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Penetration of Pharmacological Agents through Equine Skin

A report for the Rural Industries Research and Development Corporation

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Foreword

There are increasing numbers of drugs formulated for topical use (applied to the skin) due to the ease of administration, increased compliance with use, avoidance of hepatic “first-pass” effects and reduced gastric irritation. The rate and extent that drugs will pass through the skin is highly species dependent and cannot be extrapolated from studies on other species. A number of topical medications are used in horses, including anti-inflammatory, pain relief medications and antibiotics. However, few are registered for use on horses because there is little or no information about efficacy and safety.

Of equal importance to horses undertaking competition is the frequent application of strict rules that horses cannot compete while subject to the influence of drugs or their breakdown products. Any drug formulation applied topically has the potential to penetrate through the skin and reach detectable levels in the body. This potential may be exacerbated if the skin surface is damaged in any way.

The research undertaken in this project established methodologies to investigate the penetration of drugs through equine skin. Using these techniques, the region of application (e.g. applying the formulation to different parts of the body or legs), drug vehicles (i.e. what the drug is dissolved in) and changes in the integrity of the skin surface (e.g. damage, cleaning agents) were investigated for effects on the penetration of some commonly used topical drugs in horses. These results will be useful to horse owners, particularly if competing in events, horse trainers and officials associated with the equine industry, including stewards, regulators and drug testing officers.

This project was funded from RIRDC Core Funds which are provided by the Australian Government, with support from the Queensland Government Racing Science Centre.

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Peter O’Brien
Managing Director
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Acknowledgments

We would like to thank the Rural Industries Research and Development Corporation (Horse program) for funding this research and the support of the Queensland Government Racing Science Centre.

Abbreviations

ΔC	The concentration differential between compartments
A	The exposed cross-sectional area of the membrane
Al-C	Alcoholic chlorhexidine
Aq-C	Aqueous chlorhexidine
APVMA	Australian Pesticides and Veterinary Medicines Authority
AUC	Area under the curve
BSA	Bovine serum albumin
C	Control
CV%	Coefficient of variation
D	Diffusion coefficient of the drug in the intercellular lipids of the stratum corneum
dC/dt	Steady-state rate of change in the receiver concentration
ECF	Extracellular fluid
EtOH	Ethanol
h	Skin thickness
HPLC	High pressure liquid chromatography
J	Flux
J_{MAX}	Maximum flux
k_P	Permeability coefficient
MeSa	Methylsalicylate
NSAID/s	Non-steroidal anti-inflammatory drug/s
P	Partition coefficient
PBS	Phosphate-buffered saline
PG	Propylene glycol
Sa	Salicylate
SC	Stratum corneum
Sh	Shaved
µL	microlitre
µm	microns
V_R	Receiver volume

Contents

Executive Summary

What the report is about

There are an increasing number of medications being produced for topical application. Topical application, or applying drugs directly to the skin, is convenient compared to alternative routes of delivery, including oral, injectable or inhalation, particularly in animals. This in turn means that a topical medication is more likely to be given (increased compliance), directly increasing the likelihood of any medication being successful. Topical medication is also useful for delivering high concentrations of drug to a specific site, such as a wound, skin irritation, or underlying joint pain, while reducing the total amount of drug to the rest of the body. This significantly reduces the incidence of adverse drug effects and toxicity.

A major concern when developing any topical formulation is the rate and extent that the active ingredient/s and/or the vehicle (carrier agent) will penetrate into and through the skin. Extensive research shows that transdermal drug penetration will vary substantially between different species, meaning that a formulation developed for humans will have a different profile of how much drug moves through then skin and how quickly, than to other animals such as horses. These findings strongly suggest that topical medications intended for use in horses must be developed with an understanding of the kinetics and characteristics of drug penetration through equine skin. However, to date, there is very little basic information of this nature available.

Inadequate knowledge of topical drug delivery can adversely affect animals and owners following use of topical formulations. Too much drug moving through the skin can lead to toxic effects while too little drug can render the formulation ineffective. Intentional or inadvertent application of creams, sprays and gels to a horse's skin prior or during competition can lead to detectable levels of drugs in the blood or urine of the animal, which may infringe the rules and lead to penalties. Drug penetration through skin may also be exacerbated if the skin is damaged, either by physical trauma or by cleaning agents, again leading to higher systemic drug levels.

This report is intended to address the deficiency of basic knowledge of transdermal drug penetration in the horse. A centre of expertise was established both to undertake this research and to provide advice to interested stakeholders, including industry, horse owners and trainers, stewards and regulators. Models were developed and validated, particularly *in vitro* techniques, to characterise the effects of site of application, vehicle formulation and changes in the integrity of the skin surface, such as skin damage or disease, on transdermal drug penetration. This knowledge will be used to develop more effective and safer topical preparations for use in the horse.

Background

The skin is the largest organ of the body, accounting for more than 10% of body mass. It has important protective and homeostatic roles and is generally being regarded as a critical protective barrier to the external environment. Extensive studies have shown that the skin is more complex than merely a barrier which becomes apparent when agents are applied to the skin either deliberately or accidentally. The extent of absorption through the epidermis, dermis and systemically becomes important when we consider that drugs are applied to the skin for (i) local effects (e.g. corticosteroids for dermatitis); (ii) transport through the skin for systemic effects (e.g. fentanyl, nicotine, oestradiol and testosterone patches); (iii) surface effects (e.g. sunscreens and anti-infectives); (iv) to deeper target tissues (e.g. non-steroidal anti-inflammatory agents (NSAIDs) for muscle inflammation); and (v) accidental exposure (e.g. solvents in the work place, agricultural chemical, or allergens).

Investigation of human skin has revealed that the major resistance to drug penetration is the outermost layer, the stratum corneum. Several theories have been proposed for drug passage through the stratum corneum into the viable epidermis and dermis, including the "bricks and mortar" theory, representing keratinocytes held together by a lipid bilayer. However, differences in skin thickness, density of

appendages (hair follicles and glands), vascularity and metabolic enzymes mean that different regions on the same individual display different pharmacokinetics of percutaneous drug penetration. More importantly, extrapolation of percutaneous penetration data between species is not practical and use of products in veterinary practice without consideration of these interspecies variations is risky.

In this report, we examine the current knowledge of transdermal drug penetration and specifically investigate factors affecting drug movement through equine skin.

Aims/Objectives

The specific aims of this project were as follows:

- to develop *in vitro* methodologies to measure drug movement through equine skin
- to measure the regional differences (e.g. thorax vs axilla vs lower leg) in drug penetration
- to measure transdermal penetration of drugs of interest to the equine industry, including non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and analgesics (e.g. fentanyl)
- to determine the effects of vehicles (e.g. propylene glycol) on penetration of drugs in the horse
- to determine the effects of skin damage (e.g. rashes) and altered microenvironment (skin cleansers) on transdermal drug movement in the horse.

This knowledge will specifically benefit the following: Owners and trainers of horses; Pharmaceutical companies when developing topical formulations for the horse; Veterinarians; Regulators, stewards and officials associated with equine competition.

Methods used

The primary techniques used were *in vitro* models involving Franz-type diffusion cells. Skin can be harvested from horses and stored before applying it to a diffusion cell, meaning that several individual horse skins can be studied under the same conditions. Since the stratum corneum is the primary barrier to drug penetration, diffusion cells permit direct and accurate measurement of how much and how quickly any particular drug can move through skin. Diffusion cells also negate the effects of cutaneous blood flow characteristic of *in vivo* models, so regional differences and the effects of vehicles can be studied without the confounding effect of alterations in blood flow.

A number of different drugs commonly used in equine medicine were studied in this project, including hydrocortisone, fentanyl (an opioid analgesic), methylsalicylate (Dencorub®, an anti-inflammatory medication) and testosterone. Analytical techniques were also developed for each of these drugs and their metabolites using high pressure liquid chromatography (HPLC).

A final model of *in vivo* transdermal drug penetration was developed to link *in vitro* findings to *in vivo* situations. Microdialysis, where a semi-permeable probe is placed under the skin to measure drug penetrating through skin, was successfully trialled using a dog model. Further trials of this novel technique in the horse are continuing.

Results/Key findings

It was found that regional differences in the penetration of drugs through equine skin, with hydrocortisone and methylsalicylate penetrating faster and to a greater extent through skin from the leg, which is useful if applying these drugs for anti-inflammatory activity to the legs of horses. Conversely, fentanyl from a commercial TTS system (patch) penetrates better through thorax and groin regions and application of fentanyl patches to upper body regions in the horse is recommended when analgesia is required.

The results showed significant effects of the vehicle on the transdermal penetration of testosterone in the horse, with vehicles containing ethanol or propylene glycol (both commonly found in topical creams and gels) associated with greater drug amounts moving through the skin.

An important finding was the effect of perturbations to the skin surface, affecting the outermost layer, the stratum corneum. These were created using common clinical approaches to treating skin, including shaving, application then removal of adhesive tape and cleaning skin with chlorhexidine (either aqueous or dissolved in alcohol). All these treatments significantly enhanced the penetration of methylsalicylate through skin.

Implications for relevant stakeholders for

Any stakeholder in the equine industry, from trainers, owners, veterinarians, officials and regulators should be aware of the potential from any substance applied to skin to pass through this outermost barrier. They should also be aware that there are significant species differences between how much and how fast a particular formulation will penetrate skin, meaning that a formulation developed and registered for one species will be unlikely to have the same effect and safety margin when applied to another species, including the horse. This has important implications for using drugs with potential toxic effects or low margins of safety, where small changes in uptake of drugs applied topically should be avoided. Equally, stakeholders entering horses into competition should be aware of the potential for altered appearance of drugs and metabolites in the body following topical application which may lead to infringement of the rules of competition.

Stakeholders should be mindful of many of the findings in this report when applying topical drugs to the horse. There are regional differences, meaning that a formulation applied to one part of the body may have a different level of effect if applied elsewhere. Different formulations of the same drug can have significant effects on how much of the drug actually penetrates through the skin and is available for whatever purpose it was designed. Most importantly, changes in the integrity of the skin, such as rashes, abrasions, skin disease or the use of agents to clean the skin, can substantially alter the amount and rate of active drug uptake.

Importantly, stakeholders should also be aware that the basic knowledge of equine topical drug formulation is increasing, particularly with the current project, with more drugs being produced specifically for horses. Topical drugs are easy to apply and have many other advantages, meaning that the potential to treat a range of medical conditions, such as joint pain, skin conditions and cutaneous pests (i.e. fleas, biting flies), is increasing. Better understanding of transdermal drug penetration in the horse will lead to more products that are more effective for topical application to horses.

1. Introduction

1.1 Background to the project

The skin is a protective layer preserving the integrity of the internal environment. The major barrier is the outermost layer, the stratum corneum. An increasing number of pharmaceutical agents are being developed for topical use in humans and animals. Advantages of transdermal drug penetration include; (i) ease of administration; (ii) increased compliance with use; (iii) avoidance of hepatic "first-pass" effect, and (iv) reduced gastric irritation (Magnusson *et al.*, 2001; Roberts *et al.*, 2002).

It is now mandatory for any topical preparation intended for human use to undergo extensive laboratory testing prior to registration. Recent adverse developments in veterinary topical medications have shown that inadequate studies were undertaken prior to registration. The Australian Pesticides and Veterinary Medicines Authority (APVMA) have recently taken an active interest in prerequisites for registration of veterinary topical medications.

Little is known about transdermal movement of drugs through equine skin. Many of the currently available products, such as corticosteroid creams and NSAID gels, are based on results from human or laboratory animal studies, but it is widely acknowledged that extrapolation of transdermal drug movement between species is unreliable (Roberts *et al.*, 2002; Walters and Roberts, 2002). This may lead to adverse or unexpected results when these preparations are applied to horses. Indeed, an increasing number of positive swab samples collected during equine competition have been blamed on unexpected penetration through skin.

In this study, scientifically credible *in vitro* methods to investigate transdermal drug movement through equine skin was developed. Drugs that may be applied topically and are of specific interest to the equine industry were examined, including NSAIDs, corticosteroids and other analgesics (e.g. fentanyl). Potential factors affecting transdermal drug movement, particularly region of application (e.g. thorax vs groin vs legs), the effects of altered skin integrity (rashes or damage) and the effects of vehicles (e.g. propylene glycol) were also investigated.

The results from this study are of benefit to all stakeholders in the equine industry. Information about where best to apply specific drugs to maximise transdermal passage, or regions to avoid if systemic concentrations are undesirable, will be useful to veterinarians, owners and trainers. There in no information about drug movement through damaged skin and if medications are applied under bandages, yet many drugs are applied under these circumstances. The potential of inadvertent swab sample 'positives' need to the addressed by the industry and this research is an active step toward developing understanding and expertise. It may also become mandatory for laboratory testing of drugs intended for topical application and having expertise and infrastructure to undertake this task will be extremely useful.

1.2 Specific aims of the project

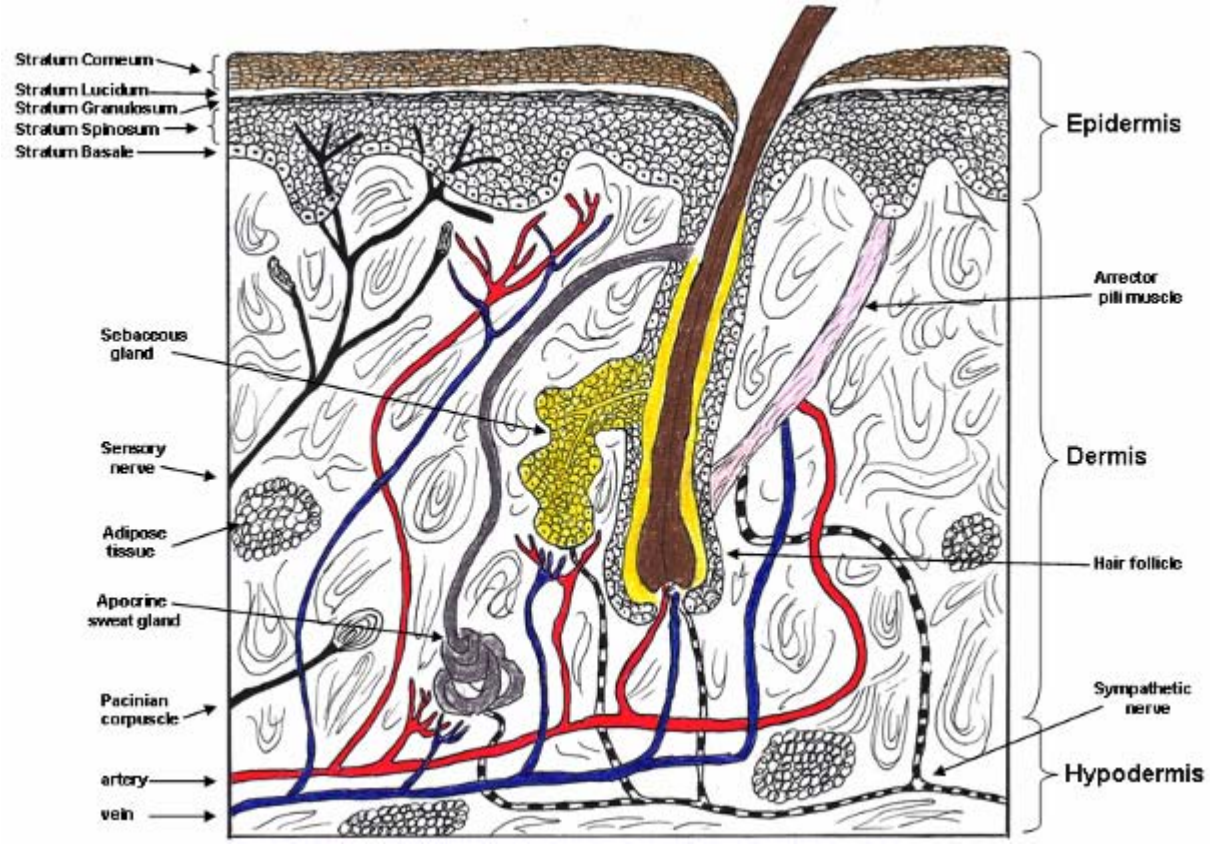
- To develop *in vitro* methodologies to measure drug movement through equine skin
- To measure the regional differences (e.g. thorax vs axilla vs lower leg) in drug penetration
- To measure transdermal penetration of drugs of interest to the equine industry, including non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and analgesics (e.g. fentanyl)
- To determine the effects of vehicles (e.g. propylene glycol) on penetration of drugs in the horse
- To determine the effects of skin damage (eg rashes) and altered microenvironment (e.g. rashes, skin cleansers) on transdermal drug movement in the horse.

2. Development of an *in vitro* model to investigate the penetration of drug formulations through equine skin

2.1 Introduction

The skin consists of the epidermis, the dermis and the subcutis or subcutaneous tissue which anchors the skin to underlying tissues. Each layer is physically and functionally distinct, with appendages, including hair follicles, sweat ducts and sebaceous glands, bridging between the layers and the skin surface (Figure 1). A more detailed account of skin structure and physiology can be found in some excellent reviews (Monteiro-Riviere, 1991; Walters and Roberts, 2002).

Figure 1. The typical structure of mammalian skin



Marked species differences have been reported in each of the skin layers and appendages and it is these differences that probably account for the observed significant differences in transdermal drug penetration between species. The stratum corneum thickness is the primary barrier to drug penetration and varies in many domestic animal species, with thinner stratum corneum structures found in small laboratory animals and values approaching that of humans (3-5 µm) seen in the pig (Monteiro-Riviere *et al.*, 1990). Similarly, the epidermis is substantially thicker in pigs, while relatively thin in rabbits, rats and mice.

2.2 Techniques used to measure transdermal drug penetration

In vivo models where the living animal is used are highly useful but are expensive and difficult to manipulate for accurate control of various physiological parameters (e.g. change in blood flow to the skin). *In vitro* techniques are more commonly used because much of the primary information about the rate and extent of drug penetration, the effects of vehicles and altered environment can be accurately assessed without risk to the animal. Two common *in vitro* techniques are:

- diffusion cells
- isolated perfused porcine skin flap.

2.2.1 Diffusion Cells

The primary technique of *in vitro* transdermal drug studies involves skin collected from the target animal species and mounted within a two-chambered diffusion cell (Figure 2). The stratum corneum side of the skin is orientated towards the donor chamber, while the underside is exposed to the receptor chamber. The diffusion cells are maintained in a water-bath at 32-35^{o}C to keep the skin at a physiological temperature (Franz, 1975; Flynn, 1990; Clarys *et al.*, 1998).

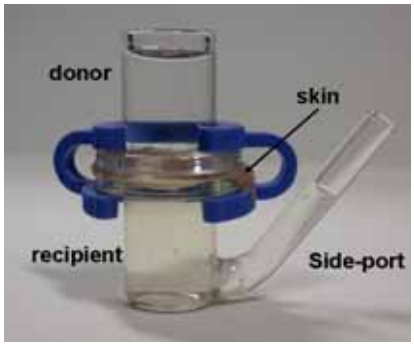


Figure 2. A typical diffusion cell

2.2.2 Isolated perfused porcine skin flap

A useful *in situ* model developed to investigate transdermal drug penetration is the Isolated Perfused Porcine Skin Flap (IPPSF) described by Riviere's group (Riviere *et al.*, 1986; Riviere and Monteiro-Riviere, 1991). This involves a flap of skin dissected from the abdomen of an anaesthetised pig with a remaining pedicle left attached to maintain the blood supply. When the flap matures, it is removed from the pig and maintained in an environment controlled for temperature and humidity, with a modified solution instead of blood circulating. This model approaches the physiological integrity of the *in vivo* system, while allowing control of several variables, such as skin blood flow. However, this model is not suitable for the horse.

2.3 Validation of the model of diffusion cells in the horse

The drug or formulation being investigated is placed in the donor cell, either as an 'in-use' quantity of 5-10 mg/cm^2 onto the exposed stratum corneum surface (finite dose) or dissolved in larger volumes of one or more liquid, semi-solid or other formulations (infinite dose). The concentration of drug can vary from trace amounts to saturated solutions. Radiolabelled drug are useful to measure trace quantities penetrating skin where analytical methods may be limited by sensitivity (Mills *et al.*, 2005), while cold (non-radiolabelled) drug is often used to quantify parent drug and metabolites (Mills *et al.*, 2004a). The receptor chamber must be filled with a suitable fluid giving sufficient solubility to investigate the kinetics of the drug of interest. For the current studies, we used phosphate-buffered saline (PBS) containing 4% bovine serum albumin (BSA). Once the amount of drug penetrating the skin over a continuous range of collection time points has been measured, there is sufficient information to solve Fick's Law of Diffusion and determination of permeation parameters of drug flux (J) and permeability coefficient, as follows (Guy and Hadgraft, 1989; Flynn, 1990):

$$\text{Flux } (J) = \frac{DP}{h} \text{ (mol/cm}^2\text{/h)}$$

where at steady state, D is the diffusion coefficient of the drug in the intercellular lipids of the stratum corneum, P is the partition coefficient of the drug between stratum corneum and the dosing medium on the skin surface and h is the skin thickness.

The permeability coefficients (k_P, cm/h), relating solute flux to the concentration gradient across the membrane (*i.e*. how fast a drug molecule travels through skin), may be calculated from the concentration versus time profile, according to the following formula (Flynn *et al.*, 1981):

$$k_P = V_R \left[\frac{dC/dt}{A.\ \Delta C} \right] \quad \text{(cm/h)}$$

where VR is the receiver volume, dC/dt is the steady-state rate of change in the receiver concentration, A is the exposed cross-sectional area of the membrane and ΔC is the concentration differential between compartments.

The maximum flux (J_{max}) or driving force (the force making the drug move through the skin) can be measured experimentally as the flux from a saturated solution or predicted from k_P multiplied by the solubility of the drug in the chosen donor phase and represents an estimation of the maximum amount of drug that can travel across a defined area of skin per unit time (Hadgraft, 1996).

There are several distinct advantages of using *in vitro* diffusion cells to study transdermal drug penetration. An important advantage over *in vivo* studies is that skin layers can be separated and studied in isolation. Some lipophilic (prefer lipid environments) drugs may pool in layers of the stratum corneum and not partition readily into the less lipophilic regions of the lower epidermis and dermis which may not be apparent from experiments using full thickness skin (Roberts *et al.*, 2004). Skin can be frozen and stored until required which is useful if a study requires skin from several different animals or animal sites or if drug penetration through skin with a particular disease process is required. Studies have shown that human skin stored frozen then defrosted is acceptable to investigate drug absorption where the stratum corneum is the primary barrier, although frozen skin is probably unsuitable if skin viability or metabolism may affect the results of the study (Harrison *et al.*, 1984; Wester *et al.*, 1998). Finally, diffusion cells are ideal to study drug penetration through artificial skin (Monteiro-Riviere *et al.*, 1997), skin alternatives (Godwin *et al.*, 1997) or model membranes, such as cellulose (Shah and Elkins, 1995) or lipids from the stratum corneum (Matsuzaki *et al.*, 1993) in the investigation of drug formulation effects on drug release or to undertake simple comparative formulation studies.

All studies in this project were approved by the Animal Ethics Committee of the University of Queensland (approval number: SVS/087/04/RIRDC).

A potential criticism of this model is the use of skin that has been frozen then defrosted as required. Grafted equine skin has been shown to retain viability when refrigerated for up to three weeks (Schumacher *et al.*, 1987), but it is generally acknowledged that frozen skin will have diminished metabolic and biochemical function (Riviere and Papich, 2001; Roberts *et al.*, 2002). However, freezing is unlikely to affect the structure of the stratum corneum (Harrison *et al.*, 1984; Roy and Flynn, 1989, 1990) which is the principal barrier to transdermal drug movement (Roberts *et al.*, 2002; Walters and Roberts, 2002). Monteiro-Riviere (1991) suggested that skin prepared for *in vitro* use offers similar barriers to topically applied compounds and diffusion cells are suitable to estimate the penetration of topically applied xenobiotics. Individual variation is also reduced as different regions on the same animal can be compared simultaneously.

3. Regional differences in drug penetration through equine skin

3.1 Introduction

Choosing a site to apply a topical medication is based on whether:

- a local effect is required on or under the site of application (e.g. an anti-inflammatory cream to the inflamed skin or a NSAID applied over a sore muscle or joint)
- a systemic effect is required and the site chosen is merely for convenience (e.g. nicotine patches).

An additional concern when applying topical medications to animals is that they may groom and/or try to remove the medication, meaning that:

- application sites are chosen for their difficult access to limit the ability of the animal to interfere with and/or remove the medication (e.g. back of the neck on dogs and cats).

One problem with this approach is that many drugs may show significant regional differences in transdermal penetration, which may be related to differences in the thickness, hair follicle density or other physiological parameters of different skin sites (Montagna, 1971; Lampe *et al.*, 1983; Monteiro-Riviere *et al.*, 1990) and/or in the degree of cutaneous blood flow (Monteiro-Riviere *et al.*, 1990; Manning *et al.*, 1991; Monteiro-Riviere *et al.*, 1993). For example, one study reported that topically applied radiolabelled hydrocortisone penetrated at different rates through human skin, with scrotum>forehead>axilla/scalp>back/abdomen>palmar/planter regions (Feldmann and Maibach, 1967). Similarly, regional differences in transdermal penetration in humans have been reported for pesticides (Maibach *et al.*, 1971), nitroglycerine (Moe and Armstrong, 1986), ketoprofen (Shah *et al.*, 1996), methyl nicotinate (Tur *et al.*, 1991), testosterone (Oriba *et al.*, 1996) and various organic compounds (Rougier *et al.*, 1987).

Little is known about regional differences in transdermal drug penetration in animals, although Qiao and co-workers observed significant differences in the absorption rate of parathion applied to different skin sites in the pig (Qiao *et al.*, 1993; Qiao and Riviere, 1995). Differences in regional penetration rate and extent of topically applied drugs can have major implications for drugs with a low therapeutic index (e.g. fentanyl) or if a relatively constant plasma drug concentration is required (e.g. testosterone).

3.2 Materials and methods

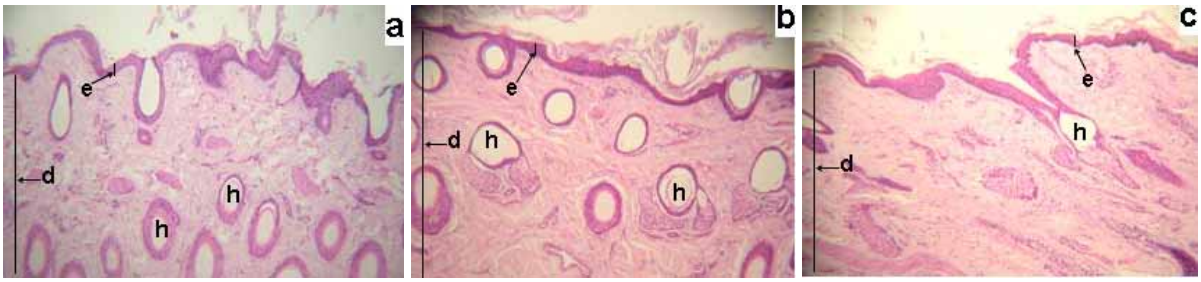
3.2.1 Skin collection

Horse skin was harvested from horses that had been presented to the University of Queensland Veterinary School for euthanasia. Skin from five different horses was used in each of these studies. Skin was harvested from the following regions:

- thorax – halfway up the ribs between ribs 6 and 16
- groin – from either side of the prepuce or udder
- leg – from the front of the foreleg between the carpus (knee) and fetlock.

The hair was removed using electric clippers and skin samples were frozen at -20°C and used within 48 hours (Mills *et al.*, 2004b). Sections from each region were prepared for microscopic examination and stained with haematoxylin and eosin to compare gross ultrastructural differences (Figure 3).

Figure 3. Skin collected from the thorax (a), groin (b) and leg (c) of a horse, showing the epidermis (e), dermis (d) and hair follicles (h)



As can be seen, there appears to be fewer hair follicles in skin from the leg region, although the stratum corneum and epidermis appear similar.

3.2.2 *In vitro* skin penetration

For each study, skin was allowed to thaw at room temperature and cut into circular sections (approximately 2 cm diameter) and mounted into with the stratum corneum side uppermost. A measured volume (approximately 3.5 mL) of PBS (pH 7.4) containing 4% BSA as a receptor fluid was added to the lower reservoir with a magnetic flea for stirring. One mL of PBS was added to the donor reservoir and the skin cell was placed in a water bath containing a magnetic stirring plate and allowed to equilibrate at 35°C for 60 min. The temperature of the skin surface in the diffusion cell was approximately 32°C. The PBS was removed from the donor reservoir and 1 mL of donor solution (this varied depending on the drug and/or formulation being studied) was added (t=0). A glass cover slip was used to occlude the donor chamber and prevent evaporative loss. A 200 µL sample was collected from the receptor fluid via a side-port of each diffusion cell, and immediately replaced with fresh solution, at 2, 4, 8, 14, 20 and 24 hr. This sample was used for analysis. The reported values represent a mean of the five horses (each time point and site represent the mean of three replicates (three different skin samples) for each horse) for each data point.

3.2.3 Analysis of radiolabelled samples

The 200 µL sample was placed in scintillation vials with 2.0 mL of scintillation fluid and the radioactivity in each vial measured using preset channels of a TriCarb 2700TR Liquid Scintillation Analyzer (Packard, Meriden, CT, USA). An aliquot (20 µL) was collected from the donor reservoir of each diffusion cell at t=24 hr to determine depletion of the radiolabelled drug, compared with the initial donor solution (donor recovery). At the completion of each study, skin samples were removed from the diffusion cell, rinsed in distilled water, placed in pre-weighed scintillation vials and accurately weighed (Sartorius CP Analytical Balance, Goettingen, Germany). Two mL of tissue solubilizer was added before incubation at 60°C for 48 hr. Two mL of scintillation fluid was then added to each sample and radioactivity was assessed as described above.

3.2.4 Analysis of non-radiolabelled drugs in samples

All cold (non-radiolabelled) drugs were measured using high pressure liquid chromatography (HPLC). A new method had to be developed for each drug since HPLC analytical methodologies are highly specific for each drug. The HPLC system consisted of: a Shimadzu 10A pumping system, a Shimadzu Sil 9A auto-injector (Sil 9A) and a Shimadzu 10AXL UV/Vis detector, using Shimadzu VP Chromatography software.

3.2.4.1 Hydrocortisone analysis

Samples were analysed for hydrocortisone at 254 nm, using a Phenomenex Luna C_{18} 5 µ 150 x 4.6 mm steel column. The mobile phase was 40% acetonitrile in water, filtered and degassed through a 0.45 µm filter, pumped at a flow rate of 1.0 mL/min. Inter- and intra-run precision (CV%) were 4.2 % and 2.7 %, respectively.

3.2.4.2 Fentanyl analysis

Samples were analysed for fentanyl using midazolam (10 mg/ml) as the internal standard. The mobile phase was 0.14% perchloric acid and 45% acetonitrile which was filtered (0.45 µm) and degassed prior to use. A Waters Symmetry Shield C_8 150 x 3.9 mm steel column was used with a flow rate of 1.0 mL /min. Linearity of the detector set at 200 nm was determined by mixing a series of samples containing fentanyl in the receptor phase (4% BSA) to final concentrations of 0.1, 0.5, 1.0, 5.0, 10.0, 20.0, 50.0 µg/mL. The detector response was linear to 50 µg /mL. The intra-run precision was determined using two concentrations of fentanyl (0.5 and 5.0 µg /mL) in receptor phase and was found to be 1.90 and 0.40 (%CV), respectively, using 10 aliquots of each solution.

3.2.4.3 Salicylate and methylsalicylate analysis

Samples were analysed for salicylate (Sa) and methylsalicylate (MeSa) at 237 nm using a mobile phase of 50:50 acetonitrile: 0.05 M potassium phosphate buffer at pH 3, with flow rates of 1.2 ml/min. The column was a µ-Bondapack C18 (3.9 x 300 mm) column with 50 µl injections.

3.3 Results

Histological examination of the skin revealed a similar thickness in the stratum corneum and dermis, but visibly less numbers of hair follicles from skin harvested from the leg region.

3.3.1 Hydrocortisone

The solubility of hydrocortisone in 50% EtOH in PBS w/w was 8.68 ± 0.49 g/L. Hydrocortisone recovery from donor solution applied to the thorax, groin and leg were 113.1 ± 4.6 %, 110.2 ± 5.1 % and 107.6 ± 3.8 %, respectively. Values for k_P and D_R were lower in the thorax (5.88 ± 0.56 x 10^{-5} cm/hr and 2.86) and groin (5.33 ± 1.56 x 10^{-5} cm/hr and 3.97) regions, compared to the leg (10.01 ± 0.89 x 10^{-5} cm/hr and 11.02; P<0.001). Similarly, J_{max} was significantly higher (P<0.001) when hydrocortisone in 50% EtOH was applied to the leg, compared to thorax and groin (Figure 4). In contrast, significantly less hydrocortisone (P<0.001) was retained within skin from the leg compared to thorax and groin regions 24 hrs post application (Figure 5).

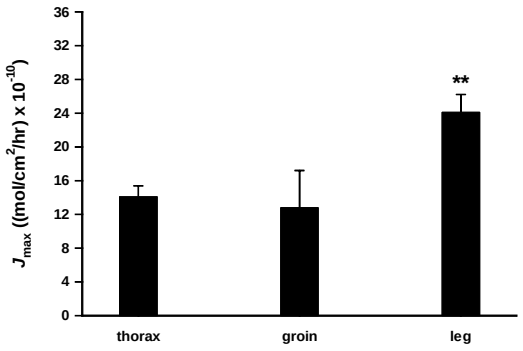


Figure 4. Maximum flux (J_{max}, mol/cm^2/h) of a saturated solution of hydrocortisone in 50% ethanol (w/w) through horse skin collected from the thorax, groin and leg (dorsal metacarpal) regions. Means ± SD (P<0.001).**

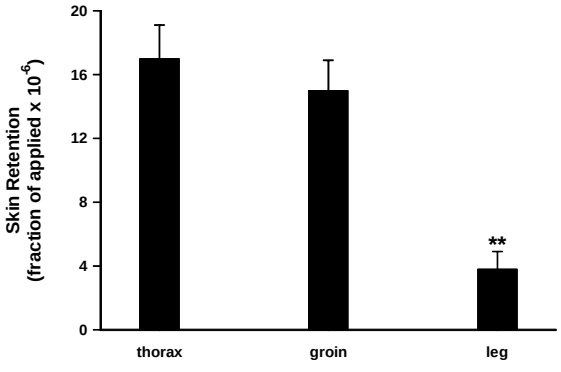


Figure 5. The skin retention (fraction of applied x 10^{-6}) of hydrocortisone after application of a saturated solution of hydrocortisone in 50% ethanol (w/w) for 24 hr to horse skin collected from the thorax, groin and leg (dorsal metacarpal) regions. Means ± SD (P<0.001).**

3.3.2 Fentanyl

The cumulative fentanyl penetration (µg /cm^2) versus time (hr) plots used to estimate steady state flux (µg /cm^2 /h) through each of the skin regions are shown in Figure 6. The fluxes and lag times determined from these regressions are detailed in Table 1. A significantly lower flux was observed following application to the leg region (P<0.05) with no significant differences detected between the thorax and groin regions. The lag time for fentanyl penetration into the receptor phase on the underside of the skin was found to be significantly slower for the groin region versus the thorax and leg sites, which were similar in magnitude (Table 1).

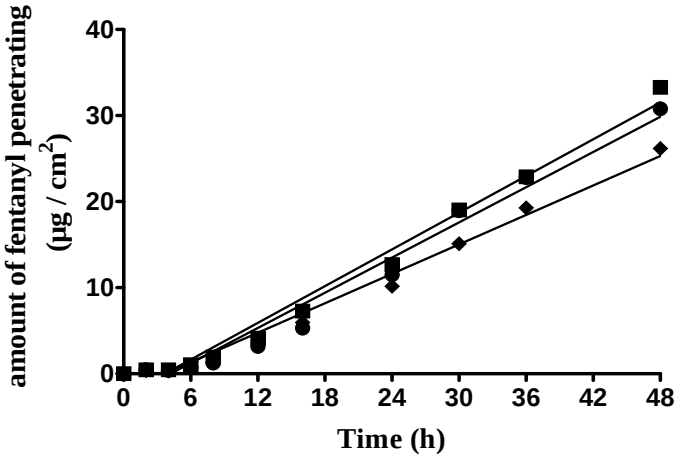


Figure 6. Cumulative fentanyl penetration versus time curves showing the steady state regression fits used to estimate flux through each of the skin regions thorax (■), groin (●) and leg (♦) regions (n=5)

Table 1. Estimated fentanyl flux (µg /cm^2/ h) and lag time (h) through each of the skin regions (n=5). Values represent Mean ± SD

Skin region	Flux (µg /cm^2/ h)	Lag time (h)
Thorax	2.32±0.17	5.66±0.97
Groin	2.21±0.11	7.87±0.51*
Leg	1.56±0.12*	5.75±0.43

* P<0.05

3.3.3 Salicylate and methylsalicylate

The total amount of MeSA and Sa penetrating the skin over the 24 h period and the steady-state rate of absorption (flux) through skin from each site are shown in Table 2. The cumulative concentration-time profiles for the appearance of MeSA and Sa in the receptor fluid, from which the steady-state flux values were estimated by linear regression, are shown in Figure 7. There was a significantly higher amount (AUC) of MeSa and Sa penetrating through skin harvested from the leg region, compared to thorax and groin, over the 24 h of the study (Table 2 and Figure 7). Similarly, a greater penetration rate in the initial 6 h after application was measured for MeSa and Sa through leg skin, compared to thorax and groin.

Table 2. AUC (hr.mg/L) over 24 h and penetration rate (mg/cm^2/hr) during the first 6 h for methylsalicylate (MeSa) and salicylate (Sa) after application of Dencorub® to horse skin harvested from the thorax, groin and leg (dorsal metacarpal) regions. Values are represented as mean ± SD.

Measurement	Region		
	Thorax	Groin	Leg
AUC (hr.mg/L)			
MeSa	2408.6 ± 231.2	2218.3 ± 204.5	3680.1 ± 245.1
Sa	1302.2 ± 121.7	1353.2 ± 99.2	1811.2 ± 133.6
MeSa + Sa	3710.7 ± 279.8	3571.5 ± 299.2	5491.3 ± 334.1
Penetration rate (mg/cm^2/hr)			
MeSa	7.75 ± 1.54	7.39 ± 1.87	12.84 ± 2.11
Sa	3.45 ± 0.67	3.31 ± 0.71	5.43 ± 1.04
MeSa + Sa	11.20 ± 2.42	10.70 ± 2.17	18.27 ± 3.86

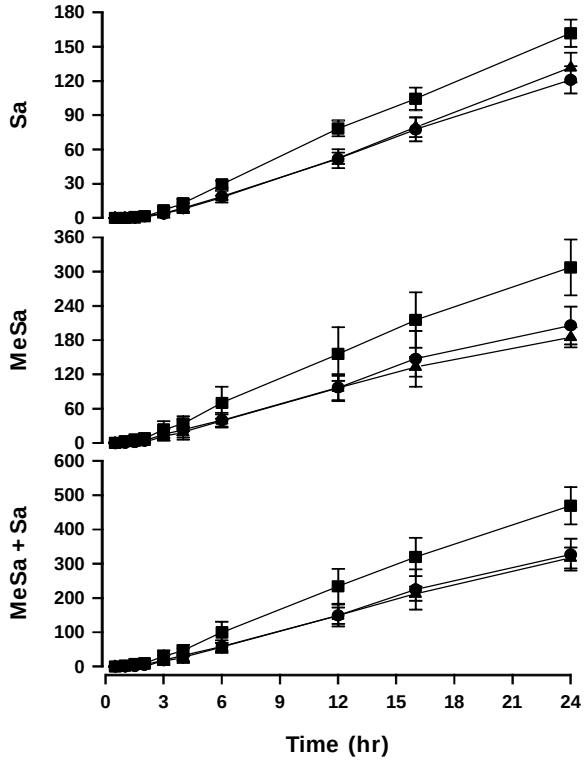


Figure 7. Concentration (mg/L) of salicylate (Sa), methylsalicylate (MeSa) and total salicylate (MeSa + Sa) penetrating through skin (mean ± SD) collected from the leg (dorsal metacarpal; (), thorax (▲) and groin (●) regions of a horse using *in vitro* diffusion cells over 24 hr.

3.4 Discussion

3.4.1 Hydrocortisone

We found a significantly higher (P<0.001) permeability rate (k_P), maximum achievable total hydrocortisone penetration (J_{max}) and estimated diffusivity (D_R) within the skin of the leg (dorsal metacarpal region) compared to other sites. Previous studies (Mills *et al.*, 2005) comparing the application of different vehicle systems containing hydrocortisone on dog skin showed that the maximum flux of hydrocortisone was significantly higher for all sites (neck, thorax and groin) when dissolved in a vehicle containing 50% EtOH, compared to PBS alone or 50% propylene glycol, with differences more prominent in skin from the neck region (P<0.0001). The findings in the horse suggest that hydrocortisone applied topically would penetrate through skin of the lower leg at almost twice the rate as through upper body regions using the vehicle and application conditions used in the current study.

Several factors may account for regional differences in transdermal drug penetration, particularly appendageal density (Tur *et al.*, 1991; Hueber *et al.*, 1992; Hueber *et al.*, 1994) and the thickness of the major barrier to drug and water movement through skin, the stratum corneum (Magnusson *et al.*, 2001; Roberts *et al.*, 2002). It has been suggested that certain drugs may penetrate via appendageal openings (Hueber *et al.*, 1992; Hueber *et al.*, 1994), although in the current study, there was no significant difference between hydrocortisone penetration through skin from the thorax (quite hairy) compared to the groin (only some fine hair visible). These results are consistent with studies performed using dog skin where hydrocortisone penetration through thorax and groin skin was similar, yet significantly higher values were observed in the neck, (Mills *et al.*, 2005). Previous studies have shown a similar thickness of stratum corneum and blood flow in the thorax and groin regions of the horse (Monteiro-Riviere *et al.*, 1990; Manning *et al.*, 1991), but thickness of the stratum corneum overlying the leg has not been reported. In addition, the number of layers of the stratum corneum may influence solute permeation due to possible lateral bilayer diffusion (Johnson *et al.*, 1997). Preliminary histological examination in the current study suggests that the stratum corneum is of a similar thickness in the three regions, although there appears to be substantially lower numbers of hair follicles visible in skin from the leg. Further studies are required to specifically measure appendage density from different regions of the horse and to compare the biochemical composition of the stratum corneum, particularly the type and amount of lipid components.

In addition to the regional differences between leg and upper body, the current study has also demonstrated the necessity to investigate transdermal drug penetration in the species of interest. Differences in skin thickness, composition and number of appendages (hair follicles and glands) make extrapolation between species impractical (Magnusson *et al.*, 2001; Walters and Roberts, 2002). A comparison of a similar study in Greyhounds (Mills *et al.*, 2005) revealed a higher (five-fold for the groin region) maximum flux of hydrocortisone in 50% EtOH in the horse. Blood flow through the groin region is similar for the dog and horse, although a thicker stratum corneum (12.20±2.12 vs 8.19±1.38 µm) in the dog has been reported (Monteiro-Riviere *et al.*, 1990). Ultrastructural differences in skin from the dog and horse have also been reported (Monteiro-Riviere, 1991). For example, the relative proportion of individual lipids within the epidermis of pigs was constant across several body regions, while greater total lipids were found on the back region (Monteiro-Riviere *et al.*, 2001). Significantly lower skin residues after application of hydrocortisone in 50% ethanol to the leg may reflect variability in dermal and epidermal constituents, compared to upper body regions. Further studies are required with commercial formulations (0.5 and 1.0 % hydrocortisone creams) to confirm this regional variation.

3.4.2 Fentanyl

We have shown a significant difference in transdermal fentanyl penetration with site of patch application in the horse, with less drug possibly available for systemic activity if patches are applied to the lower leg (dorsal metacarpal region), while penetration through the groin and thorax skin were similar. This differs somewhat from studies in the dog where faster penetration was found if a fentanyl patch was applied to skin from the groin region, compared to the thorax or neck (Mills *et al.*, 2004a). However, also inconsistent with the previous studies using canine skin, a significantly longer lag time through groin skin than the other two regions was noticed with equine skin.

In this study, histological examination shows that there appear to be substantially lower numbers of hair follicles visible in skin from the leg region, which may account for the small but significant reduction in fentanyl penetration through this region. Further studies are required to specifically measure appendageal density from different regions of the horse and to compare the biochemical composition of the stratum corneum, particularly lipid composition. The longer lag time observed through groin skin compared to the other two sites is related to a combination of the rate of partitioning from the patch into the skin and diffusion through the sample during this initial application period. Steady-state flux data (Table 1) suggests that the penetration rate of fentanyl does not appear to be particularly slower through this region and therefore could be related to the partitioning process. Further studies are required to elucidate the mechanisms responsible for these observed differences.

3.4.3 Salicylate and methylsalicylate

We have demonstrated that both MeSa and Sa will penetrate through equine skin following topical application of a commercial preparation of MeSa. The relative proportion of parent compound and any metabolite could be expected to vary between *in vitro* and *in vivo* studies because skin is metabolically active and capable of phase I and Phase II metabolism (Roberts *et al.*, 2002; Walters and Brain, 2002), meaning that more parent compound (MeSA) and less metabolite (SA) would penetrate through skin using an *in vitro* model (Megwa *et al.*, 1995). Indeed, Cross *et al.* (Cross *et al.*, 1998) reported both MeSa and Sa penetrating human skin using diffusion cells, yet only salicylate could be detected following topical application of methylsalicylate using an *in vivo* model of microdialysis in human volunteers. Conversely, a recent microdialysis study in the dog measured both MeSa and Sa in dialysate and in deeper tissues following topical application of MeSa (Mills *et al.* 2005b). These results suggest that there may be species differences in the relative metabolic activity of skin, particularly for the esterases responsible for the hydrolysis of this salicylate ester.

Both MeSa and Sa are pharmacologically active (Adams, 2001) and the total salicylate (MeSa + Sa) concentration is evaluated for anti-inflammatory activity following topical application (Megwa *et al.*, 1995; Cross *et al.*, 1998; Cross *et al.*, 1999). To specifically assess the potential anti-inflammatory activity of salicylate, it has been shown that a concentration of 130 to 150 mg/L in inflammatory exudate will inhibit the production of prostaglandin E_2, one of the primary inflammatory mediators, by 50% (Higgs *et al.*, 1987). In the current study, total salicylate concentrations approached 100 mg/L by 6 h and, in the leg region, exceeded 200 mg/L by 12 hr, which suggested that therapeutic concentrations of salicylate penetrated through equine skin following topical application of methylsalicylate.

There were significant regional differences in transdermal penetration of MeSa through equine skin. The commercial preparation used in this study passed through leg skin faster, particularly in the early stages after application, and to a greater extent. An important finding is that application of NSAIDs to extremities appears to promote relatively high local drug concentrations with relatively low systemic levels (Shah *et al.*, 1996), which suggests that topical NSAIDs may be a useful alternative for soft tissue injuries on horses legs, particularly close to competition.

3.4.4 Conclusion

In conclusion, regional differences in the penetration of hydrocortisone, fentanyl and methylsalicylate through equine skin have been found. Hydrocortisone and methylsalicylate penetrate faster and to a greater extent through skin from the leg, which is useful if applying these drugs for anti-inflammatory activity in the legs of horses. Conversely, fentanyl from a commercial TTS system (patch) penetrates better through thorax and groin regions and application of fentanyl patches to upper body regions in the horse when analgesia is required.

4. Effects of vehicle on drug penetration through equine skin

4.1 Introduction

The majority of commercial formulations applied to the skin, either intentionally or inadvertently, contain a number of substances, such as inert excipients, solvents, preservatives, fragrances and stabilizers, collectively known as the 'vehicle'. The active drug is contained within the vehicle, either dissolved or as an emulsion or suspension, at a known concentration. The activity of the commercial formulation is based on movement of the active ingredient from the vehicle into and through the skin (Ostrenga *et al.*, 1971; Polano and Ponec, 1976).

Two primary factors determine the rate and extent of active drug leaving the vehicle and moving through the skin. The first is the relative and absolute solubility of the drug in the two phases, vehicle and skin. The relative solubility determines the partition coefficient which, in turn, determines the likelihood of the drug being taken up into the SC from the vehicle, whereas the absolute solubility determines the total amount that can be contained within the SC (Roberts *et al.*, 2002). The second factor is the diffusivity. Vehicles must therefore be sufficiently soluble to contain the active drug in an aesthetically acceptable form (i.e. no granules), yet the drug must simultaneously be sufficiently soluble in the SC lipids and be able to diffuse through these intercellular lipids to reach the site of intended action (Kaplun-Frischoff and Touitou, 1997).

Theoretically, as long as neither the drug nor the vehicle affects the skin, J_{max} will be observed for a defined drug from any range of vehicles saturated with that drug. This means that, except for supersaturated solutions, J_{max} can only be increased by changing the characteristics of the skin, such as (i) increasing the diffusivity of the solute in the SC; (ii) affecting the partitioning of solute between SC lipids and other SC constituents, or (iii) increasing the solubility of the solute in the intercellular lipids (Roberts *et al.*, 2002). It is well recognized, however, that many vehicles not only interact with the applied drug, but also interact with the skin, with subsequent altered penetration of drug and vehicle, dependent on these interactions (Harrison *et al.*, 1996; Pugh *et al.*, 1996). The types of effects of the vehicle to change partitioning into and/or diffusivity of the SC barrier have been studied using inert membranes, such as silicone (Cross *et al.*, 2001), or by pre-treating the skin with the vehicle to determine penetration enhancement (Rosado *et al.*, 2003).

The effects of altering the vehicle have never been reported in the horse. In this study, we examined two common components of many vehicles, namely ethanol (EtOH) and propylene glycol (PG), compared with saline (PBS) as a control, to see how the vehicle could affect the transdermal penetration of testosterone in the horse.

.2 Materials and methods

4.2.1 Skin collection

Skin was collected from the thorax region from five different horses. The collection and preparation of the skin samples was the same as described in Chapter 3.

4.2.1.2 Testosterone analysis

Testosterone was analysed using HPLC as follows: a Shimadzu 10A pumping system, a Shimadzu Sil 9A auto-injector (Sil 9A) and a Shimadzu 10AXL UV/Vis detector, using Shimadzu VP Chromatography software. Samples collected from the diffusion cells were analysed for testosterone at 254 nm using a Phenomenex Luna C_{18} 5 µ 150 x 4.6 mm steel column. The mobile phase was 40% Acetonitrile in water, filtered and degassed through a 0.45 µm filter, at a flow rate of 1.0 mL/min. Inter- and intra-run imprecision (CV%) were 4.1% and 2.9%, respectively. Calibration samples were prepared by diluting the saturated donor solutions with the respective donor phase to 1:10, 1:100, 1:500 and 1:1000 concentrations and analyzing each for testosterone concentration, then a solubility was calculated based on the average testosterone concentration measured in each solution once the dilution factor was accounted for. Using further serial dilutions of the donor phases, a limit of detection (0.1 µmol/L) and a limit of quantification (1.0 µmol/L) could be determined.

4.3 Results

The solubility of testosterone in PBS, 50% EtOH in PBS w/w and 50% PG in PBS w/w was 0.22 mmol/L, 6.31 mmol/L and 5.14 mmol/L, respectively. Testosterone recovery in the system approached 100 % (Table 3). Penetration parameters, k_P and J_{max}, from each of the vehicles and through each of the skin sites are shown in Table I. Higher residues of testosterone were found remaining within the skin when PBS was used as a vehicle (Figure 8), compared to 50% EtOH or 50% PG in PBS (p=0.03).

Table 3. The donor recovery, k_P and J_{max} (mean ±SD; n=5) for testosterone applied *in vitro* to equine skin in PBS, EtOH (50% in PBS w/w) and PG (50% in PBS w/w).

		Vehicle		
Parameter	Unit	PBS	50% EtOH	50% PG
Donor Recovery	%	81.1	78.4	92.9
k_P x 10^{-3}	cm/hr	6.82 ± 0.71[1]	1.59 ± 0.17	2.04 ± 0.42
J_{max} x 10^{-9}	mol/cm^2/hr	1.36 ± 0.14	9.99 ± 1.08[2]	10.41 ± 2.14[2]

[1] P=0.03; [2] P=0.01.

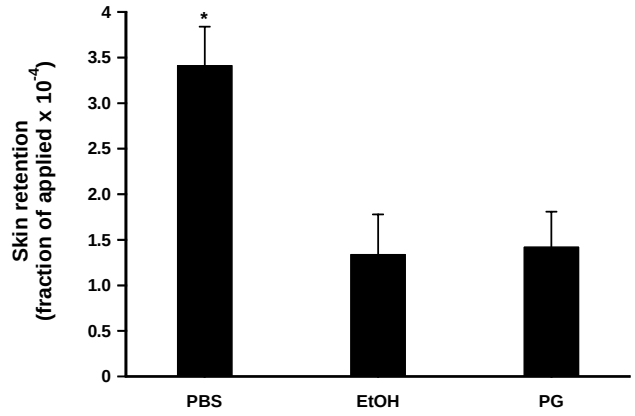


Figure 8. The skin retention (fraction of applied x 10^{-4}) of testosterone (mean ±SD; n=5) dissolved in PBS, EtOH (50% in PBS w/w) or PG (50% in PBS w/w) through equine thoracic skin (*P=0.03).

4.4 Discussion

The results in this study have shown a significant difference in transdermal penetration of testosterone dissolved in different vehicles in the horse. The dependence of transdermal drug transport on the vehicle or carrier medium is well documented (for a review see Barry (2001). Optimal penetration of a topically applied drug is achieved by formulating a vehicle-drug combination to maximise the flux (Surber *et al.*, 1990). A significantly higher J_{max} was measured in equine skin if testosterone was dissolved in 50% EtOH or 50% PG solutions, compared to PBS alone. However, a relatively higher skin retention of drug was obtained when a water-based vehicle was used, which may be suitable for treatment of specific skin diseases while minimising systemic effects. The ideal vehicle for topical application of testosterone to horses has, to these authors' knowledge, not been reported, yet many veterinary clinicians are limited to products developed for topical use in humans.

The specific effects of the different vehicles relates to solubility of the drug within the vehicle (Ostrenga *et al.*, 1971). Only solubilised drug can diffuse within the vehicle and contribute significantly to release rate (Idson, 1983). Testosterone is more soluble in EtOH and PG, compared to PBS, enhancing the penetration of testosterone through equine stratum corneum. The effect of vehicle on drug penetration cannot, however, be considered in isolation because many vehicles act by disrupting the skin surface, reducing the barrier function of the stratum corneum (Aungst *et al.*, 1990). Alcohol is known to irritate skin and delipidate surface membranes (Scheuplein and Blank, 1973; Finnin and Morgan, 1999) which may contribute to penetration of dissolved substances through the skin. Similarly, PG has been shown to diffuse into skin and improve the solubility of skin lipids to the diffusant (Hadgraft, 1996). Since many commercially available topical preparations contain alcohol and/or PG as components of the cream base (Austrailian Prescription Guide, 2002), the relative proportion of vehicle constituents may also affect the transdermal penetration of testosterone.

In conclusion, this study has demonstrated regional and vehicle differences in transdermal testosterone penetration, with vehicles containing EtOH or PG appearing superior to vehicles containing saline. This study shows that variability in clinical response to testosterone could be expected with formulation design and site of application.

5. The effects of skin damage or skin preparation on transdermal drug penetration

5.1 Introduction

Many transdermal pharmacokinetic and pharmacodynamic studies have based findings on normal skin, particularly an intact SC (Riegelman, 1974). There is limited information concerning the specific effects of skin disease on transdermal drug penetration, although it is reasonable to assume that progressive loss of the SC will greatly diminish the barrier function of skin. One study in the pig demonstrated that extraction of intercellular lipids with various solvents caused a significant reduction in the barrier function of the SC (Monteiro-Riviere *et al.*, 2001). Similarly, delipidation of skin by acetone significantly increased the *in vivo* transdermal penetration of salicylate measured using microdialysis (Benfeldt *et al.*, 1999a). Altering lipid content and fluidity could be seen as one strategy to enhance transdermal permeability. Similarly, lipid composition is known to vary with diseases of the epidermis (Yardley and Summerly, 1981), which may dramatically effect drug movement through the SC. More importantly, certain drugs, such as topical corticosteroids, intended for use in skin diseases (Wong *et al.*, 2003), are applied readily to areas of compromised epidermis, yet enhanced systemic uptake may contribute to the adverse effects of this class of drugs.

The SC could also be inadvertently damaged due to preparation of the skin prior to application of a topical agent (Wilhelm et al., 1990). For example, alcohols are known to irritate the skin by delipidising the membrane and disrupting the SC (Scheuplein and Blank, 1973; Finnin and Morgan, 1999; Mills et al., 2003a, b). Certain alcohols, such as methanol and ethanol, may be used in preparations used to clean skin in many veterinary and human applications, yet it is well known that the barrier function of skin can be greatly diminished due to loss of intercellular lipids and subsequent disruption of the SC structure (Surber et al., 1990; Inamori et al., 1994; Peck et al., 1994).

In this study, we investigated the effects of common techniques used to clean and/or prepare skin on the movement of a commonly used anti-inflammatory formation containing methylsalicylate (Dencorub®) through equine skin. The techniques considered were: shaving (Sh), cleaning with aqueous chlorhexidine (Aq-C), cleaning with alcoholic chlorhexidine (Al-C) and applying (then removing) Elastoplast tape.

5.2 Materials and methods

Horse skin was collected from the thoracic region of five different horses as described previously. The skin samples from each horse were divided into five sections using a marker pen and one of the following preparations was applied to each section using a randomly selected order for each horse: (i) no preparation (C - control); (ii) 0.1% w/v aqueous chlorhexidine (Aq-C; applied using a ball of cotton wool which was gently rubbed over the skin site three times); (iii) 0.5% w/v alcoholic chlorhexidine (Al-C; applied as for aqueous chlorhexidine); (iv) Shaving (Sh; a single bladed disposable razor was applied once over the skin site to remove the remaining hair coat); (v) Tape stripping (Ta; elastoplast adhesive tape was firmly applied to the skin section then removed).

Skin was then cut into circular sections and palced in diffusion cells as previously described. All samples were analysed for SA and MeSa using high-performance liquid chromatography (HPLC) as described previously (Cross et al., 1998). The reported values represent a mean of the five data points from each horse at each time point.

Data analysis

Area under the curve (AUC) was calculated for the MeSa and Sa measured in receptor fluid at each collection time using curve-fitting software (GraphPad Prism v 4.00 for Windows; GraphPad Software, San Diego California, USA). The Student's *t*-test for unpaired data was then used to compare the differences in AUC of MeSa and Sa using different skin pre-treatments, compared to control (clipped only). MeSa and Sa initial absorption rates (flux, mg/cm^2/hr) were calculated from linear regression analysis of the cumulative rates between 0 to 10 hr, while lag times for penetration through skin were calculated from the intercept with the 'y' axis. The two-tailed Student's *t*-test for unpaired data used to compare means using different skin pre-treatments, compared to control (clipped only). The results for the combined transdermal penetration of MeSa and Sa were calculated for illustration only with no statistical analysis performed on these results.

5.3 Results

Over the 10 hr of the study, a significantly higher proportion of MeSa penetrated through Al-C and Sh (P<0.01) and Aq-C and Ta (P<0.05) treated skin, compared to C, while significantly more Sa could be detected in the receptor phase of Ta (P<0.01), Al-C, Aq-C and Sh (P<0.05) treated skin, compared to C (Figure 9). Similarly, the absorption rate or flux of MeSa was significantly higher (P<0.05) through skin following each of the pre-treatments, compared to control, although the rate of appearance of Sa was only significantly higher than control (P<0.05) for skin pre-treated with Al-C and Ta (Table 4). A significantly shorter lag time (P<0.05) was calculated for MeSa for all pre-treatments, although a shorter lag time was only significant (P<0.05) for Sa appearance in the receptor phase following Al-C (Table 4).

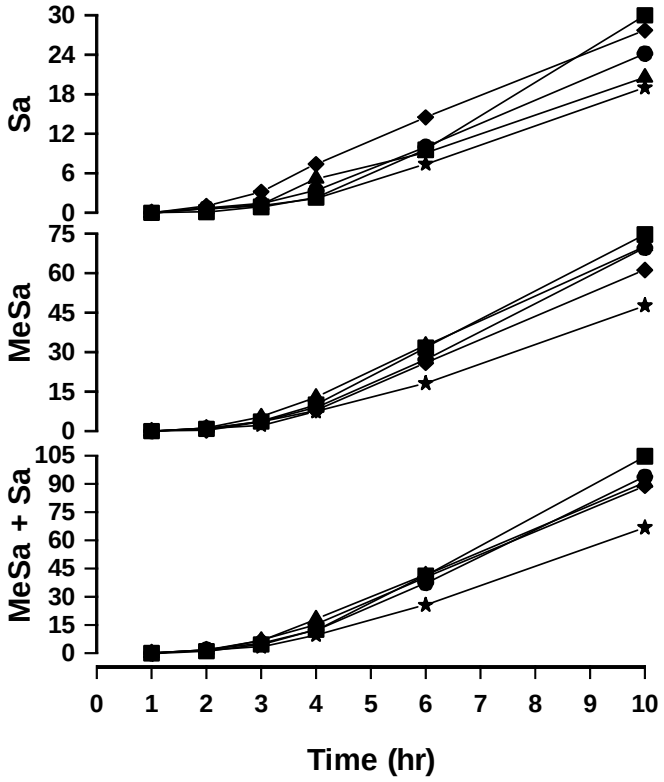


Figure 9. The penetration of MeSa (mg/L) and its active metabolite, Sa (mg/L), over 10 hr through clipped equine skin from five horses following topical application of a commercial preparation of MeSa (Dencorub®) to skin after the following pre-treatments: clipped only (control; (), cleaned with aqueous chlorhexidine (●), cleaned with alcoholic chlorhexidine (■), shaved (▲) and tape stripped (♦). Each time point represents mean of five replicates.

Table 4. A comparison of AUC (hr.mg/L), absorption rate (flux; mg/cm^2/hr) and lag Time (hr) of methylsalicylate (MeSa) and salicylate (Sa) for 10 hr after applying MeSa gel (Dencorub®) to equine thoracic skin *in vitro* following five different protocols of skin preparation.

Preparation	Absorption rate (mg/cm^2/hr)	Lag time (hr)	AUC (hr.mg/L)
Control			
Sa	2.18	-4.41	65.20
MeSa	5.49	-10.99	164.70
MeSa + Sa	7.67	-15.40	229.80
Aqueous Chlorhexidine			
Sa	2.80	-5.50	85.60
MeSa	8.05	-16.44	239.30
MeSa + Sa	10.85	-21.94	325.00
Alcoholic Chlorhexidine			
Sa	3.45	-7.80	93.45
MeSa	8.73	-17.64	264.30
MeSa + Sa	12.18	-25.45	357.50
Shaving			
Sa	2.38	-4.21	78.10
MeSa	8.21	-15.16	263.30
MeSa + Sa	10.59	-19.37	342.40
Tape stripping			
Sa	3.22	-4.99	114.20
MeSa	7.16	-14.55	215.80
MeSa + Sa	10.38	-19.54	330.10

5.4 Discussion

The results of this study conclusively demonstrate that common cleaning and preparation techniques applied to the skin can significantly affect transdermal drug penetr

Many of the transdermal pharmacokinetic and pharmacodynamic studies in the literature have based findings on normal skin, particularly an intact SC (Roberts et al., 2002). However, it is acknowledged in the literature that disease processes can alter lipid composition and disrupt the structure of the epidermis (Yardley and Summerly, 1981). Similarly, it has been shown that ation in the horse. It could be argued that clipping itself may also be a 'pre-treatment' of skin, although removal of the upper hair coat primarily removes the physical barrier function which may impede contact of a topical formulation with the SC. Histological examination of clipped equine skin revealed that hair follicles protrude marginally above the skin surface, compared to unclipped regions, with no loss or damage to the SC region (unpublished results).

the SC could be inadvertently damaged due to preparation of the skin prior to application of a topical agent (Wilhelm et al., 1990). In the current study, chlorhexidine significantly enhanced the rate and amount of MeSa penetration, and the appearance of its active metabolite Sa, which may be due to physical contact and removal of upper layers of the SC during application but is more likely to be related to a change in the constituents of the SC decreasing the barrier to this relatively hydrophilic commercial preparation. It has previously been shown that extraction of intercellular lipids with various solvents will cause a significant reduction in the barrier function of the SC (Monteiro-Riviere et al., 2001).

One group of solvents in particular that are known to irritate the skin by delipidising the membrane and disrupting the SC are alcohols, particularly the more polar members of this group (Finnin and Morgan, 1999; Mills et al., 2003b, a). Small chain alcohols, such as methanol and ethanol, may be used in preparations used to clean skin in many veterinary and human applications, yet it is well known that the barrier function of skin can be greatly diminished due to loss of intercellular lipids and subsequent disruption of the SC structure (Peck et al., 1994). The addition of methanol to chlorhexidine induced a small but not significant increase in the rate and extent of MeSa and Sa recovery in receptor phase compared to chlorhexidine alone. It is uncertain in the current study if the methanol is synergistic with or affecting the SC by a different mechanism than chlorhexidine, although some alcohol-containing vehicles have been shown to enhance transfollicular delivery of drug molecules which has been related to the solvent nature of the alcohol acting on sebum within the follicle (Bamba and Wepierre, 1993).

Direct removal of a proportion of the SC, by tape stripping or shaving, also significantly increased the transdermal penetration of MeSa and Sa through equine skin. The use of repeated tape stripping is a useful technique to investigate transdermal drug penetration and the barrier function of different layers of the epidermis *in vivo* and *in vitro* (Cross et al., 2003; Tsai et al., 2003; Honeywell-Nguyen et al., 2004). Since progressive loss of the SC will greatly diminish the barrier function of skin (Honeywell-Nguyen et al., 2004), the results in the current study indicate that common procedures used to prepare skin sites can result in significantly higher penetration of topically applied drugs. We did not specifically measure the extent of SC loss following pre-treatment of the skin samples, although the procedures for shaving and the application then removal of adhesive tape were based on what may be expected to be used during preparation of horse skin for clinical procedures. It was further noted that relatively more Sa and less MeSa was measured in receptor fluid after tape stripping, although this was not statistically significant. The relative contribution of the different layers of skin to cutaneous metabolism and resistance to transdermal penetration, particular during models of skin damage, warrants further investigation.

The results in the current study have shown that disruption and/or loss of the SC will reduce the barrier function of skin to topically applied formulations. Increased transdermal drug penetration will result in higher systemic drug concentrations. It should therefore be acknowledged that routine pre-treatments applied to skin may result in higher efficacy and, more importantly, an increased potential for incidence of adverse effects when topical formulations are subsequently applied.

6. Relating *in vitro* results to the *in vivo* situation when applying topical drugs to horses

6.1 Introduction

The studies undertaken in this project have been invaluable to the understanding of some factors affecting drug movement into and through equine skin. These studies are, however, *in vitro* studies and there needs to be some association between the laboratory results and what will happen in the horse *in vivo*. It is obvious that blood samples could be collected to measure systemic drug and metabolite concentrations, although some drugs may not reach detectable levels when applied topically and/or may be indistinguishable from endogenous compounds, examples include testosterone and cortisol. Equally, there are no studies that specifically relate systemic blood concentrations of any drugs to what is happening locally, immediately below the site of application.

A recent and highly innovative addition to the techniques used to study cutaneous drug delivery is microdialysis (Anderson *et al.*, 1994; Benfeldt *et al.*, 1999a; Kreilgaard, 2002). This technique involves placing a microdialysis probe into the tissue of interest, such as the dermis and/or subcutis. The probe consists of a semi-permeable membrane forming a thin hollow tube which is slowly perfused (typically 0.1 – 5 µl/min) with a physiological solution (perfusate) which equilibrates with the extracellular fluid (ECF) of the surrounding tissue. Small substances, including drug molecules, can pass into the membrane as long as they are smaller than the molecular weight cut-off value of the membrane (typically 10 000 D) and these molecules can then be measured in the outflow (dialysate) from the probe continuously over time course of the study following topical application.

The advantages of microdialysis are that it is relatively non-invasive and, since the probe functions as an artificial blood vessel, it is possible to monitor topical drug penetration continuously with detailed real-time chronology (Anderson *et al.*, 1996; Benfeldt *et al.*, 1999a). Some localised 'flare' is observed at the site of probe implantation, although this quickly resolves within 30-60 min and does not appear to affect drug penetration kinetics (Anderson *et al.*, 1994; Andersson *et al.*, 1995). Some drawbacks to the technique are that it may not be suitable for larger lipophilic or highly tissue-bound molecules, especially if analytical sensitivity is limited, and recovery of drug into perfusate can be difficult (Kreilgaard, 2002).

The technique is easily adaptable to veterinary applications (Qian *et al.*, 2003) and we have developed a model of microdialysis in dogs to investigate the penetration of topically applied anti-inflammatory compounds (Mills *et al.*, 2005a). This model may then be applicable to the horse when relating *in vitro* data to the *in vivo* situation when applying topical formulations.

6.2 Preliminary results using a dog model

6.2.1 Analyte concentrations in dialysate and plasma

The time course of SA penetration into dialysate from the treated dermis was rapid and significantly higher than untreated dermis (P=0.0098). Similarly, MeSA was significantly higher in treated dermis (P=0.0323). However, SA concentrations in probes placed within muscle were low and near the limit of assay detection (10 µg/L). Analytes were also detected in the regional and systemic plasma samples within 30 min of topical application of the formulation, rising to a plateau at around 180 min (Figure 10). The amount of SA measured in the femoral vein (AUC = 1719.9 h.µg/L) was not significantly higher than measured in the cephalic vein (AUC = 1348.2 h.µg/L), although the total amount of analyte (MeSA plus SA) in the local femoral vein (AUC = 3429.2 h.µg/L) was significantly higher (P=0.003) than that in the systemic circulation (cephalic vein), where SA but not MeSA was detected.

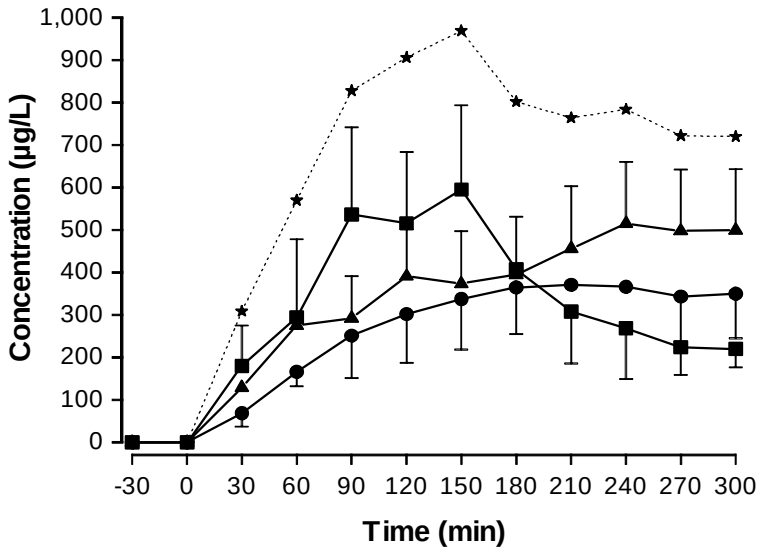


Figure 10. Plasma concentrations (mean ± SD) of SA (●) in the cephalic vein, and SA (▲) and MeSA (■) in the femoral vein of five Greyhounds. The dotted line (*) represents total analyte concentrations (SA + MeSA) in the femoral vein where the AUC (3429.2 h.µg/L) was significantly higher (*P*=0.003) than in the cephalic vein where SA (AUC = 1348.2 h.µg/L) but not MeSA was measured.

6.2.2 Analyte concentrations in tissue and synovial fluid

There were large amounts of both SA and MeSA in skin from the treated side (Table 1) and a progressive decline of both analytes with increasing tissue depth penetrating into the underlying deep muscle. SA, but not MeSA, was detected in muscle on the untreated, contralateral side, however similar concentrations of SA were found at each tissue depth. Substantial amounts of SA and MeSA were found in the synovial fluid collected from the treated joint, whilst no detectable concentration of either analyte could be measured in the synovial fluid from the untreated side or penetrating the fibrous joint capsule or articular cartilage from either site.

Table 5. Concentrations (Mean (± SD)**) of SA, MeSA and total (SA + MeSA) in tissue (µg/g) and synovial fluid (µg/L) of dogs (n=5) following topical application of 20% MeSA to the left (treated) hip joint compared with the right (untreated) hip joint.**

	Treated			Untreated	
Tissue	**SA**	**MeSA**	**Total**	**SA**	**MeSA**
Skin	38 330[1] (20 560)	143 500[2] (107 900)	182 300[3] (115 600)	0.0 (0.0)	0.0 (0.0)
Muscle					
2 mm	3 975[4] (1 735)	5 923[5] (2 776)	9 896[6] (3 756)	346.1 (330)	0.0 (0.0)
5 mm	2 471[7] (1 163)	2 159[8] (1434)	4 639[9] (2 527)	289.2 (263)	0.0 (0.0)
15 mm	1 318[10] (573)	0.0 (0)	1 318 (573)	306.4 (252.3)	0.0 (0.0)
Joint					
Synovial fluid	256.4[11] (137.3)	284.2[12] (183.2)	539.8[13] (261.9)	0.0 (0.0)	0.0 (0.0)

[1]*P*=0.00001; [2]*P*=0.00001; [3]*P*=0.00001; [4]*P*=0.0005; [5]*P*=0.0054; [6]*P*=0.0015; [7]*P*=0.0041; [8]*P*=0.0281; [9]*P*=0.0126; [10]*P*=0.0167; [11]*P*=0.0059; [12]*P*=0.0885; [13]*P*=0.0332;

6.3 Discussion

The present study has demonstrated local direct tissue penetration of a topically applied NSAID to deeper tissues and synovial fluid. Penetration of topically applied SA formulations into underlying muscle has previously been demonstrated (Singh and Roberts, 1993a; Cross et al., 1997; Cross et al., 1998; Benfeldt et al., 1999b). Similarly, diclofenac has been measured in synovial fluid collected from the knees of humans following topical application (Dawson et al., 1988; Radermacher et al., 1991), though the mechanism by which it arrived there remains unclear. Measurement of drug concentrations in peripheral vasculature has been used to suggest systemic redistribution as the main source of drug penetration into deeper tissues and joint fluid (Singh and Roberts, 1993b, a). However, application of pharmacokinetic principles to peripheral plasma drug residues, particularly when not accounting for dilution (Cross et al., 1997), would be unlikely to apply to those concentrations occurring at local application sites (Cross et al., 1998). The present study is the first to measure direct regional vascular drug residues, demonstrating a two fold higher concentration of drug (SA and ester) in this regional drainage, compared to levels (SA only) in the systemic circulation. SA levels in untreated muscle tissue samples were comparable to systemic plasma concentrations, confirming the existence of some systemic distribution of drugs shortly after topical application. Importantly, there was no evidence of SA in the contralateral (untreated) joint, yet both the applied ester (MeSA) and SA could be clearly detected in the synovial fluid of the treated joint and in the regional vasculature.

Elucidating the exact mechanisms governing the transdermal movement of drugs is a complex issue and beyond the scope of the current study (for a review see Roberts et al. (2002)). Direct penetration of NSAIDs has previously been reported to be prominent to depths of only the first 3 to 4 mm of tissue, declining exponentially from this point in deeper tissue layers (Singh and Roberts, 1994). Studies in the rat have shown that concentrations of NSAIDs in tissues peak at 2 to 4 hr and again at 10 hr, reflecting direct absorption and systemic delivery respectively (McNeill et al., 1992; Singh and Roberts, 1993a). The use of microdialysis confirmed an early (within 30 min) penetration of topically applied MeSA. Some evidence of early drug appearance in treated muscle was apparent, although these results were around the limit of detection and may be unreliable. Previous studies have also shown that drug penetration did not necessarily correlate with microdialysis probe depth (Muller et al., 1997) suggesting that perhaps a combination of direct penetration and systemic blood redistribution could be occurring. To overcome these difficulties, drug tissue residues were determined, following drainage of systemic blood from the dog (exsanguation) to limit any contamination of tissue samples by the blood they contained. These results demonstrated a gradual decline in NSAID concentration with increasing depth towards the joint, compared to the untreated side where SA concentrations were similar in the tissues irrespective of depth.

In conclusion, this study has shown that topically applied NSAIDs can penetrate to deeper tissues and synovial fluid directly. With local blood concentrations higher than circulating systemic levels, it is suggested that both direct diffusion and local blood redistribution must be contributing to this effect. This study also demonstrates that systemic blood concentrations may be inadequate to describe the regional kinetics of topically applied drugs.

7. Conclusions

Any stakeholder in the equine industry, from trainers, owners, veterinarians, officials and regulators should be aware of the potential for any substance applied to skin to pass through this outermost barrier. They should also be aware that there are significant species differences between how much and how fast a particular formulation will penetrate skin, meaning that a formulation developed and registered for one species will be unlikely to have the same effect and safety margin when applied to another species, including the horse. This has important implications for using drugs with potential toxic effects or low margins or safety, where small changes in uptake of drugs applied topically should be avoided. Equally, stakeholders entering horses into competition should be aware of the potential for altered appearance of drugs and metabolites in the body following topical application which may lead to infringement of the rules of competition.

Stakeholders should be mindful of many of the findings in this report when applying topical drugs to the horse. There are regional differences, meaning that a formulation applied to one part of the body may have a different level of effect if applied elsewhere. Different formulations of the same drug can have significant effects on how much of the drug actually penetrates through the skin and is available for whatever purpose it was designed. Most importantly, changes in the integrity of the skin, such as rashes, abrasions, skin disease or the use of agents to clean the skin, can substantially alter the amount and rate of active drug uptake.

Importantly, stakeholders should also be aware that the basic knowledge of equine topical drug formulation is increasing, e.g., the current project, with more drugs being produced specifically for horses. Topical drugs are easy to apply and have many other advantages, meaning that the potential to treat a range of medical conditions, such as joint pain, skin conditions and cutaneous pests (i.e. fleas, biting flies), is increasing. Better understanding of transdermal drug penetration in the horse will lead to more products that are more effective for topical application to horses.

8. References

Adams, H., 2001. Veterinary Pharmacology and Therapeutics, 8th Edition. Blackwell Publishers, Ames, Iowa, USA.

Anderson, C., Andersson, T., Wardell, K., 1994. Changes in skin circulation after insertion of a microdialysis probe visualized by laser Doppler perfusion imaging. *Journal of Investigative Dermatology* 102, 807-811.

Anderson, C., Andersson, T., Boman, A., Molander, M., 1996. Cutaneous microdialysis for the measurement *in vivo* of the percutaneous absorption of organic solvents. C*urrent Problems in Dermatology* 25, 37-46.

Andersson, T., Wardell, K., Anderson, C., 1995. Human *in vivo* cutaneous microdialysis: estimation of histamine release in cold urticaria. *Acta Dermato-venereologica* 75, 343-347.

Aungst, B.J., Blake, J.A., Hussain, M.A., 1990. Contributions of drug solubilization, partitioning, barrier disruption, and solvent permeation to the enhancement of skin permeation of various compounds with fatty acids and amines. *Pharmaceutical Research* 7, 712-718.

Australian Prescription Products Guide 2002. 31st Edition. Pharmaceutics Publishing Company Ltd, Hawthorn, Victoria, Australia.

Bamba, F.L., Wepierre, J., 1993. Role of the appendageal pathway in the percutaneous absorption of pyridostigmine bromide in various vehicles. *European Journal of Drug Metabolism and Pharmacokinetics* 18, 339-348.

Barry, B.W., 2001. Novel mechanisms and devices to enable successful transdermal drug delivery. *European Journal of Pharmaceutical Sciences* 14, 101-114.

Benfeldt, E., Serup, J., Menne, T., 1999a. Effect of barrier perturbation on cutaneous salicylic acid penetration in human skin: *in vivo* pharmacokinetics using microdialysis and non-invasive quantification of barrier function. *British Journal of Dermatology* 140, 739-748.

Benfeldt, E., Serup, J., Menne, T., 1999b. Microdialysis vs. suction blister technique for *in vivo* sampling of pharmacokinetics in the human dermis. *Acta Dermato-venereologica* 79, 338-342.

Clarys, P., Alewaeters, K., Jadoul, A., Barel, A., Manadas, R.O., Preat, V., 1998. In vitro percutaneous penetration through hairless rat skin: influence of temperature, vehicle and penetration enhancers. *European Journal of Pharmacy and Biopharmaceutics* 46, 279-283.

Cross, S.E., Anderson, C., Thompson, M.J., Roberts, M.S., 1997. Is there tissue penetration after application of topical salicylate formulations? *Lancet* 350, 636.

Cross, S.E., Anderson, C., Roberts, M.S., 1998. Topical penetration of commercial salicylate esters and salts using human isolated skin and clinical microdialysis studies. *British Journal of Clinical Pharmacology* 46, 29-35.

Cross, S.E., Megwa, S.A., Benson, H.A., Roberts, M.S., 1999. Self promotion of deep tissue penetration and distribution of methylsalicylate after topical application. *Pharmaceutical Research* 16, 427-433.

Cross, S.E., Pugh, W.J., Hadgraft, J., Roberts, M.S., 2001. Probing the effect of vehicles on topical delivery: understanding the basic relationship between solvent and solute penetration using silicone membranes. *Pharmaceutical Research* 18, 999-1005.

Cross, S.E., Magnusson, B.M., Winckle, G., Anissimov, Y., Roberts, M.S., 2003. Determination of the effect of lipophilicity on the *in vitro* permeability and tissue reservoir characteristics of topically applied solutes in human skin layers. *Journal of Investigative Dermatology* 120, 759-764.

Dawson, M., McGee, C.M., Vine, J.H., Nash, P., Watson, T.R., Brooks, P.M., 1988. The disposition of biphenylacetic acid following topical application. *European Journal of Clinical Pharmacology* 33, 639-642.

Feldmann, R.J., Maibach, H.I., 1967. Regional variation in percutaneous penetration of 14C cortisol in man. *Journal of Investigative Dermatology* 48, 181-183.

Finnin, B.C., Morgan, T.M., 1999. Transdermal penetration enhancers: applications, limitations, and potential. *Journal of Pharmaceutical Sciences* 88, 955-958.

Flynn, G.L., Durrheim, H., Higuchi, W.I., 1981. Permeation of hairless mouse skin II: membrane sectioning techniques and influence on alkanol permeabilities. *Journal of Pharmaceutical Sciences* 70, 52-56.

Flynn, G.L., 1990. Physico-chemical determinants of skin absorption, In: Gerity, T.R., Henry, C.J. (Eds.) Principles of Route to Route Extrapolation for Risk Assessment. Elsevier, Amsterdam, Netherlands.

Franz, T.J., 1975. Percutaneous absorption on the relevance of *in vitro* data. *Journal of Investigative Dermatology* 64, 190-195.

Godwin, D.A., Michniak, B.B., Creek, K.E., 1997. Evaluation of transdermal penetration enhancers using a novel skin alternative. *Journal of Pharmaceutical Sciences* 86, 1001-1005.

Guy, R.H., Hadgraft, J., 1989. Mathematical models of percutaneous absorption, In: Bronaugh, R.L., Maibach, H.I. (Eds.) Percutaneous Absorption: Mechanisms, Methodology and Drug Delivery. Marcel Dekker, New York, USA, pp. 13-26.

Hadgraft, J., 1996. Recent developments in topical and transdermal delivery. *European Journal of Drug Metabolism and Pharmacokinetics* 21, 165-173.

Harrison, J.E., Watkinson, A.C., Green, D.M., Hadgraft, J., Brain, K., 1996. The relative effect of Azone and Transcutol on permeant diffusivity and solubility in human stratum corneum. *Pharmaceutical Research* 13, 542-546.

Harrison, S.M., Barry, B.W., Dugard, P.H., 1984. Effects of freezing on human skin permeability. *Journal of Pharmacy and Pharmacology* 36, 261-262.

Higgs, G.A., Salmon, J.A., Henderson, B., Vane, J.R., 1987. Pharmacokinetics of aspirin and salicylate in relation to inhibition of arachidonate cyclooxygenase and antiinflammatory activity. *Proceedings of the National Academy of Sciences of the United States of America* 84, 1417-1420.

Honeywell-Nguyen, P.L., Gooris, G.S., Bouwstra, J.A., 2004. Quantitative assessment of the transport of elastic and rigid vesicle components and a model drug from these vesicle formulations into human skin *in vivo*. *Journal of Investigative Dermatology* 123, 902-910.

Hueber, F., Wepierre, J., Schaefer, H., 1992. Role of transepidermal and transfollicular routes in percutaneous absorption of hydrocortisone and testosterone: *in vivo* study in the hairless rat. *Skin Pharmacology* 5, 99-107.

Hueber, F., Schaefer, H., Wepierre, J., 1994. Role of transepidermal and transfollicular routes in percutaneous absorption of steroids: *in vitro* studies on human skin. *Skin Pharmacology* 7, 237-244.

Idson, B., 1983. Vehicle effects in percutaneous absorption. Drug Metabolism Reviews 14, 207-222.

Inamori, T., Ghanem, A.H., Higuchi, W.I., Srinivasan, V., 1994. Macromolecule transport in and effective pore size of ethanol pretreated human epidermal membrane. *International Journal of Pharmaceutics* 105, 113-123.

Johnson, M.E., Blankschtein, D., Langer, R., 1997. Evaluation of solute permeation through the stratum corneum: lateral bilayer diffusion as the primary transport mechanism. *Journal of Pharmaceutical Sciences* 86, 1162-1172.

Kaplun-Frischoff, Y., Touitou, E., 1997. Testosterone skin permeation enhancement by menthol through formation of eutectic with drug and interaction with skin lipids. *Journal of Pharmaceutical Sciences* 86, 1394-1399.

Kreilgaard, M., 2002. Assessment of cutaneous drug delivery using microdialysis. A*dvanced Drug Delivery Reviews* 54 Suppl 1, S99-121.

Lampe, M.A., Burlingame, A.L., Whitney, J., Williams, M.L., Brown, B.E., Roitman, E., Elias, P.M., 1983. Human stratum corneum lipids: characterization and regional variations. *Journal of Lipid Research* 24, 120-130.

Magnusson, B.M., Walters, K.A., Roberts, M.S., 2001. Veterinary drug delivery: potential for skin penetration enhancement. *Advanced Drug Delivery Reviews* 50, 205-227.

Maibach, H.I., Feldman, R.J., Milby, T.H., Serat, W.F., 1971. Regional variation in percutaneous penetration in man. Pesticides. *Archives of Environmental Health* 23, 208-211.

Manning, T.O., Monteiro-Riviere, N.A., Bristol, D.G., Riviere, J.E., 1991. Cutaneous laser-Doppler velocimetry in nine animal species. *American Journal of Veterinary Research* 52, 1960-1964.

Matsuzaki, K., Imaoka, T., Asano, M., Miyajima, K., 1993. Development of a model membrane system using stratum corneum lipids for estimation of drug skin permeability. *Chemical and Pharmaceutical Bulletin* 41, 575-579.

McNeill, S.C., Potts, R.O., Francoeur, M.L., 1992. Local enhanced topical delivery (LETD) of drugs: does it truly exist? *Pharmaceutical Research* 9, 1422-1427.

Megwa, S.A., Benson, H.A., Roberts, M.S., 1995. Percutaneous absorption of salicylates from some commercially available topical products containing methyl salicylate or salicylate salts in rats. *Journal of Pharmacy and Pharmacology* 47, 891-896.

Mills, P.C., Magnusson, B.M., Cross, S.E., 2003a. The effect of solute lipophilicity on penetration through feline skin. *Journal of Veterinary Pharmacology and Therapeutics* 26, 311-314.

Mills, P.C., Magnusson, B.M., Cross, S.E., 2003b. The effect of solute lipophilicity on penetration through canine skin. *Australian Veterinary Journal* 81, 752-755.

Mills, P.C., Magnusson, B.M., Cross, S.E., 2004a. Investigation of *in vitro* transdermal absorption of fentanyl from patches placed on skin samples obtained from various anatomic regions of dogs. *American Journal of Veterinary Research* 65, 1697-1700.

Mills, P.C., Magnusson, B.M., Cross, S.E., 2004b. The effect of region of application on absorption of ethanol and hexanol through canine skin. *Research in Veterinary Science* 76, 37-41.

Mills, P.C., Magnusson, B.M., Cross, C.E., 2005. The effects of vehicle and region of application on absorption of hydrocortisone through canine skin. *American Journal of Veterinary Research* 66, 43-47.

Moe, G., Armstrong, P.W., 1986. Influence of skin site on bioavailability of nitroglycerin ointment in congestive heart failure. *American Journal of Medicine* 81, 765-770.

Montagna, W., 1971. Cutaneous comparative biology. Archives of Dermatology 104, 577-591.

Monteiro-Riviere, N.A., Bristol, D.G., Manning, T.O., Rogers, R.A., Riviere, J.E., 1990. Interspecies and interregional analysis of the comparative histologic thickness and laser Doppler blood flow measurements at five cutaneous sites in nine species. *Journal of Investigative Dermatology* 95, 582-586.

Monteiro-Riviere, N.A., 1991. Comparative Anatomy, Physiology, and Biochemistry of Mammalian Skin, In: Hobson, D.W. (Ed.) Dermal and Ocular Toxicology: Fundamentals and Methods. CRC Press, Boca Raton, pp. 3-72.

Monteiro-Riviere, N.A., Inman, A.O., Riviere, J.E., McNeill, S.C., Francoeur, M.L., 1993. Topical penetration of piroxicam is dependent on the distribution of the local cutaneous vasculature. *Pharmaceutical Research* 10, 1326-1331.

Monteiro-Riviere, N.A., Inman, A.O., Snider, T.H., Blank, J.A., Hobson, D.W., 1997. Comparison of an *in vitro* skin model to normal human skin for dermatological research. *Microvascular Research Techniques* 37, 172-179.

Monteiro-Riviere, N.A., Inman, A.O., Mak, V., Wertz, P., Riviere, J.E., 2001. Effect of selective lipid extraction from different body regions on epidermal barrier function. *Pharmaceutical Research* 18, 992-998.

Muller, M., Mascher, H., Kikuta, C., Schafer, S., Brunner, M., Dorner, G., Eichler, H.G., 1997. Diclofenac concentrations in defined tissue layers after topical administration. *Clin Pharmacol Ther* 62, 293-299.

Oriba, H.A., Bucks, D.A., Maibach, H.I., 1996. Percutaneous absorption of hydrocortisone and testosterone on the vulva and forearm: effect of the menopause and site. *British Journal of Dermatology* 134, 229-233.

Ostrenga, J., Steinmetz, C., Poulsen, B., 1971. Significance of vehicle composition. I. Relationship between topical vehicle composition, skin penetrability, and clinical efficacy. *Journal of Pharmaceutical Sciences* 60, 1175-1179.

Peck, K.D., Ghanem, A.H., Higuchi, W.I., 1994. Hindered diffusion of polar molecules through and effective pore radii estimates of intact and ethanol treated human epidermal membrane. *Pharmaceutical Research* 11, 1306-1314.

Polano, M.K., Ponec, M., 1976. Dependence of corticosteroid penetration on the vehicle. A*rchives of Dermatology* 112, 675-680.

Pugh, W.J., Roberts, M.S., Hadgraft, J., 1996. Epidermal permeability - penetrant structure relationships: 3. The effect of hydrogen bonding interactions and molecular size on diffusion across stratum corneum. *International Journal of Pharmaceutics* 138, 149-165.

Qian, M., West, W., Wu, J.T., Lu, B., Christ, D.D., 2003. Development of a dog microdialysis model for determining synovial fluid pharmacokinetics of anti-arthritis compounds exemplified by methotrexate. *Pharmaceutical Research* 20, 605-610.

Qiao, G.L., Chang, S.K., Riviere, J.E., 1993. Effects of anatomical site and occlusion on the percutaneous absorption and residue pattern of 2,6-[ring-14C]parathion *in vivo* in pigs. *Toxicology and Applied Pharmacology* 122, 131-138.

Qiao, G.L., Riviere, J.E., 1995. Significant effects of application site and occlusion on the pharmacokinetics of cutaneous penetration and biotransformation of parathion *in vivo* in swine. *Journal of Pharmaceutical Sciences* 84, 425-432.

Radermacher, J., Jentsch, D., Scholl, M.A., Lustinetz, T., Frolich, J.C., 1991. Diclofenac concentrations in synovial fluid and plasma after cutaneous application in inflammatory and degenerative joint disease. *British Journal of Clinical Pharmacology* 31, 537-541.

Riegelman, S., 1974. Pharmacokinetics. Pharmacokinetic factors affecting epidermal penetration and percutaneous absorption. *Clinical Pharmacology and Therapeutics* 16, 873-883.

Riviere, J.E., Bowman, K.F., Monteiro-Riviere, N.A., Dix, L.P., Carver, M.P., 1986. The isolated perfused porcine skin flap (IPPSF). I. A novel *in vitro* model for percutaneous absorption and cutaneous toxicology studies. *Fundamentals of Applied Toxicology* 7, 444-453.

Riviere, J.E., Monteiro-Riviere, N.A., 1991. The isolated perfused porcine skin flap as an *in vitro* model for percutaneous absorption and cutaneous toxicology. *Critical Review of Toxicology* 21, 329-344.

Riviere, J.E., Papich, M.G., 2001. Potential and problems of developing transdermal patches for veterinary applications. *Advanced Drug Delivery Reviews* 50, 175-203.

Roberts, M.S., Cross, S.E., Pellett, M.A., 2002. Skin Transport, In: Walters, K.A. (Ed.) Dermatological and Transdermal Formulations. Marcel Dekker, New York, USA, pp. 89-196.

Roberts, M.S., Cross, S.E., Anissimov, Y.G., 2004. Factors affecting the formation of a skin reservoir for topically applied solutes. *Skin Pharmacology and Physiology* 17, 3-16.

Rosado, C., Cross, S.E., Pugh, W.J., Roberts, M.S., Hadgraft, J., 2003. Effect of vehicle pretreatment on the flux, retention, and diffusion of topically applied penetrants *in vitro*. P*harmaceutical Research* 20, 1502-1507.

Rougier, A., Lotte, C., Maibach, H.I., 1987. In vivo percutaneous penetration of some organic compounds related to anatomic site in humans: predictive assessment by the stripping method. *Journal of Pharmaceutical Sciences* 76, 451-454.

Roy, S.D., Flynn, G.L., 1989. Transdermal delivery of narcotic analgesics: comparative permeabilities of narcotic analgesics through human cadaver skin. *Pharmaceutical Research* 6, 825-832.

Roy, S.D., Flynn, G.L., 1990. Transdermal delivery of narcotic analgesics: pH, anatomical, and subject influences on cutaneous permeability of fentanyl and sufentanil. P*harmaceutical Research* 7, 842-847.

Scheuplein, R.J., Blank, I.H., 1973. Mechanism of percutaneous absorption. IV. Penetration of nonelectrolytes (alcohols) from aqueous solutions and from pure liquids. *Journal of Investigative Dermatology* 60, 286-296.

Schumacher, J., Chambers, M., Hanselka, D.V., Morton, L.D., 1987. Preservation of skin by refrigeration for autogenous grafting in the horse. *Veterinary Surgery* 16, 358-361.

Shah, A.K., Wei, G., Lanman, R.C., Bhargava, V.O., Weir, S.J., 1996. Percutaneous absorption of ketoprofen from different anatomical sites in man. *Pharmaceutical Research* 13, 168-172.

Shah, V.P., Elkins, J.S., 1995. In-vitro release from corticosteroid ointments. *Journal of Pharmaceutical Sciences* 84, 1139-1140.

Singh, P., Roberts, M.S., 1993a. Dermal and underlying tissue pharmacokinetics of salicylic acid after topical application. *Journal of Pharmacokinetics and Biopharmaceutics* 21, 337-373.

Singh, P., Roberts, M.S., 1993b. Blood flow measurements in skin and underlying tissues by microsphere method: application to dermal pharmacokinetics of polar nonelectrolytes. *Journal of Pharmaceutical Sciences* 82, 873-879.

Singh, P., Roberts, M.S., 1994. Skin permeability and local tissue concentrations of nonsteroidal anti-inflammatory drugs after topical application. *Journal of Pharmacology and Experimental Therapeutics* 268, 144-151.

Surber, C., Wilhelm, K.P., Hori, M., Maibach, H.I., Guy, R.H., 1990. Optimization of topical therapy: partitioning of drugs into stratum corneum. *Pharmaceutical Research* 7, 1320-1324.

Tsai, J.C., Lin, C.Y., Sheu, H.M., Lo, Y.L., Huang, Y.H., 2003. Noninvasive characterization of regional variation in drug transport into human stratum corneum *in vivo*. *Pharmaceutical Research* 20, 632-638.

Tur, E., Maibach, H.I., Guy, R.H., 1991. Percutaneous penetration of methyl nicotinate at three anatomic sites: evidence for an appendageal contribution to transport? *Skin Pharmacology* 4, 230-234.

Walters, K.A., Brain, K., 2002. Dermatological Formulation and Transdermal Systems, In: Walters, K.A. (Ed.) Dermatological and Transdermal Formulations. Marcel Dekker, New York, USA, pp. 319-399.

Walters, K.A., Roberts, M.S., 2002. The Structure and Function of Skin, In: Walters, K.A. (Ed.) Dermatological and Transdermal Formulations. Marcel Dekker, New York, USA, pp. 1-40.

Wester, R.C., Christoffel, J., Hartway, T., Poblete, N., Maibach, H.I., Forsell, J., 1998. Human cadaver skin viability for *in vitro* percutaneous absorption: storage and detrimental effects of heat-separation and freezing. *Pharmaceutical Research* 15, 82-84.

Wilhelm, K.P., Pasche, F., Surber, C., Maibach, H.I., 1990. Sodium hydroxide-induced subclinical irritation. A test for evaluating stratum corneum barrier function. *Acta Dermato-venereologica* 70, 463-467.

Wong, V.K., Della Croce, C., Schonfeld, S., Mastrangelo, A.M., Lebwohl, M., 2003. Use and abuse of topical corticosteroids in infections of the skin and related structures. *Journal of Drugs in Dermatology* 2, 268-276.

Yardley, H.J., Summerly, R., 1981. Lipid composition and metabolism in normal and diseased epidermis. *Pharmacological Therapeutics* 13, 357-383.