflammatory drug (NSAID). It is a derivative of propionic acid and widely used in the management and treatment of patients with rheumatic disease (1). The molecular structure of ketoprofen is represented below.



$C_{16}H_{14}O_3$ MM 254.3 g/mol

1.1.2. Mode of action and uses

Ketoprofen is one of the most powerful inhibitors of cyclooxygenase (COX) at concentrations well within the range of therapeutic plasma concentrations (EC50 2 μg/l) (2). It produces reversible COX inhibition by competing with the substrate, arachidonic acid, for the active site of the enzyme (3). This inhibition results in a reduction in the tissue production of prostaglandins such as PGE_2 and $PGF_{2\alpha}$ (1). In addition to its effects on COX, ketoprofen inhibits the lipoxygenase pathway of the arachidonic acid cascade. This pathway produces non-cyclized monohydroxyl acids (HETE) and leukotrienes (LKT). Of these, only leukotrienes (B4, C4, and D4) are thought to increase vascular permeability, however, both HETE and LKT synthesised within leukocytes are active in promoting leukocyte migration and activation. It has been suggested that lipoxygenase inhibitors may attenuate cell-mediated inflammation and thus retard the progression of tissue destruction in inflamed joints. Ketoprofen is also a powerful inhibitor of bradykinin, an important chemical mediator of pain and inflammation. Furthermore, it stabilises lysosomal membranes against osmotic damage and prevents the release of lysosomal enzymes that mediate tissue destruction in inflammatory reactions (1,2,4-6).

Ketoprofen has many applications: it is used for musculoskeletal and joint disorders, such as ankylosing spondylitis, osteoarthritis, and rheumatoid arthritis, and in peri-articular disorders such as bursitis and tendinitis. It is also used for postoperative pain, painful and inflammatory conditions such as acute gout or soft tissue disorders and to reduce fever (1,7,8). It is additionally indicated for the management of acute painful shoulder syndrome and juvenile rheumatoid arthritis (2).

1.1.3. Pharmacokinetics

The introduction of a topical formulation for ketoprofen raises the distinct possibility of achieving therapeutic benefit, without the risk of gastrointestinal or other side effects suffered by the oral route. To establish the validity of this hypothesis requires that:

i. percutaneous absorption occurs in sufficient amounts to achieve therapeutic concentrations in the target tissues,
ii. clinical efficacy is demonstrable in suitably controlled clinical trials,
iii. the safety profile of the topical agent is superior to that of the oral agent, particularly with regard to gastrointestinal side effects and
iv. the topical agents are cost-effective (9).

Pharmacokinetic data have demonstrated that, following percutaneous administration of ketoprofen gel, the anti-inflammatory agent penetrates into the general circulation slowly (10,11) and reaches underlying tissues including the synovial fluid (6). This low systemic diffusion, combined with good local tolerance, would ensure an improved systemic tolerance for the local treatment of rheumatic conditions (6,10-16).

Ketoprofen is extensively metabolised in the liver. Only 1% of the dose is excreted unchanged in the urine. The main pathway of metabolism is glucuronic acid conjugation, with hydroxylation as a minor pathway (1,6,7). The metabolites are excreted mainly in the urine with 80% of the dose as the glucuronide. 10-20% of the dose is excreted in the bile. Some enterohepatic circulation is probable. The metabolic products of ketoprofen appear to be pharmacologically inert (1).

1.1.4. Adverse effects

For topical preparations, delayed hypersensitivity dermatitis may occur at the site of the application, but this is uncommon (9,15). Cases of contact erythema and photocontact dermatitis have also been reported (14). It is advised that they should not be used on broken or inflamed skin (15). The skin reactions are reversible on discontinuation of therapy (6). Cross-sensitisation of topical ketoprofen and other propionic NSAIDs have been reported (9).

1.1.5. Contraindications

Administration should be avoided in persons who exhibit hypersensitivity to ketoprofen or to any other NSAID or to any ingredient in the preparation.

A copy of the product information leaflet of the topical formulation to be used in this study is located in Appendix RP A.

1.2. Dermal microdialysis sampling

1.2.1. Rationale for dermal microdialysis sampling

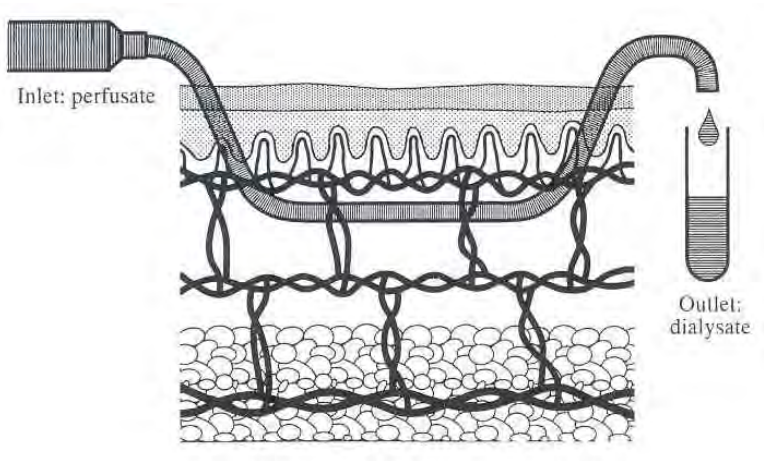
During recent decades the advantages of cutaneous and percutaneous drug delivery has gained increasing attraction for novel drug formulations. The percutaneous route may be an attractive solution for systemic delivery of very potent drugs with low oral bioavailability, low systemic clearance and narrow therapeutic window, due to the avoidance of hepatic first-pass metabolism and potential of long-term controlled release. However, the greatest potential for the topical administration route is targeted drug delivery to the skin itself, where dramatically higher skin-to-plasma ratios are attained compared to systemic drug delivery. This route of

administration can therefore maintain therapeutically effective drug concentrations in the target organ without the risk of inducing side-effects due to high systemic exposures (17).

In the clinical setting, there are currently no sampling techniques which can be used to demonstrate dermal bioavailability or bioequivalence of topically applied agents which are both minimally invasive and which also give an indication of tissue concentration at a target site in the skin with time. Dermal microdialysis has the potential to address the gap in available sampling techniques due to its minimally invasive nature and its ability to generate concentration-time profiles at the target site with good resolution provided a sufficiently sensitive analytical method is available (18).

1.2.2. Overview of microdialysis

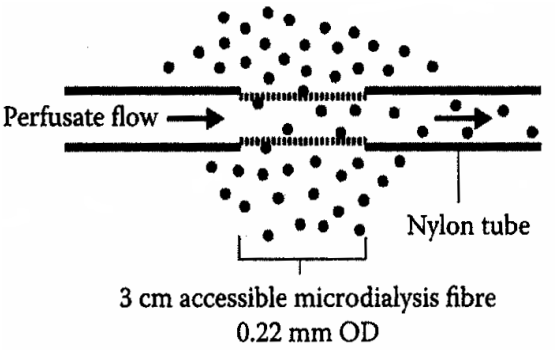
Microdialysis is a technique for sampling of endogenous and exogenous substances in the extracellular space in living tissue (19,20). The technique was originally developed in neuropharmacological sciences but has now been used extensively in other tissues in animal models and human studies (18,20). In human studies, this technique has been employed in adipose tissue, brain, heart, lung, solid tumours, skin etc (17,21,22). Initial clinical studies using microdialysis have demonstrated the suitability of this method for the measurement of drug concentrations in the interstitium of target tissues and allow for the description of relative changes of concentration-time courses of solutes against a baseline (22). The figure below shows a linear microdialysis probe inserted into the dermis prior to topical application (19).



1.2.3. Principles of microdialysis

A microdialysis fibre consists of a semipermeable membrane forming a thin hollow ‘tube’ (typically 0.22 mm ID), which functionally resembles a blood vessel. The fibre allows the passage of molecules with a volume smaller than the cut-off value of the membrane. The fibre has 1 end connected to an impermeable tube which leads to a micropump and the other end to an efferent sampling tube. For dermal microdialysis, the probe is implanted in the dermis of the skin via a guide cannula. The microdialysis fibre is slowly perfused with physiological solution, which equilibrates with extracellular fluid of the surrounding tissue exchanging substances smaller than the cut-off value of the membrane during the passage through the fibre. Entering the microdialysis fibre, the solution is termed perfusate and following dialysis of substances, the solution exiting the

fibre is termed dialysate (17). The diffusion of substances across the dialysis membrane will occur by passive diffusion, driven across the membrane by the concentration gradient; the speed of equilibrium is consequently proportional to the size of the gradient and diffusion rate of the medicine in the medium, in addition to the surface area of the fibre membrane. Using continuous flow through the probe, compounds will be recovered by the membrane and sampled for further analysis. As substances are able to diffuse in both directions through the membrane, microdialysis can principally be used to both extract and deliver substances in tissues (20).



The figure above depicts the principle of microdialysis sampling by a linear probe (20). The perfusate is pumped through the probe at a preset, low flow rate. During the passage through the membrane, the diffusion of small molecules will take place.

1.2.4. Insertion trauma and invasiveness of dermal microdialysis sampling

Dermal microdialysis sampling has been described by authors as a minimally invasive technique (18,20). The insertion of microdialysis probes creates slight but reversible trauma which initially causes an acute inflammatory reaction characterised by increased blood flow, erythema and skin thickness. This is caused by the use of a 21G guide cannula/hypodermic needle for insertion of the linear microdialysis probe. An equilibration time of 60 min has been reported to be sufficient to allow the skin to normalise (20). Histological examination of skin biopsies showed no signs of an inflammatory reaction in the tissue around the probe 8-10 h after probe insertion. Ice induces anaesthesia during probe insertion to provide minimum discomfort to the human volunteer. No significant discomfort is observed when the cannulae have been removed. Hydrocortisone (0.1%, m/m) cream applied twice daily for 2 weeks is used to arrest any residual inflammation after the completion of the study. Sterile techniques will be employed during this study to ensure the minimum risk of skin infections.

1.3. Tape stripping technique

1.3.1. Overview of tape stripping

Tape stripping is a non-invasive technique employed to study the penetration, the distribution and the dermatopharmacokinetics of topically applied drugs and cosmetics products (23-26) within the *stratum corneum*. It is commonly used to disrupt the epidermal barrier to enhance the delivery of drugs *in vivo* (27) and to obtain information about *stratum corneum* function (25). Tape stripping has been used extensively in dermatological and pharmaceutical fields to measure the *stratum corneum* mass and thickness, to collect *stratum corneum* lipids and protein samples, detect proteolytic activity

associated with the *stratum corneum*, quantitatively estimate enzyme levels and activities in the *stratum corneum* and allow the detection of metal in the *stratum corneum* (25). Although in use for over 5 decades there are no universally accepted protocols for tape stripping (28), however it has been identified to be of sufficient utility to have been proposed by the FDA as part of a standards method to evaluate the bioequivalence of topical dermatological dosage forms (29).

1.3.2. Principles of tape stripping

Tape stripping involves the sequential removal of microscopic layers (~ 0.5-1 μm) of the *stratum corneum* (24,30) by placing a strip of adhesive tape onto the skin surface with uniform pressure, which is then removed (23,24). Drug uptake into the *stratum corneum in vivo* can be measured by harvesting the *stratum corneum* previously exposed to a topical product with adhesive tapes, which are subsequently extracted and quantified for drug concentration by a validated analytical method (31). The number of tape strips needed to remove the *stratum corneum* varies with age, gender, anatomical site, skin condition and possibly ethnicity (25). Tape stripping is putatively simple, inexpensive and has been described as a minimally invasive technique (23).

1.3.3. Invasiveness of tape stripping

Although tape-stripping is considered to be essentially non-invasive, stripped sites in certain dark-skinned individuals may remain hyperpigmented for several months after healing. This effect will be communicated to the human volunteers before entering the study (24).

2. STUDY PRODUCTS

2.1. Description

	Test product
Commercial name	Fastum® gel
Generic name	Ketoprofen gel
Dosage form	Gel
Strength	2.5%, m/m
Manufacturer	Adcock Ingram, South Africa
MCC registration details	Z/3.1/165
In-house batch #	RNO1-01
Expiry date	09/2010
Description	A mucilaginous, colourless almost transparent gel with an aromatic odour
Dosage	90 μl (± 30 mg) per application site

2.2. Supply, storage and use

Sufficient test products will be timeously supplied to the clinic by the principal investigator prior to the study. On arrival the quantity, batch numbers and expiry dates of the test products will be recorded in the appropriate register and products stored in a secure cabinet. Dispensing and administration of test products will be recorded and administered only to subjects for the purpose of this study. Ketoprofen is available in gel, cream, ointment

and gel-spray preparations (32). However, only the gel preparations will be utilised in this study. It will be used externally.

3. OBJECTIVES

The objective of this study is to measure dermal concentrations of ketoprofen using both microdialysis and tape stripping to assess *in vivo* bioavailability from a topical gel formulation and therefore obtain valuable information which will lay the foundation in an attempt to determine bioequivalence of ketoprofen topical formulations using these 2 techniques. In addition, the 2 techniques will be compared for their effectiveness in the assessment of dermal bioavailability.

4. STUDY POPULATION AND MEDICAL ASSESSMENT

4.1. Number of subjects

Similar studies have made use of 6-18 human subjects for this type of study (27,33-37) with equal numbers of male and female subjects. This study will make use of 10 human subjects and will make use of an equal number of male and female subjects. Four application sites on the left arm of each subject will be used for the microdialysis study whereas 2 application sites on the right arm of each subject will be used for the tape stripping studies. Preliminary statistical analysis (Section 8) will be performed on the subjects completing the study. If needed, an add-on study of up to 10 subjects will be conducted to obtain a degree of variability of less than 30% if possible. If no further study is required, preliminary statistical analysis will be finalised and presented in the final report. If an add-on study is conducted, data for all evaluable subjects from both Group 1 and Group 2 will be used in preparation of the final report. Homogeneity testing will be conducted between the 2 groups.

4.2. Inclusion/Exclusion criteria

Inclusion criteria

Only those subjects meeting the following criteria will be included in the study:

i. Female subjects who are using reliable contraception or abstaining.
ii. Subjects who are aged between 18 and 50.
iii. Subjects who are in general good health.
iv. Subjects who will be available for the entire study period.

Exclusion criteria

Subjects meeting the following criteria will be excluded from the study.

i. Female subjects who are breast feeding.
ii. Female subjects who are contemplating becoming pregnant in the time immediately following the study.
iii. Female subjects who are pregnant.
iv. Subjects who have a known allergy/hypersensitivity to ketoprofen or any NSAID including aspirin.
v. Subjects who have any history of drug or alcohol abuse.

vi. Subjects who have any mental deficiency or handicap.
vii. Subjects who have hairy ventral forearm surfaces and/or abrasions on the underside of their forearms.
viii. Subjects who have engaged in any sun-tanning or taken any sunny vacations within the last month.
ix. Subjects who have participated in another NSAID dermal microdialysis or tape stripping study within 2 months of the study date.
x. Subjects who have used any NSAIDs within the last 3 months.
xi. Subjects who suffer from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema).
xii. Subjects who suffer from any skin disorder such as psoriasis, eczema or other relevant skin disorder.
xiii. Subjects who take regular medicine or tablets or used any creams within the last week (contraceptive pills excluded).
xiv. Subjects who test positive for HIV and Hepatitis B.
xv. Subjects with a history of any neurological, kidney or liver disorders.

4.3. Subject restrictions

i. No prescription medication and OTC medication (e.g. cold preparations, vitamins, natural products used for therapeutic benefits, antacids, herbal or traditional remedies) will be allowed for at least 1 week prior to the study.
ii. With the exception of study product no concomitant medication may be taken by subjects during the study.
iii. No alcohol may be taken by subjects from 24 h prior to product application and during the study.
iv. No strenuous physical activity may be undertaken by subjects from 12 h before product application and during the study.
v. Subjects must not smoke more than 10 cigarettes per day and will not be allowed to smoke during the study.
vi. Subjects must refrain from applying emollients, skin conditioning creams and/or tanning lotions to their forearms for a period of 24 h prior to the scheduled time of product application.

Subjects will be informed of the above restrictions and each subject will be specifically questioned on these points prior to product application. Any deviations from the above restrictions which are made known to the investigators either voluntarily or on questioning will be recorded on the appropriate Case Report Form (CRF) (Appendix RP F). A decision as to whether the affected subject continues with the study will be taken by the principal investigator and the supervisor in consultation with the study nurse.

4.4. Criteria for removal from the study

Any subject may be withdrawn from the study at any time due to the following:

i. Voluntary withdrawal by the subject due to any reason.

ii. Illness or injury during the study if regarded as clinically significant by the principal investigator and the supervisor in consultation with the study nurse.

iii. Any adverse event or signs of toxicity if regarded as clinically significant by the principal investigator and the supervisor in consultation with the study nurse.

iv. Failure of the subject to comply with or be uncooperative towards any study requirements or restrictions if regarded as clinically significant by the principal investigator and the supervisor in consultation with the study nurse.

Subjects withdrawn or dropped out of the study will be fully documented and accounted for in the CRF.

5. STUDY PROCEDURE

5.1. Pre- and post-study medical screening

Pre-study screening will be conducted not more than 30 days prior to the start of the study. Post-study follow-up will be conducted within 48 h after removal of the microdialysis probes from the forearm of the subject either at the end of the study or termination of the study or withdrawal from the study. Pre-study and post-study evaluations will be conducted as listed in the table below.

Screening test	Pre-study	Post-study
Medical tests	√[1]	x
Medical history	√[2]	√[5]
Dermatological assessment	√[3]	√[6]
Adhesive sensitivity	√[4]	x

1. Medical tests: HIV, Hepatitis B and pregnancy test
2. Medical history: Demographic data (date of birth, age, sex, origin), skin (dermatological), allergies, alcohol consumption, smoking habits, dietary habits and sporting commitments.
3. Dermatological: General assessment of the volar aspect of the forearm and any dermatological condition which may influence the barrier function of the skin and impact on the absorption of topically applied NSAIDs.
4. Adhesive sensitivity: Assessment of subject's sensitivity to adhesive on application site demarcation tape.
5. Medical history: Since start of study.
6. Dermatological: Examination of forearms and application sites.

If any study related abnormalities are observed at the post-study medical, appropriate follow-up action will be taken and re-examination and re-testing conducted until the abnormality returns to normal or until the study nurse considers the abnormality to be clinically insignificant.

5.2. Medical tape sensitivity screening

No less than 30 days prior to the start of the study, volunteers will undergo an assessment of their tolerance to the adhesive on medical tape used to demarcate application sites and for tape stripping procedures.

The following screening protocol will be utilised.

i. The forearm of the subject will be washed 1 h prior to the adhesion of the medical tape.

ii. Adhesion of the medical tape will occur at 0700 h.

iii. Two different types of medical tapes to be employed as templates and for tape stripping will be used on each side of the arm.

iv. Six h after application, the medical tapes will be removed.

v. The forearm of the subject will be assessed for allergies to the adhesive medical tapes.

Subjects who exhibit allergies will not be eligible as candidates for the study.

5.3. Check-in and confinement

Subjects will check-in at the clinic (Room T17) in the Faculty of Pharmacy building, Rhodes University, Grahamstown at 0700 h on the morning of the study day when they will undergo a brief medical examination, inclusion and exclusion criteria check and study restriction check. Subjects accepted into the study will then be prepared for product application. Subjects will remain in the study room for the entire duration of the study.

5.4. Study design

The study will comprise a single phase sequential design conducted on 10 subjects in the first instance, followed by another 10 subjects if necessary (see section 4.1). Only a single subject will be studied at any one time on any study day and the time between the first and the tenth subject is expected to be approximately 6 weeks. A total of 4 probes and application sites will be employed on the left arm for the microdialysis study whereas 2 application sites will be employed on the right arm on each subject for the tape stripping study. The duration of the study for each subject will be approximately 10 h.

Microdialysis probes prepared as described in section 5.6 will be inserted approximately 0.6 to 0.8 mm beneath the skin surface for a distance of 3 cm as described in section 5.7. Probes will be inserted parallel to each other across the volar aspect of the left forearm approximately 3 cm apart using 21G x 1½" hypodermic needles as guide cannulae. Application of an ice pack will anaesthetise the area of cannulation. No other anaesthetic will be necessary and the procedure during and after probe insertion is essentially pain-free (20). Probes will remain in place for no longer than 8 h.

At the end of the microdialysis study, templates for use with the tape stripping study will be affixed on the right arm of the subjects with pieces of Micropore™ 1530 tapes. These will be used to demarcate (2 x 2 cm) the *stratum corneum* sampling site. The templates will be designed to have 2 sampling sites, 1 site for the application of the formulation and the other for use as control. The test formulation will be applied to 1 application site and after a 30 min exposure period the excess drug will be removed by wiping with a cotton swab. Adhesive tapes (Transpore™ 1527 tapes) will be applied concurrently to both application sites with uniform pressure and then subsequently removed. Fifteen (15) successive strips will be made from both sites. Transepidermal water loss measurements will be recorded from the control application site. This procedure will take approximately 1 h.

5.5. Pre-study day activities and procedures

This study is dependent on a number of preparations prior to the study. The following is a list of items that will be made available at least 24 h prior to the study.

i. The linear microdialysis probes will be manufactured in-house no more than 24 h before the study (Section 5.6).
ii. Microlitre glass (300 µl) inserts for use with the analytical procedure will be soaked overnight with chromic acid, rinsed thoroughly with double-distilled water and then soaked in methanol. This will be done no more than 48 h before the study.
iii. Calibration standards and mobile phase for ketoprofen analysis will be prepared.
iv. Pre-weighed sampling vials or centrifuge tubes will be labelled and ready for sample collection.
v. Sterilisation solution (70%, v/v ethanol) will be prepared.
vi. Microdialysis syringes, a pair of stainless steel scissors, a pair of tweezers, spatula, and a glass rod will be left overnight in sterilisation solution.
vii. The checklist will be performed (Appendix RP B).
viii. The general setup of the clinic will be ensured.

5.6. Linear microdialysis probe manufacture

The linear probes used for the study will be manufactured in-house by the principal investigator. The probes will be prepared no more than 24 h prior to each study day. The following manufacturing protocol will be used to prepare the linear probes for the study.

i. The desired lengths of the Portex® tube (30 cm), Hemophane, (8 cm) and stainless steel wire (10 cm) will be cut.
ii. Stainless steel wire will be degreased with acetone.
iii. Hemophane fibres will be carefully threaded with the stainless steel wire, which will provide mechanical strength to the probe assembly.
iv. A length of Portex® tube will be glued with cyanoacrylate glue (Loctite®) to one end of the Hemophane fibre-stainless steel wire setup. This assembly will now be referred to as the probe.
v. Probes will be left to dry for 2 h.
vi. Preliminary leak tests will be performed with double-distilled water.
vii. Blue tubing connectors will be attached to the Portex® end of the probes.
viii. Probes will be stored in a clean glass petri dish with cover and conveyed to the clinic.
ix. Six probes will be manufactured in the unlikely event that a probe fails the preliminary leak test.
x. The probes will be sterilised by soaking in ethanol solution (70%, v/v) 20 min before use in subjects.

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5.7. Study day activities and procedures

5.7.1. Microdialysis study

i. Study sessions will begin at approximately 0700 h each study day.

ii. Verification by history of adequate washout of excluded drugs.

iii. Checklist will be confirmed (Appendix RP B).

iv. CMA microdialysis syringes pre-sterilised over night (Section 5.5) will be flushed and filled with sterile normal saline (perfusate).

v. Probes will be connected to the syringes and a final 20 drop leak test will be manually performed using perfusate prior to installing the syringes into the CMA microdialysis pumps.

vi. The syringes connected to the probes will be housed in the CMA microdialysis pump and aligned.

vii. Subjects will be advised to take a bathroom visit.

viii. The left forearm of the subject will be rinsed with mild soap and water and then blotted dry with a non-abrasive towel.

ix. Subjects will be requested to either lie on a bed or sit in an arm chair.

x. The wrist will be loosely restricted using a bandage to prevent any sudden jerk during and after the insertion of the probes.

xi. Four parallel application sites aligned in a straight line from the elbow joint to the wrist will be demarcated on the ventral surface of the forearm of the subject using adhesive labels with a 1.0 cm x 3.0 cm application area cut out.

xii. Entry and exit points through which the guide cannulae (21G x 1½") will be inserted will be marked on the forearm at the ends of each application site.

xiii. Ice packs will be placed on each application site and, by employing sterile technique, guide cannulae will be inserted between 0.6 and 0.8 mm below the surface of the skin for a distance of 3 cm transversally across the volar forearm in parallel at the 4 sites. The application of ice pack at each site on the forearm may have to be repeated during the insertion process.

xiv. Care will be taken to maintain placement in the superficial dermis in order to minimise intra-probe depth variability.

xv. Probes which have been sterilised by soaking into ethanol solution (70%, v/v) will be taken from the ethanol bath and the Hemophane portion introduced through the guide cannulae.

xvi. The guide cannulae will then be removed leaving the Hemophane portion of the probe in the dermis with the Hemophane/Portex® junction abutting the dermal insertion point.

xvii. Portex® tubing at the end of each probe will be secured on the arm with Micropore™ tape.

xviii. Entry and exit points will be sealed with a drop of cyanoacrylate glue.

xix. Adhesive labels (templates) will be placed over the probes to demarcate the product application area.

xx. Perfusion of the probes will commence at 0.5µl/min for 15 min followed by 1.25 µl/min for 45 min.

xxi. Blank samples will be collected for 30 min.

xxii. Approximately 30 mg of the product will be applied to each site from a preloaded Eppendorf® pipette (Section 5.8). Individual doses of the product will be spread evenly over the application site using a glass rod.

xxiii. Samples of perfusate will be collected continuously in 30 min aliquots in a period of 5 h, i.e. 10 samples.

xxiv. At the end of the last sampling period, the Portex® tubing will be severed from the pump setup and if necessary will be further secured on the forearm of the subject.

xxv. Templates will be removed from the forearm of the subjects.

xxvi. Ultrasound scanning will be conducted to determine the exact depth of each probe underneath the surface of the skin.

xxvii. Probes will be carefully removed from the forearm of the subject ensuring that both the Hemophane fibre and the stainless steel wire are removed.

xxviii. The application site area will be dressed with alcohol swabs and small adhesive plasters.

xxix. Subjects will receive a tube of hydrocortisone cream (0.1%, m/m) to be used twice daily for 2 weeks on the forearm to reduce any swelling caused by the insertion of the probes.

xxx. The CMA microdialysis syringes, stainless steel scissors, tweezers, spatula, and glass rod will be sterilised overnight for use with the next subject.

NB: There will no re-use of apparatus inserted into subjects. Such apparatus will be discarded as biological waste.

5.7.2. Tape stripping study

i. Adhesive tape strips (Transpore™ 1527) for the tape stripping study will be pre cut.

ii. The template for use with tape stripping will be affixed onto the right forearm with Micropore™ (1530) tapes to delineate the application sites on the *stratum corneum*.

iii. The test formulation will be applied to 1 of the 2 application sites while the other site will be used as control.

iv. Excess test formulation will be removed from the application site after a 30 min exposure period using a cotton swab.

v. Tape strips will be applied with uniform pressure to both sites and then subsequently removed.

vi. Fifteen (15) successive strips will be removed from each site.

vii. The removal of strips will be done in a clockwise manner (N, E, S, W) ensuring that equal amount of *stratum corneum* is removed in each of the 4 sides.

viii. Transepidermal water loss measurements will be measured from the control site

ix. The template will be removed from the subject.

x. The forearm will rinsed to remove any residual drug.

The actual time of study procedures and/or results/comments obtained during the study will be recorded on the '*Registration of Data during Microdialysis Form*' (Appendix RP C). The tape stripping procedure will commence after the microdialysis procedure is complete.

5.8. Product application

Just prior to the product application, an Eppendorf® (0.5 ml) pipette will be filled with the test product. The setting will be adjusted (3 on the dial of the Eppendorf® dispenser) to ensure that the correct amount (30 µl) of the test product required for application on each site will be dispensed. The test product will be dispensed 3 times at each application site to allow 90 µl (± 30 mg) of test product (corresponding to 0.75 mg of ketoprofen) to be applied at each site. The Eppendorf® dispenser will be used to ensure that an accurate amount of test product is dispensed to each application site for each subject. Application of the test product will be done by the principal investigator.

5.9. Posture and physical activity

Subjects will be expected to lie on a bed or sit in an arm chair for the duration of the microdialysis and tape stripping procedure with the forearm restricted loosely with a bandage. Subjects will not be able to walk around from the time of the first probe insertion until the disconnection of the probes from the microdialysis pump. This will be a period of approximately 8 h. Strenuous exercises will not be permitted as described in section 4.3.

5.10. Food and fluids

Subjects will not be restricted with respect to food and fluid intake. However subjects will be advised to keep fluid intake to a minimum, so as to not develop the urge to visit the bathroom during the study.

5.11. Subject monitoring

The principal investigator will be present at all times during the study. Subjects will be asked open-ended questions about their health at the time of each assessment and any discomfort observed during the experience will be recorded in the CRF (Appendix RP F). The study nurse will be contactable by phone for the duration of the study.

6. SAMPLE ANALYSIS

Samples will be analysed for ketoprofen using a validated extraction procedure and UPLC/MSMS analytical method. Analysis will be done within 24 h after sample collection.

7. DATA ANALYSIS

For the microdialysis study, concentration-time profiles will be generated and the AUC will be calculated using the trapezoidal rule. The C_{max} and T_{max} will also be calculated. The data used to estimate AUC will be reported. Normalised concentration-strip profiles will be generated for the tape stripping study. Deletion of data from analysis will be justified. All individual subject data will be documented and the individual dermal concentration-time and concentration-strip curves will be presented in linear/linear and log/linear scale.

7.1. Statistical analysis

Pharmacokinetic and statistical parameters to determine bioequivalence will be calculated with a statistical package SAS® (SAS Institute Inc., Cary, North Carolina, USA). AUC_{0-5} will be tested for comparative bioavailability using a 2 one-sided test procedure and bioequivalence concluded if the 90% CI of the log

transformed AUC_{0-5} data for pairs of test/reference application areas are within the acceptance range of 80-125%.

8. ETHICAL AND REGULATORY REQUIREMENTS

8.1. Ethical and institutional review

Approval by the Rhodes University Ethical Standards Committee (RUESC) will be obtained before the study commences. The original signed copy of the ethical approval will be retained by the principal investigator.

The study will be conducted in accordance with the recommendations of the guidelines as set out in the Declaration of Helsinki (1964) and its amendments and carried out in compliance with the guidelines on the conduct of clinical trials in South Africa.

8.2. Written informed consent

Preceding the study, the nature, purpose and risk of participating in the study will be explained to all volunteers. If volunteers desire, they will be given time to consider the information and any questions that they might have will be answered. The nature of the insurance cover will also be explained. They will also be informed that they may withdraw from the study at any time without penalty to themselves (other than a reduced remuneration) but that they will be encouraged to be committed to completing the study prior to their enrolment. They will sign a consent form in the presence of a witness. Subjects will receive written, detailed instructions concerning the study performance and restrictions.

8.3. Case report form

The Case Report Form (CRF) for this study will be designed and supplied by the principal investigator (Appendix RP F). All case report forms will be quality assured and all major events such as final acceptance of a subject, adverse events and final release from the study will be signed by both the principal investigator and the study nurse.

8.4. Record retention

All source documents, study reports and other study documentation for which the principal investigator is responsible will be archived and retained by the Faculty of Pharmacy, Biopharmaceutics Research Group. Results will be published in the scientific journals and/or presented in a thesis submitted to Rhodes University in fulfilment of the requirement for the degree of Doctor of Philosophy (Pharmacy) after the completion of the study.

8.5. Insurance

Subjects will be insured against any permanent adverse effect on their health which may arise in connection with the conduct of the study. A copy of the certificate of insurance (Appendix RP G) will be provided to RUESC as part of the application to conduct this study. Adequate insurance cover in the event of negligence on the part of principal investigator, the supervisor and the study nurse will be ensured.

8.6. Termination of the study

The principal investigator reserves the right to terminate the study in the interests of subject welfare following consultation with the supervisor and the study nurse. The supervisor may terminate the study at any time for scientific or safety reasons. If the study is prematurely terminated or suspended for any reason the principal investigator will promptly explain to the subjects, take appropriate steps as deemed necessary under the circumstances to assure the subjects and where applicable follow up with therapy and inform the RUESC.

8.7. Adherence to protocol

Excluding an emergency situation in which proper treatment is required for the protection, safety and well-being of study subjects, the study will be conducted as described in the approved protocol. Any deviation from the protocol will be recorded and explained.

Should amendments to the protocol be required, the amendments will be documented and signed by the principal investigator and the supervisor. If the protocol amendment(s) has an impact on the safety of subjects, such as a change in dosing regimen or additional formulations, the amendment will be submitted to the RUESC for approval.

8.8. Blinding

Subjects will not be blinded and will be informed about the test product for use at the application sites.

8.9. Adverse events/Adverse drug reactions

Subjects will be questioned on their health status at check-in, during the course of the study and before leaving the clinic at the end of the study. During the study, open-ended questions will be asked. If any adverse events are reported, the principal investigator together with the study nurse will monitor the adverse event, initiate appropriate treatment if required and decide whether or not to withdraw the subject from the study. Signs and symptoms of any adverse events which occur during the study will be fully documented in the appropriate CRF (Appendix RP F). If necessary, adverse events will be referred to a suitably qualified medical practitioner for assessment and follow up.

Adverse events (AEs) (which include illnesses, subjective and objective signs and symptoms that have appeared or worsened during the course of the study) will be assessed by the principal investigator and the supervisor in consultation with the study nurse during and after the study to determine whether or not they are adverse drug reactions (ADR) to the investigational test product, related to the study procedure or other. The outcome of this assessment will be recorded in the appropriate CRF (Appendix RP F).

AEs classified as severe or serious will be reported to the supervisor/study nurse, Rhodes University Ethical Standards Committee within 24 h.

ADRs classified as serious and unexpected will be subject to expedited reporting as detailed in the ICH E2A and E2B guidelines on Clinical Safety Data Management and Data Elements for Transmission of Individual Case Report Forms respectively.

9. REPORTS

A full report on the study will be compiled by the principal investigator in the format requested by the supervisor and submitted to the supervisor. The analytical report will include results for all standard and quality control samples. A representative number of chromatograms or other raw data will be included covering the whole concentration range for all standards and quality control samples. The validation report will also be submitted.

ABBREVIATIONS

ADR	Adverse Drug Reaction
AE	Adverse Effect/Event
AUC	Area Under the Curve
BRG	Biopharmaceutics Research Group
C_{max}	Maximum Concentration
CRF	Case Report Form
GCP	Good Clinical Practice
ICH	International Conference of Harmonisation
RUESC	Rhodes University Ethical Standards Committee
SOP	Standard Operating Procedure
T_{max}	Maximum Time
UPLC/MSMS	Ultra Pressure Liquid Chromatography Mass Spectroscopy

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APPENDIX I: RP A

PRODUCT INFORMATION LEAFLET

SCHEDULING STATUS: S1

PROPRIETARY NAME (and dosage form):

Fastum® gel

COMPOSITION:
Each 100 g contains 2,5 g ketoprofen i.e. 2,5 % m/m
Preservatives: p-hydroxybenzoic acid esters 0,1 % m/m

PHARMACOLOGICAL CLASSIFICATION:
A 3.1 Antirheumatics (anti-inflammatory agents)

PHARMACOLOGICAL ACTION:
Ketoprofen is a non-steroidal anti-inflammatory agent. Since ketoprofen is an inhibitor of prostaglandin synthesis it provides for anti-inflammatory, analgesic effects. Fastum Gel is ketoprofen in an excipient suitable for allowing it to reach the site of inflammation by transcutaneous route, providing the local treatment of painful joints, tendons, ligaments and muscles.

INDICATIONS:
For the relief of localised pain and inflammation associated with acute musculo-skeletal injuries.

CONTRA-INDICATIONS:
Known hypersensitivity to any of the ingredients.
Safety of Fastum Gel during pregnancy and lactation has not been established.
Safety in children has not been established.

WARNINGS:
The prolonged use of products for topical application may cause hypersensitivity phenomena. In such cases the treatment should be discontinued and a suitable alternate therapy instituted.
Since the application of Fastum Gel may provoke photosensitisation, the treated skin area should not be exposed to the sun.

DOSAGE AND DIRECTIONS FOR USE:
Treatment should not exceed 7 days.
Persons 12 years and older: Apply to the affected area once or twice daily by gently massaging in order to help absorption. Apply 5 to 15 cm of gel with each application (100 - 300 mg ketoprofen).

SIDE EFFECTS AND SPECIAL PRECAUTIONS:
Considering the very low systemic absorption by topical application, systemic side effects are not expected, but cannot be excluded.
Side effects experienced with systemically absorbed ketoprofen include:

Gastro-intestinal, peptic ulceration, gastro-intestinal bleeding, headache, dizziness, nervousness, skin rashes, pruritus, tinnitus, oedema, depression, drowsiness, insomnia, blurred vision and other ocular reactions.
Sensitivity reactions, abnormalities of liver function tests, impairment of renal function including interstitial nephritis or nephrotic syndrome, agranulocytosis and thrombocytopenia may occur less frequently.
Fastum Gel should be used with caution in patients with asthma or bronchospasm, bleeding disorders, cardiovascular disease, peptic ulceration or a history of such ulceration, renal failure and in those who are receiving coumarin anticoagulants.
Fastum Gel should not be applied to open wounds or lesions of the skin, or near the eyes.
Do not apply to mucous membranes.

KNOWN SYMPTOMS OF OVERDOSAGE AND PARTICULARS OF ITS TREATMENT:
Treatment is symptomatic and supportive.

IDENTIFICATION:
A mucilaginous, colourless almost transparent gel with an aromatic odour.

PRESENTATION:
50 g and 100 g tubes.

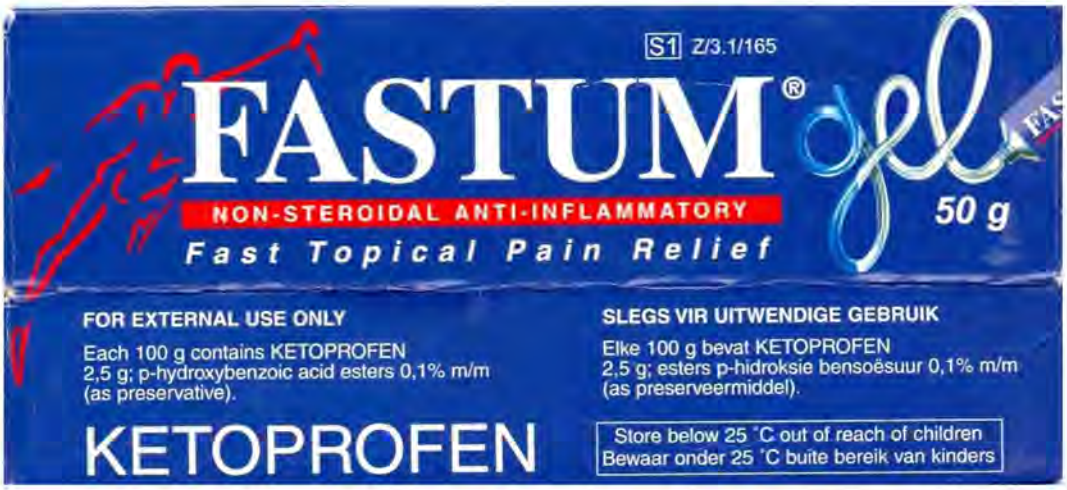
STORAGE INSTRUCTIONS:
Store below 25°C.
Keep out of reach of children.

REGISTRATION NUMBER:
Z/3.1/165

NAME AND BUSINESS ADDRESS OF THE APPLICANT:
MENARINI SA (PTY) LTD
Epsom Downs Office Park, Sloane St., Bryanston, 2152

Date of publication of this package insert: 13.12.91

Marketed by **RESTAN LABORATORIES**



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APPENDIX I: RP B

CHECKLIST

SUBJECT NAME		**SUBJECT INITIALS**		**DATE**	
STUDY NUMBER	**RNO_01_2007**	**VOLUNTEER REFERENCE**			
2.5 ml microdialysis syringes (labelled were necessary)					
21G 1½" intravenous needles					
3M Micropore™ tape (1530)					
3M Transpore™ tape (1527)					
Alcohol swabs					
Bandages					
Biological waste container (yellow)					
Calculator					
CMA 400 microdialysis pumps setup					
Cotton swabs					
Cyanoacrylate glue (Loctite® glue)					
Electrical cables and power supply					
Eppendorf® preparations					
Glass rod					
Glue remover-Bostix®					
Green cloth underlay					
Human subject under study					
Ice packs					
Pen/marker					
Perfusate solution-*Normal saline*					
Prepared probes attached to blue connectors (leaks pre-checked with water) in glass dish					
Pre-weighed and labelled sampling vials/centrifuge tubes					
Ruler					
Scissors					
Snack/Lunch/Supper					
Soap and towel					
Sterile gloves					
Sterilisation ethanolic solution (70%, v/v)					
Sterilisation trays					
Templates for sampling					
Thermometer					
Timer					
Tweezers					
Ultrasound scanner setup					
CHECKED BY:			**SIGNATURE:**		

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APPENDIX I: RP C

REGISTRATION OF DATA DURING MICRODIALYSIS EXPERIMENTS

Date		**Subject initials**	
Subject/Volunteer name		**Volunteer Reference**	
Age		**Study number**	**RNO_01_2007**
Sex			
Race			
Formulation			
Applied dose			
Site			
Principal Investigator			
Assistant principal investigator			
Room temperature			
Relative Humidity			

TIME SCHEDULE

Task	**Time start**	**Time stop**	**Comments**
Confirm Inclusion and Exclusion criteria			
Confirm consent form			
Confirm checklist			
Fill syringes with perfusate			
Connect probes to syringes			
Preliminary leak check (20 drops)			
Flush and align syringes in CMA 400 pump			
Bathroom visit (volunteer!!)			
Forearm wash			
Wrist bandage to be placed			
Mark entry and exit points on arm			
Probes put in ethanol soak (20 min)			
Remove probes from ethanol soak			
Ice pack on arm			
Insertion 1			
Ice pack on arm			
Insertion 2			
Ice pack on arm			
Insertion 3			
Ice pack on arm			

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Insertion 4			
Probe insertion (4 probes)			
21G intravenous needle removal			
Seal entry and exit points (15 min)			
Place test area (templates) over probes			
Secure Portex® on the arm with Micropore™			
Stabilise flow at 0.50 µl/min (15 min)			
Stabilise flow at 1.25 µl/min (45 min)			
Collect blank solutions			
Application of test formulation and commence sampling every 30 min			
End of microdialysis sampling			
Cut the Portex® tubing to the pump			
Remove the templates from the arm			
Ultrasound scanning			
Probes out of arm			
Dress area with alcohol swabs and bandages			
Ethanol soak of equipment			
End of study			

ULTRASOUND SCANNING

	Probe 1 (mm)		**Probe 2 (mm)**		**Probe 3 (mm)**		**Probe 4 (mm)**	
	Skin	**Probe**	**Skin**	**Probe**	**Skin**	**Probe**	**Skin**	**Probe**
Scan 1(a)								
Scan 2(b)								
Scan 3(c)								
Mean								
Std Dev								

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APPENDIX I: RP D

INFORMATION FOR VOLUNTEERS BROCHURE

APPLICATION OF DERMAL MICRODIALYSIS AND TAPE STRIPPING FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF KETOPROFEN IN HEALTHY VOLUNTEERS

STUDY NUMBER: RNO_01_2007

PROTOCOL VERSION 1

BROCHURE VERSION 1

JULY 2007

1. Study objective

The study involves research to assess the rate and extent to which ketoprofen diffuses into the skin from a generic product, using dermal microdialysis and tape stripping as a measure of efficacy. This study will provide valuable insight for use in the assessment of sameness of generic and proprietary ketoprofen formulations.

Test product	**Fastum® Gel**
Drug	Ketoprofen (2.5%, m/m)
Company	Adcock Ingram Limited
	South Africa

The study will involve 10 subjects.

2. Ethical considerations and standards of practice

Innovator drug companies who develop a new drug have patent protection for the drug for a number of years, typically 15-20. Once the patent has expired, generic drug companies are permitted to make and market their own products as competitors to the innovator's product, usually at a substantially reduced cost. However, a prerequisite for registration with national drug registration bodies (and subsequent marketing) is that the rate and extent to which the drug is absorbed from the generic product is shown to be equivalent to that from the innovator product. This is an indirect way of demonstrating that the generic product will be as effective clinically as the innovator and is demonstrated by what are known as 'comparative bioavailability or bioequivalence studies'.

2.1. Ethical and institutional review

Approval by the Rhodes University Ethical Standards Committee (RUESC) is required before the study can commence. The study will be conducted in accordance with guidelines set out in the Declaration of Helsinki (1964) and its amendments of Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West (1996) and Edinburgh (2000). This Declaration sets out ethical principles, which protect your rights for participating in such studies. Guidelines on the conduct of clinical trials in South Africa will be adhered to.

The study will also be conducted according to the ICH (International Conference on Harmonisation) Guidelines for GCP (Good Clinical Practice), the Biopharmaceutics Research Group's Standard Operating Procedures (SOP) and the requirements of RUESC.

2.2. Written informed consent

Preceding the study, the nature, purpose and risk of participating in the study will be explained to you. Should you wish, you will be given time overnight to consider the information and any questions that you might have will be answered. The nature of the insurance cover will also be explained. If you decide to participate in the study you will sign a consent form in the presence of a witness. You are encouraged to consult your parents or personal medical doctor for approval to partake in this study.

3. Voluntary nature of participation

Your participation in this study is entirely voluntary and you may withdraw from the study at any time, without prejudice. Should you decide to participate, we ask that you try to be committed to completing the study if at all possible. Should you encounter any problems along the way, please speak to me so that every effort can be made to assist you.

4. Dates and duration of the study phases

The study consists of 1 phase only which will run over a day. The study will be conducted from 0700 h until 1800 h.

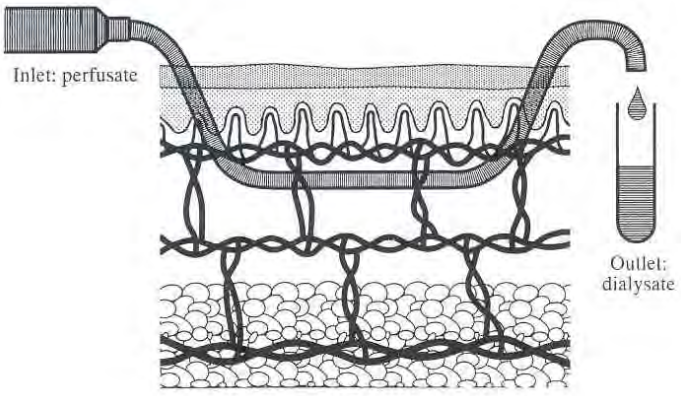
5. Place of study

The study will be conducted in the clinic (Room T17) in the Faculty of Pharmacy building at Rhodes University. Room T17 is on the top floor of the Pharmaceutical and Chemical Sciences building in Artillery Road on the Rhodes University campus. The principal investigator, supervisor and the study nurse will be suitably qualified, trained and experienced to perform the study procedures.

6. Background information

Ketoprofen is used in the management and treatment of patients with inflamed joints and muscles. In the treatment of adults, ketoprofen gel is usually applied to the affected area once or twice daily.

Microdialysis is a technique for sampling of natural and foreign substances in the space surrounding cells in living tissue. The technique was originally developed for brain research but has now been used extensively in other tissues in animal models and human studies. In human studies, this technique has been employed in adipose tissue, brain, heart, lung, solid tumours, skin etc. A microdialysis fibre consists of a semipermeable membrane forming a thin hollow 'tube' (~ 0.22 mm i.d.), which acts like a capillary. The fibre has one end connected to a tube which leads to a micropump and the other end to sampling tube. The probe is implanted in the dermis or the middle layer of the skin via a needle. The microdialysis fibre is slowly perfused with normal saline and substance in the skin will diffuse across the membrane into the saline which is collected and analysed. Substances move across the dialysis membrane by passive diffusion.



Dermal microdialysis sampling is described as a minimally invasive technique. The insertion of the microdialysis probes creates acute inflammatory reaction characterised by increased blood flow, erythema and swelling demonstrated with the use of a linear microdialysis probe by a 21G needle. This procedure has been shown to be safe and no irritation with the probes has been noted to date. Ice is used as an anaesthetic to provide minimum discomfort. No more discomfort is observed when the needle has been removed. You will be supplied with a tube of hydrocortisone (0.1%, m/m) cream to apply twice daily for 2 weeks to arrest any residual inflammation after the completion of the study. Sterile techniques will be employed during the study to ensure the minimum risk of skin infections.

Tape stripping is a technique also employed to study the penetration of drugs to the skin. Tape stripping involves the sequential removing of microscopic layers (typically 0.5-1 μm) of outer dead layers skin (*stratum corneum*) by placing an adhesive tape strip onto the skin surface with uniform pressure which is then subsequently removed. Tape stripping is simple and has been described as a minimal invasive technique. Although tape-stripping is considered to be essentially non-invasive, stripped sites in certain dark-skinned individuals may remain darker the usual for several months after healing.

7. Study design

On the left arm, 4 very thin microtubules which are 0.2 mm in diameter (*microdialysis probes*) will be inserted approximately between 0.6 and 0.8 mm beneath the skin and approximately 3 cm apart. Ice will be used as an anaesthetic to allow for minimum discomfort when the probes are inserted into the skin by means of a needle. Following this there will be no pain. Once the microdialysis probes are in place, you will not be able to move freely, but will have to remain lying on a bed or sitting in an arm chair with the opportunity to watch TV or read. During the study, the washout liquid which is pumped through the probes will be collected every 30 min. The microdialysis study will conclude at 1700 h, after the position of the probes under the skin has been ascertained by means of an ultrasound scanner. This is a quick pain free non-invasive technique which uses high frequency sound to measure the depth of the probe in the skin. Thereafter the probes will be removed from the skin and the area where the probes were inserted will be covered by small adhesive plaster strips.

At the end of the microdialysis study, templates for use with the tape stripping study will be affixed on your right arm with pieces of Micropore™ 1530 tapes. These will be used to demarcate (2 cm x 2 cm) the *stratum*

corneum sampling site. The templates will be designed to have 2 sampling sites, 1 site for the application of the formulation and the other for use as control. The test formulation will be applied to 1 application site and after a 30 min exposure period the excess drug will be removed by wiping with a cotton swab. Adhesive tapes (Transpore™ 1527 tapes) will be applied concurrently to both application sites with uniform pressure and then subsequently removed. 15 successive strips will be made from each site. The amount of moisture loss measurements will be recorded from the control application site using a small instrument which is placed on the skin. This is also entirely pain free. This procedure will take approximately 1 h. The entire study will conclude at 1800 h.

8. Adverse effects

For topical preparations, delayed hypersensitivity dermatitis may occur at the site of the application, but this is uncommon. Cases of reddening of the skin and inflammation of the skin have also been reported. It is advised that ketoprofen gel preparations should not be used on open wounds or lesions on the skin or near the eye. The skin reactions are however reversible on discontinuation of therapy. Ketoprofen must not be administered to healthy or ill individuals who have an allergy to this compound or to any of the ingredients in the formulation. In this study, a single application to a limited area is unlikely to invoke any adverse effects but procedures will be in place to address any discomfort noted during and after the study.

Microdialysis and tape stripping techniques are safe and no adverse reactions such as irritation or allergic reactions to the probes have been reported so far. The insertion of microdialysis probes creates slight but reversible swelling. This is caused by the use of a 21G needle for insertion of the linear microdialysis probe. No reactions have been reported to date in tissue around the probe 8-10 h after probe insertion. Ice is used to induce anaesthesia during probe insertion to provide minimum discomfort. No significant discomfort is observed when the needles have been removed. Sterile techniques will be employed during the study to ensure the minimum risk of skin infections. Although tape-stripping is considered to be essentially non-invasive, stripped sites in certain dark-skinned individuals may remain hyperpigmented for several months after healing.

9. Conditions of participation in the study

To participate in this study you must:-

i. Undergo and pass a medical assessment which includes giving a general medical history, recording of vital signs, body height and weight measurements and an assessment of your skin.
ii. Undergo a skin test to assess any possible allergy or sensitivity to the adhesive in the medical tape that will be used. After 6 h the tape is removed, the forearm washed and the skin inspected for any signs of reaction to the tape adhesive.
iii. Fulfil certain inclusion and exclusion criteria which have been set out in the protocol.
iv. Agree to be fully committed to the study and conscientiously abide by the restrictions required of you as listed in section 11.

NB: It is extremely important that you divulge any past medical history and abide by the rules for participation in this study. This is to protect you from unnecessary risks and to help ensure the reliability of the data gathered.

10. Volunteer inclusion/exclusion criteria

You will be considered for this study if you are aged between 18 and 50, in general good health, available for the entire study period and, if female, be on reliable contraception or abstaining from sex.

Please do not consider participating in this study if you

i. are breast feeding.
ii. are contemplating becoming pregnant in the time immediately following the study.
iii. are pregnant.
iv. have a known allergy/hypersensitivity to ketoprofen or any NSAID including aspirin.
v. have any history of drug or alcohol abuse.
vi. have any mental deficiency or handicap.
vii. have hairy ventral forearm surfaces and/or abrasions on the underside of their forearms.
viii. have engaged in any sun-tanning or taken any sunny vacations within the last month.
ix. have participated in another NSAID dermal microdialysis or tape stripping study within 2 months of the study date.
x. have used any NSAIDs within the last 3 months.
xi. suffer from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema).
xii. suffer from any skin disorder such as psoriasis, eczema or other relevant skin disorder.
xiii. take regular medicine or tablets or used any creams within the last week (contraceptive pills excluded).
xiv. test positive for HIV and Hepatitis B.
xv. have a history of any neurological, kidney or liver disorders.

11. Study restrictions

Restricted item	Duration of restriction	Examples of restriction	Comments
Prescription drugs	From 1 week before the start of the study, until the end of the study.	- all medication obtained on prescription - antibiotics, vaccinations - anti-inflammatories - anti-asthmatic drugs - anti-acne drugs etc.	This includes all long term medication.
Over-the-counter (OTC) drugs	From 1 week before the start of the study, until the end of the study.	- anti-flu drugs, sports supplements, antacids, paracetamol - vitamins, minerals -homeopathics	This includes herbs, natural products & all medications that can be bought without a prescription.
Alcohol	From 24 h before the start of the study and during the study. **No alcohol for a total of just over 2 days**	All alcoholic drinks and alcohol containing foods.	**It is important that this requirement is taken seriously and observed, as alcohol can significantly affect the liver.**
Strenuous Physical Exercise	From 12 h before the start of the study. **No strenuous exercise for a total of almost 2 days.**	Rugby Squash Rowing Gym Tennis etc.	Light exercise such as walking is permitted.
Smoking	No smoking will be permitted during the study.	Cigarettes	No cigars or pipe smoking.
Moisturising creams	You must refrain from applying any type of skin conditioning creams to their forearms from 24 h before the study until the end of the study	All skin creams, e.g. moisturizers, Vaseline, medicated creams, aqueous cream and tanning lotions.	This could interfere with the absorption of the study gel.

NB: Random checks will be done to ascertain whether you have managed to adhere to the above restrictions. If you have not been able to adhere to the study restrictions please inform us immediately or else you may not be able to participate and remuneration will be reduced as detailed in section 15.

12. Procedures and duration of the study

The study consists of a single phase. The procedures and duration of the study are detailed below.

i. On the morning of the study you must check into the clinic (Room T17) in the Faculty of Pharmacy building at 0700 h. At check-in you will be:-
   a. questioned and undergo a brief medical examination to establish whether you still fulfil all the inclusion and exclusion criteria since your pre-study medical examination and that you have complied with the study restrictions.
   b. prepared for product application by having medical tape applied to your forearms.

ii. You will be instructed to use the bathroom because when the study starts your wrist will be restricted and you will not be able to leave the bed or the chair for approximately 6 h.

iii. The study will commence at 0800 h and you will be expected to remain in the clinic throughout the course of the study.

iv. Entertainment and lunch will be provided.

v. You are not restricted with regard to food and drink during the study period although minimum fluid intake will be advised so as not to develop an urge to visit the bathroom during the study.

vi. Professional medical assistance (registered study nurse and/or a registered nurse) will be available throughout the course of the study.

vii. Should any new and significant information about the study medication become available during the course of the study, this will be communicated to you.

viii. A tube of hydrocortisone cream (0.1%, m/m) will be given to you to self medicate your arms twice daily for 2 weeks.

13. Benefits

Since you have been screened as healthy before the start of the study, there is no medical benefit to you from participating in this study.

14. Financial compensation

You will receive a gratuity of **R 500.00** for full participation in the study. Payment following withdrawal from the study will be calculated on a pro-rated basis from the start of the study at 0700 h to the end of the study at 1800 h.

15. Adverse medical events

You will be monitored prior to, during and after each study day for any adverse events whether or not they are thought to be related to the investigational products or procedures. If any adverse events are reported, the study nurse will decide whether or not to withdraw you from the study and what treatment is appropriate. In addition, the Rhodes University Ethical Standards Committee will be notified of any serious adverse events (SAEs). All adverse events will be monitored and treated until recovery to your pre-study status. You will be referred to a medical doctor if necessary and all medical costs will be covered by the BRG.

16. Insurance

You are covered by an insurance policy, taken out by the BRG, in case of claims arising from the medication or procedures as outlined in the protocol and in case of negligence on the part of the BRG. If you have personal insurance, e.g. life insurance/assurance, your participation in this study may affect your policy. You are advised to determine this prior to participating in the study. If you need any assistance in this regard please feel free to contact me. During the clinical study you are not entitled to participate in other studies and you should not participate in any other study/s for thirty (30) days after completion of this study to ensure that there are no interactions. Insurance policies will be available from the principal investigator for scrutiny.

17. Confidentiality

Your medical history and physical examination records and any other information or data generated during this study will be kept confidential. However, you must agree that all the above mentioned documentation and data can be released for any lawful purpose and released for publication in scientific journals and/or presentation in a thesis submitted to Rhodes University in fulfilment of the requirement for the degree of Doctor of Philosophy (Pharmacy) after the completion of the study. In such cases your name will be removed from all documentation to ensure your anonymity. In signing the consent form for the study, you agree to the granting of access to your medical data. Your medical data will be provided to you upon request and you will be informed of significant abnormalities identified before or after the study.

Your consent is also required to permit the medical staff at the BRG to consult with any study nurse who is normally responsible for your care if the need arises. The onus, however, is on you to inform such a practitioner of your intention to participate in such a study, should you so wish.

18. Amendments/Changes

Should there be any changes made to the trial protocol these will be communicated to you verbally and in writing in time to enable you to reconsider your decision to participate in the study.

19. Withdrawal

You may withdraw from the study at any time due to the following:

i. Voluntary withdrawal by yourself due to any reason.
ii. Illness or injury during the study if regarded as clinically significant by the principal investigator and the supervisor in consultation with the study nurse.
iii. Any adverse event or signs of toxicity if regarded as clinically significant by the principal investigator and the supervisor in consultation with the study nurse.
iv. Failure to comply with or being uncooperative towards any study requirements or restrictions if regarded as clinically significant by the principal investigator and the supervisor in consultation with the study nurse.

It is your right to withdraw from the study at any time. However, by signing the Informed Consent form and participating in the study, you agree to be committed to completing the study if at all possible.

20. Termination of the study

The BRG reserves the right to terminate the study prematurely in the interests of your welfare.

21. Emergency contacts

You are obliged to notify the principal investigator as soon as possible if you are unable to follow the procedures or if you suffer any adverse event we have not told you about. In particular, you should make every effort to contact me if you suffer a Serious Adverse Event (SAE) or need to take additional medication of any kind. This applies to out of h, as well as to normal work time.

In such cases of medical emergencies during the study, or if you have any urgent questions relating to adverse effects or unrelated illness, please feel free to telephone the study nurse, Sr. Kay Wentworth, at any time at the following phone numbers:

Work 046 603 8189 Home 046 622 6384 Cell 072 299 8230

22. Contacts for additional information

The following individuals are responsible for conducting this study and as such may be approached for more information:

Portfolio	**Name**	**Contact details**	
Principal Investigator	**Ralph Nii Okai Tettey-Amlalo** *BPharm, MSc, PhD Candidate (Rhodes)*	Work Home Cell Email	046 603 8142 046 603 8041 082 831 5320 r.tettey-amlalo@ru.ac.za
Supervisor	**Professor Isadore Kanfer** *BSc (Pharm), BSc (Hons), PhD (Rhodes)*	Work Email	046 603 8381/8382 i.kanfer@ru.ac.za

If you have questions about this study which have not been answered adequately by the principal investigator, supervisor or the study nurse, you should first discuss them with your doctor. After you have consulted your doctor and still dissatisfied you may contact the Rhodes University Ethics Standards Committee at:

Professor Roy Jobson
Chairman: Rhodes University Ethics Standards Committee
RHODES UNIVERSITY
Tel: 046 603 8399
Fax: 046 636 1205
Email: r.jobson@ru.ac.za

APPENDIX I: RP E

STUDY PARTICIPATION INFORMED CONSENT FORM

APPLICATION OF DERMAL MICRODIALYSIS AND TAPE STRIPPING FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF KETOPROFEN IN HEALTHY VOLUNTEERS

STUDY NUMBER: RNO_01_2007

PROTOCOL VERSION 1

CONSENT VERSION 1

JULY 2007

I ..... Born on.....

Present address .....

.....

hereby give permission that the necessary probes may be inserted into the surface of my skin and the microdialysis and tape stripping procedure conducted as described to me by the principal investigator and that the formulation stated below be applied to my skin during the course of this study.

Test product: Fastum® Gel (Ketoprofen 2.5%, m/m)-Adcock Ingram, South Africa

My consent is given freely and I realise that it may be withdrawn at any time, without penalty to me. Furthermore, I understand that I do not give up any of my legal rights by signing this consent form.

I have been fully informed by ..... regarding the possible adverse effects of the medication, procedures to be used in this study and the risks thereof, as detailed in the "Information for Volunteers Brochure". I will receive a copy of the information brochure and signed consent form for my records.

I undertake to comply with all the relevant conditions contained in the Information to Volunteers Brochure and confirm that I understand that it is important not to withhold or misrepresent any information asked of me. I agree to undergo the necessary pre- and post-study medical investigations as listed in the protocol.

I undertake to inform the study nurse and the principal investigator immediately of any symptoms-expected or unexpected-which I might experience.

I agree to my medical records being reviewed in the event of an audit, enquiry, monitoring and/or inspection on the understanding that my anonymity will be maintained.

I agree and consent that the BRG may consult with any study nurse who is normally responsible for care of study subjects. _____ Initial

I have been informed that if I do not adhere to the protocol, it may result in my exclusion from the study and forfeiture of the agreed upon remuneration. I acknowledge that instructions relating to my participation in this study have been communicated to me both verbally and in writing, and that I understand them.

I also declare that I have made the necessary arrangements regarding the attendance of lectures and other academic activities.

I understand that a policy to cover volunteers in clinical studies against death or disablement arising as a direct result of participation in such clinical studies has been taken out by the Biopharmaceutics Research Group. I accept the conditions of the policy as set out in the insurance policies.

I acknowledge that I will receive **R 500.00** for full participation in this study and that I will receive a pro-rated amount if I withdraw from the study before it has been completed.

_____ _____

Signature of volunteer **Date (yyyy-mm-dd)**

_____ _____

Signature of a witness **Date (yyyy-mm-dd)**

_____ _____

Principal Investigator **Date (yyyy-mm-dd)**
Ralph Nii Okai Tettey-Amlalo
BPharm, MSc, PhD Candidate (Rhodes)

Contact details of subject	
Telephone number	
Cell number	

APPENDIX I: RP F

CASE REPORT FORM

APPLICATION OF DERMAL MICRODIALYSIS AND TAPE STRIPPING FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF KETOPROFEN IN HEALTHY VOLUNTEERS

STUDY NUMBER: RNO_01_2007

PROTOCOL VERSION 1

CASE REPORT FORM VERSION 1

JULY 2007

Test product	**Fastum® Gel (ketoprofen 2.5%, m/m)-Adcock Ingram, South Africa**
Principal Investigator	**Ralph Nii Okai Tettey-Amlalo**
	BPharm, MSc, PhD Candidate (Rhodes)
Supervisor	**Professor Isadore Kanfer**
	BSc (Pharm), BSc (Hons), PhD (Rhodes)

Subject initials	
Volunteer reference number	
Study subject number	
Date of screening medical	

Pre-study examiner:	
Name and initials	
Qualifications	

MEDICAL HISTORY (to be completed by the examining study nurse)

DEMOGRAPHIC DATA

Birth Date	Age in Years	Sex (M/F)	Origin				Other
			White	Black	Oriental	Indian	

Height (m)	Weight (kg)	BMI (Body Mass Index) To be calculated by BRG Investigators	Not required

REVIEW OF PAST ILLNESS

System/Site	Illness? Y/N	Describe Abnormalities
Skin -Connective Tissue Any other illness?		

PHYSICAL EXAMINATION (to be completed by the examining study nurse)

VITAL SIGNS

Blood Pressure-Supine (mmHg)	Pulse (beats/min)	Oral Temperature °C

GENERAL SYSTEMS EXAMINATION

System/Site	Illness? Y/N	Describe Abnormalities
Skin-General Skin-Forearms		
Medical tape allergy pass		

INCLUSION/EXCLUSION CRITERIA

(NB: Unshaded areas-acceptable: Shaded areas-unacceptable)

	YES	NO
Will you be available for the entire study period?		
Are you in general good health?		
Are you aged between 18 and 50?		
Do you have eczema or scratch marks on the underside of your forearms?		
Do you suffer from any skin disorder such as psoriasis or other relevant skin disorder?		
Do you suffer from any neurological, kidney or liver disorders?		
Do you suffer from any allergic conditions (hayfever, allergic rashes, asthma or childhood eczema)?		
Have you engaged in any sun-tanning or taken any sunny vacations within the last month?		
Do you take any regular medicine (prescription or OTC)?		
Have you taken any medicine or tablets or used any creams within the last week (contraceptive pills excluded)?		
Have you participated in another NSAID dermal microdialysis study within 2 months of the study date?		
Have you used any NSAIDs within the last 3 months?		
Do you have any mental deficiency or handicap?		
Are you pregnant?		
Have any history of drug or alcohol abuse?		
Are you contemplating becoming pregnant in the time immediately following the study?		
Are you breastfeeding?		
Are you using reliable contraception (The contraceptive pill, minipill, IUD or abstinence)?		
Do you have a known allergy/hypersensitivity to ketoprofen or any NSAIDs including aspirin?		
Have you ever had a reaction to a local anaesthetic injection at the dentist or in casualty?		

VOLUNTEER ACCEPTABILITY /COMMENTS WITH REGARDEXAMINATION/INCLUSION/ EXCLUSION CRITERIA

Acceptable		Not Acceptable	
Notes/Comments			
Sign Date _____ Study nurse (dd/mm/yy)			
Sign Date _____ Principal investigator (dd/mm/yy)			

POST-STUDY REPORT/MEDICAL

SUBJECT/STUDY STATUS

Study Completed		Study Not Completed	
Comments (If "no" give reasons and procedure/follow-up undertaken)			
Sign Date _____ Study nurse (dd/mm/yy)			

CLINICAL EXAMINATION/SUBJECT RELEASE

Test		**Status/Comments**	
Medical Update			
Application Site Assessment			
Subject Released		Subject Retained for Further Investigation	
Sign Date _____ Study nurse (dd/mm/yy)			
Sign Date _____ Principal investigator (dd/mm/yy)			

APPENDIX I: RP G

CERTIFICATE OF INSURANCE

Biopharmaceuticals Research Institute
e-mailed

Alexander Forbes
RISK SERVICES

Attention: Mike Skinner 31 July 2007

Dear Mr Skinner

SUBJECT: Confirmation of Insurance

TO WHOM IT MAY CONCERN

This is to confirm that clinical trial volunteers are insured for accidental bodily injury and / or illness as a direct result of participation in clinical study no RNO-01-2007 subject to the terms and conditions of certificate 10/725/113 underwritten by Lloyds of London. This policy is renewable annually on the 1 January. The next renewal date is therefore 1 January 2008.

The compensation limits are as follows (per volunteer but not exceeding R5,000,000 in respect of all claims arising from one common test):

Death	: R500,000
Permanent Disability	: Such percentage of R500,000 as is specified for the particular disability
Medical Expenses	: R100,000 (first amount payable R250)

If you have any queries, please call.

Yours sincerely

Debra Tuohy
Direct Line: 041 -3928514

A Division of Alexander Forbes Group (Pty) Ltd
Co. Reg. No : 1969/018487/07
An Authorised Financial Services Provider - FSB/FSP Licence No : 9299
P O Box 27155, Greenacres, Port Elizabeth, 6057
270 Cape Road, Newton Park, Port Elizabeth, 6045
Tel: +27 (41) 392 8300 (s/b)
For your convenience, please refer to our direct lines
Website: www.alexanderforbes.com

A copy of the original certificate will be included in the application.

APPENDIX I: RP H

SCREENING MEDICAL AND HIV TESTING CONSENT FORM

APPLICATION OF DERMAL MICRODIALYSIS AND TAPE STRIPPING FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF KETOPROFEN IN HEALTHY VOLUNTEERS

STUDY NUMBER: RNO_01_2007

PROTOCOL VERSION 1

HIV CONSENT FORM VERSION 1

JULY 2007

I ..... Born on.....

Present address .....

.....

hereby:

i. Confirm that my consent is given freely and I realise that it may be withdrawn at any time, without penalty to me.

ii. Confirm that I have read the information contained in Appendix RP H.1 and have been informed of the tests to be undertaken.

iii. Confirm that I have been informed of the general procedure for clinical studies undertaken by the BRG.

iv. Confirm that I have attended an informed consent session specific to the clinical study for which I am volunteering at which the contents of the 'Information for Volunteers' Brochure, Version … were explained to me and a copy given to me to take home and read before making my decision.

v. Recognise that undergoing a screening medical does not ensure automatic inclusion into the clinical study.

vi. Agree to my medical records being reviewed in the event of an audit or enquiry on the understanding that my anonymity will be maintained.

vii. Agree to be tested for Hepatitis B.

viii. Agree to be tested for the Human Immunodeficiency Virus (HIV), which causes Acquired Immunodeficiency Syndrome (AIDS), and with respect to this test:

   a. I understand the information contained in the attached three-page HIV Informed Consent Document (Appendix RP H.1)

   b. I freely consent to the withdrawal of blood from me.

   c. I freely consent to the testing of that blood.

   d. I understand that the results will be kept confidential, except for the disclosure by the BRG of any reactive result to the doctor who performed the screening medical or a doctor of my choice.

   e. I have read the information in this document about what a test result means.

   f. I understand that the BRG will pay for 1 session of pre- and post-test counselling which will be conducted by a member of staff of the St Raphael Centre at 11 Donkin Street, Grahamstown 6139.

g. I understand that I should contact the study nurse who performed the screening medical or a doctor of my choice for further information and counselling concerning the HIV test if required.

h. I understand that I have the right to request and receive a copy of this form.

_____ _____

Signature of volunteer **Date (yyyy-mm-dd)**

_____ _____

Principal Investigator **Date (yyyy-mm-dd)**

Ralph Nii Okai Tettey-Amlalo

BPharm, MSc, PhD Candidate (Rhodes)

Appendix I: RP H.1 HIV SCREENING TEST INFORMATION SHEET

ALL CLINICAL STUDY VOLUNTEERS MUST READ THIS INFORMATION SHEET BEFORE THE TEST FOR HIV IS DONE

INTRODUCTION

This document contains the information that you have a right to be given before agreeing to be tested for HIV antibodies. The HIV antibody test (sometimes called the "AIDS test") is a test that will tell you whether or not you have been infected with HIV, the "AIDS virus". Below we set out your rights with respect to this test, information about HIV and AIDS and the AIDS test, any why the BRG wants to test you for HIV antibodies before it decides whether or not to include you in a clinical study.

WHAT ARE MY RIGHTS?

Your rights are:

i. Not to be tested for the AIDS virus without your free and informed consent.
ii. To be given all material information on the harms, risks and benefits of taking, or not taking, the AIDS test.
iii. To refuse to take the test. If you do this, you will not be able to participate in a clinical study.
iv. You will receive pre-test counselling which is private and confidential, and which will inform you more about the test and it's implications before you consent to the test. Pre-test counselling will be at the expense of the BRG.
v. To have your test result treated confidentially. The result will be made available to your doctor only with your prior consent. A test result will also be stored by the BRG. You have the right to access this information to check that it is correct.
vi. To 1 session of post-test counselling at the expense of the BRG, whether the test is positive or negative. After this you will be referred to a healthcare provider who will provide you with further counselling and treatment if a positive result is obtained.

WHAT IS HIV?

HIV is the virus that causes AIDS, and is sometimes called "the AIDS virus". While infected with HIV, and before a person develops AIDS, he or she will feel well or healthy. During this time, the person will be able to infect other people with the virus.

WHAT IS AIDS?

AIDS is the name for a number of illnesses that develop as a result of being infected with HIV. The AIDS virus attacks the immune system and leaves it unable to fight various illnesses. More than half of the people infected with the AIDS virus will get AIDS within 10 years of infection.

When you are sick with AIDS, you can usually no longer work. AIDS is a serious disease that eventually leads to death.

WHAT IS THE HIV TEST?

The HIV test checks your blood for antibodies to the AIDS virus. The test cannot tell you the date you were infected, or by whom you were infected. A sample of blood will be drawn from you. It will be sent to a pathologist's laboratory, where it will be tested.

HOW DO I BECOME INFECTED WITH THE VIRUS THAT CAUSES AIDS?

Almost all cases of infection result from sexual intercourse. The AIDS virus is transmitted in this way from one person to another through semen and vaginal fluids. The AIDS virus can also be passed on to babies through the mother's blood or through breast-feeding. Although rare, the AIDS virus can be transmitted by contact with infected blood-for example, through blood transfusions, through sharing needles during drug-of-abuse use or by inadvertent needle-stick injuries or spillage, where health care workers are especially at risk. Most cases of infection are transmitted from women to men, or from men to women. Men and women of all ages, races and religious beliefs can be infected with the AIDS virus. Homosexual transmission also occurs.

IS THERE A CURE FOR HIV AND AIDS?

There is no known cure for HIV or AIDS. Modern medical science as well as traditional healers have searched for cures for the AIDS virus. So far these efforts have been unsuccessful.

However, should you be HIV positive, by adopting a healthy life-style and having your HIV managed properly by health care workers, you can greatly enhance your quality of life before AIDS sets in. It is therefore of the utmost importance that you keep yourself both mentally and physically healthy in spite of being HIV positive. It is also possible that a cure may be found over this time.

WHY DOES THE BRG NEED TO TEST FOR THE AIDS VIRUS?

The BRG conducts clinical tests (called bioequivalence tests) on pharmaceutical formulations. These tests involve administering medications to study subjects and taking blood samples from each subject at various intervals during the study. Blood samples are then centrifuged and prepared for analysis, when the concentration of medication in each sample is measured. During the clinical and analytical process numerous people handle each blood sample e.g. the nurse who takes the sample, the technician who prepares the sample immediately after it is taken and the analyst who prepares the sample for analysis. The BRG is duty bound to ensure that the individuals who handle these blood samples are not at risk from contracting the AIDS virus. The BRG must also ensure that subjects who participate in the study are not at risk from contracting the HIV virus through any possible accidental contact with any blood while participating in the study. In addition, the BRG must satisfy import/export authorities in South Africa and the receiving country that samples in any shipment across international borders are non-infectious.

IS THE TEST ALWAYS CORRECT? CAN THERE BE MISTAKES?

The tests are usually very accurate, and are performed by registered pathology laboratories. If your test result shows that you are infected with the AIDS virus, you can have this confirmed by having further tests done at your own expense, or by going to the nearest clinic or public hospital for a free HIV test.

WHAT DOES IT MEAN IF THE TEST IS NEGATIVE?

If your test result is negative, this does not mean that you may not become infected in the future. If you engage in unprotected sex, you may be infected at some time in the future. You should think very seriously about the ways in which you can ensure that you are not infected in the future. In particular, you should consider using safer sexual practices, for example, a condom.

There is a time of approximately 6 weeks after infection when an HIV test will not detect the AIDS virus. This happens because the test for antibodies cannot detect them for a short while after infection. This time is called the “window period”. If you are in the “window period” your test results will be negative, although you are actually infected with the AIDS virus.

The chance of being in the “window period” is very small. If you suspect that you may have become infected recently and are in the “window period”, you can arrange to be tested again in about 3 or more months’ time at your own expense, or go to the nearest clinic or public hospital for a free test and counselling.

WHAT DOES IT MEAN IF THE TEST IS POSITIVE?

If your test result is positive, this means that you have been infected with the AIDS virus. A positive test result will mean that you will not be able to participate in any clinical study conducted by the BRG. The implications of a positive test result should be discussed with your doctor or the doctor who conducted your screening medical. The BRG will pay for 1 session for you to discuss these implications with a doctor.

WHAT ARE THE HARMS AND RISK OF THE AIDS TEST?

Many people do not understand the facts about infection with the AIDS virus. This has led to people infected with the AIDS virus being stigmatised and isolated by their families and communities. Some people have committed suicide. A positive test can lead to difficulties in seeking housing bonds, employment, as well as medical and dental treatment. Psychological difficulties might also arise. For these reasons, the BRG will keep your test results confidential.

WHAT ARE THE BENEFITS OF THE AIDS TEST?

If the test is negative, this can reassure you and help you make sure you do not become infected with the AIDS virus. A positive test result can offer an opportunity to get early treatment, to change life plans and to prevent infection of your sexual partners.

NOTIFICATION OF TEST RESULTS

If your test result is negative: You will be considered for inclusion into the clinical study.

If your test is positive: Because a trained person should deliver that information so that you can understand clearly what the test result means, you are asked in Appendix RP H.2 of this document to designate the doctor who conducted the screening medical or a doctor or clinic of your choice to deliver the test result to you.

Consequently it is of the utmost importance that you think carefully about the person who should receive the results. Should you not know who to name, please ask someone for assistance or suggestions.

You will be advised to contact the designated doctor or clinic, so that they can discuss the meaning of the test result with you. Please note that if you are asked or receive a letter to contact the designated doctor, that this does not automatically mean that the AIDS test result is positive, as many other medical impairments may lead to you not being able to participate in the clinical study and which should require further medical follow-up for your benefit.

Appendix I: RP H.II

This page must be detached from the document and taken with you when you go for your HIV test.

Section 1 must be completed by you, the clinical study volunteer.
Sections 2 and 3 must be completed by the person drawing the blood sample.

Section 1: **To be completed by the clinical study volunteer.**

I designate: i. The doctor who conducted the screening medical as the person to deliver a positive test result to me.

Signature of person being tested _____ Date _____

OR

I designate: ii The following doctor or clinic to deliver a positive test result to me.

Name _____

Address _____

_____

Signature of person being tested _____ Date _____

To be completed by the person drawing the blood sample.
(Please retain this document and forward to the BRG together with the HIV test results)

Section 2: **Identification of applicant for all pathological tests (must always be completed)**

Passport/Identity Number of person being tested: | | | | | | | | | | | | | |

Name of person being tested _____

Address _____

_____

Signature of person being tested _____ Date _____

Section 3: **Identification of and declaration by person drawing the sample.**

Name of person drawing the sample _____

Practice number _____

Address _____

_____

Signature of person being tested _____ Date _____

I have satisfied myself that the person being tested has received the Informed Consent Document, and I have verified the identity of the applicant and that he/she has freely consented to have the sample drawn and tested for HIV antibodies.

I have inspected the following document to verify the identity of the volunteer:

☐ valid South African identity document

☐ valid South African passport

☐ valid temporary South African identity document

☐ foreign passport

Signature of person drawing the sample _____ Date _____

APPENDIX I: RP I
VERBAL DELIVERY OF INFORMATION TO STUDY VOLUNTEERS

The announcement will be posted around campus.

Students then contact the principal investigator directly and relay their interest in participating in the study. A short and factual explanation is given of the study and female participants are specifically asked whether they are using reliable contraception (the contraceptive pill, minipill or an IUD) and whether any participant suffers from eczema or psoriasis.

The principal investigator then mails the participants information brochure to the participants at the place of residence of the study volunteer. As the announcement is made 1-2 weeks before the commencement of the study, ample time is allowed for the reading through of this information.

At the first meeting in the Seminar Room in the Faculty of Pharmacy building 30 min are allocated to a verbal explanation of the study and any questions. This provides an undisturbed physical environment and the time allocation of 30 min is realistic.

Study volunteers are then shown the clinic unit where the study will take place as well as the needles for the insertion of the probes etc. Thereafter questions from the study volunteers are answered.

Study volunteers will be given approximately 7 days in which to consider the written information and formulate questions. Should there be any additional questions regarding the preceding verbal information, study volunteers have an opportunity to consider these answers before a final decision is made with regard to participation.

Should the study volunteer continue to be interested in participating, the 2 days on which the study will be conducted are arranged. Thereafter the study volunteer signs the study participation informed consent form as well as the screening medical and HIV testing consent form which are also signed by the principal investigator and in the presence of a witness if required. Study volunteers are hereby included in the study and are given a copy of the Information for Volunteers Brochure and the signed consent forms as well as written instructions regarding the 2 pre-arranged study days.

The study volunteers will then be contacted to see the study nurse for medical screening as outlined in the protocol.

04 December 2007

Professor Roy Jobson
Chairman
Rhodes University Ethical Standards Committee
RHODES UNIVERSITY
GRAHAMSTOWN

Dear Professor Roy Jobson,

APPLICATION OF DERMAL MICRODIALYSIS AND TAPE STRIPPING FOR THE ASSESSMENT OF THE BIOAVAILABILITY OF KETOPROFEN IN HEALTHY VOLUNTEERS

STUDY NUMBER: RNO_01_2007, APPROVAL DATE 22/08/2007

PROPOSED AMENDMENTS TO THE PROTOCOL

The outcome of the first study showed some degree of variability with respect to the diffusion pattern of ketoprofen through the skin. In an attempt to minimise variability, a number of key suggestions have been identified and discussed, one of which involves increasing the amount of formulation applied onto the skin during the study.

We are of the opinion that the amount of formulation applied onto the skin is insufficient to generate reproducible results and the spreading of the formulation within the designated surface area may invariably cause alterations in diffusion pattern due to the rubbing effect of the principal investigator. Increasing the amount of applied formulation from 90 µl (± 30 mg) to 150 µl (± 50 mg) per application site will sufficiently occupy the surface area but more importantly will not require the principal investigator to spread the formulation over the application site with a glass rod.

It is unlikely that an increase to 150 µl (± 50 mg) representing 1.25 mg of ketoprofen will cause any topical or systemic toxicity since this amount only represents 0.625% of an oral solid dosage form of ketoprofen (200 mg ketoprofen tablet).

We are also of the opinion that the widespread use of the forearm may also contribute to variability.

The following amendments to the protocol relating to study RNO_01_2007 are submitted herewith for your consideration.

Section 2.1 Description

Current Dosage: 90 µl (± 30 mg) per application site

Proposal Dosage: 150 µl (± 50 mg) per application site

Section 2.2 Supply, storage and use

Current Approximately 30 mg of the product will be applied to each site from a preloaded Eppendorf® pipette (Section 5.8.). Individual doses of the product will be spread evenly over the application site using a glass rod.

Proposal Approximately 50 mg of the product will be applied to each site from a preloaded Eppendorf® pipette (Section 5.8.). The product will be dispersed evenly across the entire surface area directly from the Eppendorf® dispenser

Section 5.4. Study design

Current Probes will be inserted parallel to each other across the volar aspect of the left forearm approximately 3 cm apart using 21G x 1½" hypodermic needles as guide cannulae.

Proposal Probes will be inserted parallel to each other across the volar aspect of the left forearm approximately 1.5 cm apart using 21G x 1½" hypodermic needles as guide cannulae.

Section 5.8. Product application

Current Just prior to the product application, an Eppendorf® (0.5 ml) pipette will be filled with the test product. The setting will be adjusted (3 on the dial of the Eppendorf® dispenser) to ensure that the correct amount (30 µl) of the test product required for application on each site will be dispensed. The test product will be dispensed 3 times at each application site to allow 90 µl (± 30 mg) of test product (corresponding to 0.75 mg of ketoprofen) to be applied at each site. The Eppendorf® dispenser will be used to ensure that an accurate amount of test product is dispensed to each application site for each subject. Application of the test product will be done by the principal investigator.

Proposal Just prior to the product application, an Eppendorf® (1.0 ml) pipette will be filled with the test product. The setting will be adjusted (5 on the dial of the Eppendorf® dispenser) to ensure that the correct amount (150 µl) of the test product required for application on each site will be dispensed. The test product will be dispensed 6 times at each application site to allow 150 µl (± 50 mg) of test product (corresponding to 1.25 mg of ketoprofen) to be applied at each site. The Eppendorf® dispenser will be used to ensure that an accurate amount of test product is dispensed to each application site for each subject. Application of the test product will be done by the principal investigator.

All these above changes are reflected in the relevant sections.

Thank you.

Yours sincerely,

……..
Ralph Nii Okai Tettey-Amlalo
BPharm, MSc, PhD Candidate (Rhodes)
Principal Investigator

Rhodes University
Faculty of Pharmacy
Biopharmaceutics Research Group
PO Box 94
Grahamstown 6140
South Africa
Tel: +27 82 831 5320
Fax: +27 46 636 1205

06 August 2007

Professor Roy Jobson
Chairman
Rhodes University Ethical Standards Committee
RHODES UNIVERSITY
GRAHAMSTOWN

Dear Professor Roy Jobson,

APPROVAL FOR ADVERTISEMENT

The Biopharmaceutics Research Group (BRG) wishes to conduct a dermal microdialysis and tape stripping study on healthy human volunteers for the assessment of the bioavailability of ketoprofen within the next 6 months. We would like to post advertisements around Rhodes University campus in order to obtain volunteers for these studies. The BRG wishes to seek the approval from the Rhodes University Ethical Standards Committee.

Attached is a copy of the advert.

Thank you.

Sincerely,

……..
Ralph Nii Okai Tettey-Amlalo
BPharm, MSc, PhD Candidate (Rhodes)
Principal Investigator

Rhodes University
Faculty of Pharmacy
Biopharmaceutics Research Group
PO Box 94
Grahamstown 6140
South Africa
Tel: +27 82 831 5320
Fax: +27 46 636 1205

BRG

ADVERTISEMENT

Valid - 01 July 2007 to 30 Dec 2007

CLINICAL STUDY VOLUNTEERS

The **B**iopharmaceutics **R**esearch **G**roup is looking for

HEALTHY FEMALE and MALE VOLUNTEERS

to participate in clinical studies on a medicated gel applied to the skin using microdialysis and tape stripping

If You Are:

1. Over 18 years
2. Generally healthy and currently medicine free
3. Willing to spend a day in the clinic

Then You May Be Eligible to Participate

You Will Be Remunerated For Participating

If You Are Interested Please Contact **Ralph** as soon as possible for More Information

Preferably by E-mail at: r.tettey-amlalo@ru.ac.za

Or by Phone at: 046 603 8412/8041

This advert has been approved by the Rhodes University Ethical Standards Committee

Please take a tear-off slip below

BRG Microdialysis Study
r.tettey-amlalo@ru.ac.za
046 603 8412/8041

BRG Microdialysis Study
r.tettey-amlalo@ru.ac.za
046 603 8412/8041

BRG Microdialysis Study
r.tettey-amlalo@ru.ac.za
046 603 8412/8041

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046 603 8412/8041

BRG Microdialysis Study
r.tettey-amlalo@ru.ac.za
046 603 8412/8041

APPENDIX II

Dermal microdialysis (DMD) data

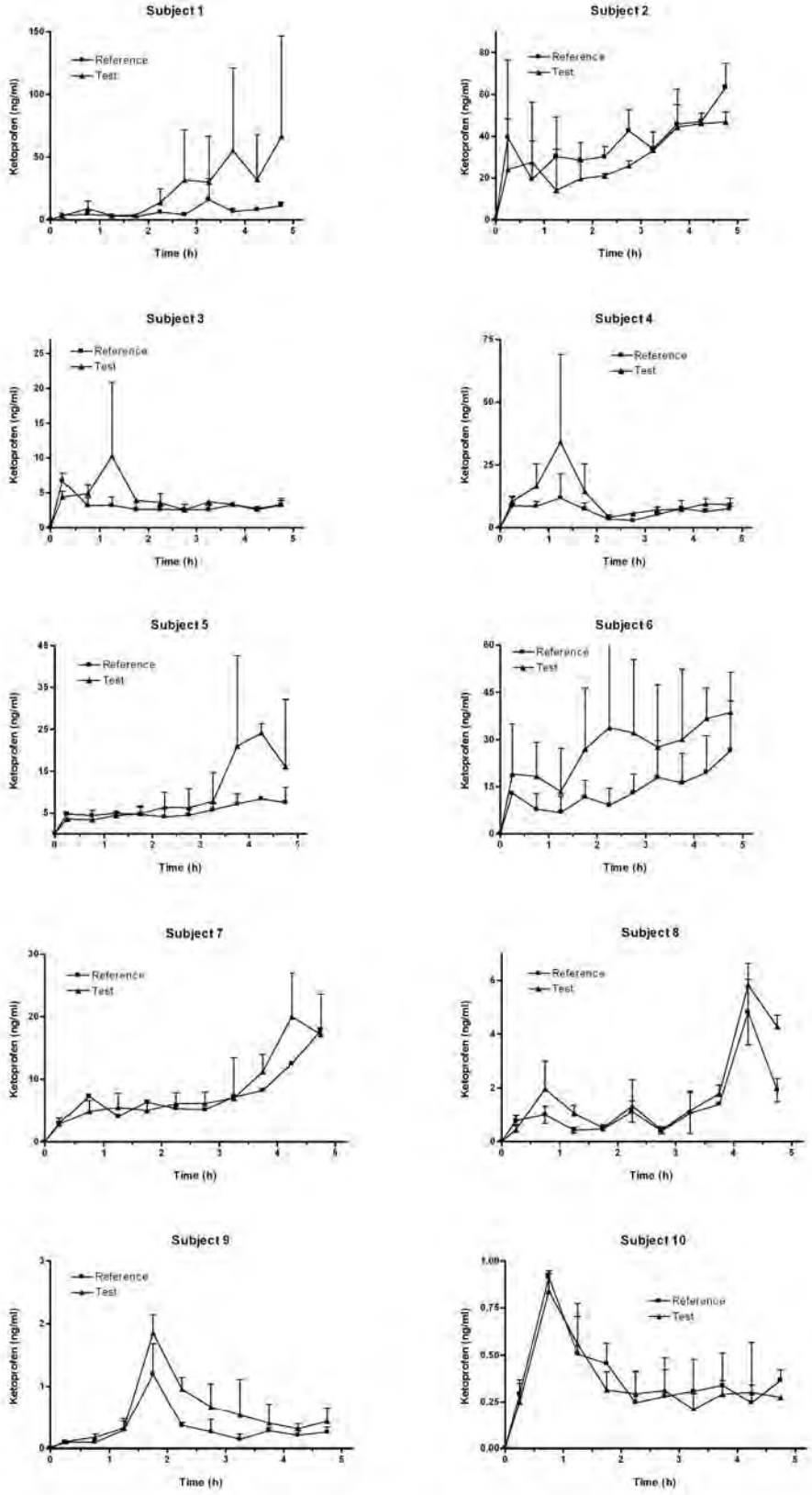
HUMAN STUDY I: RAW DATA

SUBJECTS 1-10

Subj.	Probe	Seq.	0.00	0.50	1.00	1.50	2.00	2.50	3.00	3.50	4.00	4.50	5.00	AUC	C_{max}	T_{max}
01	**01**	**T**	0.0000	4.2416	13.3215	2.8012	3.9082	21.5950	59.9007	55.9513	101.5902	57.6006	122.9518	**191.1931**	**122.9518**	**5.0**
01	**02**	**T**	0.0000	2.4882	4.5263	4.0683	3.3559	6.0308	3.6054	4.3639	8.3811	6.9381	9.4782	**24.2485**	**9.4782**	**5.0**
01	**03**	**R**	0.0000	2.4882	4.5263	4.0683	3.3559	6.0308	3.6054	4.3639	8.3811	6.9381	9.4782	**24.2485**	**9.4782**	**5.0**
01	**04**	**R**	0.0000	4.9653	4.5016	2.2994	1.9398	6.3765	4.6980	28.0982	5.3051	9.4794	13.2773	**37.1510**	**28.0982**	**3.5**
02	**01**	**T**	0.0000	41.2054	47.8023	28.1066	25.3921	20.0423	27.5562	32.0817	57.0918	46.8488	50.1682	**175.6056**	**57.0918**	**4.0**
02	**02**	**R**	0.0000	65.5830	32.3122	16.9837	34.4546	33.7039	49.8111	39.7452	52.3429	49.8521	55.4569	**201.2586**	**65.5830**	**0.5**
02	**03**	**T**	0.0000	7.0033	7.1050	0.1083	13.9701	21.9657	24.1591	34.1787	31.5818	45.2110	43.5205	**103.5216**	**45.2110**	**4.5**
02	**04**	**R**	0.0000	13.2518	6.5506	43.7009	22.5465	26.8118	35.4272	28.1740	39.3617	44.2079	71.4772	**147.8855**	**71.4772**	**5.0**
03	**01**	**T**	0.0000	4.9982	5.7805	2.8012	3.9082	4.4959	1.9811	3.5065	3.5053	2.7347	3.9681	**17.8479**	**5.7805**	**1.0**
03	**02**	**R**	0.0000	5.8953	3.9849	4.0683	3.3559	2.2909	2.6420	2.9999	3.4244	2.8935	3.7336	**16.7110**	**5.8953**	**0.5**
03	**03**	**R**	0.0000	7.5154	2.3574	2.2994	1.9398	2.9539	2.5893	2.2183	3.2060	2.3261	2.7979	**14.4023**	**7.5154**	**0.5**
03	**04**	**T**	0.0000	3.8554	4.0434	17.7612	3.9221	2.7423	3.1461	3.9326	3.2338	2.7422	2.7184	**23.3692**	**17.7612**	**1.5**
04	**01**	**R**	0.0000	11.4475	7.3044	18.6599	9.2060	4.3332	3.4222	7.4222	6.9839	5.7292	6.0152	**38.7580**	**18.6599**	**1.5**
04	**02**	**T**	0.0000	9.0760	10.4612	58.7778	22.3461	4.9031	6.1848	7.8330	9.8994	10.9356	11.0619	**72.9740**	**58.7778**	**1.5**
04	**03**	**T**	0.0000	11.8058	22.8661	9.4412	6.6323	3.3578	5.3242	6.3950	5.0577	8.0104	7.4699	**41.3127**	**22.8661**	**1.0**
04	**04**	**R**	0.0000	5.8746	9.8954	5.1546	5.7921	2.6552	2.5166	3.1203	8.2365	7.2369	9.2156	**27.5450**	**9.8954**	**1.0**
05	**01**	**R**	0.0000	5.1251	3.5658	5.2209	3.4340	2.5229	3.2698	4.7119	5.4670	8.6188	5.0276	**22.2251**	**8.6188**	**4.5**
05	**02**	**T**	0.0000	3.2392	2.9698	4.9668	3.5251	3.9158	3.0454	2.9094	5.7313	22.5646	4.7361	**27.6177**	**22.5646**	**4.5**
05	**03**	**R**	0.0000	4.4246	5.2554	4.6800	5.8964	5.5425	5.7572	6.7485	8.8779	8.1018	10.0665	**30.1588**	**10.0665**	**5.0**
05	**04**	**T**	0.0000	3.8297	3.7674	3.7802	6.1777	8.8891	9.4378	12.6351	36.2514	25.7147	27.3977	**62.0910**	**36.2514**	**4.0**
06	**01**	**R**	0.0000	12.5534	11.2573	3.1873	15.4034	12.8707	17.2727	26.1776	22.8822	27.8161	37.6048	**84.1117**	**37.6048**	**5.0**
06	**02**	**R**	0.0000	13.2206	4.1903	10.3879	7.8645	5.2455	8.9168	9.6126	9.3852	11.1867	15.3221	**43.8356**	**15.3221**	**5.0**
06	**03**	**T**	0.0000	30.2682	10.5672	3.6304	13.1248	13.2086	15.6476	13.3737	14.1294	29.7661	29.6661	**79.2745**	**30.2682**	**0.5**
06	**04**	**T**	0.0000	7.7996	25.8956	23.1164	40.7211	54.2914	48.5452	41.6920	45.8549	43.5809	47.7053	**177.6749**	**54.2914**	**2.5**
07	**01**	**T**	0.0000	2.7130	3.6225	3.8738	3.9097	4.8910	4.8431	2.3660	9.2589	15.1107	12.5857	**28.4409**	**15.1107**	**4.5**
07	**02**	**T**	0.0000	3.0937	6.0791	7.0871	6.1372	7.3391	7.4555	11.5846	13.2130	24.9413	21.7426	**48.9010**	**24.9413**	**4.5**
07	**03**	**R**	0.0000	3.6091	7.1515	3.9853	6.2581	5.2236	5.0512	7.0418	8.0920	12.2771	17.4810	**33.7151**	**17.4810**	**5.0**
07	**04**	**R**	0.0000	2.4260	7.4140	4.1320	6.4878	5.4155	5.2368	7.3002	8.3888	12.7269	18.1210	**34.2942**	**18.1210**	**5.0**
08	**01**	**T**	0.0000	0.5835	1.2351	0.8501	0.5867	2.0193	0.5249	0.6510	2.0200	6.4042	3.9738	**8.4309**	**6.4042**	**4.5**
08	**02**	**R**	0.0000	0.6593	0.7751	0.3535	0.4084	1.3999	0.3258	1.6071	1.3831	5.6825	2.2207	**6.8525**	**5.6825**	**4.5**
08	**03**	**T**	0.0000	0.3144	2.6974	1.2695	0.4074	0.6612	0.3537	1.6727	1.5405	5.2612	4.5916	**8.2369**	**5.2612**	**4.5**
08	**04**	**R**	0.0000	0.9256	1.2360	0.4971	0.5472	0.8486	0.4685	0.5294	1.4085	3.9656	1.6116	**5.6161**	**3.9656**	**4.5**
09	**01**	**T**	0.0000	0.1163	0.1389	0.2097	1.6435	1.0873	0.4034	0.9425	0.1779	0.2505	0.5871	**2.6317**	**1.6435**	**2.0**
09	**02**	**R**	0.0000	0.1086	0.0950	0.3882	1.5416	0.3970	0.4028	0.1980	0.2457	0.1518	0.3149	**1.8430**	**1.5416**	**2.0**
09	**03**	**R**	0.0000	0.0755	0.0913	0.1892	0.8485	0.3292	0.1181	0.0819	0.3128	0.2577	0.1988	**1.2017**	**0.8485**	**2.0**
09	**04**	**T**	0.0000	0.0962	0.2126	0.4266	2.0607	0.8293	0.9354	0.1206	0.6226	0.3688	0.2798	**2.9064**	**2.0607**	**2.0**
10	**01**	**R**	0.0000	0.2418	0.8918	0.6961	0.3794	0.3666	0.3827	0.4274	0.3560	0.3123	0.4045	**2.1281**	**0.8918**	**1.0**
10	**02**	**T**	0.0000	0.3213	0.9078	0.6615	0.3830	0.2124	0.4355	0.2707	0.4469	0.4888	0.2696	**2.1314**	**0.9078**	**1.0**
10	**03**	**T**	0.0000	0.1815	0.7721	0.4554	0.2479	0.3763	0.1900	0.1517	0.1385	0.1135	0.2810	**1.3836**	**0.7721**	**1.0**
10	**04**	**R**	0.0000	0.3468	0.9384	0.3149	0.5317	0.1281	0.1766	0.1862	0.3205	0.1803	0.3278	**1.6438**	**0.9384**	**1.0**

HUMAN STUDY I: DIALYSATE CONCENTRATION-TIME PROFILES

SUBJECTS 1-10



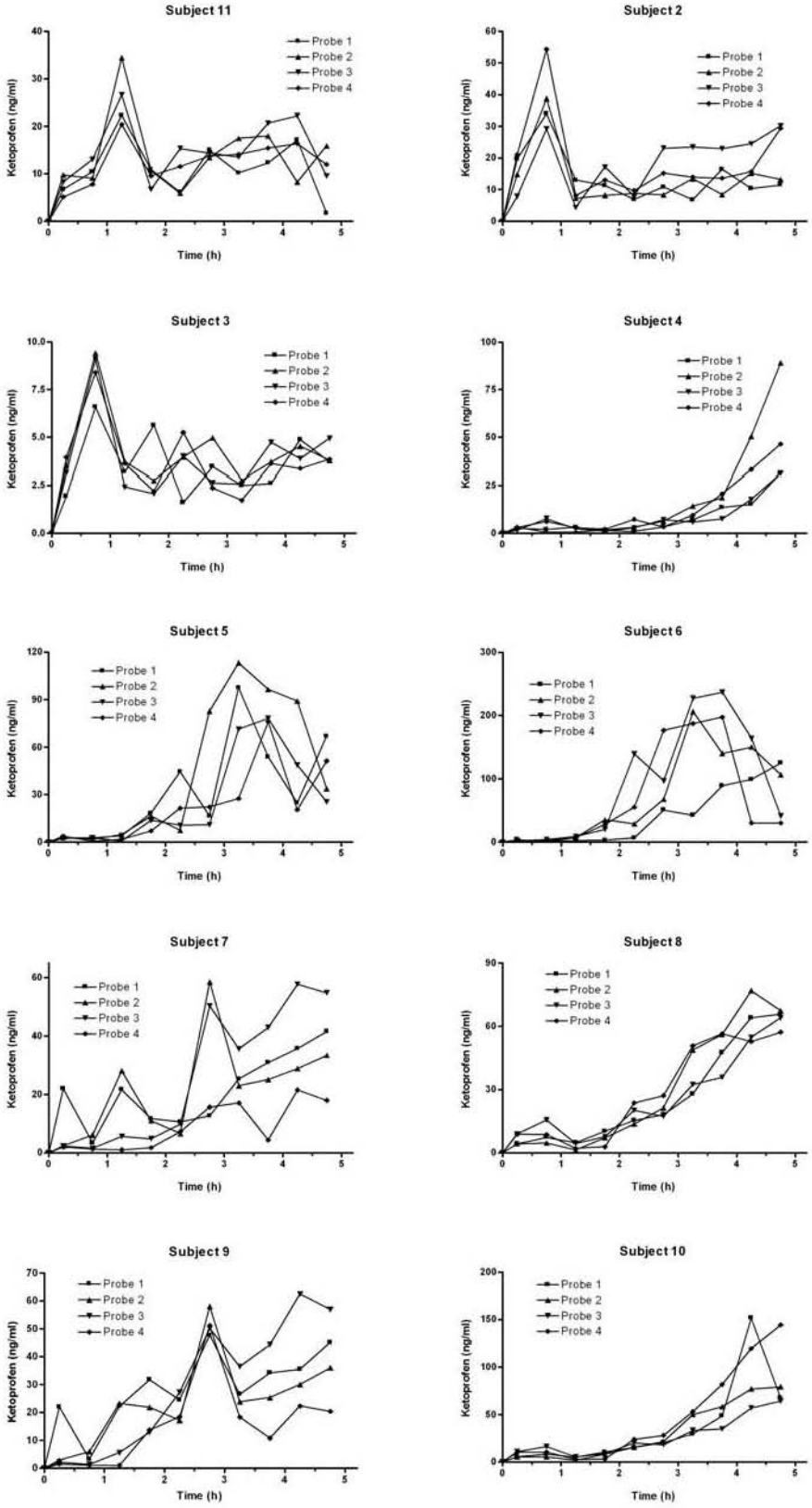
HUMAN STUDY II: RAW DATA

SUBJECTS 1-10

Subj.	Probe	Seq.	0.00	0.50	1.00	1.50	2.00	2.50	3.00	3.50	4.00	4.50	5.00	AUC	C_{max}	T_{max}
1	**01**	**T**	0.0000	6.7276	10.4586	22.3493	10.9305	6.1350	15.0188	10.1972	12.3049	17.1054	1.7470	**56.0504**	**22.3493**	**1.5**
1	**02**	**T**	0.0000	9.7485	9.0833	34.4454	10.6966	5.9267	13.4572	17.4782	17.9788	8.2443	15.9143	**67.5081**	**34.4454**	**1.5**
1	**03**	**R**	0.0000	8.3261	13.0650	26.6472	6.7616	15.3397	14.3951	13.5278	20.6545	22.2416	9.5310	**72.8621**	**26.6472**	**1.5**
1	**04**	**R**	0.0000	5.1168	7.7864	20.3816	9.6006	11.5565	13.7572	14.0845	15.4197	16.3877	11.9878	**60.0425**	**20.3816**	**1.5**
2	**01**	**T**	0.0000	19.7273	33.9988	13.0571	11.2770	6.8826	10.8528	6.7661	16.4792	10.3395	11.4770	**67.5594**	**33.9988**	**1.0**
2	**02**	**R**	0.0000	14.8239	38.7934	7.3047	8.2162	8.8179	8.4140	13.4556	8.4862	15.1435	13.2411	**65.0380**	**38.7934**	**1.0**
2	**03**	**T**	0.0000	7.9099	29.2352	4.3944	17.1835	7.9338	23.0997	23.4757	22.9498	24.4874	30.1433	**87.8705**	**30.1433**	**5.0**
2	**04**	**R**	0.0000	21.0471	54.3318	8.1127	13.0321	9.7274	15.1783	13.9299	13.6397	15.6432	29.4574	**89.6854**	**54.3318**	**1.0**
3	**01**	**T**	0.0000	1.9458	6.5936	3.1815	5.6285	1.6081	3.4648	2.4885	2.6084	4.8664	3.7042	**17.1189**	**6.5936**	**1.0**
3	**02**	**R**	0.0000	3.5155	9.4307	3.7156	2.7562	3.9629	4.9623	2.7596	3.7114	4.5238	3.7817	**20.6145**	**9.4307**	**1.0**
3	**03**	**R**	0.0000	3.1409	9.0993	2.4181	2.0668	4.0135	2.6388	2.5761	4.7306	3.8777	4.9407	**18.5161**	**9.0993**	**1.0**
3	**04**	**T**	0.0000	3.9451	8.4003	3.6232	2.2024	5.2545	2.3682	1.7294	3.6272	3.3583	3.8417	**18.2148**	**8.4003**	**1.0**
4	**01**	**R**	0.0000	2.5120	2.0746	3.1419	1.4367	1.0671	3.4195	7.0912	13.5946	15.2104	31.3607	**32.6142**	**31.3607**	**5.0**
4	**02**	**T**	0.0000	3.3111	0.6220	0.7561	1.7387	3.0389	6.2862	14.3329	18.6103	50.3687	89.0471	**71.7943**	**89.0471**	**5.0**
4	**03**	**T**	0.0000	1.5605	7.7509	2.2715	1.1075	2.8349	7.1236	5.9290	7.6118	17.6618	31.1881	**34.7229**	**31.1881**	**5.0**
4	**04**	**R**	0.0000	2.9655	6.1997	2.8731	2.1762	7.3537	3.4061	9.5004	20.7125	33.0971	46.4004	**55.7423**	**46.4004**	**5.0**
5	**01**	**R**	0.0000	2.5878	2.6612	4.1436	18.2829	44.4740	16.5822	97.5562	53.7450	24.7688	66.9927	**149.1491**	**97.5562**	**3.5**
5	**02**	**T**	0.0000	3.1581	1.8326	4.6545	16.4457	7.5844	82.7465	113.2915	96.5684	89.2353	33.7471	**216.1954**	**113.2915**	**3.5**
5	**03**	**R**	0.0000	2.3332	2.7087	0.7356	13.6731	10.7012	11.0176	71.3438	78.1871	48.7607	25.4548	**126.0942**	**78.1871**	**4.0**
5	**04**	**T**	0.0000	3.7243	0.5770	1.8964	7.0309	21.4253	22.1799	27.5853	76.2709	20.4192	51.3912	**103.4025**	**76.2709**	**4.0**
6	**01**	**R**	0.0000	3.9930	0.9097	2.0378	3.1720	6.6872	50.8585	42.3540	89.1891	99.0291	125.0330	**180.3735**	**125.0330**	**5.0**
6	**02**	**R**	0.0000	2.9800	0.5901	7.2347	35.1691	28.7840	67.7161	206.0861	140.1496	150.0479	106.5921	**346.0268**	**206.0861**	**3.5**
6	**03**	**T**	0.0000	1.9173	4.3615	8.8464	20.3191	139.4382	96.8757	227.4555	236.6054	163.9448	41.1038	**460.1579**	**236.6054**	**4.0**
6	**04**	**T**	0.0000	2.0412	2.8341	7.2560	28.3400	55.3103	176.5206	187.2228	197.4352	30.3264	30.2230	**351.1991**	**197.4352**	**4.0**
7	**01**	**T**	0.0000	21.9967	3.3500	21.6660	11.6452	10.6278	12.6777	25.3603	30.9824	35.7411	41.5445	**97.4097**	**41.5445**	**5.0**
7	**02**	**T**	0.0000	2.4354	6.1485	28.1112	11.0024	6.6134	58.5008	22.9639	25.0788	28.8848	33.3987	**103.2192**	**58.5008**	**3.0**
7	**03**	**R**	0.0000	2.3648	1.5717	5.6320	4.8520	9.9087	50.2571	35.5165	42.9050	57.7418	54.7519	**119.0628**	**57.7418**	**4.5**
7	**04**	**R**	0.0000	1.9832	1.2916	1.0747	1.7686	7.3056	15.6421	17.1010	4.3453	21.5160	17.9565	**40.5032**	**21.5160**	**4.5**
8	**01**	**T**	0.0000	4.3155	7.3875	4.8644	10.3280	15.3663	18.1394	27.8698	47.5847	64.0113	65.5789	**116.3283**	**65.5789**	**5.0**
8	**02**	**R**	0.0000	4.2991	4.6686	1.4536	7.1888	13.6949	21.3460	48.7009	56.1584	76.8715	67.3351	**134.0247**	**76.8715**	**4.5**
8	**03**	**T**	0.0000	8.9104	15.6116	4.3818	7.6764	20.2143	17.4092	32.3651	35.7410	54.7883	64.0000	**114.5490**	**64.0000**	**5.0**
8	**04**	**R**	0.0000	8.8031	8.7040	2.1417	2.8629	23.6611	27.1350	50.7068	56.4882	52.8060	57.1799	**130.9494**	**57.1799**	**5.0**
9	**01**	**T**	0.0000	21.9803	3.2583	22.1567	31.7638	24.5229	47.7787	26.5781	34.2195	35.4851	45.1539	**135.1602**	**47.7787**	**3.0**
9	**02**	**R**	0.0000	2.9493	5.9844	23.2296	21.8831	17.2168	58.0215	23.8387	25.3814	30.0817	36.0519	**113.3061**	**58.0215**	**3.0**
9	**03**	**R**	0.0000	2.2377	1.4122	5.5760	12.9027	27.2437	50.1988	36.4921	44.3918	62.5535	56.9179	**135.7337**	**62.5535**	**4.5**
9	**04**	**T**	0.0000	1.5833	1.1011	1.0519	13.7456	18.4784	51.2317	18.3563	10.8117	22.3617	20.3914	**74.4587**	**51.2317**	**3.0**
10	**01**	**R**	0.0000	5.5589	8.0602	5.1420	10.2798	15.3650	20.5945	29.5438	48.6304	151.5543	67.4964	**164.2385**	**151.5543**	**4.5**
10	**02**	**T**	0.0000	5.4741	5.3888	1.7199	7.9957	15.4752	21.4875	49.9931	58.2558	76.9537	79.1699	**141.1643**	**79.1699**	**5.0**
10	**03**	**T**	0.0000	10.9555	16.3444	5.3762	8.6144	20.1386	18.1768	33.2716	34.8640	56.9841	64.1825	**118.4084**	**64.1825**	**5.0**
10	**04**	**R**	0.0000	10.0651	10.3732	2.4990	3.0979	23.9031	28.0318	53.0289	81.6866	119.4402	144.2851	**202.1342**	**144.2851**	**5.0**

HUMAN STUDY II: DIALYSATE CONCENTRATION-TIME PROFILES

SUBJECTS 1-10



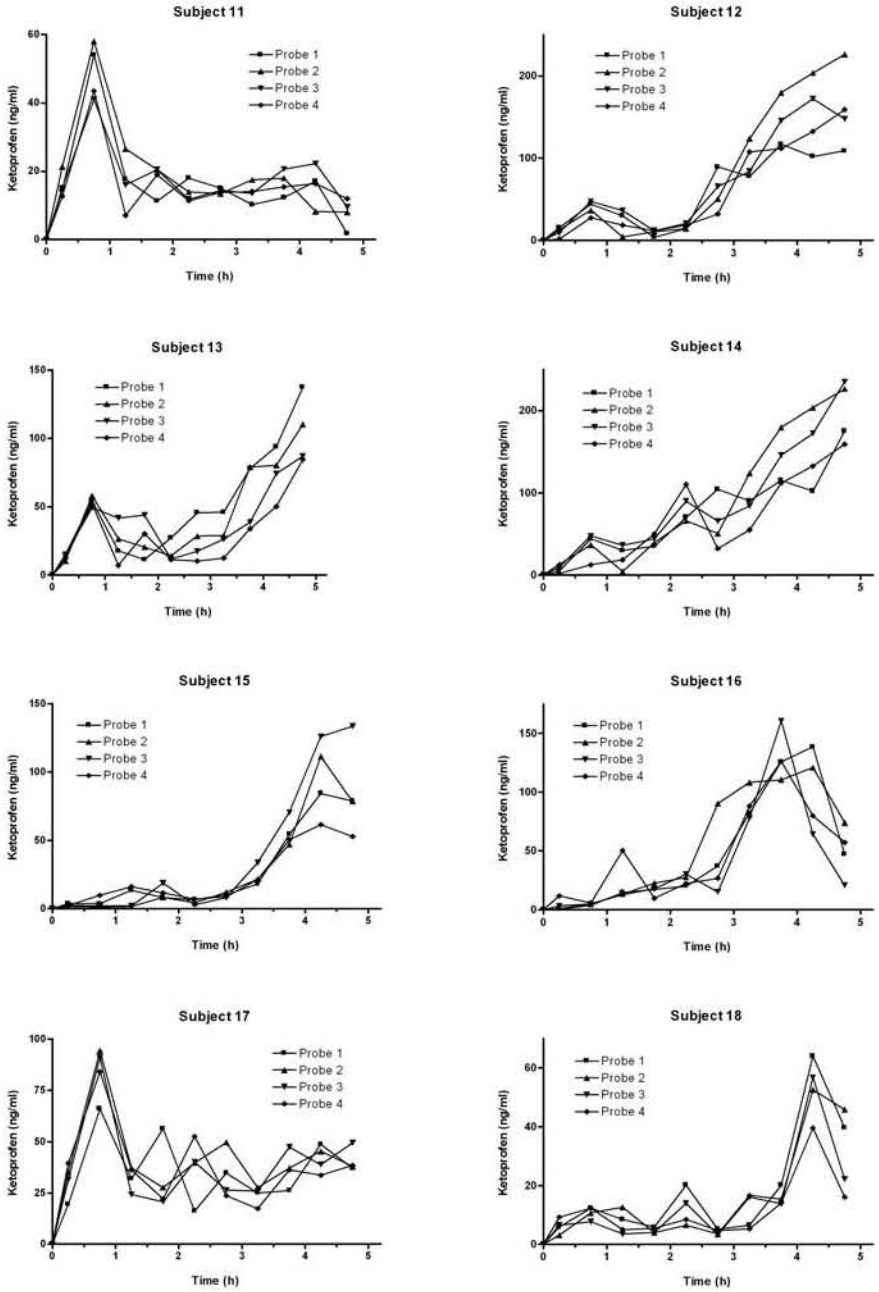
HUMAN STUDY III: RAW DATA

SUBJECTS 11-18

Subj.	Probe	Seq	0.00	0.50	1.00	1.50	2.00	2.50	3.00	3.50	4.00	4.50	5.00	AUC	C_{max}	T_{max}
11	**01**	**T**	0.0000	14.4310	54.0495	17.7209	11.2525	18.0000	15.0188	10.1972	12.3049	17.1054	1.7470	**85.4768**	**54.0495**	**1.0**
11	**02**	**R**	0.0000	21.3363	58.0341	26.4813	20.4475	13.9641	13.4572	17.4782	17.9788	8.2443	8.0000	**100.7109**	**58.0341**	**1.0**
11	**03**	**R**	0.0000	15.0805	41.0000	16.0000	20.5303	11.8565	14.3951	13.5278	20.6545	22.2416	9.5310	**90.0260**	**41.0000**	**1.0**
11	**04**	**T**	0.0000	12.6682	43.4703	7.1019	19.0000	11.3750	13.7572	14.0845	15.4197	16.3877	11.9878	**79.6292**	**43.4703**	**1.0**
12	**01**	**R**	0.0000	15.4522	44.1218	29.9852	4.0592	14.4009	89.5496	77.7944	116.9299	102.1406	108.9380	**274.4514**	**116.9299**	**4.0**
12	**02**	**T**	0.0000	13.2034	36.8828	4.4767	10.9855	14.4851	50.4903	123.7180	179.5443	203.6156	226.0511	**375.2137**	**226.0511**	**5.0**
12	**03**	**T**	0.0000	9.4033	47.5746	36.4060	12.1246	21.1022	65.7159	84.1857	145.6124	172.2625	147.7076	**334.1205**	**172.2625**	**4.5**
12	**04**	**R**	0.0000	1.8034	27.4433	18.6027	11.7935	18.6950	32.1461	107.6487	111.7778	132.5374	159.0367	**270.9831**	**159.0367**	**5.0**
13	**01**	**R**	0.0000	15.2369	54.0495	17.7209	11.2525	27.1768	45.6953	45.9393	77.8985	93.9606	137.6628	**228.8808**	**137.6628**	**5.0**
13	**02**	**T**	0.0000	10.2568	58.0341	26.4813	20.4475	13.9641	28.7027	28.8543	79.0475	80.2690	110.2598	**200.5936**	**110.2598**	**5.0**
13	**03**	**R**	0.0000	15.0805	49.2635	41.6252	43.9233	11.8565	17.3966	26.2769	38.7245	74.2339	87.0576	**180.9549**	**87.0576**	**5.0**
13	**04**	**T**	0.0000	12.3695	52.0123	7.1019	30.2506	11.3750	10.1895	12.4199	33.9552	50.1609	84.6237	**131.0733**	**84.6237**	**5.0**
14	**01**	**R**	0.0000	5.2695	44.1218	29.9852	35.0256	70.2698	104.3034	90.2650	115.2360	102.1406	175.2369	**342.1176**	**175.2369**	**5.0**
14	**02**	**R**	0.0000	13.2034	36.8828	4.4767	39.2654	66.0664	50.4903	123.7180	179.5443	203.6156	226.0511	**415.1443**	**226.0511**	**5.0**
14	**03**	**T**	0.0000	9.4033	47.5746	36.4060	44.2657	90.1260	65.7159	84.1857	145.6124	172.2625	235.0125	**406.5292**	**235.0125**	**5.0**
14	**04**	**T**	0.0000	1.8034	12.6044	18.6027	50.1260	110.2360	32.1461	55.1884	111.7778	132.5374	159.0367	**302.2702**	**159.0367**	**5.0**
15	**01**	**T**	0.0000	3.6812	3.5633	13.3667	8.0988	6.4356	10.0538	18.0588	54.3802	84.2468	78.6770	**120.6119**	**84.2468**	**4.5**
15	**02**	**T**	0.0000	1.4946	1.2369	1.4740	8.3003	4.1675	11.9873	21.5909	46.9825	111.1997	78.3863	**123.8134**	**111.1997**	**4.5**
15	**03**	**R**	0.0000	2.2771	1.8989	2.0341	18.6449	2.7904	8.0868	33.6313	70.3735	125.7261	133.3561	**166.0705**	**133.3561**	**5.0**
15	**04**	**R**	0.0000	2.3608	9.7041	16.2343	11.4844	6.8756	8.6912	21.1018	50.2946	61.3612	52.6881	**107.2260**	**61.3612**	**4.5**
16	**01**	**T**	0.0000	0.6167	3.8177	14.7619	17.4697	20.1776	37.2315	82.0543	125.6404	138.6396	47.1296	**231.9872**	**138.6396**	**4.5**
16	**02**	**R**	0.0000	1.1404	4.9341	13.5456	22.6711	27.6619	90.3323	108.2506	110.6190	120.9815	73.9598	**268.5582**	**120.9815**	**4.5**
16	**03**	**T**	0.0000	3.3164	4.9368	12.8874	17.9032	30.2919	15.1921	78.0820	160.7158	64.3005	20.8619	**199.0285**	**160.7158**	**4.0**
16	**04**	**R**	0.0000	11.8234	5.7665	50.4250	9.8400	22.4978	26.9038	88.2617	126.2654	79.9087	57.3235	**225.1771**	**126.2654**	**4.0**
17	**01**	**T**	0.0000	19.4579	65.9358	31.8152	56.2850	16.0814	34.6482	24.8855	26.0838	48.6645	37.0421	**171.1892**	**65.9358**	**1.0**
17	**02**	**R**	0.0000	35.1551	94.3074	37.1559	27.5618	39.6294	49.6233	27.5960	37.1140	45.2377	37.8175	**206.1447**	**94.3074**	**1.0**
17	**03**	**R**	0.0000	31.4087	90.9931	24.1812	20.6682	40.1351	26.3885	25.7613	47.3061	38.7770	49.4067	**185.1612**	**90.9931**	**1.0**
17	**04**	**T**	0.0000	39.4512	84.0029	36.2323	22.0243	52.5454	23.6817	17.2936	36.2724	33.5832	38.4172	**182.1478**	**84.0029**	**1.0**
18	**01**	**R**	0.0000	5.8347	12.3512	8.5013	5.8672	20.1931	5.2489	6.5097	20.2004	64.0421	39.7381	**84.3088**	**64.0421**	**4.5**
18	**02**	**T**	0.0000	3.1436	10.6987	12.6950	4.0738	6.6123	3.5366	16.7269	15.4051	52.6123	45.9159	**74.2311**	**52.6123**	**4.5**
18	**03**	**T**	0.0000	6.5927	7.7508	3.5347	4.0839	13.9986	3.2576	16.0714	13.8315	56.8253	22.2070	**68.5250**	**56.8253**	**4.5**
18	**04**	**R**	0.0000	9.2563	12.3600	4.9714	5.4718	8.4864	4.6850	5.2936	14.0846	39.6557	16.1161	**56.1614**	**39.6557**	**4.5**

HUMAN STUDY III: DIALYSATE CONCENTRATION-TIME PROFILES

SUBJECTS 11-18



APPENDIX III

Tape stripping (TS) data

HUMAN STUDY IV: RAW DATA

SUBJECTS 1-10

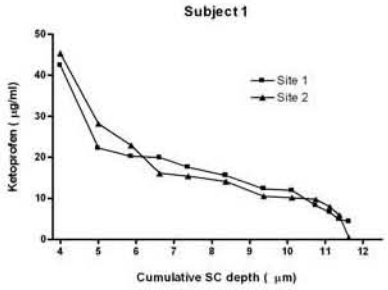
Subj.	Site	1	2	3	4	5	6	7	8	9	10	11	12	AUC
1	1	42.3912	22.2378	20.2322	19.9153	17.5882	15.5646	12.3254	11.8592	8.2439	6.6036	4.8727	4.3852	**131.2627**
1	2	45.3275	28.1802	22.8971	16.0467	15.4081	14.1061	10.4792	10.1751	9.7598	7.9889	5.8869	0.4032	**132.3731**
2	1	42.2022	31.7573	19.7553	14.1761	9.2882	6.4688	5.5492	4.9958	4.8870	4.4387	3.1589	2.2188	**91.5679**
2	2	122.1778	73.4519	32.0932	22.8971	15.0465	12.6567	11.8129	11.5983	11.2316	8.4899	7.4075	6.7258	**198.9422**
3	1	140.9083	114.5712	112.7614	78.9718	76.5177	49.0711	32.1645	14.9009	7.2278	6.3471	3.8810	3.2837	**203.0251**
3	2	51.6552	48.5940	38.0230	32.0932	31.5500	29.9840	27.2041	25.7322	10.1344	5.3775	3.8936	3.4743	**99.0895**
4	1	59.4629	46.5917	15.9614	8.3744	6.5541	4.6055	4.1700	3.4347	2.3641	2.1332	1.9022	1.4475	**88.9335**
4	2	28.2395	21.4225	11.0227	9.7269	8.3412	6.8537	3.8331	2.8203	2.2024	1.7856	1.4017	1.3803	**56.7731**
5	1	69.4183	55.2788	45.3756	35.8250	34.0491	31.4760	13.1795	12.9820	6.7086	4.8072	4.5904	3.6875	**271.3461**
5	2	32.3637	20.0972	15.8519	13.3936	11.2729	10.0381	4.6313	3.6579	2.5552	1.8645	1.3807	1.1263	**98.8002**
6	1	105.9720	93.3092	46.1362	32.8487	21.9377	17.1670	11.0729	10.7796	6.8727	4.9146	3.1869	2.6728	**205.2040**
6	2	86.8081	39.9175	26.2624	16.0280	14.4263	10.3056	8.4425	6.4182	2.9986	2.5535	1.9930	1.9906	**117.9588**
7	1	59.6675	31.6140	20.0465	13.5737	9.8199	5.2165	2.9667	1.7894	1.3643	0.8533	0.6778	0.6659	**110.2500**
7	2	34.3950	29.0770	16.9121	7.3560	5.9262	5.1185	3.2219	2.6415	2.3676	1.6971	1.0435	1.0254	**84.5746**
8	1	33.6617	18.1776	12.2061	10.2937	8.9318	6.9893	5.9556	2.7128	2.5626	2.2555	2.0350	1.0389	**75.8011**
8	2	30.1484	20.1330	10.9333	10.1908	5.6715	5.4660	3.9538	3.2340	3.0054	2.1852	1.7169	1.1699	**68.4090**
9	1	49.0579	27.7417	17.9987	14.6675	12.2302	6.8619	5.5389	4.6327	3.6138	3.2054	2.1959	1.7833	**138.0394**
9	2	42.2077	29.2921	23.2276	16.8906	10.9904	7.8001	5.7957	4.7277	4.0990	3.4220	2.2141	2.1177	**145.7501**
10	1	22.0920	12.8668	8.4156	4.3085	4.2487	3.3625	2.7241	1.3610	1.2644	0.6856	0.6602	0.4665	**33.8621**
10	2	17.5629	9.8697	5.4628	4.0396	2.8176	2.3594	1.6464	1.5618	1.0546	0.7616	0.6618	0.3366	**25.8316**

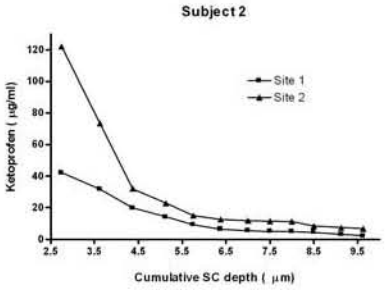
Cummulative SC depth (µm)

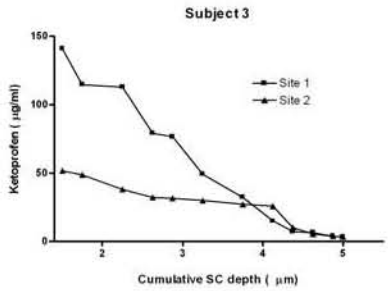
1	4.0000	5.0000	5.8750	6.6250	7.3750	8.3750	9.3750	10.1250	10.7500	11.1250	11.3750	11.6250
2	2.7500	3.6250	4.3750	5.1250	5.7500	6.3750	7.0000	7.5000	8.0000	8.5000	9.1250	9.6250
3	1.5000	1.7500	2.2500	2.6250	2.8750	3.2500	3.7500	4.1250	4.3750	4.6250	4.8750	5.0000
4	2.3750	3.1250	3.8750	4.6250	5.3750	5.8750	6.2500	6.7500	7.3750	8.0000	8.5000	8.7500
5	3.8750	5.0000	6.2500	7.0000	7.6250	8.7500	10.0000	10.6250	11.2500	11.8750	12.2500	12.6250
6	3.8750	4.6250	5.3750	6.0000	6.6250	7.2500	7.8750	8.5000	8.8750	9.2500	9.6250	10.1250
7	3.7500	4.8750	5.8750	6.6250	7.3750	8.2500	8.7500	9.0000	9.8750	10.6250	10.7500	11.0000
8	3.7500	4.6250	5.1250	5.7500	6.7500	7.7500	8.8750	9.8750	10.8750	11.8750	12.8750	14.2500
9	4.3750	5.5000	6.7500	7.8750	8.8750	9.7500	11.1250	12.3750	13.2500	14.1250	15.0000	16.1250
10	1.5000	2.0000	2.5000	3.0000	3.8750	5.1250	6.1250	7.2500	8.2500	8.8750	9.6250	10.2500

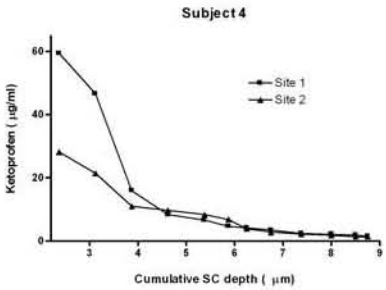
HUMAN STUDY IV: CONCENTRATION-CUMULATIVE SC DEPTH PROFILES

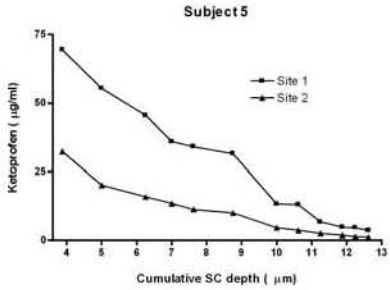
SUBJECTS 1-10

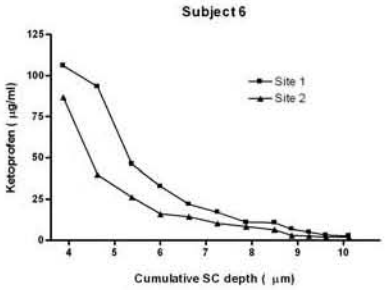


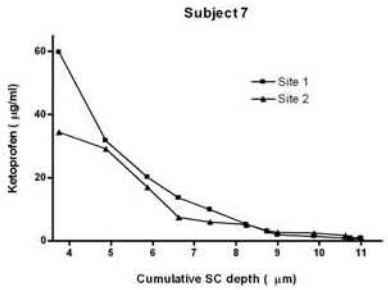


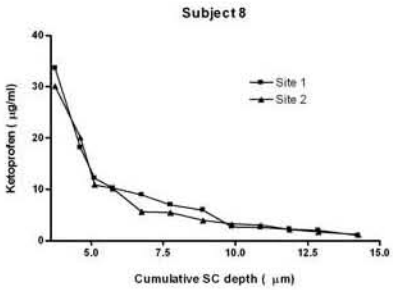


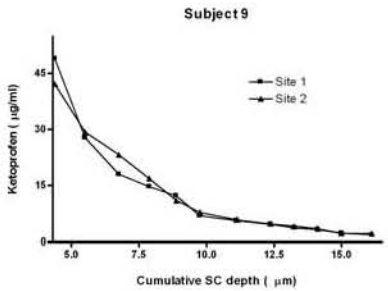


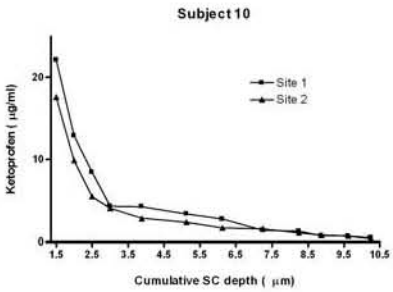












HUMAN STUDY IV: RAW DATA

SUBJECTS 11-18

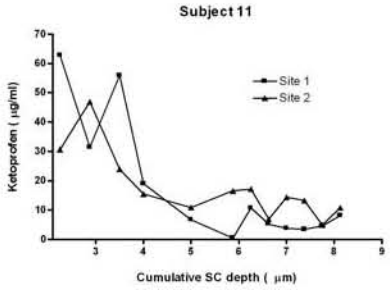
Subj.	Site	1	2	3	4	5	6	7	8	9	10	11	12	AUC
11	1	62.7730	31.4381	55.8472	18.9890	6.7054	0.4217	10.7004	5.2171	3.7641	3.4089	4.4799	8.1498	**103.3381**
11	2	30.7047	46.9034	23.8961	15.4773	10.8756	16.5818	17.1667	6.8353	14.3680	13.2615	4.9188	10.7576	**107.7423**
12	1	131.0436	87.2089	95.3867	82.9926	48.7392	36.3891	16.9528	15.5088	9.1806	31.9967	11.1789	7.4538	**367.0119**
12	2	133.6703	75.6023	44.2009	56.5078	68.4375	35.1663	14.4826	8.6967	5.3538	61.0152	5.2183	4.6798	**321.0700**
13	1	67.2966	37.8960	18.6483	11.4733	8.2789	9.4231	4.4584	6.8066	5.9758	7.6001	8.4742	6.3691	**80.6341**
13	2	79.7733	46.1193	22.2166	19.8708	13.3266	11.3126	3.5773	4.5303	4.6273	2.8860	2.7146	2.4775	**86.0027**
14	1	24.7770	32.5169	24.0181	8.6038	7.2995	4.5179	5.0678	5.3162	2.2581	1.8154	2.4669	1.0513	**57.7974**
14	2	23.8071	15.2052	7.9046	5.4723	4.6951	2.8913	2.1379	2.7807	2.9257	1.4080	0.9770	1.0029	**32.5623**
15	1	111.4051	44.2826	22.8765	11.2939	5.2808	3.5288	3.5407	2.5191	3.0675	3.1025	1.4495	1.0125	**79.4227**
15	2	22.6535	9.9890	5.6583	5.6237	4.8352	2.5328	2.5955	1.8582	1.4093	1.4201	1.0625	1.0459	**24.4455**
16	1	65.3988	48.6410	54.0851	26.7441	38.3166	12.7369	28.2692	5.9002	6.9254	9.1722	23.1058	3.3504	**153.1223**
16	2	69.8760	40.3289	26.4994	12.1203	9.5975	8.2887	7.9250	4.2797	3.6014	5.2646	6.0068	6.7095	**84.2191**
17	1	32.6484	17.7581	10.7503	8.3699	7.5381	4.0714	4.7087	3.4558	3.1986	2.7664	1.4136	1.3101	**47.8668**
17	2	136.8702	53.8567	27.2024	14.8591	18.7783	15.7288	8.7271	3.9700	4.0382	2.9155	3.9233	3.7792	**127.0460**
18	1	69.8760	40.3289	26.4994	12.1203	9.5975	8.2887	7.9250	4.2797	3.6014	5.2646	6.0068	6.7095	**84.2191**
18	2	65.3988	48.6410	54.0851	26.7441	38.3166	12.7369	28.2692	5.9002	6.9254	9.1722	23.1058	3.3504	**153.1223**

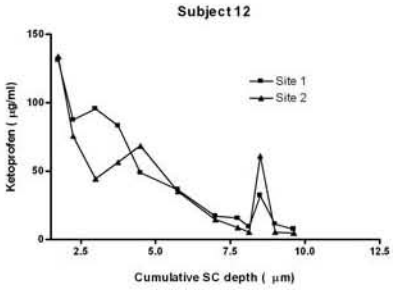
Cummulative SC depth (µm)

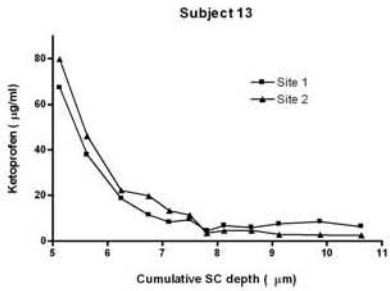
11	2.2500	2.8750	3.5000	4.0000	5.0000	5.8750	6.2500	6.6250	7.0000	7.3750	7.7500	8.1250
12	1.7500	2.2500	3.0000	3.7500	4.5000	5.7500	7.0000	7.7500	8.1250	8.5000	9.0000	9.6250
13	5.1250	5.6250	6.2500	6.7500	7.1250	7.5000	7.8125	8.1250	8.6250	9.1250	9.8750	10.6250
14	1.7500	2.3750	2.8750	3.2500	3.7500	4.2500	4.8750	5.6250	6.2500	6.7500	7.3750	8.0000
15	2.0000	2.3750	3.1250	3.8750	4.2500	4.7500	5.1250	5.5000	6.0000	6.3750	6.8750	7.5000
16	2.7500	3.2500	3.7500	4.2500	4.7500	5.2500	6.1250	6.8750	7.1250	7.5000	7.8750	8.5000
17	4.0000	4.5000	5.0000	5.6250	6.2500	6.8750	7.7500	8.5000	9.2500	10.1250	10.6250	11.0000
18	2.7500	3.2500	3.7500	4.2500	4.7500	5.2500	6.1250	6.8750	7.1250	7.5000	7.8750	8.5000

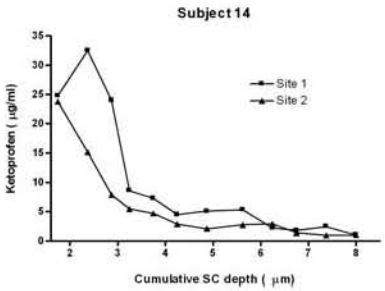
HUMAN STUDY IV: CONCENTRATION-CUMULATIVE SC DEPTH PROFILES

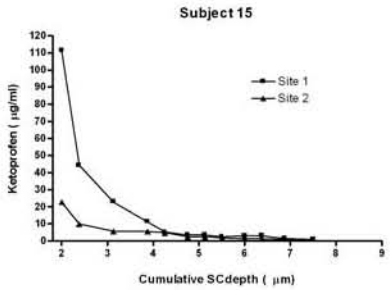
SUBJECTS 11-18

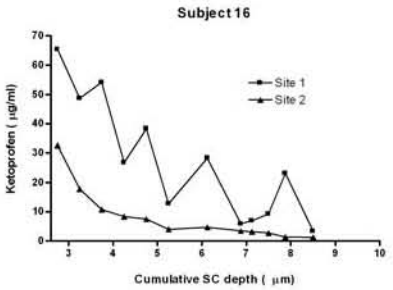


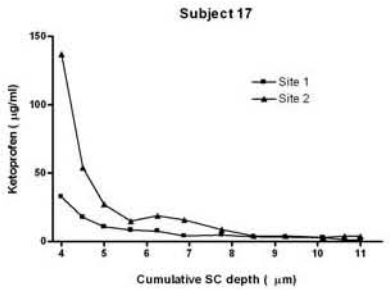


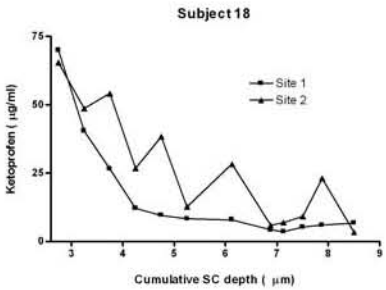












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