EVALUATION OF PERCUTANEOUS PENETRATION OF INSECT REPELLENTS AND SUNSCREENS

BY

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A Thesis
Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Faculty of Pharmacy
University of Manitoba
Winnipeg, Manitoba
CANADA

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FACULTY OF GRADUATE STUDIES

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THIS THESIS IS DEDICATED TO

MY PARENTS

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STATEMENT OF ORIGINALITY

The work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and the materials have not been submitted, either in whole or in part for a degree at this or any other university.

Sreeneeranj Kasichayanula

November, 2006

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LIST OF ABBREVIATIONS

AUC:	Area under the concentration-time curve
C_{max}:	Maximum concentration observed in plasma
CDC:	United States Center for Disease Control
DEET:	N,N-diethyl-*m*-toluamide
DHB:	2,4-Dihydroxybenzophenone
DHMB:	N,N-diethyl-*m*-hydroxymethylbenzamide
DMB:	2,2'-Dihydroxy-4-methoxybenzophenone
DMSO:	Dimethyl sulfoxide
DPT:	N,N-Dipropyl-*m*-toluamide
EPA:	United States Environmental Protection Agency
ET:	N-ethyl-*m*-toluamide
FDA:	Food and Drug Administration
GCD:	γ-Cyclodextrin
HPLC:	High performance liquid chromatography
HPDCD:	Hydroxypropyl-β-cyclodextrin
LOD:	Limit of detection
LOQ:	Limit of quantification
MED:	Minimum effective dose
MED:	Minimum erythema dose
MRT:	Mean residence time
OTC:	Over the counter
OXY:	Oxybenzone

PDMS:	Poly(dimethyl) siloxane
PEG:	Polyethylene glycol 400
PG:	Propylene glycol
RF:	*In vitro* receptor fluid
SC:	Stratum corneum
SPF:	Sun Protection Factor
THB:	2,3,4-Trihydroxybenzophenone
T_{max}***:***	Time to achieve C_{max}
UV:	Ultraviolet
UVA:	Ultraviolet radiation – Type A
UVB:	Ultraviolet radiation – Type B
UVC:	Ultraviolet radiation – Type C
UVR:	Ultraviolet radiation
WNV:	West Nile virus

LIST OF SYMBOLS

C: Concentration gradient across the barrier *(g/ml)*

C_S: Concentration of test compound in donor solution *(g/ml)*

D: Diffusion coefficient *(cm^2/h)*

J: Flux *(g/cm^2h)*

K: Partition coefficient *(cm/h)*

M: Amount of permeant *(g)*

p: Permeation coefficient *(cm/h)*

S : Surface area of membrane *(cm^2)*

t_L: Lag time *(h)*

x: Membrane thickness *(cm)*

ABSTRACT

Insect repellents and sunscreens are over-the-counter consumer care products that have been extensively used by the general public for decades. With an increasingly awareness of mosquito-borne diseases and detrimental effects of UV radiation from the sun, concurrent use of both repellent and sunscreen preparations has become prevalent. Repellent DEET and sunscreen oxybenzone had been investigated individually for their percutaneous properties in the past; however, no study has been carried out to assess their transdermal characterization from a concurrent application.

In this thesis, *in vitro* diffusion experimentation was utilized to systematically evaluate transmembrane behaviors of DEET and oxybenzone across three different membrane models. Permeation properties were assessed with individual DEET or oxybenzone, as well as with the combination of both compounds. The influence of dissolving solvent, application concentration and temperature on diffusion process was also studied. Further *in vivo* studies were performed using three commercially available repellent and sunscreen products in a piglet model. Percutaneous penetration and systemic absorption of DEET and oxybenzone after topical skin application were evaluated using tape stripping. Concentrations of DEET, oxybenzone and their primary metabolites in biological samples were analyzed using a validated HPLC assay.

DEET and oxybenzone permeated across all study membranes *in vitro*. A synergistic permeation was observed when the two compounds were present simultaneously. This penetration enhancement was also dependent on the type of dissolving solvent and the concentration of both DEET and oxybenzone used. The penetration patterns were found to be identical among three membranes tested. *In vivo* studies indicated percutaneous penetration and systemic absorption of DEET and oxybenzone from commercial preparations. Combined repellent/sunscreen formulation demonstrated a significant higher percutaneous penetration of DEET and oxybenzone than its individual counterparts. DEET penetrated through the skin at a faster rate than oxybenzone. Oxybenzone and its major metabolites were detected in the urine samples 48 hours after the topical administration; however, no DEET was found in the urine samples.

Percutaneous permeation and systemic absorption of repellent DEET and sunscreen oxybenzone was systematically investigated in this thesis. Transdermal enhancement of DEET and oxybenzone from a concurrent application was first observed and reported. As topical preparations, both insect repellents and sunscreens should remain on the surface of skin with minimal systemic absorption; an increased percutaneous permeation observed in this thesis would potentiate undesirable characteristics and compromise protection efficacy of repellent and sunscreen products.

Chapter 1

Introduction

1.1. Human Skin Structure

Human skin is a remarkable and useful organ, the largest of all body parts and the outmost protective barrier against the external world. The skin takes 10-15% of body mass and provides our body with numerous mechanisms to interact with the external environment. Structurally, the skin is composed of four distinctive functional layers: the stratum corneum (SC, nonviable epidermis), epidermis (viable epidermis), dermis, and subcutaneous tissues (*Figure 1.1*). Stratum corneum is the top skin layer that is made of dead keratinized cells in stratified configuration. Each cell in the stratum corneum is approximately 40 μm in diameter and 0.5 μm in thickness; the total thickness of stratum corneum amounts to around 10-20 μm. The viable epidermis consists of metabolically active layers beneath stratum corneum, which can be further categorized as stratum basale, stratum spinosum, and stratum granulosum. Blood and lymphatic vessels, nerve endings and skin appendages (hair follicles and sweat glands) are all originated in dermis, while subcutaneous tissues bridge the overlying dermis and the underlying body constituents. Under healthy and intact conditions, human skin is capable of preventing most foreign substances from entering into the body.

The skin performs many biological and physiological functions that are essential to the survival of the human body in a highly hostile environment. For example, the skin keeps most external substances such as chemical compounds, bacteria and sunlight from reaching and influencing the internal body organs or tissues. The skin not only maintains the homeostasis of the body, but also acts as the largest sensory organ, adjusting and adapting the body against various environmental parameters such as temperature,

pressure, pain, and physical shock. In addition, the skin is in a continuous state of restoration and regeneration. Two to three billion skin cells are replaced and replenished on a daily basis. The human body executes this effort to rejuvenate the skin every month so that it can well maintain the first line of defense against dehydration, infection, injury and many other environmental extremes. Skin cells also detoxify harmful substances with many of the same enzymatic processes that the liver uses *in vivo*. As a gatekeeper, the skin absorbs and utilizes nutrients that are topically applied or deposited. Because it cannot completely discriminate, the skin takes many synthetic chemicals that are often present in skin care products such as soaps, body lotions and cosmetic preparations, which may have no benefits at the best or could be irritating or even toxic at the worst.

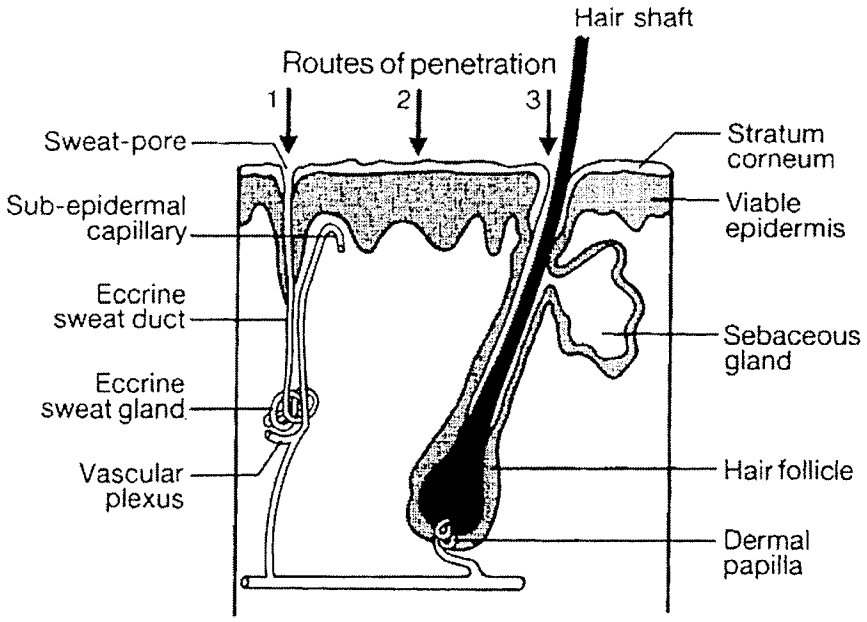


Figure 1.1. Simplified structure of the skin layers
(Barry BW: Novel mechanisms and devices to enable successful transdermal drug delivery. Eur J Pharm Sci 2001; 14(2):101-114)

1.1.1. Stratum Corneum (Non-Viable Epidermis)

Stratum corneum is composed of 15-25 flattened, stacked, hexagonal and cornified dead cells. At a thickness of approximately 10-20 μm, each cell of non-viable epidermis is roughly 40 μm in diameter and 0.5 μm in thickness. The thickness of stratum corneum varies significantly according to the functions of individual body part that it covers; body areas that are exposed frequently to direct and substantial physical interactions with the external environment tend to have thicker stratum corneum than those that are less exposed. As a result, stratum corneum of the palms and the soles are the thickest of the skin surface, while stratum corneum of the interior of the thighs and the arms are the thinnest of the skin surface. The barrier properties of stratum corneum against foreign substances result from the combination of high density (1.4 g/cm^3 in the dry state), low hydration (15-20% compared to normal 70% for the body), and wide intercellular regions (less than 0.1 μm) of this skin layer of the skin [1].

Cells of stratum corneum are composed of approximately 70% of insoluble bundled keratins and 20% of lipids encased in a cell envelope, which accounts for about 5% of the mass of stratum corneum. The intercellular regions of stratum corneum are filled with lipids and desmosomes for corneocyte cohesion. The barrier function of stratum corneum is further strengthened by a continuous desquamation of this horny layer, which renews completely once every 2-3 weeks. Stratum corneum also acts as a barrier in preventing the loss of internal body constituents, particularly water content to the external surroundings. It is estimated that a normal healthy adult would lose about 250 ml of water every day due to "insensible perspiration" [2].

Six classes of ceramides (designated ceramides 1-6) have been isolated and identified from stratum corneum in humans [3]. These molecules contain long-chain bases, such as sphinganine and 4-hydroxysphinganine, N-acetylated by different fatty acids. It is also evident that the intercellular lipid lamellae are further stabilized by the chemical links between the long chain ceramides and glutamate residues. Overall, the intercellular lipid lamellae appear to be highly structured and stable; they constitute an extremely effective blocking barrier to penetration and permeation of foreign substances through the skin.

The ability of stratum corneum to control water loss from within, and influx of potentially harmful components from outside, arises from the evolution of a unique mixture of protein and lipid materials that collectively constructs a coherent and protective membrane with morphologically distinctive domains. These domains are principally proteinaceous (the keratinocytes) and lipophilic (the intercellular spaces). Because of the lack of nuclei in the keratinocytes, stratum corneum is generally considered a non-viable tissue with no metabolic effects, and yet it is a highly effective rate-controlling barrier to the penetration of any foreign compound [4].

1.1.2. Viable Epidermis

Cells of stratum corneum originate in the viable epidermis and undergo numerous morphological changes before desquamation. *Figure 1.2* demonstrates epidermis of cell strata at varying levels of differentiation. The origin of epidermis cells lies in the basal lamina between the dermis and the viable epidermis. There are melanocytes, Langernhan cells, Merkel cells and two types of keratinic cells; the first type of keratinic cell

functions as stem cells that exhibit the capacity of dividing and producing new skin cells. The second type of keratinic cell serves to anchor the epidermis to the basement membrane [5]. Development of stratum corneum involves several steps of differentiation, which have resulted in a structure-based classification of the epidermis layers above the basal layer (stratum basale). These cells further progress through stratum spinosum, stratum granulosum, and stratum lucidum, finally turning into stratum corneum on the exterior of the skin surface as a protective layer against percutaneous penetration and permeation [6].

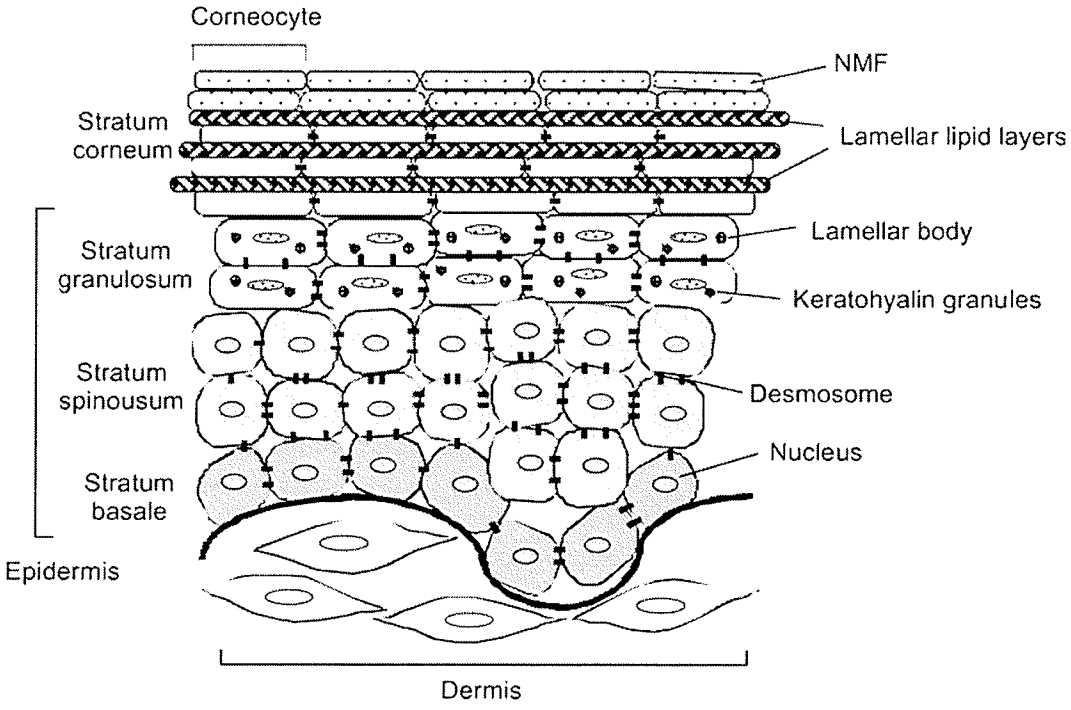


Figure 1.2. The differentiation of epidermis (www.biolcell.org, 2006)

The viable epidermis performs a number of physiological functions in addition to the generation of stratum corneum as previously described. Metabolism of substrates and foreign substances after their percutaneous permeation generally takes place in this layer of the skin; synthesis of melanin from melanocytes for skin pigmentation also occurs within epidermis. There are other cells present in viable epidermis. For example, Langerhans cells are important in recognizing the presence of antigens and initiating immune responses of the body against foreign attacks; Merkel cells are responsible for sensory perception, and keratinocyte cells produce keratin, the fibrous proteins in the skin.

1.1.3. Dermis and Subcutaneous Tissues

The main structural components of the dermis are referred to as a network of robust collagen fibers with a fairly uniform thickness of regularly spaced cross-striations. Dermis is approximately 0.1-0.5 cm in thickness and consists of 70% of collagenous fibers, which provide the body a scaffold of support and cushioning with its elastic connective tissues. Major cells present in dermis include fibroblasts, which produce the connective tissue components of collagen, laminin, fibronectin, vitronectin, and mast cells. These fibroblasts are also responsible for immune and inflammatory reactions on the skin surface, and production of melanocytes to generate skin pigment melanin. Beneath dermis, the fibrous tissues merge with the fat layers known as subcutaneous tissues. The upper portion of the dermis is formed into ridges (or papillae) projecting into the epidermis, where blood and lymphatic vessels and nerve endings are located. The blood flow rate within the skin is approximately 0.05 ml/min, providing a vascular

exchange to the papillary layer, the hair follicles, the sweat and apocirine glands, and the subcutaneous area.

1.1.4. Skin Appendages

There are four types of skin appendage: hair follicles and associated sebaceous glands, eccrine sweat glands, apocrine sweat glands, and nails. Hair follicles are distributed across the entire body surface, except for the soles of the feet, the palms of the hands and the lips. Each hair follicle is associated with sebaceous glands, which vary in size between 200-2000 μm in diameter. The sebum secreted by sebaceous glands, which consists largely of triglycerides, free fatty acids, and waxes, protects and lubricates the skin surface and maintains a healthy skin pH (approximately 5.0). Eccrine sweat is produced through merocrine secretion in the coiled gland and is composed of water, sodium chloride, potassium lactate, urea, ammonia, serine, ornithine, citrulline, aspartic acid, heavy metals, organic compounds and proteolytic enzymes. The primary physiological function of the eccrine units is thermoregulation, which is accomplished by the cooling effects of evaporation of eccrine sweat on the skin surface. Apocrine glands are outgrowths of the superior portions of pilosebaceous units (hair follicles). The exact biological function of apocrine glands is still unclear; it was postulated that they might represent as scent glands [7]. Nails are produced by living skin cells in the areas of fingers and toes. They are composed primarily of keratin, a hardened protein also found in skin and hair. Nails themselves consist of several different parts, including nail plate, nail bed, matrix, lunula, and cuticleand nail folds. Overall, the role of skin appendages in transdermal drug delivery and absorption is minimal and therefore negligible.

1.2. Skin Transport

Stratum corneum is the predominant barrier that limits the rate and extent at which a foreign agent permeates and diffuses into deeper layers of the skin [8]. Intercellular lipids of stratum corneum are essential to not only normal skin barrier homeostasis but also healthy tissue hydration levels [9,10]. The architecture of stratum corneum (*Figure 1.3*) has been regarded as a "mortar and brick" configuration, where the flattened interdigitated keratinized cells represent the bricks, while the interstitial lipid-rich regions between the cells and on the surface represent the mortar. A majority of passive drug diffusion takes place through the hydrophobic skin barriers [11]. Recent studies on the characterization of intercellular lipids under pathological skin conditions have confirmed the important function of the hydrophobic barrier as a major component of the skin transport [12-14].

The primary route of drug permeation across the stratum corneum is via the continuous intercellular lipid spaces. As such, the rate of permeation is largely dependent on the physicochemical characteristics of the penetrant. Two major variables are responsible for the differences in the rate and extent that a compound permeates across the skin; the concentration gradient of the permeant between the stratum corneum and the vehicle, and the diffusivity of the molecule within the stratum corneum.

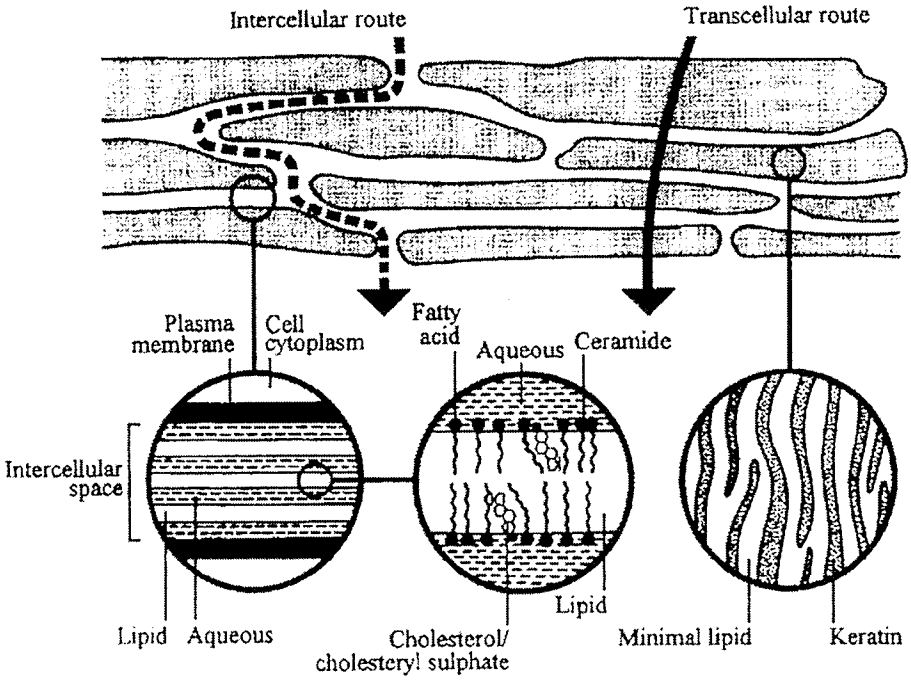


Figure 1.3. The stratum corneum as "brick and mortar" structure (Williams AC & Barry BW: Penetration enhancers. Adv Drug Deliv Rev 2004; 56(5):603-618)

Lampe *et al* had tried to provide new quantitative data of human epidermal lipids by proposing that the composition of lipids changed from a polar character to a neutral mixture during epidermal differentiation [15]. For whole stratum corneum, the major fractions are neutral lipids (78%) and sphingolipids (18%), together with a small amount of polar liquid. There are considerable quantities of non-polar materials present, such as squalene and N-alkanes (11%). The findings by Lampe *et al* have provided a better understanding of the stratum corneum lipids as well as the permeability nature of the skin membrane.

1.2.1. Chemical Penetration Enhancement

Skin penetration enhancers are substances that reversibly remove the barrier resistance of the stratum corneum. They facilitate various chemical substances in penetrating more readily across the viable skin tissues and subsequently reaching the systemic blood circulation. There has been an increased interest in novel drug delivery through human skin, and transdermal devices have been commercially developed for drugs such as clonidine, estradiol, fentanyl, nitroglycerine, scopolamine and testosterone [16-21]. Major obstacles in modifying drug influx through the skin have been stemmed from highly impermeability of the human skin and its biological and physiological variability. It would be both beneficial and practical to circumvent these problems by including in the formulations molecules that would reversibly alter the barrier characteristics of stratum corneum. For most penetrants, permeation through the bulk of stratum corneum constitutes the rate-limiting step and therefore stratum corneum is considered as the primary skin barrier in transdermal drug delivery. Both the structured lipid environments between the cells and the hydrated proteins within the corneocytes play a major role in skin permeability. Cell membranes themselves are probably of less significance in skin penetration since the cells are predominantly dead *(Figure 1.4)*. There are two potential routes for drug permeation, between the cells (intercellular route), and through the protein-filled cells and across lipid-rich regions in tandem (transcellular route). For each penetrant, the mechanism of penetration through these dual permeation routes depends upon its solubility, polarity, partition coefficient, and diffusivity within these phases [22]. Overall, polar compounds predominantly take the transcellular percutaneous route. As a penetrant becomes less polar, the likelihood of an intercellular

route increases. Under any circumstance, lipids in stratum corneum are one of the very significant parts of the barrier function of the stratum corneum. An understanding of their nature and organization is crucial in designing effective skin penetration enhancers and modifying the interactions between a drug compound and the skin membranes, with the ultimate goal of improving the pharmacological effects of the active ingredients.

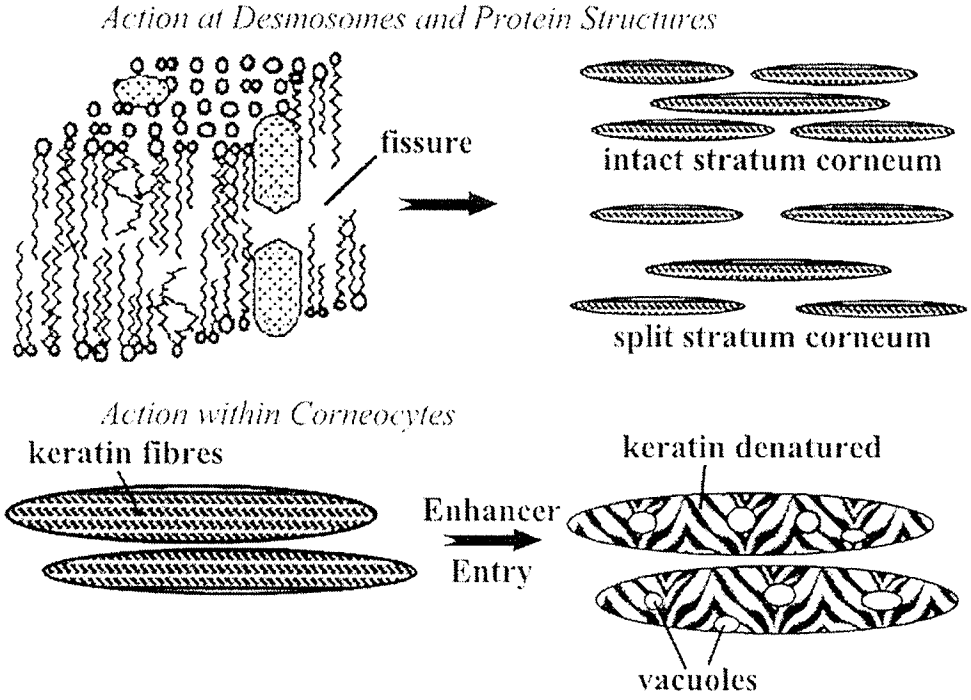


Figure 1.4. Action of penetration enhancers on the integrity of stratum corneum (Williams AC & Barry BW: Penetration enhancers. Adv Drug Deliv Rev 2004; 56(5):603-618)

Formation of bilayers by intercellular lipids at physiological pH has been demonstrated, either directly in pig epidermis [23] or indirectly from other mammalian species, including humans [24]. It was proposed that within the intercellular route accelerants may interact at the polar head groups of the lipids or within the aqueous region between the hydrophobic tails of the bilayer. Inside the corneocyte the major site of action would be the keratin fibrils and their associated water molecules. A penetration enhancer may also exert a direct action on regions where the tissues change their bulk constitution. In addition, high concentration of solvents in a formulation, such as propylene glycol, ethanol, the pyrrolidones or dimethylsulfoxide, may penetrate into skin tissues and subsequently change the partition coefficient (stratum corneum vs. vehicle) of the other penetrants. *Table 1.1* lists some common methods of skin penetration enhancement reported in the literatures.

The application of chemical penetration enhancers is considered the most feasible technique to facilitate permeation of a compound across the skin, leading to increased systemic absorption and bioavailability. There are numerous percutaneous mechanisms for different chemical enhancers, and *Figure 1.5* shows part of permeation enhancement by both chemical substances and molecular supersaturation. Williams and Barry have proposed several mechanisms of enhancement by chemical enhancers, 1) they interact with the intercellular lipids by disorganizing their highly ordered structures, thus enhancing the paracellular diffusivity of the compounds through the membranes, and 2) they may also interact with intercellular proteins embedded in the corneocytes to increase transcellular permeation and/or increase partitioning of the active ingredients into stratum corneum *(Figure 1.6)*.

Table 1.1. Chemical and physical approaches of penetration enhancement

Chemical Enhancement	
Dimethylsulfoxide	Extraction of soluble components of SC, denaturization of proteins
Ethanol, octanol	Enhancement of the solubility of drugs, disruption of SC integrity through extraction.
Propylene glycol	Solubility modification
D-limonene, menthol	Disruption of lipid packing within bilayers, disturbance in the stacking of the bilayers
Anionic: sodium laurate Cationic: cetyl ammonium Nonionic: lecithin	Alteration of the SC barrier functions, removal of water soluble agents, emulsification of sebum
Supersaturated solutions	Elevation of thermodynamic activity of drugs
Microemulsions	Disruption of SC lipids, increase of drug portioning in skin
Physical Enhancement	
Microneedle array	Creation of transient pores in epidermal layer with needles
Iontophoresis	Facilitation to charged species by electrical repulsion
Ultrasound	Disruption of SC lipid packing by cavitation
Electroporation	Creation of transient pores through SC by electrical probe

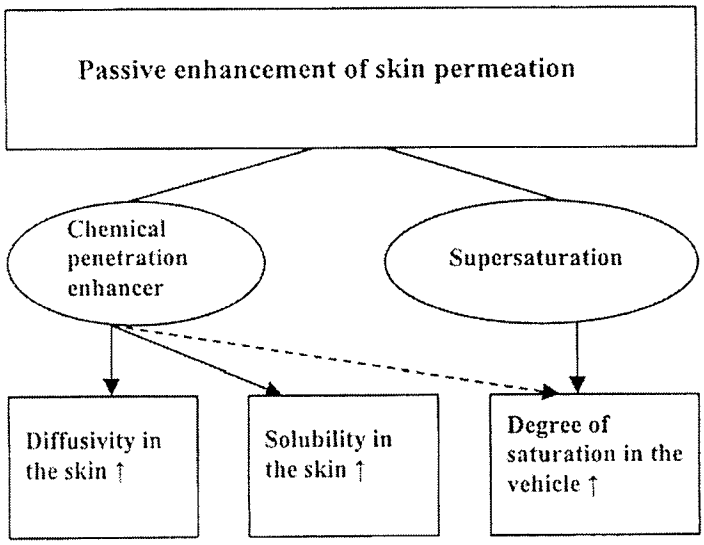


Figure 1.5. Strategies of skin permeation enhancement by chemical penetration enhancers or supersaturation (Moser K et al: Supersaturation: enhancement of skin penetration and permeation of a lipophilic drug. Pharm Res 2001; 18(7):1006-1011)

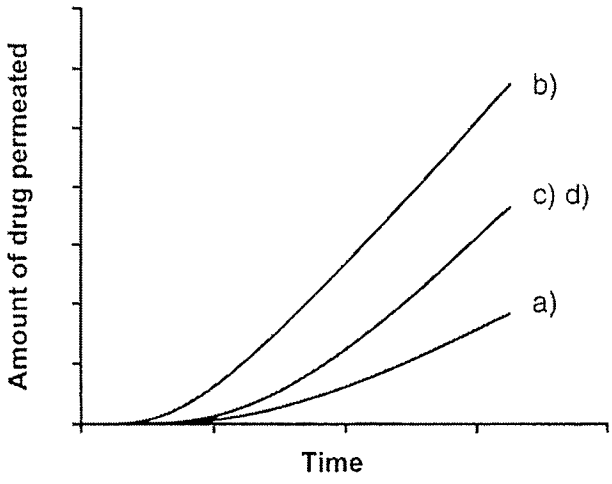


Figure 1.6. Skin permeation of a drug substance following the Fick's Laws (Moser K et al: Supersaturation: enhancement of skin penetration and permeation of a lipophilic drug. Pharm Res 2001; 18(7):1006-1011)

Other proposed mechanisms of enhancement include the formation of polar channels for drug diffusion on long-term tissue hydration [25], and the ability of some enhancers, notably oleic acid and terpenes, to exist as pools within the lipid domains [26,27]. Any combination of these percutaneous enhancement mechanisms may perturb the barrier properties of stratum corneum, leading to an increased rate and extent of percutaneous absorption of drugs that are co-administered with the enhancers.

1.2.2. Alternative Means of Penetration Enhancement

Permeation enhancement with chemical substances may not always accomplish the desirable therapeutic efficacy. There are various physical enhancement approaches that may provide alternative and/or synergistic efficacy in systemic transdermal permeation. *Figure 1.7* lists several mechanisms that have been widely used in transdermal drug research and development.

Iontophoresis is a physical permeation enhancement technique that applies a small electrical current to the skin to deliver ionized drug molecules and peptides/proteins at an elevated rate and extent of transdermal permeation [28] *(Figure 1.8)*. One of the unique advantages of iontophoretic delivery is that the flux of the permeant can be effectively controlled by altering the applied current, thus tailoring drug levels for specific medical conditions. Transport of chemical molecules by iontophoresis is enhanced by three mechanisms, 1) charged molecules are driven across the membranes, primarily by electrical repulsion, 2) electric current flow increases the permeability of the skin by creating artificial gaps within stratum corneum, and 3) electroosmosis affects uncharged molecules and large polar peptides by facilitating the diffusion of the charged particles

across stratum corneum. Numerous studies have confirmed that pretreatment with chemical enhancers increases the effectiveness of iontophoresis in skin delivery; chemical enhancers act as lipid-bilayer disrupters, creating permeability defects in stratum corneum, which in turn decreases the electrical impedance and resistance of the skin, and complements iontophoretic transdermal drug delivery [29]. Considerable research in iontophoresis has been demonstrated in the possible transdermal delivery of therapeutic proteins, peptides and oligonucleotides, and many other small compounds such as lidocaine and dexamethasone phosphate [30,31].

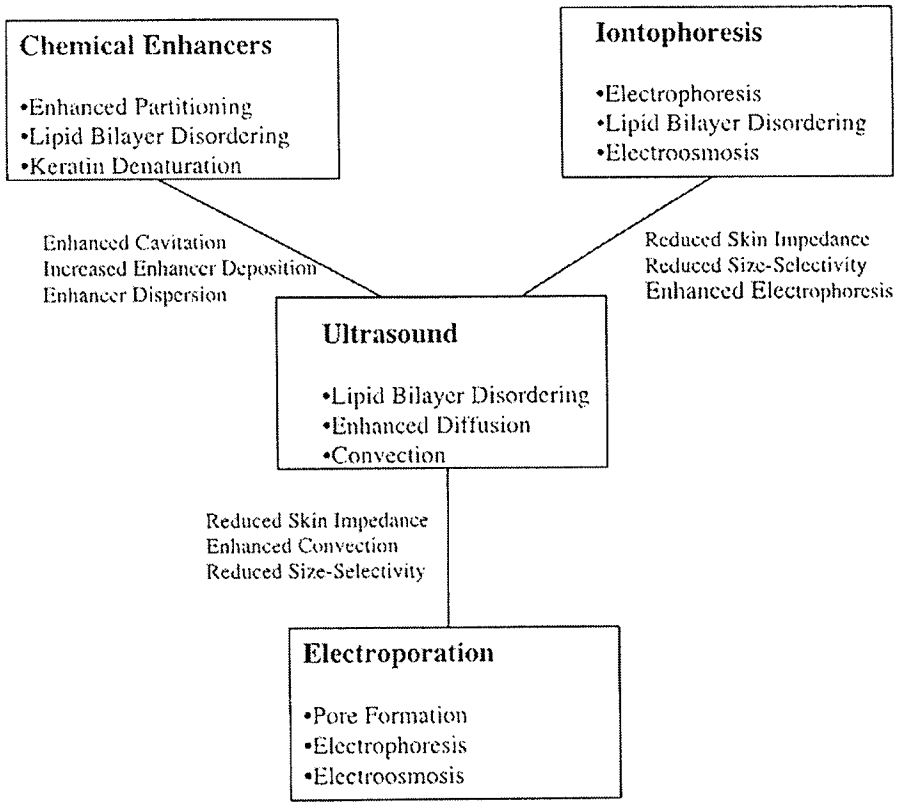


Figure 1.7. Possible mechanisms of enhancement synergy (Mitragotri S & Kost J: Low-frequency sonophoresis: a review. Adv Drug Deliv Rev 2004; 56(5):589-601)

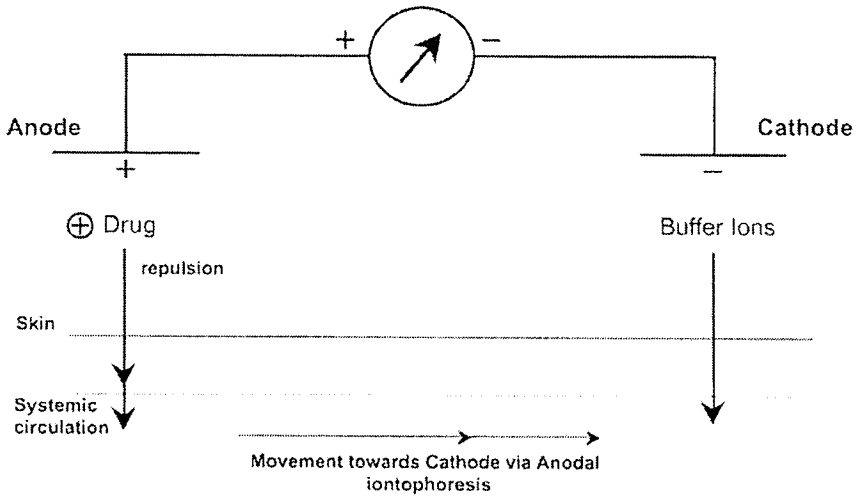


Figure 1.8. Diagram of iontophoretic technique
(Wang Y et al: Transdermal iontophoresis: combination strategies to improve transdermal iontophoretic drug delivery. Eur J Pharm Biopharm 2005; 60(2):179-191)

Phonophoresis or sonophoresis is defined as the movement of a compound through intact skin into underlying soft tissues under the influence of an ultrasonic perturbation. Ultrasound at various frequencies has been used for sonophoresis; the ultrasonic energy (at low frequency) is able to disturb the lipid packing in stratum corneum by cavitation [32,33]. The synergistic effects of ultrasound and chemical enhancers have resulted in improved penetration and dispersion of chemical enhancers within the stratum corneum, leading to better absorption of the active ingredients in delivery preparations. In addition, for the treatment of joint diseases and bursites, phonophoresis has been tested for its ability to aid drug penetration in a variety of drug-ultrasound combinations, mainly for localized effects.

In electroporation, an exponentially decaying high voltage is pulsed for a very short time (10^{-6} to 10^{-3} seconds) to produce transient changes in cell membranes or lipid bilayers. It is hypothesized that an electroporation pulse destabilizes the lipid matrix of cell membranes, resulting in the creation of transient hydrophilic pores in stratum corneum and epidermis [34]. Percutaneous transport is possibly enhanced as a result of the local driving force for ionic drugs, leading to increased diffusion and electroosmosis. Researchers are actively testing and developing transdermal electroporation for commercial clinical application. Nevertheless, there are still several obstacles that need to be overcome for this innovative delivery technology, such as regeneration of the functional skin barrier after the treatment and feasibility of the preparation size and cost [35].

1.3. Drug Permeation Principles

The passive diffusion process by which a substance permeates through the skin can be typically described mathematically with the Fick's First and Second Laws of Diffusion. The primary theory underlying the permeation of a compound across a barrier is that the rate of transfer (flux J) at which a permeant diffuses across a section of unit area is proportional to the amount of permeant (M), the surface area (S) and the time (t) at a steady state, as shown in *Equation 1*,

$$J = \frac{dM}{Sdt} \qquad \textit{(Eq. 1)}$$

Fick's First Law of Diffusion can also describe flux in terms of concentration gradient, which is commonly applied to pharmaceutical applications, as in *Equation 2*,

$$J = -\mathrm{D}\frac{dC}{dx} \qquad \textit{(Eq. 2)}$$

in which D is the diffusion coefficient of the permeant, C is the concentration gradient across the barrier, and x is the thickness of the barrier through which the substance permeates. The diffusion coefficient illustrates that the ability of a permeant to pass through a barrier is a function of system variables such as pressure, temperature, solvent, and natures of the barrier and the permeant. The negative sign in the equation indicates that the flux decreases in the direction of the concentration, i.e., down along the concentration gradient.

Fick's Second Law of Diffusion describes the rate of change in diffusant concentration with time at a particular location, i.e., the change in concentration with time in a particular region is proportional to the change in the concentration gradient at that point in the system,

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} \qquad \textit{(Eq. 3)}$$

Both Fick's Laws of Diffusion can be combined to describe the steady-state conditions of diffusion, in which the flux and the concentration gradient across the barrier become constant, and an equilibrium in diffusion process has been reached,

$$J = \frac{dM}{Sdt} = DK\frac{C_0}{x} \qquad \textit{(Eq. 4)}$$

in which the concentration gradient approaches C_S, which is defined as the saturated concentration of the compound in the donor solution under "sink condition", K is the

partition coefficient of the permeant between the barrier and the receptor solution, D is the diffusion coefficient, and x is the barrier thickness.

The diffusion coefficient and partition coefficient of a substance can also be combined into a further diffusion parameter, permeation coefficient (p), which describes the rate of diffusion of a permeant in terms of linear velocity as length per unit time,

$$p = \frac{DK}{h} = \frac{dM}{C_d S dt} \qquad (Eq.\ 5)$$

in which M is the cumulative amount of the permeant that passes per unit membrane area as the function of the time. The slope of the linear portion of the curve represents a zero-order rate of drug delivery, i.e., the flux J while the x intercept of the slope is the lag time (t_L). This is a measure of the time required for the permeant to establish its concentration gradient across a barrier.

1.4. Study Methods of Skin Transport

Demands for data describing the rate, extent and mechanism of percutaneous penetration of various compounds have been steadily increasing over the years. Transdermal and topical drug administration are not only considered valuable and feasible alternatives to more traditional approaches of drug delivery such as oral and parenteral administration, but also have the potentials to improve the overall healthcare outcomes through innovative formulation research and development. *In vitro* investigation has focused on the evaluation and collection of toxicological characterization and risk implications of everyday use of a wide range of potentially harmful materials in the agrochemical, chemical, cosmetic, households, and

pharmaceutical sectors, whose information had been lacking for decades. While drug delivery development and toxicological considerations are two of the most important factors to researchers working in the field, it is also obvious that increasing the overall database on percutaneous permeation will benefit their understanding of the mechanisms and kinetics of this unique drug delivery process.

1.4.1. Diffusion Cell Design

In vitro diffusion techniques have been extensively applied to assess percutaneous absorption of drugs and other chemicals in both industry and academia. There is also an increasingly tendency for the pharmaceutical manufacturers to submit *in vitro* data together with *in vivo* data to the regulatory bodies for compliance and documentation purposes for all established and newly developed pharmaceutical preparations. Hence *in vitro* diffusion assessment has become a common form of evaluation for all transdermal and topical delivery systems.

One of the major advantages of an *in vitro* diffusion study is that the experimental conditions can be precisely controlled and reproduced, so that the variables are limited only to the test membranes and the test materials. *In vitro* diffusion systems range in complexity from a simple two compartment "static" diffusion cell [36] to multi-jacketed flow-through cells [37] *(Figure 1.9)*. Construction materials must be chemically inert and stable; glass is mostly common, although Teflon and stainless steel are also used sometimes. Efficient and uniform mixing of the receptor phase (sometimes the donor phase as well) is essential; sample removal and replenishment should be simple and accurate. None of these processes should interfere with the diffusion of permeant being

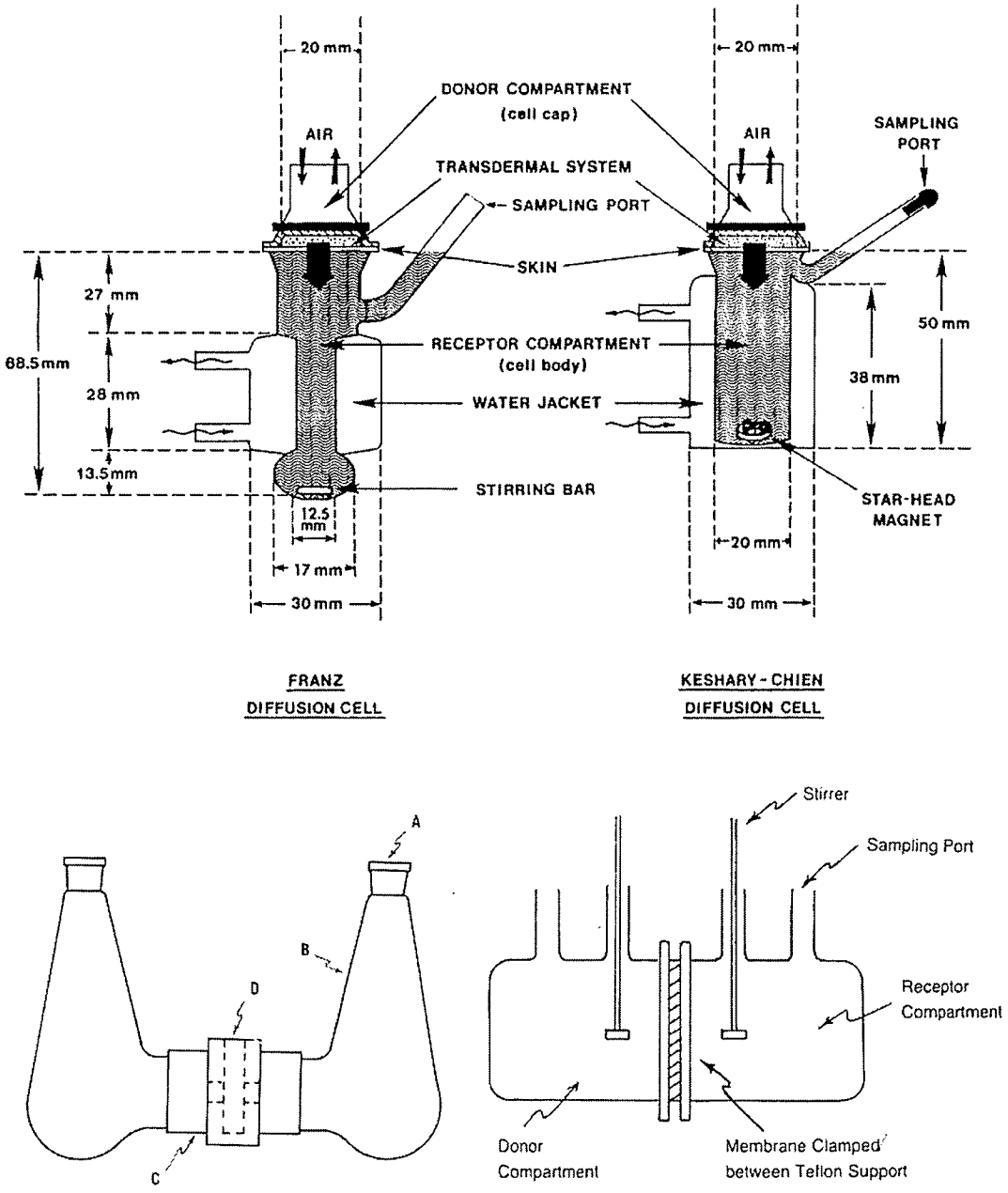


Figure 1.9. Diffusion cells of different designs (top: vertical, bottom: horizontal) (Martin A: Physical Pharmacy, Lea & Febiger, 1993)

tested. Mixing of the receptor phase ensures the homogeneity of the medium while simple sampling will ensure the reliability and reproducibility of the experimental results.

Static diffusion cells are usually the upright (Franz-style) or side-by-side type with a receptor chamber volume of approximately 2-10 ml and a diffusion surface area of approximately 0.2-2 cm^2. Cell dimensions should be accurately measured, and precise values used in subsequent calculations, with due regard to analyte dilution resulting from sampling and replenishment. Experimental errors could result in significant inaccuracy in data interpretation. The main difference in the application of the two cell types is that side-by-side diffusion cells are used for the measurement of permeation from one stirred solution, through a membrane, and into another stirred solution. This is of particular advantage when flux from a saturated solution with excessive presence of solid permeant is to be examined, where accumulation of extra solid substance on the membrane surface must be prevented. Side-by-side cells can also be modified to evaluate absorption characteristics of a permeant in a vapor phase. Franz-style cells are especially useful for studying absorption from semisolid formulations spread on the membrane surface, which simulates *in vivo* performance under real-life application situations. The donor compartments can be either capped to provide an occlusive condition, or left open, according to variable objectives of the studies.

Flow-through diffusion cells are useful when the permeant has a limited solubility in the receptor medium. Such a system normally contains diffusion cells, fraction collectors, and a heater/recirculator. A controller permits the operator to simultaneously collect nine to twelve samples for up to several hours from all study cells. Sink conditions are maximized as the receptor fluid is continuously replaced using a peristaltic pump.

The dimension of a receptor chamber is somehow constrained due to requirements to achieve a "sink condition" in the receptor phase such as a large receptor compartment. While a large receptor volume may ensure sink conditions for the diffusion study, it could compromise analytical sensitivity when there is limitation in concentration measurement. In addition, large receptor chambers do not realistically represent physiological situations in clinical applications, where skin conditions are generally dry with very low moisture content. As a result, diffusion surface area and donor cell size are subsequently constrained to match with the configuration of the relevant receptor cells.

An ideal receptor phase provides an accurate simulation of the conditions pertaining to *in vivo* permeation of the test constituent. As a general rule, the concentration of permeant in the receptor fluid should not exceed above 10% of the saturation solubility [38]. Excessive concentrations in the receptor phase could lead to a decrease in the rate of transmembrane diffusion, which would consequently result in an underestimate of the transdermal absorption and bioavailability. The most commonly used receptor fluid is phosphate-buffered saline (PBS, pH 7.4), which is considered capable of mimicking *in vivo* physiological conditions in humans. It has been postulated that if a test compound had a water solubility of less than 10 μg/ml, a wholly aqueous receptor medium would be unsuitable and the addition of solubilizers became necessary [39]. Receptor fluids described in the literature [40,41] ranged from water to isotonic phosphate buffers containing albumin and preservatives such as benzalkonium chloride. Albumin increases the solubility of various permeants [42] while the preservatives inhibit microbial growth in the receptor fluid for a prolonged period of time.

It is important to recognize that solubilizers may potentially interfere with the barrier functions of the skin membranes by extracting lipids from the epidermis. Several commonly used receptor media were examined and a number of recommendations were made [43]. One of the most useful receptor phases was an aqueous solution containing a nonionic polyethylene glycol-20-oleyl ether surfactant. The flux of either water or urea across the rat skin was not significantly changed in the receptor fluid when compared to normal saline, suggesting that this surfactant did not disrupt the barrier functions of rat skin to hydrophilic compounds.

Some of the critical technical elements for accuracy and reproducibility in any *in vitro* experiment include continuous agitation of the receptor medium; sampling from bulk liquid rather than side arm of the diffusion cells and accurate replenishment of receptor medium after sampling are important practical considerations to maintain continuous sink conditions by stirring the receptor medium. It is essential that air bubbles not be introduced below the membrane during sampling as this may form an unwanted barrier between the diffusion membrane and the receptor medium. The unwanted barrier could inhibit the permeation of solute and thereby undermining the penetration levels in the receptor media.

1.4.2. Skin Membranes

One of the major design considerations for for any *in vitro* skin diffusion experiment is the nature of the test skin model. Animal skin specimens have been widely used as substitutes for human skin [44], due primarily to the difficulty in obtaining sufficient human skin tissues. Southwell *et al* investigated both *in vitro* and *in vivo*

variations in the permeability of human skin among different specimens (interspecimen) and between same specimens (intraspecimen).

How age and gender would affect skin permeability in humans has been inadequately addressed to date. Limited studies have concluded that skin permeability is negligibly independent upon age, gender and storage conditions *in vitro* [45]. However, numerous studies have documented differences in skin permeability based on racial origins. Skin specimens from Caucasians are slightly more permeable than those from Africans [46,47], which correlated with observations that dark skin has more cell layers within the stratum corneum and a higher lipid content than light skin [48]. A recent study on skin specimens from Caucasian, Hispanic, Black, and Asian origin ranked them in order of skin permeability to methyl nicotinate as Black < Asian < Caucasian < Hispanic [42].

Different skin preparation methods may also yield variable results from *in vitro* diffusion experiments. Full-thickness skin, which includes stratum corneum, viable epidermis, and dermis, is normally used for studying metabolic fate of drug molecules. In order to achieve satisfactory and reliable data from full skin specimens, it is necessary to fully understand the physicochemical characteristics of a test solute as well as its transmembrane penetration profiles. In addition, sensitivity and reproducibility of the analytical method are also essential to ensure accuracy of the experimental results. In general, penetration rate and penetration extent of a compound across full-thickness skin are relatively limited.

Epidermal skin membrane, which includes both the stratum corneum and viable epidermis, but not the lower dermis layer, is more commonly used for *in vitro* diffusion

studies. Two preparation methods can be used for the samples. With the heat separation technique, skin specimens are immersed in a water bath at 60 °C for one minute, and epidermis is then scrapped out using a sharp scalpel. Care must be taken to avoid overexposure of the samples to high temperature conditions. Epidermal skin membrane can also be obtained by using a surgical dermatome, in which the membrane thickness can be easily adjusted and changed. When only stratum corneum is needed for the experiment, heat-separated epidermis is further treated in phosphate buffer solution containing 0.0001% (w/v) bovine pancreas trypsin type III at 37 °C overnight. After washing the separated stratum corneum sheet several times with deionized water, it is air-dried and stored under vacuum at room temperature. Before use, the sample is rinsed twice with ice-cold hexane to remove extra skin lipids [49].

The selection of an appropriate testing membrane model for diffusion experimentation is directly associated with the nature of the test permeant. While the continuously perfused subcutaneous vasculature *in vivo* is able to rapidly remove any permeant reaching the epidermal-dermis junction, these vessels, even if still present *in vitro*, will not perfuse in a simple diffusion method. In addition, the relatively aqueous environment of dermis *in vitro* inhibits easy penetration of most lipophilic compounds, but this barrier is circumvented by the capillary bed *in vivo*. Hence use of dermatomed epidermal or stratum corneum membranes is considered more appropriate in testing lipophilic molecules than hydrophilic molecules, because the affinity of a lipophilic compound to skin membrane is longer than that of a hydrophilic compound, subsequently its retention within the skin is also longer [50,51].

1.4.3. In Vitro Assessment of Skin Permeation

The manner in which a substance is applied to the surface of skin can be a major determinant of its subsequent percutaneous absorption. For *in vitro* diffusion studies, several factors must be carefully considered in selecting a suitable application procedure. These may include the nature of the formulation vehicle, the permeant concentration, the amount of preparation applied, the exposure time and the sampling method. In addition, *in vitro* experimental conditions should simulate as closely as possible the *in vivo* situation.

There are two basic approaches in applying test substances to the donor cell. The infinite dosing technique involves the application of a sufficient amount of test permeant to make concentration changes from molecular diffusion in donor cells negligible. In other words, the applied dose of a test substance in a donor cell is effectively infinite. This condition of saturation is desirable when the objective of experimentation involves either calculation of diffusion parameters such as permeability and diffusivity, or investigation of mechanisms of permeation enhancement [52]. The finite dosing technique is designed for monitoring in-use conditions, which involves application of a dose that may show marked depletion of the test substance during experiment [53]. This simulates various real-life applications, for example, when a significant portion of the permeant entering the skin is being carried away by the general circulation, or the applied dose is removed from the skin surface by rinsing or washing procedures. Direct comparison of finite and infinite dosing application for the same testing substances is generally uncommon, because there are differences in experimental methodology and study goals and objectives, even though a few studies did predict and assess

transmembrane behaviors and effects by using variable doses of the same model compounds [54,55].

In vitro diffusion experiments are usually conducted at a temperature of 32 °C, which is equivalent to that of healthy skin *in vivo*. This is achieved by maintaining the receptor solution at 37 °C, by either immersing the cells in a water bath of that temperature, or using cell jackets perfused at the required temperature. A wider range of temperatures (2-45 °C) may also be used to simulate certain specific applications, as long as the stratum corneum does not significantly change its barrier properties under the testing temperature. Nevertheless, most substances exhibit increased percutaneous penetration at higher temperatures, resulting from increased molecular diffusivity and enhanced skin permeability. Katz and Poulsen concluded that there was a direct and realistic correlation between temperature and skin permeability [56].

While some investigators did extend the duration of *in vitro* diffusion experiments for up to 120 hours, largely for assessing drug release from transdermal delivery patches [57], the recommended duration of a diffusion experiment is generally between 24-48 hours [58]. Degradation of skin barrier functions over a prolonged period of time is the primary concern. In addition, an extended diffusion period is not representative of actual drug administration on the skin. On the contrary, many topical drug applications and transdermal drug delivery preparations sustain drug release for less than 24 hours. Therefore, a shorter experimental duration (e.g., 6-12 hours) would be more appropriate and realistic for evaluation purposes. The skin integrity can be checked either qualitatively by visual examination, or quantitatively by measurement of the flux of marker compounds such as tritium water and sucrose [59]. The generally accepted

permeability coefficient for water diffusion through human skin is less than 1.5×10^{-3} cm/h.

The quality of data derived from any *in vitro* or *in vivo* experiment is ultimately dependent on the reliability of the analytical methods employed. Every aspect of the analytical procedures should be carefully considered and optimized in the experimental design [60]. Replication and multiple time point sampling are common in experimentation. The integrity of the method must also be well understood. High-performance liquid chromatography (HPLC) is particularly useful as relatively large aqueous samples (approximately 200 µl) can often be handled without preliminary processing or concentration.

1.4.4. In Vivo Assessment of Skin Permeation

Topical and transdermal drug delivery has been used as one of the effective routes of administration for both local and systemic outcomes. Subsequently, dermatopharmacology and dermatotoxicology deal with characterization of pharmacological and toxicological profiles of various substances applied to the skin surface. Most compounds that are of interest and concern in dermatopharmacology and dermatotoxicology are lipophilic in nature. The stratum corneum, the supposed skin barrier to percutaneous absorption, is also a tissue full of saturated lipids. The lipid-rich stratum corneum acts as a "sink" to topically applied lipophilic materials, closely approximating a sponge capable of absorbing and retaining a large amount of liquid.

In vivo evaluation of drug permeation through the skin is much more complicated than that of *in vitro* diffusion studies. Initial permeation of a test compound into the skin

is dependent on the chemical and physical properties of preparation vehicles and the barrier/sink properties of stratum corneum in a living subject. Once the compound transports into deeper skin layers, the vitality of living skin will then determine the metabolism, distribution and excretion of the compound through the skin and in the body [61].

Tape stripping is a relatively non-invasive technique to measure drug permeation through the skin. Following application of the test formulation, different layers of the stratum corneum are collected by successive tape applications and removals from the application skin site *(Figure 1.10)*. The collected tape strips are then assayed for the content of the active ingredient. This method is a useful supplement to the calculation of percutaneous pharmacokinetics and pharmacodynamics, where urinary and fecal drug excretion is also evaluated. In addition, regular compounds rather than radio-labelled molecules can be used, because the skin strippings contain adequate drug amount that is sufficiently detectable with common assay methodology. In particular, tape stripping is advantageous and practical when skin metabolism is not being assessed.

Skin stripping methods generally determine the concentration of a substance in the stratum corneum at the end of a certain application interval (e.g., 30 minutes); linear extrapolation of the results is capable of predicting percutaneous absorption characteristics of that specific compound for longer application intervals. Rougier, Lotte and coworkers have established a linear correlation between drug reservoir content in the stratum corneum and percutaneous absorption characterization using a standard urinary excretion method [62]. They concluded that the influence of a vehicle on the overall

absorption of a drug could be predicted by measuring the amount of the compound present in the stratum corneum at around 30 minutes after the topical application.

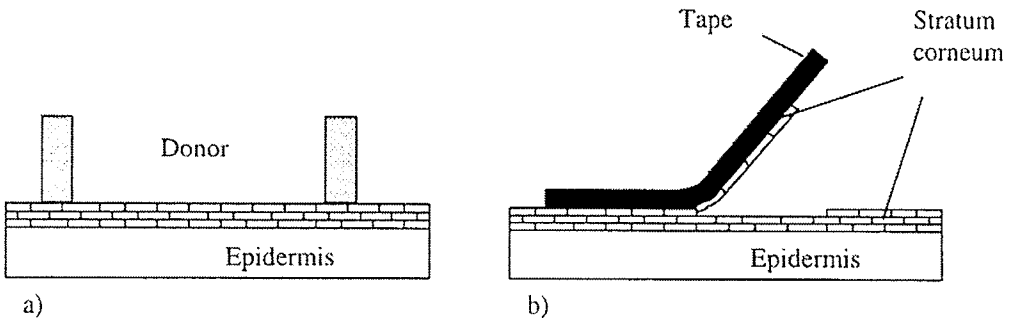


Figure 1.10. Schematic drawing of the tape-strip technique (Moser K et al: Passive skin penetration enhancement and its quantification in vitro. Eur J Pharm Biopharm 2001; 52(2):103-112)

It has been generally accepted that the most appropriate *in vivo* evaluation technique for percutaneous drug absorption is to monitor systemic drug concentrations over the time; this is particularly applicable to transdermal drug delivery systems in which effective therapeutic concentration can achieve only after an adequate amount of drug has reached the general blood circulation percutaneously. For pharmacokinetic and pharmacodynamic evaluation, it is also necessary to monitor how drug levels change after the removal of a transdermal patch. Extra blood flow to the application site may take place once a drug patch is removed, which would subsequently promote drug transport

from the skin reservoir and result in more drug uptake. This phenomenon has been noted in trials with β-blocker timolol [63].

Measurement of biological responses to transdermal drug administration is another approach to evaluate *in vivo* drug efficacy from transdermal drug delivery. However, this parameter is highly variable and unpredictable because it is difficult to establish a direct relationship between plasma drug level and therapeutic effect. For topically applied medications or other substances, measuring residual drug concentration on the surface of the skin is also inconvenient as skin biopsies may be required for such purposes. Determination of a drug and its metabolites in blood, skin and other biological samples is made possible using validated analytical assays such as chromatography with UV, fluorescence or mass spectrometric detection.

There are simple equations such as Fick's Laws of Diffusion to predict intrinsic ability of a substance to permeate through the skin. Nevertheless it might not be easy to reliably and accurately predict how a preparation would influence or change percutaneous permeation of a compound from the formulation and beyond. Until we gain adequate information on the molecular interactions involved in a transdermal transport, we will have to rely mainly on various *in vitro* diffusion experiments and *in vivo* animal models to guide novel topical and transdermal formulation development programs.

1.5 Sunscreens

Skin cancer has become increasingly common around the world for the past two decades, largely attributed to an increased exposure of the earth to ultraviolet radiation from the sun. Over 60,000 Canadians develop skin cancer each year [64]; approximately half of the all new cancer diagnosed in the US in 1996 was skin cancer related [65]. Even though most skin cancers are benign and treatable if diagnosed on an earlier stage, protection of the skin is the ultimate objective for the general public. There are numerous practical and preventive approaches that can be used to minimize the incidence of skin cancer, regular application of sunscreens being among one of the mostly applied and economical methods by the general public for decades.

Skin damage from UV radiation builds up with continued exposure, regardless of whether sunburn actually occurs. It has been estimated that approximately 80% of the skin damage induced by UV exposure from the sun that one experiences throughout his or her entire lifetime actually takes place by the time when one reaches 21 years of age. Skin damage can subsequently lead to skin conditions such as wrinkling, premature skin aging and leathery appearance of the skin; these become more prominent with aging [66]. Dermatological experts believe that individuals who have suffered one or more severe sunburns during childhood are at double the risk of developing malignant melanoma. It has been predicted that even one blistering sunburn before the age of 20 might more than double chances of inducing the deadliest types of skin cancer. That is the reason why sunscreen products are widely promoted to the general public as a safe and economic approach to minimize sunburn and skin damage [67].

A rise in the incidence of melanoma has been exceptionally high in Queensland, Australia, where medical establishments have been vigorously promoting the use of sunscreens for years. There have been more reported cases of melanoma per capita in Queensland than any other region in the world [68,69]. Generally the closer to the equator a person is, the more potent the UV radiation from the sun is, and consequently the higher the number of cases of melanoma will result [70]. In addition, exposure duration and intensity also affect the outcome of skin cancer. There are two types of sunscreens. Sun blocks are physical agents that reflect and scatter the UV rays. Although most physical sunscreens are opaque materials, new product development has produced brands that are almost invisible after application, which can not only improve consumer acceptance but also enhance administration compliance [71]. Chemical sunscreens have the ability to protect against UVA rays, UVB rays, or both. Most sunscreen products contain 2-5% of chemical sunscreen substance oxybenzone, which is one of the most powerful free radical generators known to humans. Free radicals are thought to be a critical factor causing cancers and other adverse effects.

1.5.1 Types of Skin Cancer

There are three major types of skin cancer: malignant melanoma, basal cell carcinoma, and squamous cell carcinoma [72]. Actinic keratosis, an abnormal skin growth, is considered a pre-cancerous condition that may further develop into skin cancer [73].

While malignant melanoma is the least common of all three types of skin cancer, it is the most life-threatening skin cancer. Malignant melanoma maifests in varying

shades of brown to black, or in multicolored patches of red, white and blue. It may have an asymmetrical outline with notched or scalloped edges, and is usually larger than 6 mm (1/4 inch) in diameter. Melanoma with a thickness of larger than 2 mm is curable in a large proportion of patients, but the risk of lymph node and/or systemic metastasis increases with increasing thickness of the primary skin lesion. Local treatment of malignant melanoma is by surgical excision with the margins based on the thickness and anatomic location [74].

Basal cell carcinoma can appear in various stages, ranging from a red patch, shiny pearly or translucent bump in pink, red, or white, to crusty, open skin sores that will not heal. There may be a rolled border with an indented center in basal cell carcinoma. Imiquimod and fluorouracil have been approved for the treatment of superficial basal cell carcinoma, which can also been used to treat actinic keratosis (AK), the most common skin precancer. In addition, surgical removal is also quite common for basal cell carcinoma. Using a scalpel, a surgeon removes the entire cancerous growth along with a surrounding border of apparently normal skin as an extra safety precaution. The incision is closed, and the excised specimen is sent to a pathological laboratory to verify that all cancerous cells have been removed.

Squamous cell carcinoma can take the form of a persistent scaly red patch that sometimes crusts or bleeds, an open skin sore that does not heal, or a raised, wart-like growth that may bleed. Treatments for squamous cell carcinoma are similar to those for basal cell carcinoma, and include both medicinal and surgical approaches.

The principal cause of melanoma is thought to be overexposure to ultraviolet (UV) radiation. Those who either have fair skin or tend to sunburn easily, especially

Caucasians with red or blond hair, may be at a greater risk of developing skin cancer because their skin cells contain less melanin (a dark biological pigment). In addition, those who are regularly exposed to large amount of sunlight would have more chances of developing skin cancer. As a result, employees or farmers who work outdoors year-around, and residents who live in areas where sunlight is very strong, such as the Southwest US and Australia, should regularly check for symptoms of skin abnormalities. It is therefore prudent to protect against overexposure to the sunlight; the most practical and common approach currently available is to dress appropriately and to avoid sun exposure during UV peak hours in the summer months. In addition, using sunscreens regularly might also be beneficial in long-term skin care and health.

1.5.2 Action of Sunscreens

Light is classified into three basic types, i.e., ultraviolet (UV) light, visible light, and infrared (IR) light; UV radiations are further classified into UVA, UVB and UVC, based on their range of wavelengths (*Table 1.2*). UVC from solar radiation does not generally penetrate through the atmosphere of the earth, but both UVA and UVB do reach us and contribute to skin aging. Since UVA radiation includes longer wavelengths, it is able to penetrate deeper into the skin *(Figure 1.11)*. It was once thought that UVB rays were the most significant contributor to skin wrinkling, skin cancers and other skin diseases. However, recent studies have shown that UVA radiation also results in the development of solar damage and skin diseases, such as lupus erythematosus, melanoma and nonmelanoma skin cancers. Sunscreen products with broad protection spectrum have been formulated to protect the skin from both UVB and UVA radiations.

Table 1.2. Classification of UV light

Light Type	Wavelength (nm)	Possible Effects on Skin Damage
UVC	200-290	Mostly absorbed in ozone, without reaching surface of the earth
UVB	290-320	Responsible for 90% of sunburns and skin damage, primary cause of skin cancer
UVA	320-400	Responsible for skin photoaging and drug photosensitivity, also capable of skin damage

The causative relationship between UVB exposure and non-melanoma skin cancer has been well documented through research [75]. In the meantime, numerous epidemiological studies have also addressed contributing factors of UVB and UVA to photocarcinogenesis, in particular with regards to melanoma development, which has seen a dramatic rate of increase worldwide for the last decade and is currently causing over 7000 cases of death each year in the United States alone [76]. In addition, studies have proven that UVB radiation induces squamous cell carcinoma in mice [77] while UVA radiation acts as a skin carcinogen in rodents [78]. No one is immune to sunburn or other skin disorders regardless of skin complexion. Nevertheless, new genetic techniques have been able to facilitate assessment of one's risk of developing skin damages from sun exposure. Those with a family history of skin cancer, melanoma in particular, are at an increased risk of developing skin cancer during their lifetime [79]. Certain diseases and medical conditions also increase skin reactions to sun radiations, which can be further

enhanced by treatment medications. Many drugs are able to increase photosensitivity of the skin; exposure to the sun should therefore be limited appropriately when one is taking these medications.

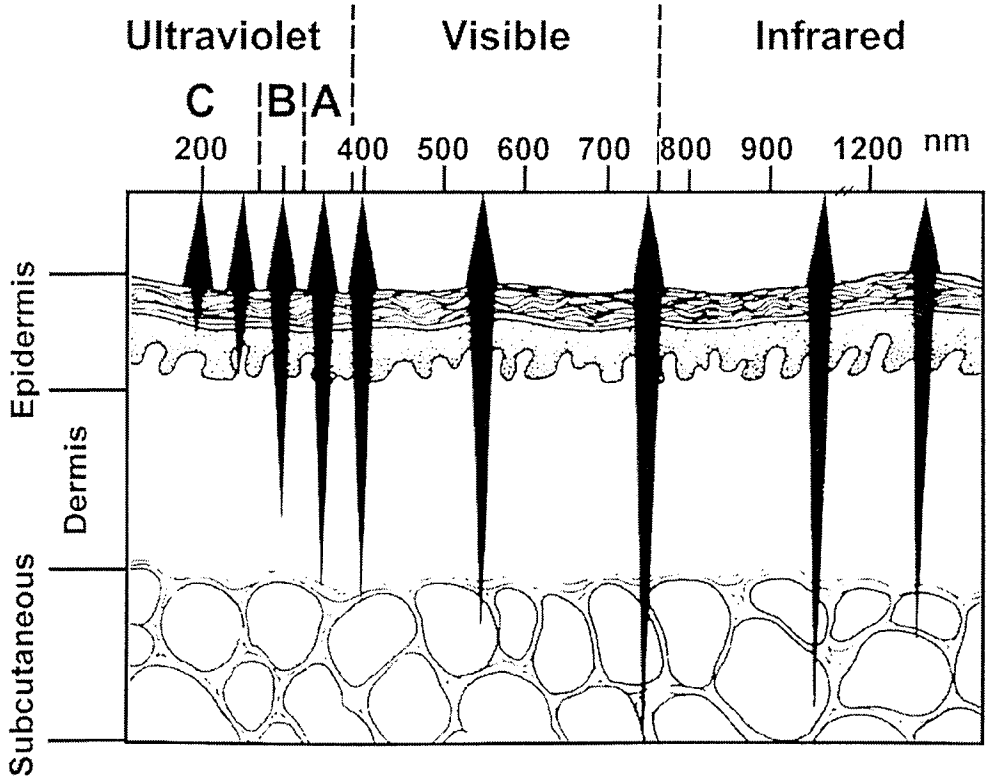


Figure 1.11. Schematic representation of light penetration into the skin (Hornung RL & Downham TF: www.easi.org/nape/sc_slides/skca_slides.ppt 2006)

Sunscreens are traditionally divided into chemical absorbers and physical blockers (*Table 1.3*), based on their mechanisms of protection on the skin surface. Chemical sunscreens are generally aromatic compounds conjugated with carbonyl group; in many cases, an electron-releasing group such as an amine or a methoxyl group is substituted in the ortho- or para- position of the aromatic ring [80]. Compounds of this

configuration are capable of absorbing harmful short-wave UV rays (254-340 nm, high energy) and converting them into innocuous long-wave radiations (above 380 nm, low energy), subsequently minimizing the negative effects of UV radiations on the skin. Physical blockers, on the other hand, reflect or scatter UV rays through their lower refractive indices. Recent research indicates that newer micro-sized forms of physical blockers may function partly by absorbing UV rays. Sometimes referred to as "non-chemical" sunscreens, they might be more appropriately designated as inorganic particulate sunscreen ingredients.

The efficacy of a sunscreen product is indicated by the Sun Protection Factor (SPF). SPF is a parameter that predicts the protection efficacy offered by a sunscreen preparation against sunburn and is dependent on the content of UV filters present in the formulation [81]. It is defined as the ratio of time that it would take to develop skin erythema with or without the application of a sunscreen product [82]. The higher the SPF value of a preparation, the longer it protects the skin from burning radiation. For example, a product with an SPF value of 8 protects twice as long as a product with an SPF value of 4. The SPF rating applies only to UVB protections; those with protection against UVA might list no SPF values. As a general guideline, dermatological experts recommend the use of broad-spectrum sunscreen products with an SPF value of 15 or higher.

Table 1.3. FDA-approved category 1 sunscreens

UVA Absorbers	Butylmethoxydibenzoylmethane
	Oxybenzone
	Sulisobenzone
	Dioxybenzone
UVB Absorbers	p-aminobenzoic acid
	2-ethoxyethyl p-methoxycinnamate
	Diethanolamine p-methoxycinnamate
	Digalloyl trioleate
	Ethyl 4-bis (hydroxypropyl) aminobenzoate
	2-ethylhexyl-2-cyano-3,3-diphenylacrylate
	Ethylhexyl p-methoxycinnamate
	2-ethylhexyl p-methoxycinnamate
	2-ethylhexyl salicylate
	Glyceryl aminobenzoate
	Homomenthyl salicylate
	Lawsone with dihydroxyacetone
	Octyl-dimethyl PABA
	2-phenylbenzimidazole-5-sulfonic acid
	Triethanolamine salicylate
Physical Blockers	Red petrolatum
	Titanium dioxide
	Zinc oxide
	Talc

1.5.3 Oxybenzone

Oxybenzone or benzophenone-3 is one of the most widely used sunscreens available to the market today [83]. Its chemical structure and principal properties are shown in *Figure 1.12* and *Table 1.4* respectively. There are 12 types of benzophenone, which are all derived from 2-hydroxybenzophenone and designated as benzophenone 1 through 12. These congeners of 2-hydroxybenzophenone have all been used as active sunscreen ingredients. Unlike other sunscreen agents available, benzophenones are the only class of compounds that belong to aromatic ketone category. While benzophenones are primarily UVB absorbers, oxybenzone absorbs well through both UVA and UVB, and is generally considered as a broad-spectrum sunscreen absorber. Oxybenzone has been extensively used in medicine for the treatment of photo-dermatoses for over 40 years; in agriculture it is used as a photostabilizer in the manufacture of various agricultural films. The use of oxybenzone in commercial sunscreen preparations has increased geometrically over the years, due mainly to increasingly awareness of deleterious effects of UV radiations on human skin and promotion in sunscreen applications for the general public [84]. Investigators have focused on elucidating mechanisms of photocarcinogenesis and photoaging for years, and significant progress has been made recently [85]. However, there has been little information available on pharmacology and toxicology of individual active sunscreen compounds. Neither is there information on detrimental potentials of sunscreens from transdermal systemic absorption.

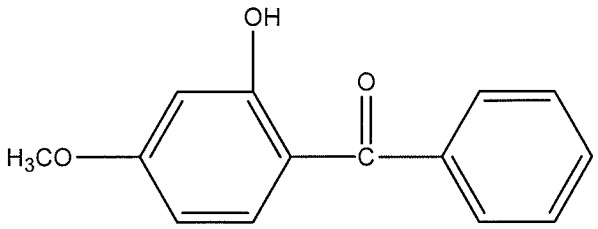


Figure 1.12. Chemical structure of oxybenzone

Table 1.4. Physical and chemical properties of oxybenzone

Parameter	Value / Property
Molecular Weight	228.8
Purity	Oxybenzone, 98% minimum
Specific Gravity (25/25°C)	1.32
Color/Form	White to yellow powder
Odor	Faint
Water Solubility (25°C)	Insoluble
Vapor Pressure (20°C)	Not determined
Toxicity	Oral rat LD_{50}: 7400 mg/kg
Boiling Point	150 - 160 °C (1 mmHg)
Stability	Stable under normal conditions

* http://www.spectrumchemical.com/

In vivo biotransformation of oxybenzone has been proposed by some investigators. Okereke *et al* studied the disposition of oxybenzone from dermal absorption in rats [86]. After applying an oxybenzone dose to the skin of Sprague-Dawley rats, blood samples were collected, and the parent compound as well as its metabolites was analyzed using an HPLC assay. It was found that oxybenzone rapidly permeated through rat skin. The biphasic mode of elimination in rats suggested distribution of the parent compound to some compartments other than plasma. The bimodal distribution of these metabolites over the time observed could also be related to distribution of the compounds in tissues from systemic circulation due perhaps to an initial sequestration of the compounds in tissues. Tissue distribution study revealed the formation of 2,4-dihydroxybenzophenone (DHB) and 2,3,4-trihydroxybenzophenone (THB) as principal metabolites of oxybenzone. DHB is formed via O-dealkylation of methoxy side chain at para-position of oxybenzone nucleus (*Figure 1.13*). This metabolic reaction is known to be mediated by cytochrome P-450 enzyme system, a family of enzymes that are capable of metabolizing a wide variety of xenobiotics [87]. The formation of THB involves aromatic hydroxylation, which further suggests possible roles of microsomal enzyme system in overall disposition of oxybenzone [88]. The researchers also found copious amounts of 2,2'-dihydroxy-4-methoxybenzophenone (DHMB), a bacterial mutagen, after dermal application of oxybenzone in rats, which should warrant further investigation as this might culminate in toxicity following prolonged use of the compound [89]. Major findings in all research publications have suggested significant penetration of oxybenzone into and through the skin.

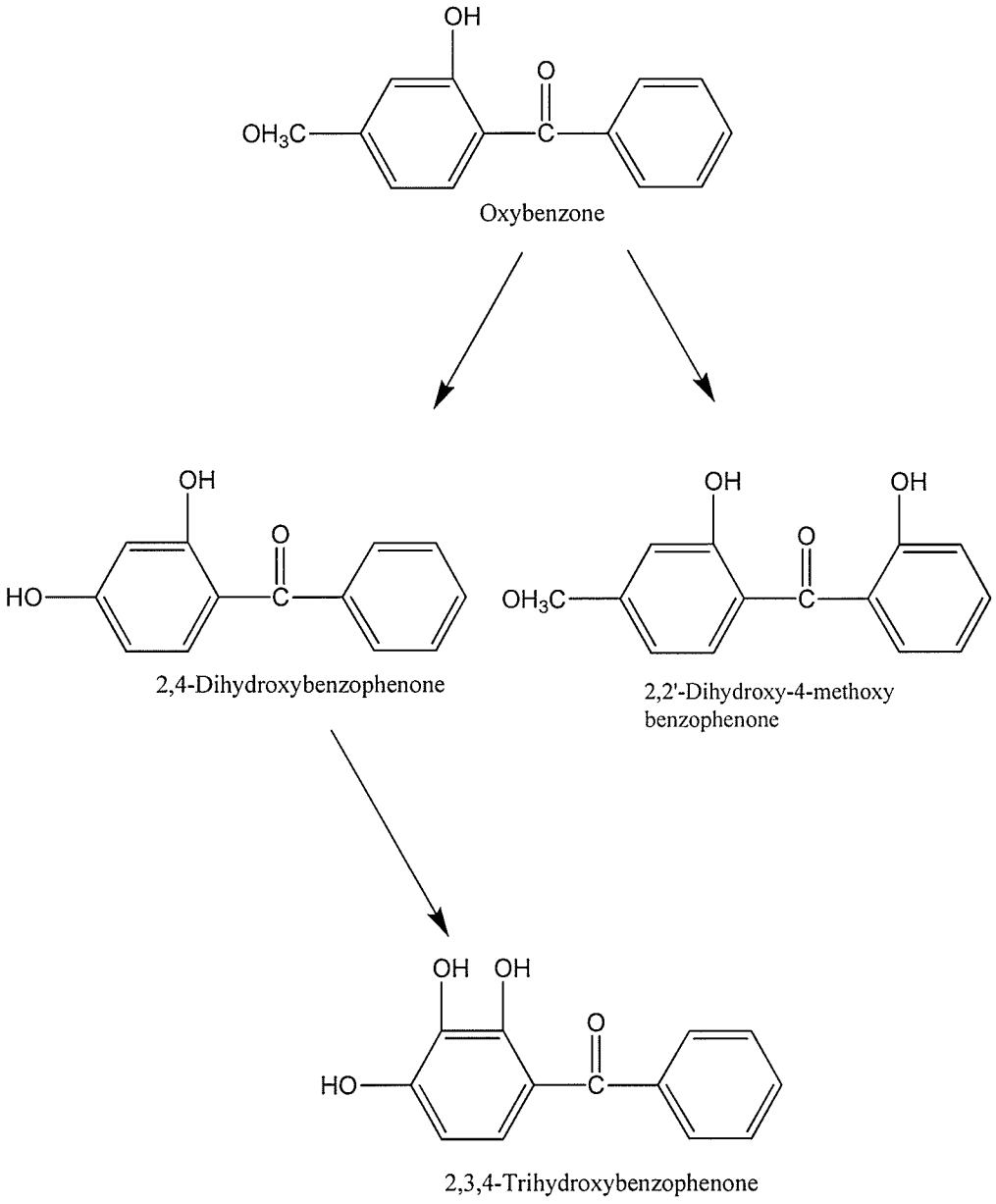


Figure 1.13. Proposed metabolic pathway of oxybenzone in rats

1.6 Insect Repellents

Mosquitoes are believed to emerge approximately 170 million years ago during the Jurassic Era (206-135 million years ago); the earliest known fossils of mosquitoes are dated back to the Cretaceous Era (144-65 million years ago). They evolved in the land mass that is now South America, spreading initially to the northern continent Laurasia and re-entering the tropics from the north. Ancestral mosquitoes were roughly three times the size of existing species, and they were a sister group to *Chaoboridae* (biting midges). Nowadays, mosquitoes are found in all continents except Antarctica. Members of the genera *Anopheles*, *Culex* and *Aedes* are most commonly responsible for biting in humans. There are approximately 170 species of mosquitoes in North America alone.

1.6.1. Vector-borne Diseases

Mosquitoes transmit numerous diseases to more than 700 million people annually around the globe; they are responsible for the deaths of 1 in every 17 people living on the earth [90]. For instance, malaria is caused by infection with a protozoan carried by mosquitoes, and results in 30 million cases of death every year. In addition, mosquitoes transmit arboviruses that are responsible for yellow fever, dengue hemorrhagic fever, epidemic polyarthritis, and several forms of encephalitis [91].

West Nile Virus (WNV) is a mosquito-borne virus that can induce a range of symptoms of varying severity in humans. The virus was first isolated and identified in 1937 in a febrile patient in the West Nile district of Uganda, after which the virus was hence named. Prior to 1999, the virus was primarily found in the eastern hemisphere,

with wide distributions in Africa, Asia, the Middle East and Europe. There had been infrequent reports of human outbreaks, mainly associated with mild febrile illnesses in groups of soldiers, children and healthy adults, in Israel and some African countries. However, the virus had been relatively unknown to the Americas until the summer of 1999 when it staged an outbreak in the State of New York. Since 1999 the virus has rapidly spread westwards across the North America. Currently, the virus has been found in all forty eight continental US states, seven Canadian provinces and throughout Mexico. In addition, its activity has been detected in Puerto Rico, the Dominican Republic, Jamaica, Guadeloupe and El Salvador. According to reports from U.S. Centers for Disease Control and Prevention (CDC), more than 15,000 people in the U.S. have tested positive for WNV infection since 1999, which also included over 500 casualties. Many more people may have likely been infected with WNV, but have experienced mild or no symptoms.

Although the exact mechanism of illness is unclear, West Nile virus is thought to enter the bloodstream of a host through the sting of a mosquito; it multiplies in the blood and then moves up to the brain by crossing the blood-brain barrier. Once the virus infects the brain or its linings, an inflammatory response occurs and symptoms arise. The more severe symptoms of virus infection include high fever, headache, neck stiffness, stupor, disorientation, coma, tremors, convulsions, muscle weakness, vision loss, numbness and paralysis. These symptoms may last for several weeks, and the neurological adverse effects could become permanent. The virus transmission occurs mostly during warm weather conditions, particularly in summer months when mosquito populations are active. The incubation period ranges between 3-14 days. Mosquito infestation and

potential transmission of West Nile virus have, therefore, become an imminent health hazard to the general public, especially to children and geriatric population who tend to have weaker immune systems [92].

1.6.2. Mechanisms of Insect Repellents

Insect repellents are considered one of the most convenient approaches for personal protection against insect bites; they are as economical and feasible as large-scale vector control operations. Use of insect repellents has been proven effective in reducing insect bites and subsequently minimizing risk of vector-borne diseases. Nevertheless, repellent usage is not free of possible adverse effects, especially if applied in large amounts or administered improperly. Little information has been available in the effectiveness of insect repellents at lower concentrations and their potential adverse effects on children because of their larger body surface area.

The factors involved in attraction of mosquitoes to a host are complex, and have not been fully elucidated by entomologists [93-95]. Mosquitoes use a combination of visual, thermal and olfactory stimuli to locate a target. For example, wearing dark-colored clothing may initiate the orientation of a mosquito towards a human host [96]. In addition, it is estimated that 300-400 compounds are released from human body as byproducts of various metabolism, some of which play important roles in attracting mosquitoes and other insects [97]. Carbon dioxide and lactic acid have been the two chemical compounds that have been best studied as mosquito attractants. Mosquitoes possess chemoreceptors within their antennae that are stimulated by lactic acid. It was shown recently that the *Anopheles gambiae* protein AgOr1, a female-specific member of

a family of putative odorant receptors, responds to a component of human sweat. Compounds designed to activate or block receptors of this type could function as either attractant or repellent to trap and deter mosquitoes and other biting insects [98].

Despite the obvious desirability of finding an effective insect repellent for oral administration, no success has been achieved due to numerous complex factors in insect repellence [99,100]. Topical application still remains as the safest and most feasible approach for repellent products. Ideally, a repellent agent would not only repel multiple species of biting arthropods, but also exert no systemic toxicity or topical irritation. In addition, a good preparation should also facilitate active repellent ingredient in extending protection duration, resisting loss of activity and enhancing application convenience. No repellent product currently available in the market has met all these criteria. Abrasion from clothing, evaporation of the components through higher temperature or windy conditions, percutaneous absorption from the skin surface, washing-off by sweating or rain, can all decrease effectiveness of the repellent products [101,102]. An increase of 10 °C in temperature would lead to as much as 50% of reduction in protection time. Mosquito density also affects protection efficacy, as more repellents would have to be applied when mosquito population becomes higher [103] .

1.6.3. DEET

N,N-diethyl-m-toluamide (DEET) has remained as the most preferable insect repellent for decades (*Figure 1.14*). This substance was initially discovered and developed by scientists at the U.S. Department of Agriculture, and was patented by the U.S Army for military use in 1946. It was subsequently registered for civil use by the

general public in 1957 [104]. *Table 1.5* lists basic physical and chemical properties of DEET.

DEET is a broad-spectrum repellent that is effective against mosquitoes, biting flies, chiggers, fleas and ticks. Numerous studies have been published to assess the efficacy of DEET versus various other insect repellents [105-107]. For occasional protective use, there is no need for high concentration of DEET. Mathematical modeling has indicated that the effectiveness and persistence of a mosquito repellent are proportional to the dose or concentration of the active ingredient in the product. However, this curve tends to form a plateau at higher repellent concentrations, providing a smaller additional protection for each incremental dose of the active ingredient. On the other hand, undesirable pharmacological and toxicological potentials could arise with the increase in dosing. For example, 100% pure DEET had been used by military personnel in heavily infested regions for personal protection; products that contain higher than 50% of DEET do not significantly increase protection efficacy or prolong protection duration. On the contrary, products with 10-35% of DEET are able to provide adequate protection under most conditions. The American Academy of Pediatrics recommends that repellents to be used in children contain no more than 10% of DEET [108]. While repellents may be applied directly to the skin, or to clothing, window screens, mesh insect nets, tents and sleeping bags, DEET-based repellents can damage plastics, rayon, spandex, and other synthetic fabrics. Leather, painted or varnished surfaces should also be carefully managed. Consumers who apply both DEET and sunscreens should aware that the repellent can reduce the protective efficacy of the sunscreens; however it was found that the repellency of DEET was not altered by sunscreens [109].

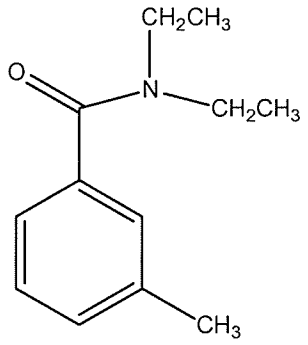


Figure 1.14. Chemical structure of N,N-diethyl-m-toluamide

Table 1.5. Physical and chemical properties of DEET

Parameter	Value / Property
Molecular Weight	191.26
Purity	N,N-diethyl-*m*-toluamide, 95% Minimum
Specific Gravity (25/25°C)	0.992-0.999
Color/Form	Colorless to off-white, light-yellow, amber liquid
Odor	Nearly odorless
Water Solubility (25°C)	Practically insoluble
Partition Coefficient (K_{ow})	100
Soil Sorption Coefficient (K_{oc})	300
Vapor Pressure (20°C)	5.6×10^{-3}mmHg
EPA Toxicity Classification	Class III
Boiling Point	111°C (1mmHg)
Viscosity (30°C)	13.3cps

* http://www.rand.org/publications/

1.7. Percutaneous Absorption of Sunscreens/Repellents

Human skin forms a complex protection barrier to the external environment, maintaining body fluids within the system and excluding harmful substances from the outside world. The ability to quantitatively predict absorption of substances through the skin has implications in the pharmaceutical, chemical and cosmetic industries as well as in the environmental health. A topical dosage form can be effective only if the active ingredient is capable of reaching the site of skin disorder or infection. The existence of rich blood capillaries within the skin also allows it to be used as a site for systemic drug administration. For repellent and sunscreen products, however, percutaneous absorption of the active ingredients might be undesirable because they are solely intended for external protection effects.

1.7.1. Sunscreens

Systemic permeation and absorption of the commonly used sunscreen oxybenzone after topical application to human volunteers has been reported [110]. It was found that 1-2% of the applied dose of oxybenzone in a commercial sunscreen product was excreted in the urine over a period of 48 hours following a single application. Jiang *et al* reported that limited absorption across the skin was observed for a majority of common sunscreen compounds; oxybenzone however demonstrated sufficiently high percutaneous penetration [111]. Sunscreen products are advised to be applied to large areas of skin in a repeated manner, in particular for summer outdoor activities for better skin protections. Most cosmetic manufacturers have now also incorporated sunscreen

ingredients into their products for regular applications. In order to provide effective sun protection to the skin, sunscreens should reside on or near skin surface; there is no therapeutic requirement for systemic absorption of sunscreen active ingredients. Determination of skin absorption of sunscreens is an essential element in the risk assessment of commercial sunscreen products [112]. Watkinson and co-workers suggested that significant skin absorption of some sunscreen compounds might occur [113]. Nevertheless, various factors including the nature of formulation vehicles and coexistence of excipients can all influence skin permeation and absorption of topically applied chemicals. Therefore it is essential that sunscreen absorption into the skin is determined in well designed *in vitro* and *in vivo* studies. In any case, sunscreen products and other topical cosmetic preparations are effective only while they remain on the surface of the skin. The systemic transdermal absorption of these products, if any, should be minimal and negligible.

Gupta *et al* reported that penetration of sunscreens oxybenzone and octyl methoxycinnamate across Yucatan pig skin was formulation-dependent [114,115]. Percutaneous absorption of the compounds was higher when both were applied in combination than when individually applied. Flux and permeability coefficient of oxybenzone was also related to vehicle solubility parameter; alcohol as a solvent enhanced skin and membrane diffusivity of oxybenzone. While numerous common sunscreens are capable of penetrating into stratum corneum, the extent and rate at which they permeate to deeper dermal tissues over the time have not been well established or elucidated [116]. The affinity of highly lipophilic sunscreens to stratum corneum confers their ability to resist removal by water, which is important in retaining protection efficacy

of the active ingredients after swimming or sweating [117]. This may also minimize diffusion of the sunscreens into viable epidermis and deeper tissues.

Overall assessment of possible health risks associated with sunscreen usage requires a better understanding of the skin absorption and distribution at clinically relevant dosing, plus the influences of formulation parameters on percutaneous absorption and distribution in skin tissues. As little information is available regarding penetration enhancement of common sunscreen ingredients such as oxybenzone, it is necessary that systematic investigations be carried out to fully evaluate penetration characteristics of oxybenzone with or without co-administration of other widely used topical preparations.

1.7.2. Insect Repellents

The percutaneous characterization of the insect repellent DEET has been studied for decades; yet no definite conclusions have been drawn regarding pharmacological and toxicological properties of this widely used compound. Even though over 90% of commercially available repellent products are DEET-based, there remains debate as to its safety and effectiveness. Generally, transdermal permeation of DEET across the skin is closely related to the repellent formulations. It was found that commercially available DEET-based repellent products showed a 6-100% cumulative permeation in human skin *in vitro*, depending upon different repellent preparations tested [46]. Earlier research suggested that 9-56% of applied DEET dose permeated through the skin, although only about 15% was systemically absorbed. However, *in vitro* studies involving infinite-dose applications of DEET to human skin obtained close conclusions, Stinecipher and Shah

calculated the steady-state flux of DEET at 21-63 μg/cm^2/hr [118] while Moody *et al* calculated the value as 20-60 μg/cm^2/hr [119].

To accurately determine DEET absorption, it is necessary to recover all applied dose and achieve mass balance. Few studies had been successful with this testing approach. One study reported a satisfactory mass balance of 88.7-94.3% from ^{14}C-labeled DEET [120]. In this study, formulations containing 100% and 15% of ^{14}C-DEET in ethanol were applied to the forearms of two groups of six human volunteers. The application site was washed 8 hours after the application, followed by tape stripping at 1, 23 and 48 hours. Blood, urine and fecal samples were also collected and analyzed in order to estimate biodistribution of DEET *in vivo*.

Plasma radioactivity indicated absorption of DEET as early as 2 hours after the application, but elimination was rapid and complete. Most of the applied dose was washed off the skin, while the absorbed dose was metabolized. Six major metabolites were detected in the urine, the primary route of excretion for DEET. Based on the percentage of applied DEET recovered in the total excreta, dermal absorption of DEET was found to range from 3-8% of 100 % DEET preparation and 4-14% of 15% DEET preparation.

Several studies have reported the absorption, metabolism and excretion of DEET in animals and humans. Selim *et al* studied the dermal application of ^{14}C-DEET in male human volunteers [121]. The study revealed that DEET was metabolized completely prior to elimination in the urine. The metabolic profile of the compound in human urine was also compared with the metabolic profile previously reported in rats [122] (*Figure 1.15*).

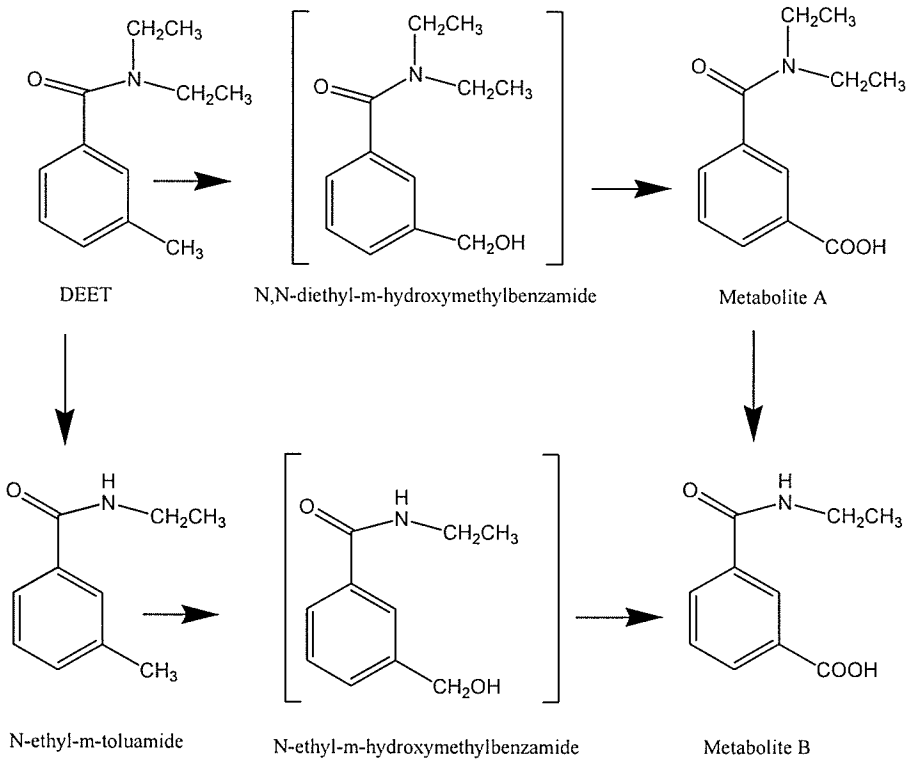


Figure 1.15. Proposed metabolic pathway for DEET in rats
Metabolite A: m-[(N, N-diethylamino) carbonyl]-benzoic acid, Metabolic B: m-[(ethylamino) carbonyl]-benzoic acid

Two major metabolites of DEET have been identified in rats. Metabolite A, m-[(N,N-diethylamino) carbonyl] benzoic acid, accounts for 46-60% of the administered dose. Metabolite B, m-[(ethylamino) carbonyl]-benzoic acid, accounts for 10-17% of the administered dose. Other minor metabolites include N,N-diethyl-m-hydroxymethylbenzamide, N-ethyl-m-toluamide, and N-ethyl-m-hydroxymethylbenzamide. While the overall metabolic profile of the compound is similar in both sexes, male rats seem to excrete a lower percentage of metabolite B than female rats. Metabolite A is formed by oxidation of the aromatic methyl group to a carboxylic acid group; metabolite B is formed by oxidation of the same aromatic methyl group to a carboxylic acid group, plus dealkylation on the amide group of DEET molecule. Taylor and Spooner also found that these two unconjugated metabolites accounted for the major portion of a topical dose of DEET in rat urine [123]. Shoenig *et al* attempted to use *in vivo* study data to determine the applicability of rat toxicology studies. The studies were conducted by oral route of administration to evaluate potential exposure by the dermal route of administration. The authors concluded that DEET and/or its metabolites are absorbed into the systemic circulation by both oral and dermal routes of administration in rats. No major differences in tissue distribution, route of excretion, or metabolism were noted in rats when DEET was administered by oral or dermal route of administration. However, rats given DEET orally absorb DEET and/or its metabolites more rapidly and attain higher tissue levels of DEET and/or its metabolites. Therefore, rat toxicity studies using the oral route of administration represent a qualitatively similar but quantitatively more severe exposure scenario than studies conducted by the dermal route of

administration. The authors inferred that this was an important observation, because the oral route of administration was normally used to evaluate DEET toxicity [124].

It has been reported that formulation vehicles had a marked impact on DEET permeation from topical solution or gel emulsion *in vitro*; stable formulations are necessary to minimize DEET permeation through the skin [47]. Also, the incorporation of skin humectants does not appear to significantly lower DEET skin permeability [125]. Santhanam *et al* tried to study the effects of DEET volatility on its transdermal penetration [126]. They studied DEET disposition on skin using a diffusion/evaporation model having parameters that can be correlated to molecular weight, volatility, and lipid solubility. Under normalized conditions in the fumehood, 49-72% of the dose penetrated in the lab bench studies and 11-34% of the dose penetrated in the fumehood studies. They concluded that difference in penetration was dependent on evaporation rate and airflow [127]. The authors also concluded that DEET enhanced not only its own penetration through the skin, but also that of other compounds [128]. Proniuk *et al* investigated the utility of cyclodextrins as a safer alternative to the commonly used cosolvent ethanol in topical DEET formulations [129]. They prepared DEET formulations containing up to 20% (w/w) of gamma-cyclodextrin (GCD) or 30% (w/w) of hydroxypropyl-beta-cyclodextrin (HPβCD). These formulations were then evaluated by determining the drug release rate through a synthetic membrane. It was concluded that the reduced release rate of DEET from these formulations might minimize the toxic potential of the compound. While such reports are promising, the repellency of DEET in novel formulations has not been very well documented. Also, the authors did not test the

formulations in skin which raises concerns as DEET is known to be a penetration enhancer possibly by extracting the lipids from the skin.

Although there are several studies of the formulation and penetration nature of DEET, there is a significant lack of information on the nature of DEET when combined with other widely used topical compounds. DEET can only function to its optimum when it stays on the surface of skin. Any systemic absorption is not desirable. The formulation characteristics and systemic study on penetration enhancement effect is also lacking in the literature. Since DEET is the most widely used insect repellent and its safety profiles are not fully elucidated in any reports, it would be prudent to conduct more studies with DEET alone and/or DEET with other compounds in order to evaluate the transdermal absorption characteristics of this poorly studied repellent substance.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1. Chemicals

The following chemicals were used for HPLC analysis, *in vitro* diffusion studies and *in vivo* pharmacokinetic studies in an animal model:

Acetonitrile (HPLC Grade): Fischer Scientific, Fair Lawn, NJ, USA

Brij 20 Oleyl Ether (Brij® 98): Sigma Aldrich Co., St. Louis, MO, USA

2,4-Dihydroxybenzophenone (DHB) (Analytical Grade): Sigma Aldrich Company, Oakville, ON, Canada

2,2'-Dihydroxy-4-methoxybenzophenone (DMB) (Analytical Grade): Sigma Aldrich Company, Oakville, ON, Canada

Ethanol (HPLC grade): Fisher Scientific, Fair Lawn, NJ, USA

Glacial Acetic Acid (Analytical Grade): Mallinckrodt Specialty Chemical Company, Paris, KY, USA

Isopropyl Alcohol (Analytical Grade): Fischer Scientific, Fair Lawn, NJ, USA

Methanol (HPLC Grade): Fischer Scientific, Fair Lawn, NJ, USA

N-Ethyl-m-toluamide (ET): Agriculture and Agri-Food Canada, Saskatoon, SK, Canada

N,N-Diethyl-m-hydroxymethylbenzamide (DHMB): Agriculture and Agri-Food Canada, Saskatoon, SK, Canada

N,N-Diethyl-m-toluamide (DEET) (Analytical Grade): Fluka Chemika GmbH, Buchs, Switzerland

N,N-Dipropyl-m-toluamide (DPT): Agriculture and Agri-Food Canada, Saskatoon, SK, Canada

Oxybenzone (OBZ) (Analytical Grade): Riedel-de Haën GmbH, Seelze, Germany

Polyethylene Glycol 400 (Analytical Grade): Fisher Scientific, Fair Lawn, NJ, USA

Potassium Phosphate Monobasic (Analytical Grade): Fisher Scientific, Fair Lawn, NJ, USA

Propylene Glycol (Analytical Grade): Fisher Scientific, Fair Lawn, NJ, USA

Sodium Hydroxide (Analytical Grade): Fisher Scientific, Fair Lawn, NJ, USA

2,3,4-Trihydroxybenzophenone (THB) (Analytical Grade): Sigma Aldrich Company, Oakville, ON, Canada

2.1.2. Insect Repellent and Sunscreen Products

Three commercially available insect repellent and sunscreen products were purchased from a local pharmacy. They were used as obtained without further manipulation. The names and major active ingredients of the products are listed as follows:

OFF!® Skintastic Insect Repellent Lotion: S.C. Johnson and Son, Ltd., Brantford, ON, Canada, containing 10% DEET as the active ingredient

Coppertone® UVA/UVB Protection Sunscreen Lotion (SPF30): Schering-Plough Healthcare Products Canada, Mississauga, ON, Canada, containing 5% oxybenzone as one of the active ingredients

OFF! ® Skintastic Insect Repellent with Sunscreen Lotion (SPF 15): S.C. Johnson and Son, Ltd., Brantford, ON, Canada, containing 10% DEET as active repellent ingredient and 5% oxybenzone as one of the sunscreen components

2.1.3. Membrane Models

Both artificial and biological membranes were used in the diffusion experiments to assess penetration characteristics of DEET and oxybenzone *in vitro*. The selection and use of these testing membranes were based on standard practices, literature reviews and regulatory publications.

Polydimethylsiloxane (PDMS) membrane (Pillar Surgical Inc., San Diego, CA, USA) was a medical-grade inert silicone membrane commonly used in various medical applications. This type of membrane was provided in a vulcanized form with a woven or double knit polyester fabric for the reinforcement of dimensional stability and tear resistance. The technical parameters of PDMS membrane are listed in *Table 2.1*.

Pig skin was collected from 3-week old piglets (Glenlea Research Station, University of Manitoba, Winnipeg, MB, Canada). The Animal Use Protocol was approved by the University of Manitoba Fort Garry Campus Protocol Management and Review Committee, and conducted according to current guidelines published by the Canadian Council on Animal Care (CCAC). The freshly excised skin samples were kept at –20 °C immediately after the collection, and thawed at 4 °C overnight before each experiment.

Human skin specimens were obtained from the Department of Surgery, St. Boniface General Hospital, Winnipeg, MB, Canada. The Human Research Ethics was

approved by the Research Ethics Boards at both the University of Manitoba and St. Boniface General Hospital. The skin samples were kept at –20 °C after receiving from the hospital and thawed at 4 °C overnight before experiment.

Table 2.1. Specification of PDMS membrane

Parameter	Value/Property
Membrane Thickness (inches)	0.010
Visual Appearance	Clear to translucent
Durometer	50
Tensile Strength (PSI)	1600
Specific Gravity	1.16
Tear Strength	250
Elongation %	850
Cytotoxicity	Non toxic
USP Class	Class VI

2.1.4. Instrumentation

An *in vitro* diffusion system was used for the evaluation of permeation characterization of DEET and oxybenzone across various membrane models. The diffusion system consisted of 15 vertical Franz-style diffusion cells (PermeGear Inc., Bethlehem, PA, USA), a thermostatic water bath (Blue Island, IL, USA), and an externally controlled multiple-station magnetic stirring plate (Variomag, Daytona Beach, FL, USA). The volume of the Franz-style donor and receptor cell was 1.0 ml and 3.5 ml, respectively. The surface area available for drug diffusion was 1.1 cm^2.

The quantification of DEET and oxybenzone in all experiments was performed using a Waters® HPLC system (Waters Corporation, Milford, MA, USA). The HPLC was composed of a Waters® 2690 Alliance® solvent delivery system, a 996 photodiode array detector and a Symmetry® C_{18} column (3.9×150 mm, 4 μm). The HPLC system was operated by Millennium® 32 Software.

Other instruments that were used in the experimentation included, a pH meter (Fisher Scientific, Fair Lawn, NJ, USA) for the measurement of various buffer solutions, a Milli-Q® water system (Millipore, Nepean, ON, Canada) for the production of deionized water, and an electric dermatome (Padgette Instruments, Kansas City, MO, USA) for the preparation of biological skin samples used in all *in vitro* diffusion studies.

2.2. Methodology

2.2.1. HPLC Assay

An HPLC assay was developed to simultaneously measure DEET, oxybenzone and their major metabolites. Test compounds included N,N-diethyl-m-toluamide (DEET), oxybenzone (OBZ), N,N-diethyl-m-hydroxymethylbenzamide (DHBM), N-ethyl-m-toluamide (ET), 2,4-dihydroxy-benzophenone (DHB), 2,2'-dihydroxy-4-methoxybenzophenone (DMB), 2,3,4-trihydroxy-benzophenone (THB), and N,N-dipropyl-m-toluamide (DPT).

2.2.1.1. *HPLC Conditions*

The mobile phase was composed of methanol and water (pH 2.83, adjusted with glacial acetic acid). To achieve optimal separation and resolution, three stages of solvent gradient were used as following, 0-3 minutes: 50% methanol/50% water (v/v); 3-9.5 minutes: 70% methanol/30% water; 9.5-14 minutes: 90% methanol/10% water. A 2-minute interval was allowed between two consecutive injections to condition the column. The flow rate was maintained at 0.8 ml/min throughout the sample run. DEET, its two metabolites and the internal standard DPT were detected at 254 nm, while oxybenzone and its three metabolites were detected at 289 nm.

2.2.1.2. *Assay Validation*

The standard stock solutions were prepared by accurately weighing each compound and then dissolving in individual 10 ml volumetric flasks with methanol at 1.0 mg/ml. The working solutions of each compound were prepared by further dilution to 1 μg/10ul, 2 μg/10μl and 5 μg/10μl of the stock solutions with methanol before use. Calibration curves were constructed by spiking 1.0 ml of blank plasma specimen with working solutions to concentrations of 0.1, 0.5, 1.0, 2.0, 4.0 and 5.0 μg/ml.

Assay optimization was carried out based on previous isocratic reversed-phase HPLC methods that had been developed and used in our laboratory for *in vitro* diffusion of DEET and oxybenzone. DEET and oxybenzone showed best detection sensitivity at 254 nm and 289 nm. Since six new compounds were to be measured for biological samples and there were additional biological components present, the isocratic elution was not sufficient enough for the complete separation of the analytes in biological

specimens from *in vivo* assessment. Steps of solvent gradient were therefore employed to improve separation and detection of each individual compound.

The robustness and reproducibility of the assay were validated after satisfactory elution of the analytes had been achieved. Method validation was performed by injecting six replicates of spiked plasma samples at 0.1 and 5.0 μg/ml of each compound during same working day (intra-day variation) and on six different days (inter-day variation). Peak area ratios between the individual analyte and the internal standard were calculated and the variations of the results compared. In addition, extraction recovery was determined by measuring spiked plasma and urine samples at known concentrations of 0.5 and 5.0 μg/ml; six replicates were also used in the determination of extraction recovery. Limit of detection (LOD) was defined as the concentration corresponding to a signal three times the noise level of the background. Limit of quantification (LOQ) was defined as the concentration corresponding to a signal ten times the noise level of the background. Millennium® software was used to automatically calculate and compare background noise to signal ratio in all studies.

2.2.2. Preparation of Samples

Samples of plasma, urine and tape strip were treated before the HPLC analysis. Extraction procedures for both plasma and urine samples were generally similar; tape strips were treated differently with additional steps of removing the adhesive glue. The extraction methods were optimized in order to achieve best analytical accuracy and sensitivity for each sample type.

2.2.2.1. *Plasma Samples*

Samples were stirred for 30 minutes at 500 rpm on a Variomag® multi-station magnetic stirrer, and then vigorously vortexed for 1 minute. 300 µl of mixture of methanol and acetonitrile (1:2, v/v) were added to the samples, and the solutions were further vortexed for 1 minute, followed by centrifugation at 10,000 g for 10 minutes. The supernatants were transferred to sample vials, and 10 µl were injected to the HPLC system for drug analysis.

2.2.2.2. *Urine Samples*

Samples were stirred for 30 minutes at 500 rpm on a Variomag® multi-station magnetic stirrer, and vigorously vortexed for 1 minute. 300 µl of mixture of methanol and acetonitrile (1:2, v/v) were then added to the samples. The solutions were further vortexed for 1 minute, followed by centrifugation at 10,000 g for 10 minutes. The supernatants were transferred to sample vials, and 10 µl were injected to the HPLC system for drug analysis.

2.2.2.3. *Tape Strip Samples*

Tape strips were extracted for DEET and oxybenzone using a two-step process adapted from earlier publication [132]. This involved immersion of the adhesive tape in isopropanol for two hours to dissolve the polymeric glue, followed by mixing the solution with acetonitrile. The mixture was then centrifuged at 10,000 g for 10 minutes. The supernatants were transferred to sample vials, and 10 µl were injected to the HPLC system for drug analysis.

2.2.3. *In Vitro Diffusion Studies*

2.2.3.1. *PDMS Membrane*

No special pretreatment was used for the artificial PDMS membrane. Prior to each diffusion study, the membrane was cut into small pieces (2×2 cm). They were soaked in deionized water for 30 min before being mounted onto diffusion cells.

The dissolving vehicles tested in the diffusion study included ethanol (50%), PEG-400, and propylene glycol. The objectives of the experiment were to compare permeation characteristics among different solvents and to obtain detailed information on transmembrane profiles of DEET and oxybenzone. Concentrations tested were 1, 2.5, 5 and 10 mg/ml for DEET and oxybenzone, either individually or in combination. Experimental temperatures were set at 37 °C and 45 °C; each experiment was conducted in 5 replicates.

The test solution (1.0 ml) was carefully placed in the donor cell, while 3.5 ml of receptor medium was added to the receptor cell. The receptor fluid was composed of pH 7.4 phosphate buffer containing 4% Brij 98. Use of a surfactant in the receptor medium was to facilitate solubilization of the highly lipophilic DEET and oxybenzone, thus maintaining "sink conditions" in the diffusion experiments. The receptor fluid was stirred continuously at 650 rpm to maintain homogeneity in the receptor cells. Receptor fluid (100 μl) was collected hourly for six hours, followed by replenishment of the same volume of fresh, pre-heated buffer medium after each sampling interval. Samples were directly injected to the HPLC for concentration analysis.

2.2.3.2. Pig Skin

Piglet skin was immediately kept at –20 °C after the collection. Before each study, the skin specimen was thawed, rinsed with deionized water and dried by blotting the surface with paper towels. The hair was shaven with a razor to provide a smooth skin surface, and the fat and underlayer skin tissues were removed with a scalpel. The skin was then placed on a plastic cutting board, and epidermis was dermatomed to a thickness of 250 μm using an electric dermatome. The integrity of the skin was carefully examined and only undamaged skin section with even thickness was selected for the diffusion studies. The epidermis was further cut into small pieces (2×2 cm) and presoaked in deionized water before being mounted onto the static Franz-style diffusion cells. The cell consisted of a 2 ml donor compartment and a 3.5 ml receptor compartment, with a 1.1 cm^2 surface available for diffusion.

The dissolving vehicles tested in the diffusion study included ethanol (50%), PEG-400, and propylene glycol. These solvents were selected because of their common use in the formulation of commercial insect repellents and sunscreens. DEET and oxybenzone were dissolved in the vehicles at 1, 2.5, 5 and 10 mg/ml, either individually or as a combination. Four replicates were conducted for each experiment at 45 °C.

The test solution (1.0 ml) was carefully placed in the donor cell, while 3.5 ml of receptor medium was added to the receptor cell. Receptor fluid was composed of pH 7.4 phosphate buffer containing 4% Brij 98. Use of a surfactant in the receptor medium was to facilitate solubility of the highly lipophilic DEET and oxybenzone, thus maintaining "sink conditions" in the diffusion experiments. The receptor fluid enhanced the solubility of test components without affecting membrane or transmembrane diffusion

characteristics [130]. Receptor fluid was stirred continuously at 650 rpm to maintain homogeneity in the receptor media. Receptor fluid (100 µl) was collected hourly for six hours, followed by replenishment of the same volume of fresh buffer medium after each sampling interval. Samples were directly injected to the HPLC for concentration analysis.

2.2.3.3. Human Skin

Human skin specimens were obtained from plastic surgeries after breast reduction surgeries and immediately kept at –20 °C after the collection. Before each study, the skin sample was thawed, rinsed with deionized water and dried by blotting the surface with paper towels. Fat and underlayer skin tissues were carefully removed with a scalpel. The skin was then placed on a plastic cutting board, and epidermis was dermatomed to a thickness of 250 µm using an electric dermatome. The integrity of the skin was carefully examined and only undamaged skin section with even thickness was selected for the diffusion studies. The epidermis was further cut into small pieces (2×2 cm). The prepared samples were presoaked in deionized water before being mounted to the diffusion cells for dosing.

The dissolving vehicles tested in the diffusion study included ethanol (50%), PEG-400, and propylene glycol. DEET and oxybenzone were dissolved in each vehicle at 1, 2.5, 5 and 10 mg/ml, either individually or as a combination. Four replicates were conducted for each experiment at 45 °C.

The test solution (1.0 ml) was carefully placed in the donor cell, while 3.5 ml of receptor medium was added to the receptor cell. Receptor fluid was composed of pH 7.4 phosphate buffer containing 4% Brij 98. Receptor fluid was stirred continuously at 650

rpm to maintain homogeneity in the receptor medium. Receptor fluid (100 μl) was collected hourly for six hours, followed by replenishment of the same volume of fresh buffer medium after each sampling interval. Samples were directly injected to the HPLC system for concentration analysis.

2.2.3.4. Solubility of DEET and Oxybenzone

Separate tests were carried out to determine solubility of DEET and oxybenzone in ethanol (50%), PEG-400, propylene glycol and the receptor fluid. In a single component test, excessive amount of DEET or oxybenzone was added to 10 ml solvent to maintain two phases, i.e., liquid phase for DEET and solid phase for oxybenzone. In a two-component test, only one compound was added to 10 ml solvent with excessive amount to achieve a two-phase stage, while the amount of the other compound was maintained constant at 10 mg/ml, i.e., the maximal test concentration in all diffusion studies. The mixture was kept at 37 °C under continuous agitation for 48 hours. Samples were centrifuged for 10 minutes afterwards, and the supernatant was further diluted to appropriate concentration for content analysis. Four replicates were conducted for each solubility study, and the concentrations of DEET and oxybenzone in each solvent were determined by the HPLC method.

2.2.4. In Vivo Studies in Piglets

Transdermal penetration and systemic absorption of DEET and oxybenzone were tested in 18 three-week old piglets. The animal use protocol was approved by the University of Manitoba Fort Garry Campus Animal Use Protocol Review Committee,

and conducted according to guidelines published by the Canadian Council of Animal Care (CCAC). References have indicated that pig skin demonstrates relatively similar skin configurations and physiological characteristics to human skin in terms of percutaneous permeation and absorption; piglets were also of suitable size and blood volume for intended dose application and sample collection. Young piglets were hence selected as an animal model for *in vivo* evaluation [131].

2.2.4.1. Catheterization

Piglets were purchased from Swine Unit of Glenlea Research Station, University of Manitoba. The age of animals at purchase was approximately 17 days. The piglets were checked by a University Veterinarian for general health conditions upon arrival at the Department of Animal Science Holding Facility; they were housed individually in a holding pan and fed special food for the following 3 days.

On the day of catheterization, the health condition of each study animal was checked again by the Veterinarian. The animal was then given an intramuscular injection of atropine sulphate (0.05 mg/kg), and sedated with an intramuscular injection of acepromazine (0.2 mg/kg) and ketamine (10 mg/kg). To maintain anesthesia during the surgical procedure, the piglet was intubated, and received oxygen (2 L/min) containing 2% of halothane. The depth of anesthesia was monitored by checking eye and appendage movement upon administration of a pressure. The reaction to pain stimulus was tested using a toe pinch method.

After complete anethesia had been achieved, a 5-cm incision was made at the site of jugular vein location on the back of the animal, and the external jugular vein was

isolated and exposed. A venous catheter was introduced into the blood vessel, using aseptic surgical technique, and secured afterwards. The incision was then sealed; the catheter was placed subcutaneously, keeping the exit on the dorsal side of the animal.

Following the surgery, the piglet was administered Trivetrin (antibiotic, 120 mg) and Banamine (analgesic, 1 mg/kg) by intramuscular injection to prevent potential infection. The exposed section of the catheter was placed in a custom designed nylon pouch and secured. The animal was released back to the holding pan and put under continuous monitoring of health conditions for the first three hours post surgery. Signs of post surgical discomfort (behavioral observations) or infection (rectal temperature over 39.9 °C) were also checked during the following 3-5 days. Catheter patency was monitored daily by judging the ability to aspirate from the catheter. Actual experiments were conducted only after the animals had fully recovered from surgical catheterization procedures.

2.2.4.2. Dose Application

Before dermal application of the selected repellent or sunscreen preparations, an area of 300 cm^2 (30×10 cm) on the piglet back was shaven with an electric clipper to clean the hair. One gram of three commercially available repellent or sunscreen products, i.e., a repellent lotion that contained 10% DEET, a sunscreen lotion that contained 5% oxybenzone, and a combined repellent/sunscreen lotion that contained 10% DEET and 5% oxybenzone, was applied evenly to the animals in three study groups. The animals were kept in individual pens in a room with controlled temperature and humidity. Play time was provided when not sampled, and food and water were supplied *ad libitum*

during the entire study period. The University Veterinarian was notified regularly of health conditions of the animals to ensure accuracy of the studies as well as well-being of the study animals.

2.2.4.3. Blood and Urine Sampling

Blood samples (2 ml) were collected from the jugular vein catheter at 0, 2, 4, 6, 8, 12, 24 and 48 hours after the dermal application. The catheter was flushed with 2 ml of 0.9% saline after each sampling time to replace fluid loss and to prevent blood from coagulating within the catheter. Plasma was separated by centrifugation at 2500 rpm for 10 minutes, and immediately stored at –20 °C until drug analysis. Urine sample was collected at 48 hours after euthanasia of the animals. The volume of urine samples was measured and a portion of the urine was immediately stored at –20 °C until drug analysis. Plasma and urine samples were treated as previously described in *Section 2.2.2.*

2.2.4.4. Skin Stripping

To evaluate dermal penetration of DEET and oxybenzone, tape strips were collected at 2, 12 and 48 hours after the topical application. Before skin stripping, a small area of the application site on the back (2×4 cm) was cleaned twice with alcohol swipes to remove the applied dose. After complete drying of the skin surface, 12 strips of Scotch® tape were evenly applied to the skin and pressed for 10 seconds, and then removed. Each tape strip was placed individually in marked polyethylene tubes, and stored at –20 °C until drug analysis. Three different application areas were used for

stripping samples in order to realistically represent dermal penetration of the applied dose. Samples were treated as previously described in *Section 2.2.2*.

2.3. Data Analysis

2.3.1. HPLC Assay

Six replicates were used for the validation of the HPLC method. Mean values and standard deviations were calculated. The relative standard deviation (RSD) of the variations was also calculated using the formula, $RSD = 100 \times S / X$, in which S was the standard deviation of the samples and X was mean of the samples. Linear regression was performed to correlate linearity between the peak ratio of each analyte against the internal standard and the test concentration used.

2.3.2. In Vitro Diffusion Studies

Permeation amount and percentage of DEET and oxybenzone across various membrane models were calculated, based on their concentrations at each sampling interval. Steady-state diffusion flux was obtained from the linear portion of the diffusion curve [133]. Statistical analysis was performed using two-way ANOVA and Tukey's test (PC-SAS® 8.02, SAS Institute Inc., Cary, NC, USA). The following statistical comparisons of the data were conducted, 1) the overall permeation percentage of DEET and oxybenzone across PDMS membrane among the four test concentrations at 37 °C and 45 °C; 2) the overall permeation percentage and steady-state flux of DEET and oxybenzone across the piglet skin and human skin among the three test vehicles; 3) the

overall permeation percentage of DEET and oxybenzone across the piglet skin and human skin among the four test concentrations at 45 °C; 4) the solubility of DEET and oxybenzone among the four test solvents. All differences were considered statistically significant at $p \leq 0.05$.

2.3.3. In Vivo Animal Studies

Concentrations of DEET and oxybenzone in plasma, urine and tape strip samples at different time intervals were calculated and summarized. The presence of five principal metabolites from both DEET and oxybenzone in plasma, urine and skin strip samples was also checked, and quantified where detected. Plasma concentrations of DEET and oxybenzone were subjected to non-compartmental pharmacokinetic simulation using WinNonlin® program (Version 5.0.1, Pharsight Corporation, Mountain View, California, USA), and various pharmacokinetic parameters calculated and compared. Transdermal penetration of DEET and oxybenzone after topical application was calculated based on dosing content recovery from skin tape strips. Statistical analysis was performed using two-way ANOVA and Tukey's test (PC-SAS® 8.02). The following statistical analyses of the data were conducted, 1) the amount of DEET and oxybenzone recovered from the skin tape strips post application of the three commercial formulations; 2) the percutaneous permeation of DEET and oxybenzone among the three application methods; 3) the area under the plasma concentration curve (AUC_{0-t}) and the maximum plasma concentration (C_{max}) of DEET and oxybenzone among the three commercial formulations. Differences were considered statistically significant at $p \leq 0.05$.

Chapter 3

Analysis of DEET, Oxybenzone and Their Relevant Metabolites

3.1. Objectives

Percutaneous penetration of DEET and oxybenzone from individual preparations has been widely studied [134-137]. Results have demonstrated the capability of both compounds in transdermal permeation and systemic absorption. However, no study has indicated interactions between DEET and oxybenzone that could lead to synergistic enhancement of percutaneous penetration. It is therefore prudent to investigate whether there is such an interaction between the two components on the surface of the skin, because concurrent application of DEET-based repellent and sunscreen products may potentiate systemic absorption of the active ingredients and subsequently lead to undesirable clinical impacts [138,139].

Developing a sensitive and selective analytical method is a prerequisite for pharmaceutical research. Numerous publications have reported individual assays for quantitative or qualitative measurement of DEET and oxybenzone [140,141]. Nevertheless, no assay has attempted to determine both DEET and oxybenzone simultaneously. Since concurrent use of DEET-based repellents and sunscreens was to be investigated for their transdermal characteristics, it would be convenient and practical to use a single and selective assay for all analytes involved, which included parent compounds DEET and oxybenzone, as well as their principal metabolites. A simultaneous and simple assay would not only allow high-throughput of a large number of samples but also produce more reliable and reproducible analytical results, which are critical parameters in biomedical analysis for *in vivo* pharmacokinetic and pharmacodynamic evaluation. Hence the objectives of initial experiment were to develop

and validate a sensitive and selective HPLC assay, for simultaneous separation and quantification of DEET, oxybenzone and six other primary metabolites of the two agents. The eight analytes to be measured were N,N-diethyl-m-toluamide (DEET), N,N-diethyl-m-hydroxymethylbenzamide (DHMB), N-ethyl-m-toluamide (ET), oxybenzone (OXY), 2,4-dihydroxybenzophenone (DHB), 2,2'-dihydroxy-4-methoxybenzophenone (DMB), 2,3,4-trihydroxybenzophenone (THB), and the internal standard N,N-dipropyl-m-toluamide (DPT). *Figure 3.1* shows the chemical structures of the analytes in this study.

3.2 Results and Discussion

Figure 3.2 shows the representative chromatograms of spiked plasma sample and tape strip respectively. DEET, oxybenzone and their metabolites in plasma were well separated with the help of a three-stage gradient step. *Table 3.1* lists the calibration linearity and detection limit of the developed assay. Individual calibration curves in the relationship between ratio of the peak areas (analyte versus internal standard) and the corresponding concentrations are presented in *Figures 3.2-3.9*. A linearity study verifies that a sample solution is within a range that the detection response is linearly proportional to the concentration. Excellent linearity of each test compound was achieved between 0.1-5.0 μg/ml of the concentrations. While the limit of detection (LOD) was calculated as 3 times greater than the baseline noise, the limit of quantification (LOQ) was calculated as 10 times greater than the baseline noise. Under current chromatographic conditions, LOD and LOQ ranged 0.5-0.7 ng and 1.7-2.3 ng for the test compounds respectively.

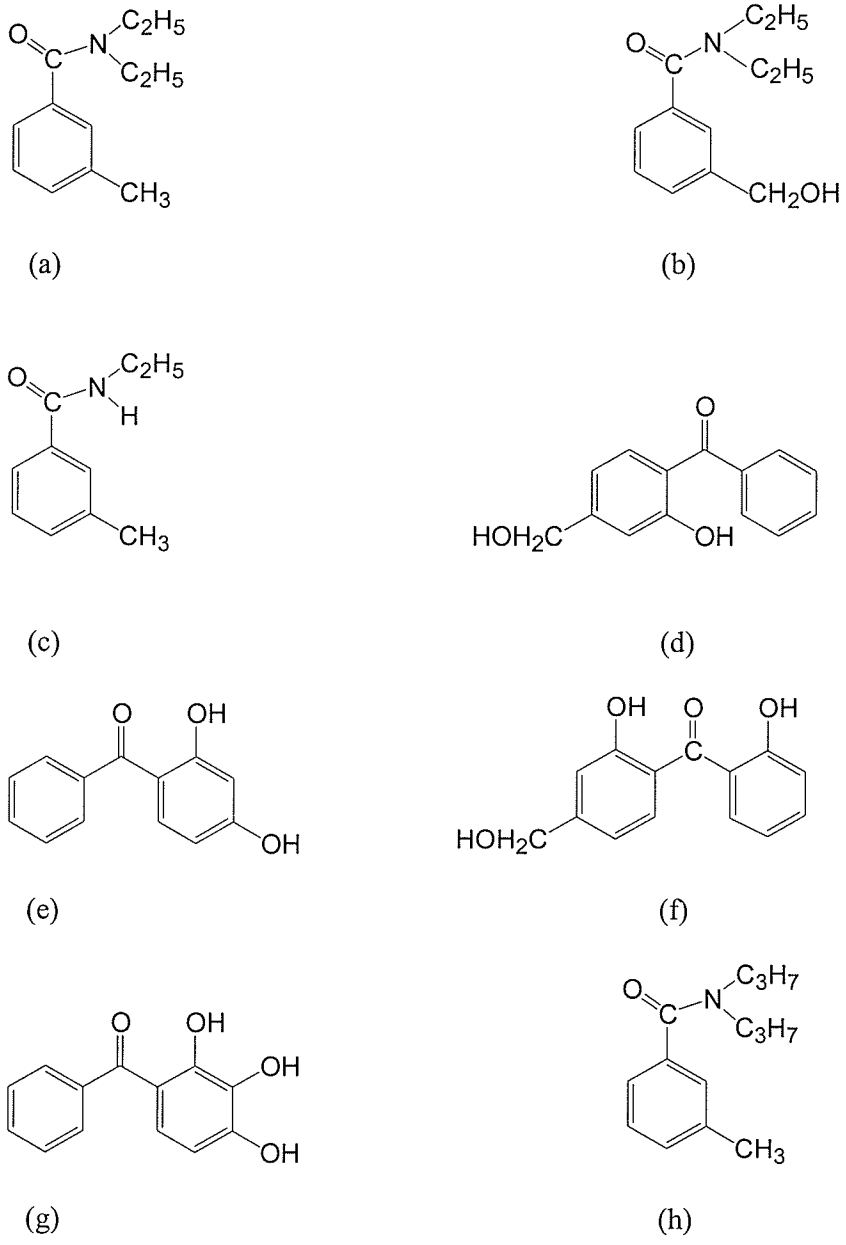


Figure 3.1. Chemical structures of the test compounds using HPLC assay (a) N,N-diethyl-m-toluamide (DEET), (b) N,N-diethyl-m-hydroxymethylbenzamide (DHBM), (c) N-ethyl-m-toluamide (ET) (d), oxybenzone (OXY) (e) 2,4-dihydroxybenzophenone (DHB), (f) 2,2'-dihydroxy-4-methoxybenzophenone (DMB),(g) 2,3,4-trihydroxybenzophenone (THB), (h) N,N-dipropyl-m-toluamide (DPT)

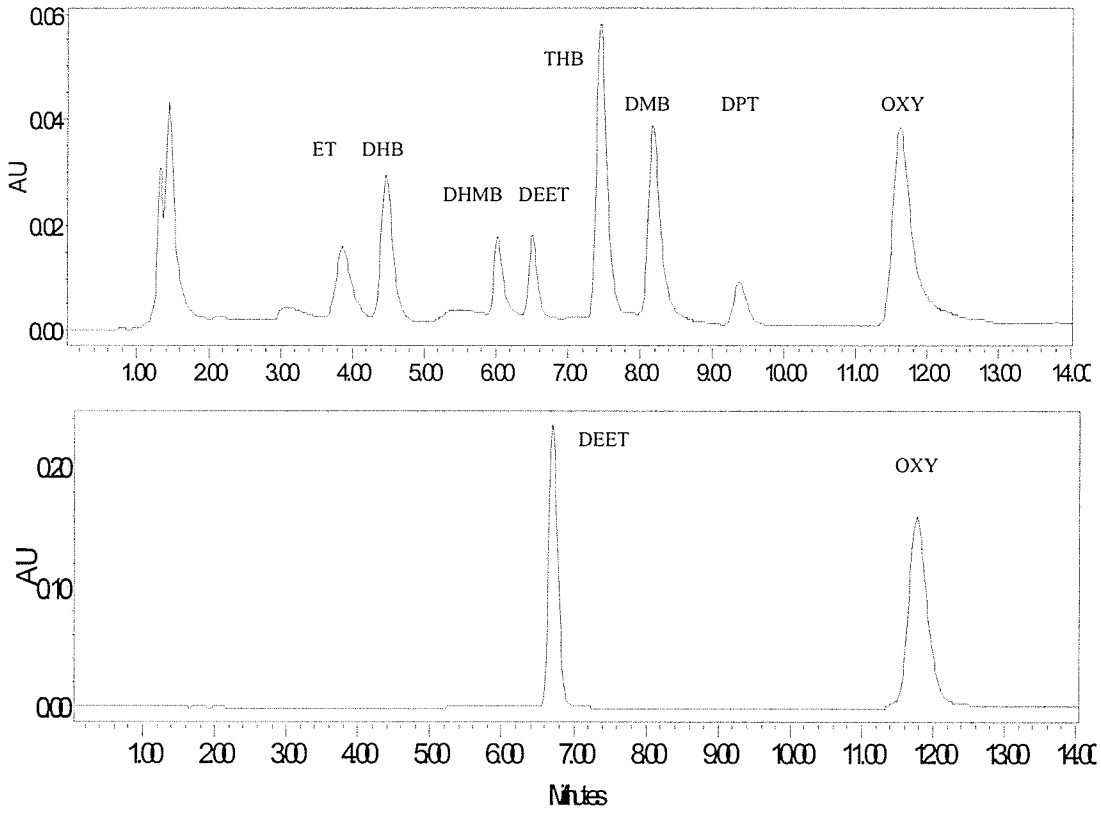


Figure 3.2. Representative chromatograms of the test compounds at 289 nm in spiked plasma (5.0 μg/ml, top) and in tape strip (bottom)

The total runtime for each injection was 14 minutes, which was considered acceptable for the analysis of biological samples. In order to maximize assay sensitivity, DEET and its metabolites were detected at 254 nm, while oxybenzone and its metabolites were detected at 289 nm. The selection of two different wavelengths for drug analysis by photodiode array detector provided further improvement in method sensitivity and selectivity. In addition, the wavelength of 289 nm illustrated the best sensitivity for

oxybenzone, because it is a known UVA and UVB absorber. Based on the retention time, the gradients were adjusted using a combination of water and methanol until oxybenzone finally eluted at 12 minutes. Modifications were further made in the solvent gradients until satisfactory retention times were obtained for clear separation of each analyte. The flow rate was kept uniform at 0.8 mL/min throughout to avoid peak surges caused by changes in flow rate. Final retention times were, 3.8 minutes for ET, 4.6 minutes for DHB, 6.1 minutes for DHMB, 6.6 minutes for DEET, 7.6 minutes for THB, 8.2 minutes for DMB, 9.5 minutes for DPT and 11.8 minutes for OXY.

Table 3.1. Calibration of test compounds in plasma samples (n = 6)

Compound	Wavelength (nm)	Linearity (μg/ml)	Correlation (r^2)	Limit (ng)
DEET	254	0.1–5.0	0.9999	0.6
DHMB	254	0.1–5.0	0.9999	0.6
DHB	289	0.1–5.0	0.9999	0.7
DMB	289	0.1–5.0	0.9999	0.6
ET	254	0.1–5.0	0.9998	0.5
OXY	289	0.1–5.0	0.9998	0.5
THB	289	0.1–5.0	0.9999	0.5

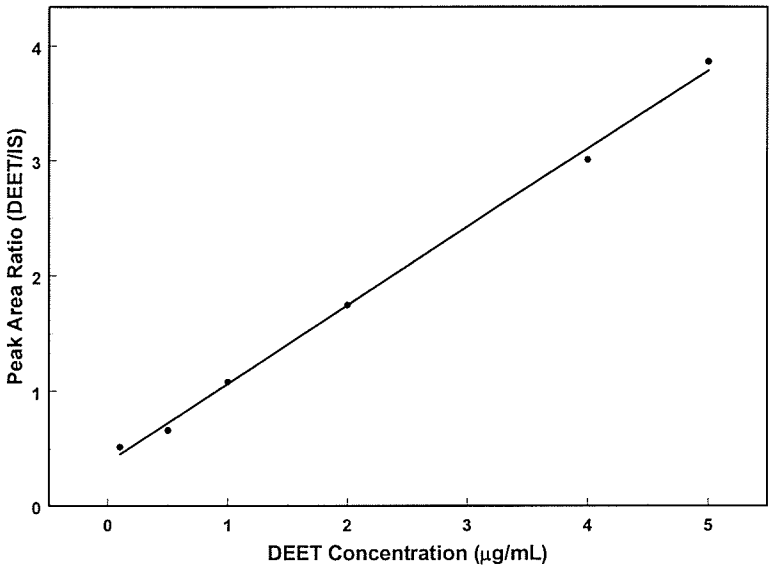


Figure 3.3. Calibration curve of N,N-diethyl-m-toluamide

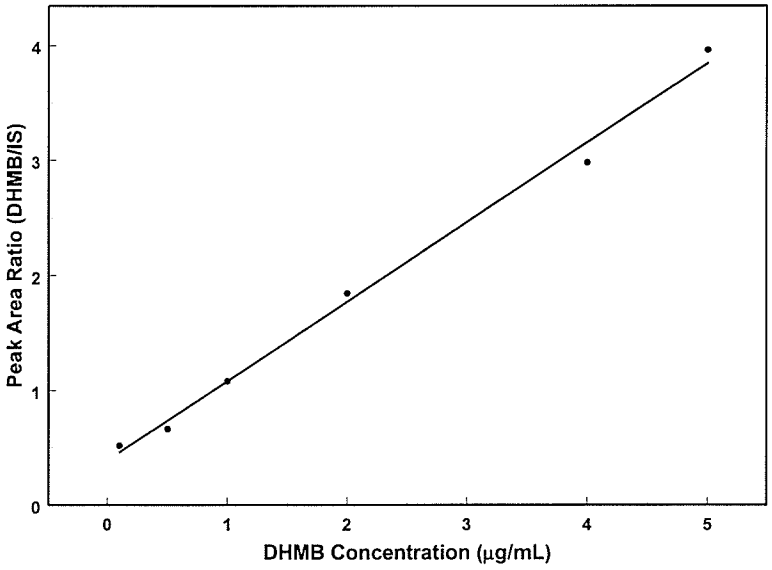


Figure 3.4. Calibration curve of N,N-diethyl-m-hydroxymethylbenzamide

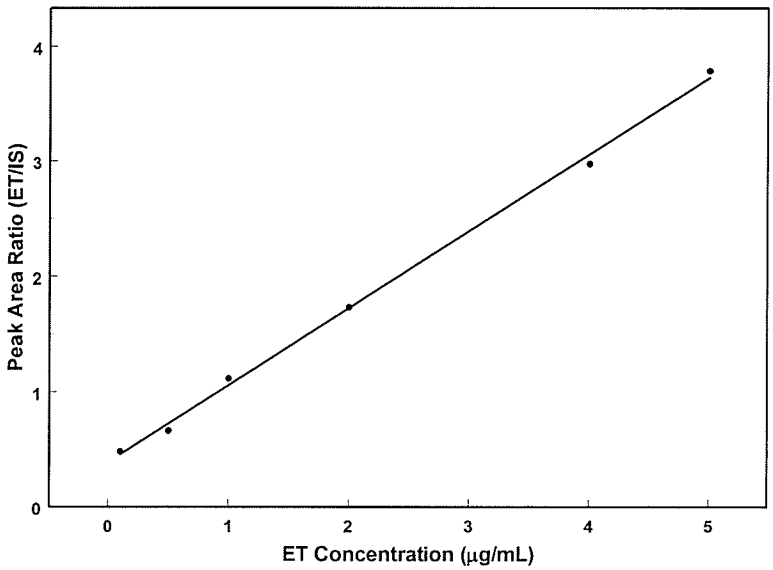


Figure 3.5. Calibration curve of N-ethyl-m-toluamide

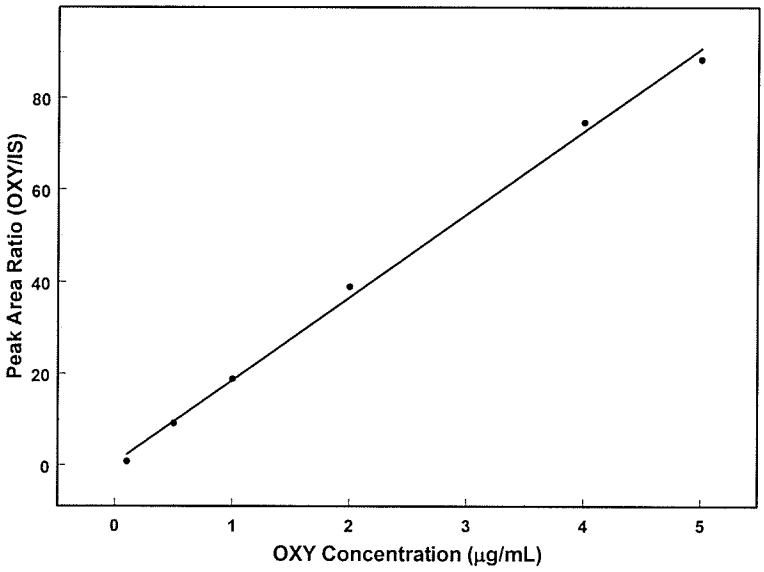


Figure 3.6. Calibration curve of oxybenzone

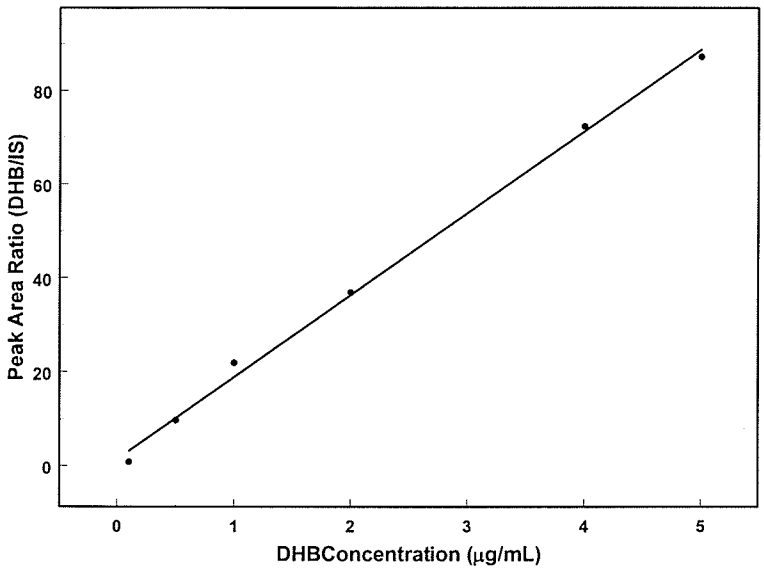


Figure 3.7. Calibration Curve of 2,4-dihydroxybenzophenone

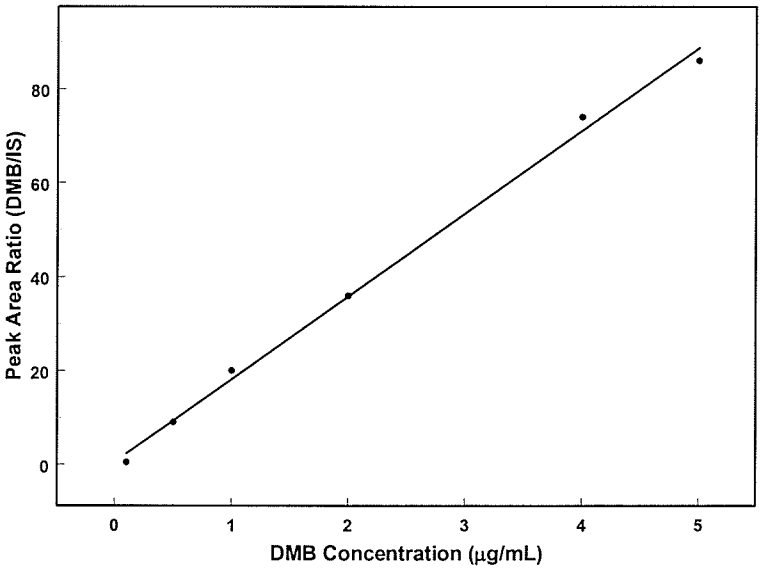


Figure 3.8. Calibration curve of 2,2'-dimethoxybenzophenone

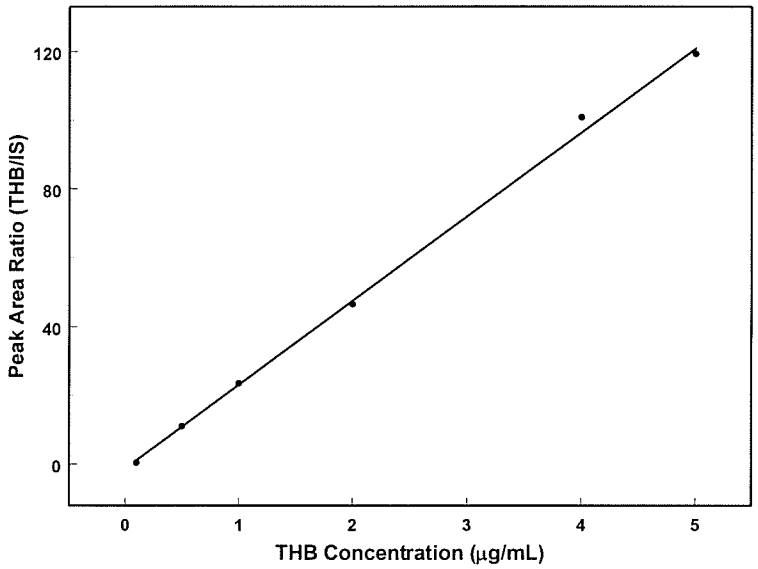


Figure 3.9. Calibration curve of 2,3,4-trihydroxybenzophenone

Assay validation was one of the necessary steps in method development to ensure the accuracy and reproducibility of the instrumentation. In addition, measurement of biological samples generally requires high robustness and sensitivity in order to compensate for drug loss due to extraction procedures. The validation was conducted at two different concentrations in spiked plasma on several days of analysis. *Tables 3.2-3.5* list the inter-day and intra-day variations of the assay. The analytical variations ranged 0.44-9.28%, indicating satisfactory accuracy and reproducibility of the instrumentation. Extraction recovery for all test compounds from plasma and urine samples was examined. It was found that the overall extraction recovery for the analytes was greater than 78% (ranging 78-99%), indicating acceptable material recovery and assay sensitivity. *Tables 3.6-3.9* show extraction recovery at two different concentrations.

Table 3.2. Inter-day variation of test compounds in plasma samples (0.1 μg/ml)

Analyte	Inter-Day Variation								
	1	2	3	4	5	6	Mean	S.D.	RSD
DEET	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	3.2
DHMB	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	3.0
DHB	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	3.0
DMB	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	9.2
ET	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	3.9
OXY	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.0	4.1
THB	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	4.9

Table 3.3. Inter-day variation of test compounds in plasma samples (5.0 μg/ml)

Analyte	Inter-Day Variation								
	1	2	3	4	5	6	Mean	S.D.	RSD
DEET	5.2	4.9	4.8	5.1	5.0	4.8	4.9	0.0	0.6
DHMB	5.2	4.9	5.1	5.4	4.9	4.8	5.0	0.4	7.8
DHB	4.9	5.2	5.0	4.9	5.0	4.8	4.9	0.6	1.2
DMB	5.2	4.9	5.1	4.8	4.8	5.1	4.9	0.4	7.9
ET	5.1	5.0	4.8	5.0	5.0	4.9	4.9	0.0	0.6
OXY	5.2	5.1	4.7	4.9	5.1	4.9	4.9	0.2	5.3
THB	5.1	4.8	5.1	5.2	4.9	4.9	5.0	0.0	0.5

Table 3.4. Intra-day variation of test compounds in plasma samples (0.1 μg/ml)

Analyte	Intra-Day Variation								
	1	2	3	4	5	6	Mean	S.D.	RSD
DEET	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	4.0
DHMB	0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.2	5.0
DHB	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	4.9
DMB	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	6.2
ET	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	7.8
OXY	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	1.0
THB	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	1.0

Table 3.5. Intra-day variation of test compounds in plasma samples (5.0 μg/ml)

Analyte	Intra-Day Variation								
	1	2	3	4	5	6	Mean	S.D.	RSD
DEET	4.8	4.9	4.9	4.9	5.0	4.8	4.9	0.0	0.7
DHMB	4.9	5.0	4.9	4.9	4.9	4.9	4.9	0.0	0.4
DHB	5.2	5.0	4.6	4.8	4.9	5.5	5.0	0.3	7.7
DMB	4.9	4.9	4.9	4.9	4.9	4.9	4.9	0.0	0.4
ET	4.8	5.0	4.6	4.6	4.8	5.4	4.9	0.3	6.1
OXY	4.9	5.4	4.6	4.7	5.5	4.9	4.9	0.3	6.0
THB	4.8	5.1	4.7	4.6	4.8	5.5	4.9	0.3	6.2

Table 3.6. Extraction recovery of test compounds from plasma samples (0.5 μg/ml)

Analyte	Plasma Extraction Recovery (%)							
	1	2	3	4	5	6	Mean	S.D.
DEET	86.5	89.4	91.4	94.5	88.5	91.4	90.3	2.7
DHMB	82.6	84.9	85.6	79.8	84.5	81.4	83.1	2.2
DHB	92.4	95.5	89.5	99.4	94.7	93.0	94.1	3.3
DMB	78.8	74.5	79.5	81.5	79.8	79.4	78.9	2.3
ET	74.4	87.5	81.4	85.9	87.5	83.2	83.3	4.9
OXY	97.0	99.4	100.2	98.5	100.4	99.2	99.1	1.2
THB	88.4	85.6	89.4	91.4	92.4	92.4	89.9	2.6

Table 3.7. Extraction recovery of test compounds from plasma samples (5.0 μg/ml)

Analyte	Plasma Extraction Recovery (%)							
	1	2	3	4	5	6	Mean	S.D.
DEET	82.5	85.4	94.5	86.4	84.9	86.4	86.7	4.0
DHMB	87.4	88.9	82.6	89.4	88.0	91.5	88.0	2.9
DHB	95.4	98.7	99.4	97.8	95.6	98.4	97.6	1.6
DMB	87.4	88.7	89.4	88.7	96.4	97.8	91.4	4.4
ET	79.5	87.5	84.5	86.3	87.4	85.6	85.1	2.9
OXY	100.2	98.2	99.4	98.4	97.4	99.4	98.6	1.0
THB	89.5	94.7	95.5	98.7	97.8	94.5	95.1	3.2

Table 3.8. Extraction recovery of test compounds from urine samples (0.5 μg/ml)

Analyte	Urine Extraction Recovery (%)							
	1	2	3	4	5	6	Mean	S.D.
DEET	85.4	84.7	87.4	84.1	84.6	87.4	85.6	1.4
DHMB	82.6	84.9	85.6	79.8	84.5	81.4	83.1	2.2
DHB	90.2	93.6	94.8	96.4	89.2	92.1	92.7	2.7
DMB	92.4	93.2	97.8	88.7	86.4	87.4	91.0	4.2
ET	78.4	76.5	74.4	80.2	79.4	77.0	77.7	2.1
OXY	98.4	99.7	102.2	97.8	99.4	97.8	99.2	1.6
THB	87.4	86.5	89.3	94.4	93.1	88.0	89.8	3.2

Table 3.9. Extraction recovery of test compounds from urine samples (5.0 μg/ml)

Analyte	Urine Extraction Recovery (%)							
	1	2	3	4	5	6	Mean	S.D.
DEET	88.8	90.5	91.4	82.4	86.9	88.1	88.0	3.1
DHMB	84.7	88.7	89.4	82.1	79.4	78.4	83.8	4.6
DHB	92.5	93.0	94.5	97.5	94.5	98.5	95.1	2.3
DMB	92.1	96.3	98.7	99.4	97.0	98.0	96.9	2.6
ET	79.8	78.2	80.1	84.5	84.0	82.5	81.5	2.5
OXY	97.4	99.4	99.8	100.2	99.0	98.0	99.0	1.0
THB	89.2	89.6	88.0	91.1	89.6	87.2	89.1	1.3

The need for better insect repellent and sunscreen products with established safety profiles is evident with increasing awareness of sun safety and mosquito borne diseases. A simple yet efficient method of quantification will not only assist in developing such better products but also establish better extraction and detection procedures for other transdermal products. Determination of DEET and oxybenzone in formulations and biological specimens has been previously reported. Nevertheless, those assays dealt with either DEET or oxybenzone separately, or DEET together with its metabolites, or oxybenzone together with other sunscreen compounds. The reversed-phase HPLC-UV assay described above was capable of simultaneous analysis of DEET, oxybenzone and five relevant metabolites. It used simple pretreatment and extraction of biological samples including plasma, urine and skin tape strips. Abu-Qare *et al* reported the separation of DEET, melathoin and permethrin simultaneously using a solid phase extraction. The authors reported the LOD and LOQ for DEET was 50 ng/ml and 100 ng/ml [154]. The LOD and LOQ obtained in our method were similar to those reported; however, only very simple and straightforward pretreatment of the samples was required. The method was believed to be the first assay to simultaneously analyze DEET, oxybenzone and primary metabolites of both compounds.

3.3. Conclusion

This developed assay not only offered simultaneous detection of major compounds that would likely be found from concurrent use of insect repellent and sunscreen products, but also provided rapid and selective detection of multiple compounds. Assay sensitivity was further improved with this method in comparison to our previous methods [151,152]. The sample runtime of the assay was also shorter than some earlier references that analyzed the components separately [140,153]. The assay demonstrated accuracy, specificity, selectivity, and versatility; it would be suitable for simultaneous quantification of DEET, oxybenzone and their metabolites in pharmacokinetic and pharmacodynamic studies *in vivo*.

Chapter 4

In Vitro Assessment of DEET and Oxybenzone

4.1. Hypothesis

Increased public awareness of the need for protection against the West Nile virus and skin cancer has led to a dramatic rise in the application of topical insect repellent and sunscreen preparations. Concurrent use of insect repellents and sunscreens has gradually become a widely accepted practice in many developed countries. Intended as "Topical Use Only" preparations, active repellent and sun-blocking ingredients such as DEET and oxybenzone (OXY) should remain on the surface of the skin with minimal percutaneous penetration and systemic absorption. This is of particular importance for insect repellents because of toxicity concerns of DEET in humans. Furthermore, insect repellents and sunscreens are mainly used at the discretion of individuals without fixed doses and application sequences. Potential physical and chemical interactions among active compounds from a concurrent application of the two preparations could not only alter percutaneous characteristics of these active components across various skin layers, but also compromise the protection efficacy of both products.

No studies have been carried out to evaluate percutaneous characterization of DEET and oxybenzone from a concurrent use. The objective of the *in vitro* assessment was to evaluate transdermal profiles of the active components from concurrent application of insect repellent and sunscreen preparations. Using human skin, excised piglet skin and artificial poly(dimethylsiloxane) (PDMS) membrane it was hypothesized that,

There would be an increase in the *in vitro* penetration of DEET and oxybenzone when applied concurrently using all the membrane models;

Transmembrane penetration of DEET and oxybenzone would be dependent on the vehicle, the concentration of the solute in the formulation and the temperature of experimental setting;

In vitro penetration of DEET and oxybenzone would be significantly different among the artificial PDMS membrane, biological piglet skin and the human skin.

The general aims of the *in vitro* diffusion studies were,

To evaluate the penetration profiles of DEET and oxybenzone when applied alone or in combination using various membrane models;

To compare the penetration amounts of DEET and oxybenzone using various formulations;

To assess the overall transmembrane flux of DEET and oxybenzone across various membrane models.

Results from these *in vitro* diffusion experiments would provide characteristic information on transmembrane permeation of DEET and oxybenzone from various solvents and across different membrane models. They would also allow for design of *in vivo* evaluation of commercially available repellent and sunscreen products in an animal model.

4.2. Results

4.2.1. Permeation across PDMS membrane

In vitro assessment using PDMS membrane showed that both DEET and oxybenzone permeated across the PDMS membrane. Permeation of the compounds was dependent upon the dissolving vehicles and test concentrations. *Figures 4.1-4.12* show the overall permeation percentage of DEET and oxybenzone after 6 hours in the three solvents. *Tables 4.1-4.12* show the overall permeation amount and the steady state flux of oxybenzone and DEET in three solvents across the PDMS membrane.

Transmembrane permeation of both DEET and oxybenzone in combination increased significantly in ethanol compared to their individual single counterparts. The increment in permeability of DEET and oxybenzone was 207% and 154% respectively. There was no change in permeation of DEET and oxybenzone in propylene glycol, with a slight increment of 107% for DEET and 105% for oxybenzone respectively. The increment of permeability of DEET and oxybenzone in PEG was 124% and 254%, respectively, which showed a pattern of increment between the other two solvents. Permeation characteristics of DEET and oxybenzone in ethanol were statistically different from those in PEG and propylene glycol. Between PEG and propylene glycol, permeation profiles of oxybenzone were statistically different, but permeation profiles of DEET were very similar.

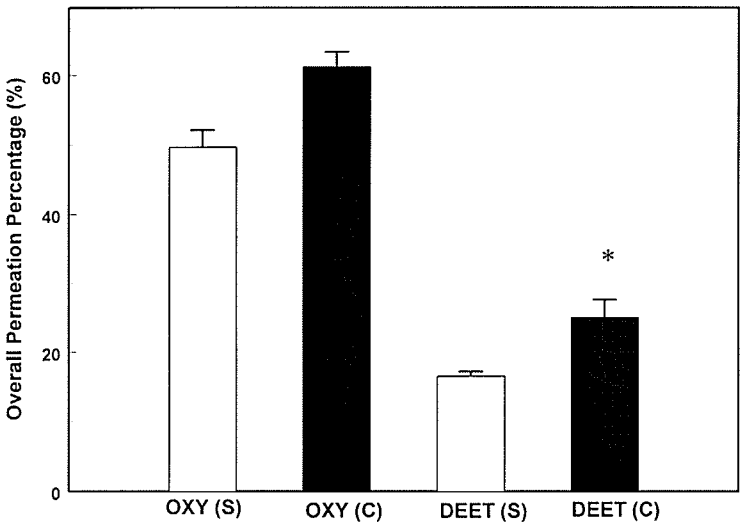


Figure 4.1. DEET and OXY (1%) in Ethanol at 37 °C through PDMS

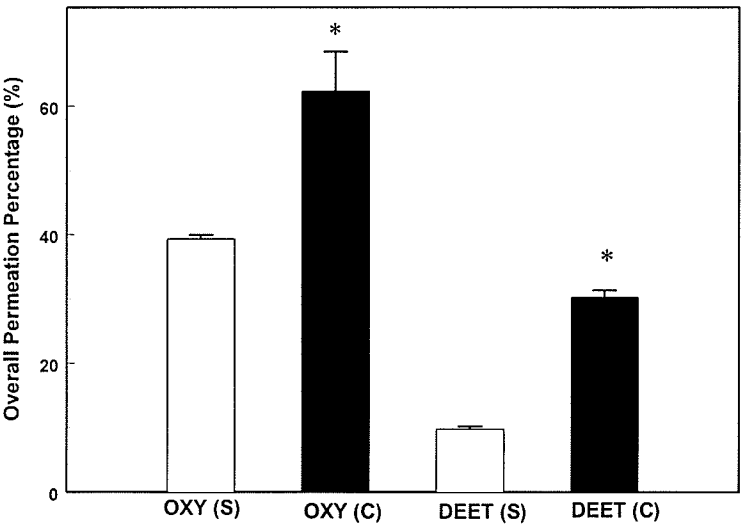


Figure 4.2. DEET and OXY (2.5%) in Ethanol at 37 °C through PDMS

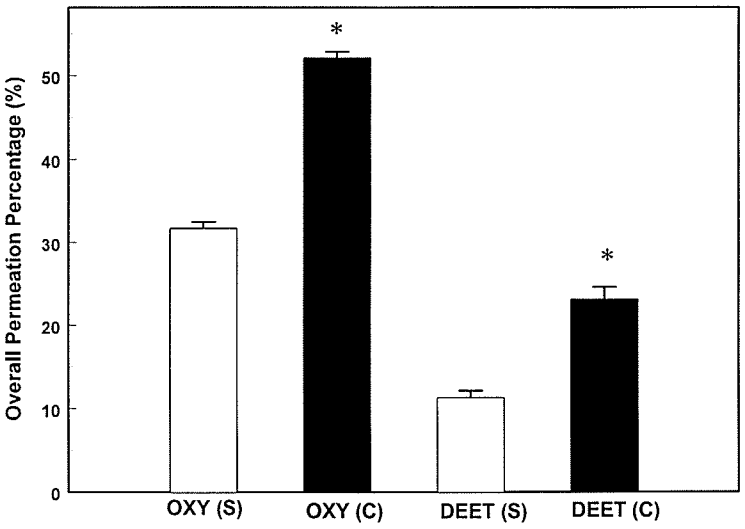


Figure 4.3. DEET and OXY (5%) in Ethanol at 37 °C through PDMS

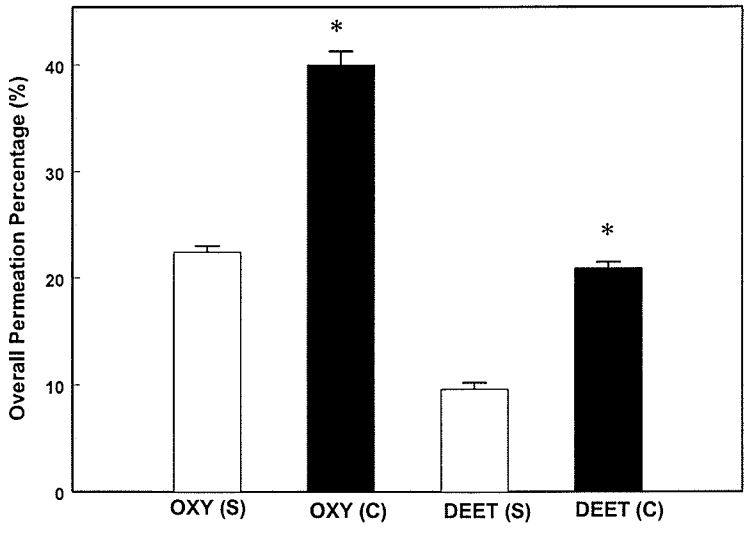


Figure 4.4. DEET and OXY (10%) in Ethanol at 37 °C through PDMS

Table 4.1. Penetration of OXY in ethanol through PDMS at 37 °C (μg/cm²)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	431.6	995.5	1428.6	2209.8	591.3	1560.9	2683.3	3625.5
2	500.3	953.2	1613.4	2036.9	538.9	1019.8	2514.5	3785.3
3	479.6	1031.4	1641.2	2396.6	638.9	1487.5	2504.8	4138.6
4	584.3	944.0	1619.5	2319.7	629.8	1788.9	2636.8	4173.9
5	491.0	990.9	1614.1	2244.3	662.9	1925.4	2694.9	4278.4
Mean	497.4	983.0	1583.3	2241.5	612.3	1556.5	2606.9	4000.3
SEM	24.8	15.8	39.0	60.4	21.7	155.4	40.9	125.2

Table 4.2. Penetration of DEET in ethanol through PDMS at 37 °C (μg/cm²)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	166.0	227.8	492.7	787.8	249.8	773.5	1041.4	1897.0
2	185.4	223.8	535.6	1064.3	176.9	753.4	933.9	2055.8
3	170.2	277.5	589.6	1022.0	217.1	656.7	1187.2	2089.4
4	160.5	254.2	704.8	1064.0	271.9	764.7	1263.8	2205.6
5	143.0	241.0	498.8	839.7	333.2	825.3	1358.9	2191.2
Mean	165.0	244.9	564.3	955.6	249.8	754.7	1157.0	2087.8
SEM	6.9	9.7	39.1	59.0	26.3	27.4	76.2	55.7

Table 4.3. Steady state flux of OXY in ethanol through PDMS at 37 °C (μg/cm² h)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	65.4	150.8	216.4	334.8	89.6	236.5	406.6	549.3
2	75.8	144.4	244.4	308.6	81.6	154.5	381.0	573.5
3	72.7	156.3	248.7	363.1	96.8	225.4	379.5	627.1
4	88.5	143.0	245.4	351.5	95.4	271.0	399.5	632.4
5	74.4	150.1	244.6	340.1	100.4	291.7	408.3	648.2
Mean	75.4	148.9	239.9	339.6	92.8	253.8	395.0	606.1
SEM	3.8	2.4	5.9	9.2	3.3	3.2	6.2	19.0

Table 4.4. Steady state flux of DEET in ethanol through PDMS at 37 °C (μg/cm² h)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	25.2	34.5	74.7	119.4	37.9	117.2	157.8	287.4
2	28.1	33.9	81.2	161.3	26.8	114.1	141.5	311.5
3	25.8	42.0	89.3	154.9	32.9	99.5	179.9	316.6
4	24.3	38.5	106.8	161.2	41.2	115.9	191.5	334.2
5	21.7	36.5	75.6	127.2	50.5	125.0	205.9	332.0
Mean	25.0	37.1	85.5	144.8	37.8	175.3	175.3	316.3
SEM	1.0	1.5	5.9	8.9	4.0	11.6	11.6	8.4

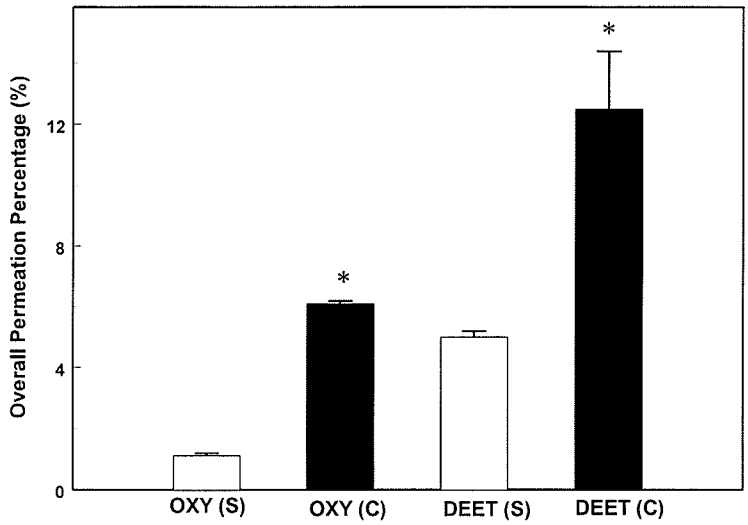


Figure 4.5. DEET and OXY (1 %) in PEG at 37 °C through PDMS

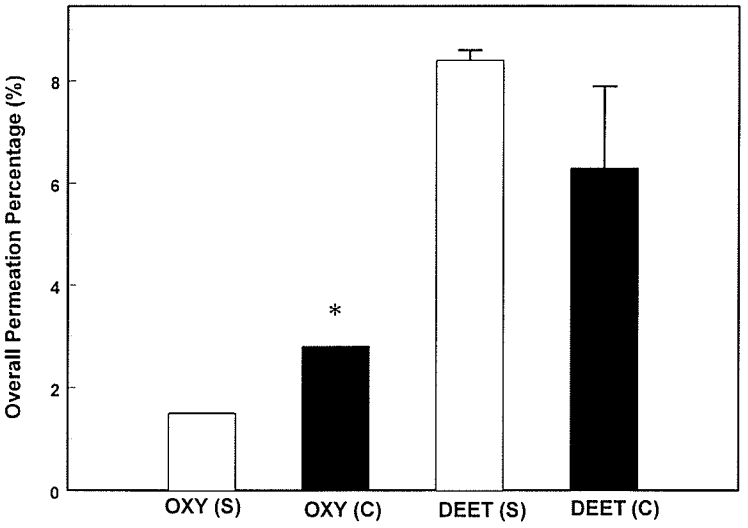


Figure 4.6. DEET and OXY (2.5%) in PEG at 37 °C through PDMS

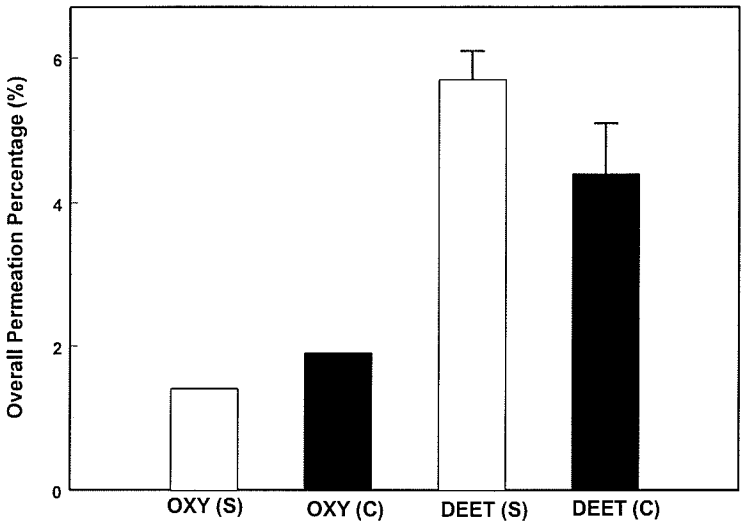


Figure 4.7. DEET and OXY (5%) in PEG at 37 °C through PDMS

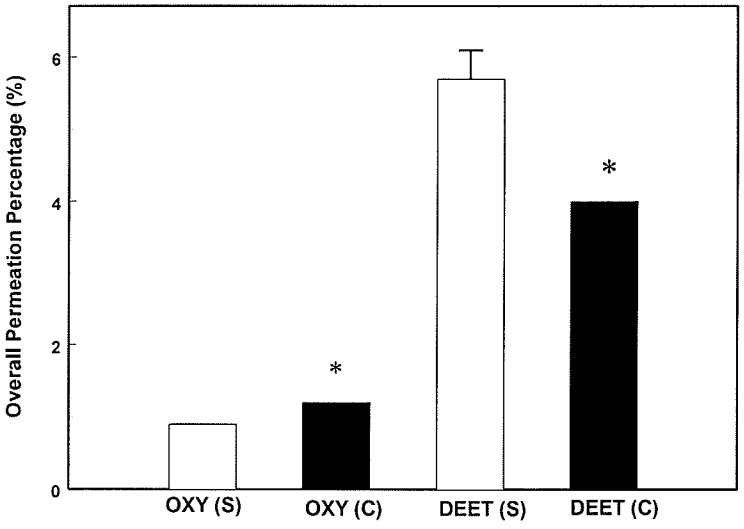


Figure 4.8. DEET and OXY (10%) in PEG at 37 °C through PDMS

Table 4.5. Penetration of OXY in PEG through PDMS at 37 °C (μg/cm²)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	8.6	34.4	67.9	92.2	60.0	67.9	99.1	119.0
2	9.5	38.1	71.1	89.9	61.0	70.3	95.6	112.5
3	10.6	37.4	68.8	92.7	63.4	68.3	87.2	113.6
4	11.6	40.1	67.2	95.6	61.1	70.8	96.0	132.9
5	14.4	40.2	73.4	96.0	61.7	67.5	87.3	119.0
Mean	11.0	38.0	69.7	93.3	61.4	69.0	93.0	119.4
SEM	1.0	1.1	1.1	1.1	0.6	0.7	2.4	3.6

Table 4.6. Penetration of DEET in PEG through PDMS at 37 °C (μg/cm²)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	52.4	198.0	372.8	623.9	194.0	70.0	347.7	382.9
2	44.9	223.8	406.8	537.8	94.8	45.4	182.6	401.8
3	44.5	214.7	474.0	688.3	96.9	232.1	153.7	401.9
4	54.7	211.2	360.6	490.7	100.4	236.9	193.8	398.8
5	54.2	196.9	345.1	505.8	141.1	199.5	214.4	396.2
Mean	50.1	208.9	391.9	569.3	125.4	156.8	218.5	396.3
SEM	2.3	5.1	22.9	37.6	19.1	41.1	33.8	3.5

Table 4.7. Steady state flux of OXY in PEG through PDMS at 37 °C (μg/cm² h)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	1.3	5.2	10.3	14.0	9.1	10.3	15.0	18.0
2	1.4	5.8	10.8	13.6	9.2	10.7	14.5	17.0
3	1.6	5.7	10.4	14.5	9.6	10.3	13.2	17.2
4	1.8	6.1	10.2	14.5	9.3	10.7	14.6	20.1
5	2.2	6.1	11.1	14.0	9.3	10.2	13.2	18.0
Mean	1.7	5.8	10.6	14.1	9.3	10.4	14.1	18.1
SEM	0.2	0.2	0.2	0.2	0.1	0.1	0.4	0.6

Table 4.8. Steady state flux of DEET in PEG through PDMS at 37 °C (μg/cm² h)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	7.9	30.0	56.5	94.5	29.4	10.6	52.7	58.0
2	6.8	33.9	61.6	81.5	14.4	6.9	27.7	60.9
3	6.7	32.5	71.8	104.3	14.7	35.2	23.3	60.9
4	8.3	32.0	54.6	74.3	15.2	35.9	29.4	60.4
5	8.2	29.8	52.3	76.6	21.4	30.2	32.5	60.0
Mean	7.6	31.7	59.4	86.3	19.0	23.8	33.1	60.0
SEM	0.3	0.8	52.3	5.7	2.9	6.2	5.1	0.5

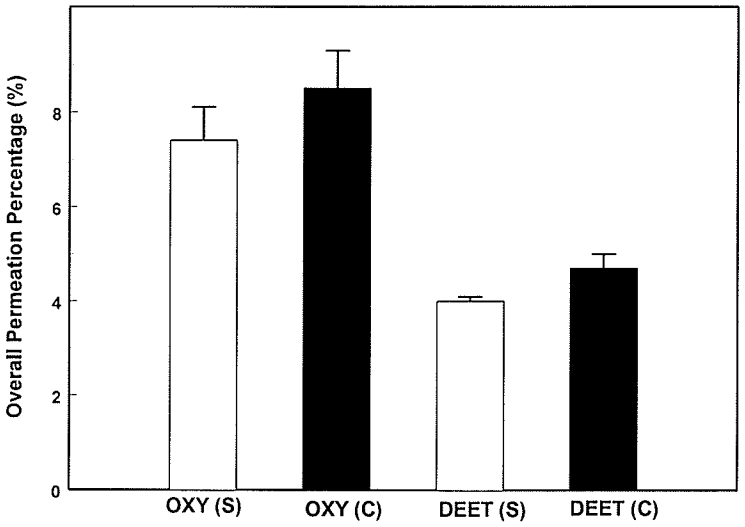


Figure 4.9. DEET and OXY (1%) in PG at 37 °C through PDMS

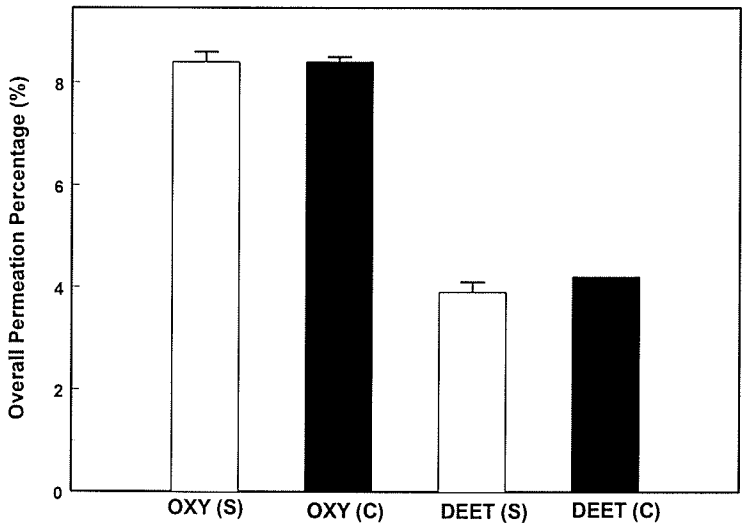


Figure 4.10. DEET and OXY (2.5%) in PG at 37 °C through PDMS

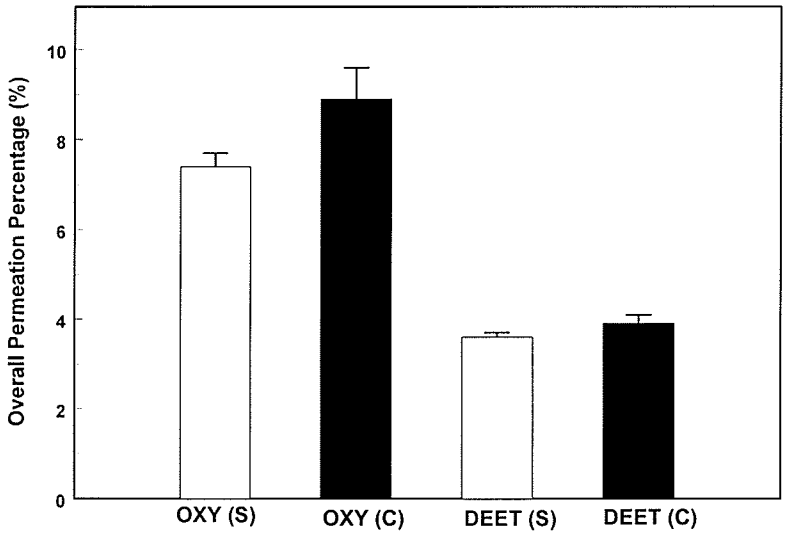


Figure 4.11. DEET and OXY (5%) in PG at 37 °C through PDMS

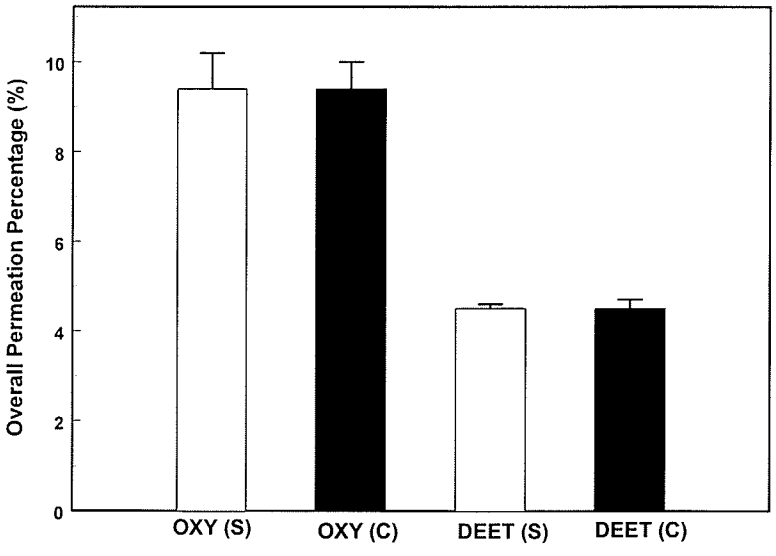


Figure 4.12. DEET and OXY (10%) in PG at 37 °C through PDMS

Table 4.9. Penetration of OXY in PG through PDMS at 37 °C (μg/cm²)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	78.5	217.8	391.5	954.2	99.5	221.1	327.8	739.2
2	52.5	196.9	395.2	880.3	100.5	217.3	419.0	901.5
3	68.4	214.5	407.8	692.3	57.5	202.8	529.9	932.6
4	76.0	221.1	323.7	950.4	77.0	205.6	488.7	1103.9
5	94.0	201.9	339.6	1220.9	91.4	205.5	465.1	1039.4
Mean	73.9	210.5	371.5	939.6	85.2	210.4	446.1	943.3
SEM	6.8	4.7	16.7	84.9	8.1	3.6	34.6	62.7

Table 4.10. Penetration of DEET in PG through PDMS at 37 °C (μg/cm²)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	38.0	101.2	188.1	449.0	50.5	106.1	164.7	377.0
2	35.2	110.6	173.3	471.6	50.4	102.7	188.2	425.5
3	42.5	100.3	166.9	483.5	37.3	105.2	223.0	437.2
4	42.4	81.6	174.0	451.0	45.1	103.5	196.9	500.6
5	41.2	97.2	185.5	415.0	49.3	108.3	199.7	489.8
Mean	39.8	98.2	177.6	454.0	46.5	105.2	194.5	446.0
SEM	1.4	4.7	4.0	11.7	2.5	1.0	9.4	22.5

Table 4.11. Steady state flux of OXY in PG through PDMS at 37 °C (μg/cm² h)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	11.9	33.0	59.3	144.6	15.1	33.5	49.7	112.0
2	7.9	29.8	59.9	133.4	15.2	32.9	63.5	136.6
3	10.4	32.5	61.8	104.9	8.7	30.7	80.3	141.3
4	11.5	33.5	49.0	144.0	11.7	31.1	74.0	167.3
5	14.2	30.6	51.4	185.0	13.8	31.1	70.5	157.5
Mean	11.2	31.9	56.3	142.4	12.9	31.9	67.6	142.9
SEM	1.0	0.7	2.5	12.9	1.2	0.6	5.2	9.5

Table 4.12. Steady state flux of DEET in PG through PDMS at 37 °C (μg/cm² h)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	5.8	15.3	28.5	68.0	7.7	16.1	24.9	57.1
2	5.3	16.8	26.3	71.5	7.6	15.6	28.5	64.5
3	6.4	15.2	25.3	73.3	5.7	15.9	33.8	66.2
4	6.4	12.4	26.4	68.3	6.8	15.7	29.8	75.8
5	6.2	14.7	28.1	62.9	7.5	16.4	30.3	74.2
Mean	6.0	14.9	26.9	68.8	7.1	15.9	29.5	67.6
SEM	0.2	0.7	0.6	1.8	0.4	0.2	1.4	3.4

The effect of temperature on *in vitro* membrane permeation was examined at 45 °C under identical experimental conditions of 37 °C. The selection of a higher experimental temperature was to simulate percutaneous permeation of the compounds in real-life applications. Sunscreen preparations are generally used under elevated body temperatures associated with outdoor activities, where sunlight and UV radiation can contribute to additional temperature increase. Insect repellents may be applied to elevated body temperatures as well when they are concurrently used in combination with the sunscreens. Temperature increase facilitates thermal activity of a diffusant, subsequently resulting in enhancement in both percutaneous diffusion and penetration. In addition, blood circulation is faster at higher temperatures, transferring more absorbed drug from the absorption site in the skin *in vivo*. It was found from the experiments that a statistically significant increase in transmembrane permeation of DEET and oxybenzone was observed with the increase in temperature. *Figures 4.13-4.24* show the overall permeation percentage of DEET and oxybenzone after 6 hours in the three solvents across the three membranes. *Tables 4.13-4.24* show the overall permeation amount and the steady state flux of oxybenzone and DEET across the PDMS membrane at 45 °C in three solvents of ethanol, PEG and PG.

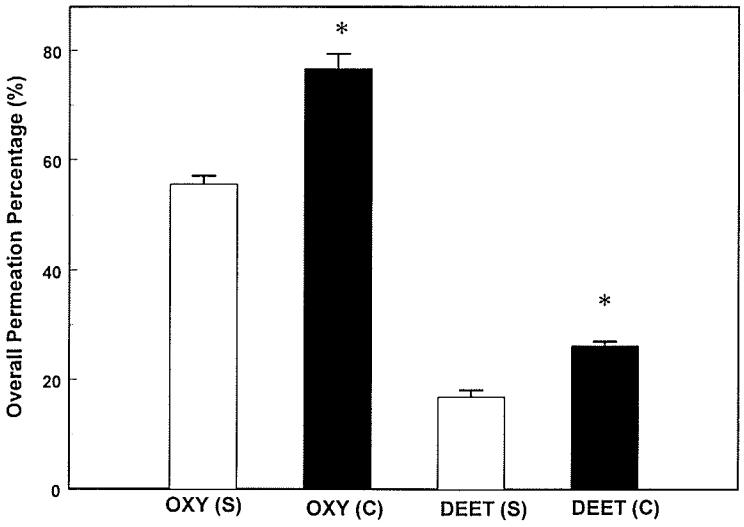


Figure 4.13. DEET and OXY (1%) in Ethanol at 45 °C through PDMS

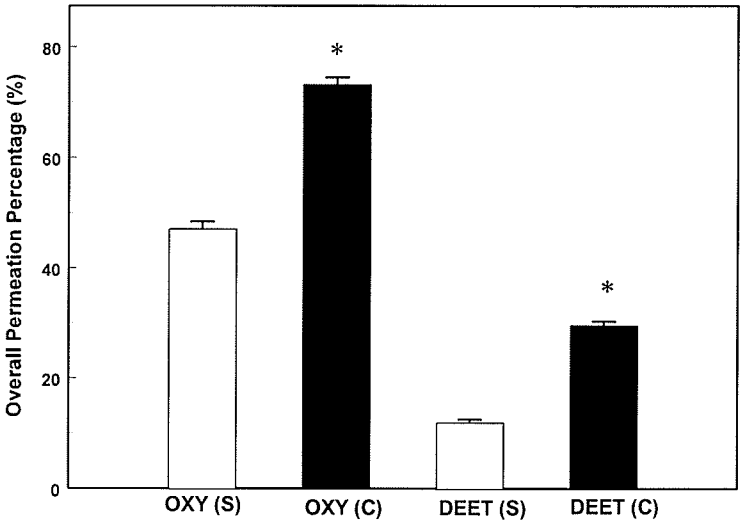


Figure 4.14. DEET and OXY (2.5%) in Ethanol at 45 °C through PDMS

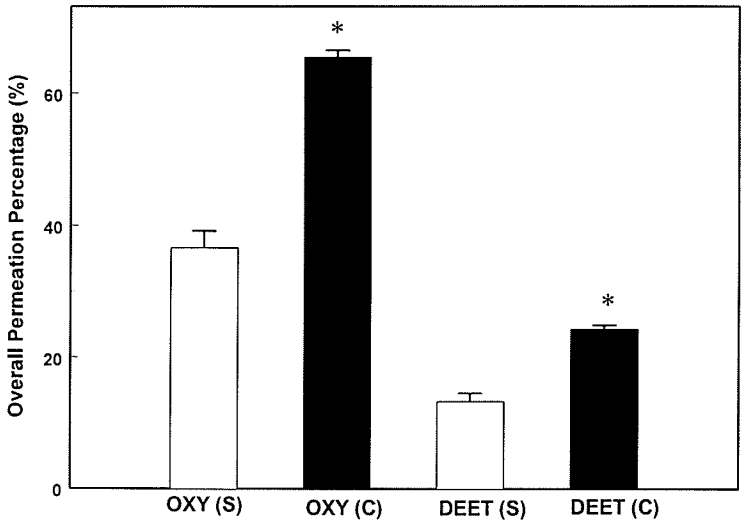


Figure 4.15. DEET and OXY (5%) in Ethanol at 45 °C through PDMS

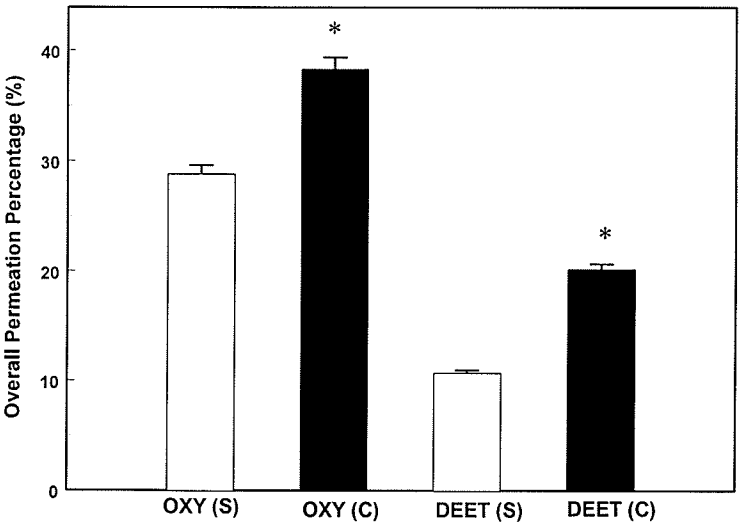


Figure 4.16. DEET and OXY (10%) in Ethanol at 45 °C through PDMS

Table 4.13. Penetration of OXY in Ethanol through PDMS at 45 °C ($\mu g/cm^2$)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	516.8	1044.6	1394.6	2856.9	689.8	1692.7	3101.1	3432.2
2	597.8	1194.0	1648.6	2583.3	717.9	1855.2	3212.2	3801.5
3	527.2	1188.6	2045.3	2918.1	799.3	1860.4	3416.4	4083.1
4	580.1	1198.9	2009.9	3027.4	842.0	1850.3	3297.5	3967.4
5	559.3	1255.0	2052.4	3014.6	783.4	1883.4	3349.4	3855.2
Mean	556.2	1176.2	1830.2	2880.1	766.5	1828.4	3275.3	3827.9
SEM	15.3	35.0	132.4	80.6	27.7	34.4	54.9	110.1

Table 4.14. Penetration of DEET in Ethanol through PDMS at 45 °C ($\mu g/cm^2$)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	203.3	241.0	777.3	968.4	245.5	718.2	1159.5	1930.7
2	175.0	312.3	775.6	1069.5	240.7	755.2	1204.0	1949.3
3	130.6	303.4	680.4	1099.8	285.5	700.1	1322.9	2200.3
4	170.5	325.4	649.3	1154.6	278.6	708.5	1245.1	1996.0
5	163.3	306.3	433.3	1076.3	257.9	812.7	1180.3	2006.5
Mean	168.5	297.7	663.2	1073.7	261.6	738.9	1222.4	2016.5
SEM	11.7	14.7	62.8	30.3	8.9	20.7	28.9	48.0

Table 4.15. Steady state flux of OXY in Ethanol through PDMS at 45 °C (μg/cm²)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	78.3	158.3	211.3	432.9	104.5	256.5	469.9	520.0
2	90.6	180.9	249.8	391.4	108.8	281.1	486.7	576.0
3	79.9	180.1	309.9	442.1	121.1	281.9	517.6	618.7
4	87.9	181.7	304.5	458.7	127.6	280.4	499.6	601.1
5	84.7	190.1	311.0	456.8	118.7	285.4	507.5	584.1
Mean	84.3	178.2	277.3	436.4	116.1	277.0	496.3	580.0
SEM	2.3	5.3	20.1	12.2	4.2	5.2	8.3	16.7

Table 4.16. Steady state flux of DEET in Ethanol through PDMS at 45 °C (μg/cm² h)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	30.8	36.5	117.8	146.7	37.2	108.8	175.7	292.5
2	26.5	47.3	117.5	162.1	36.5	114.4	182.4	295.4
3	19.8	46.0	103.1	166.6	43.3	106.1	200.4	333.4
4	25.8	49.3	98.4	174.9	42.2	107.3	188.7	302.4
5	24.7	46.4	65.7	163.1	39.1	123.1	178.8	304.0
Mean	25.5	45.1	100.5	162.7	39.6	112.0	185.2	305.5
SEM	1.8	2.2	9.5	4.6	1.3	3.1	4.4	7.3

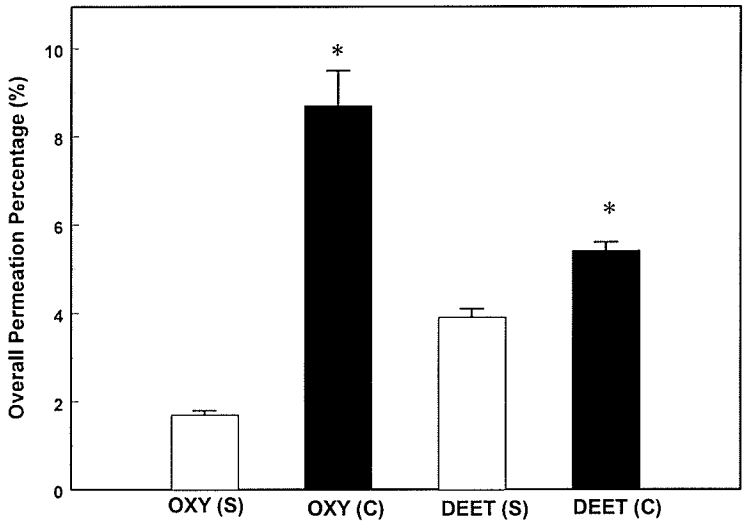


Figure 4.17. DEET and OXY (1%) in PEG at 45 °C through PDMS

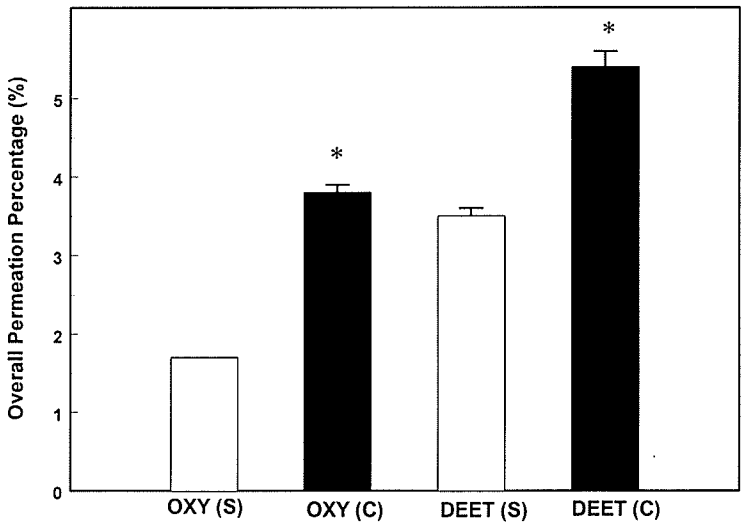


Figure 4.18. DEET and OXY (2.5%) in PEG at 45 °C through PDMS

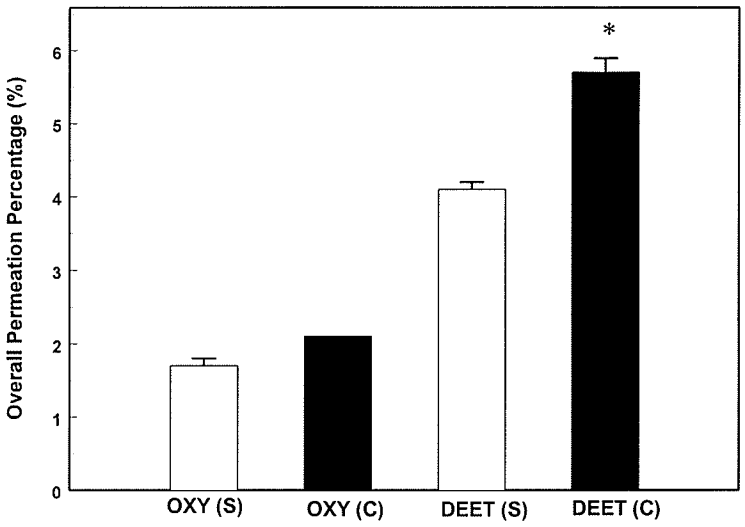


Figure 4.19. DEET and OXY (5%) in PEG at 45 °C through PDMS

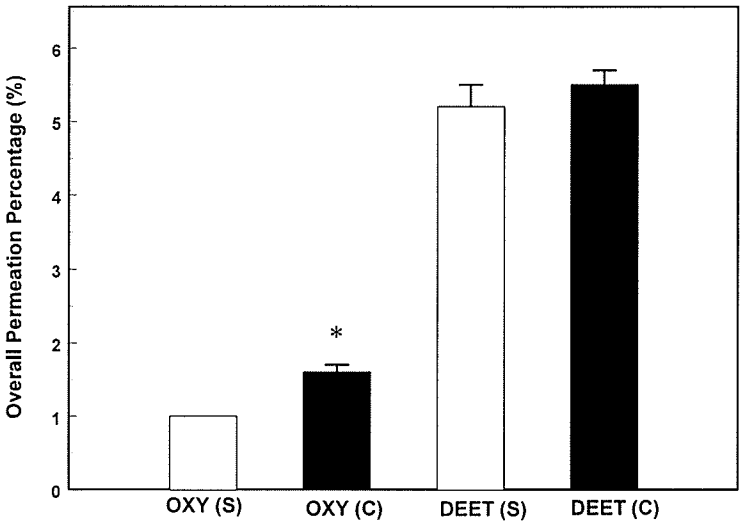


Figure 4.20. DEET and OXY (10%) in PEG at 45 °C through PDMS

Table 4.17. Penetration of OXY in PEG through PDMS at 45 °C (μg/cm²)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	18.9	41.9	77.0	104.5	59.6	93.8	104.6	141.8
2	12.9	38.1	99.2	105.4	82.8	100.4	109.5	173.1
3	17.5	41.1	81.9	98.4	97.7	84.0	108.4	175.0
4	18.0	44.1	81.6	104.1	98.1	92.4	102.8	165.8
5	18.3	43.7	82.2	102.8	98.1	102.6	111.1	162.7
Mean	17.1	41.7	84.4	103.0	87.3	94.6	107.3	163.7
SEM	1.1	1.1	3.8	1.2	7.5	3.3	1.6	5.9

Figure 4.18. Penetration of DEET in PEG through PDMS at 45 °C (μg/cm²)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	43.2	76.5	197.2	460.7	48.0	130.0	258.0	499.8
2	42.5	95.1	220.0	427.4	53.3	124.4	269.0	594.3
3	34.5	93.0	201.8	616.7	58.1	132.3	294.2	555.8
4	38.4	82.5	201.5	563.9	57.0	136.8	301.5	549.3
5	34.8	85.0	198.6	549.1	54.7	149.9	295.2	535.9
Mean	38.7	86.4	203.8	523.5	54.2	134.7	283.6	547.0
SEM	1.8	3.4	4.1	34.7	1.8	4.3	8.5	15.3

Table 4.19. Steady state flux of OXY in PEG through PDMS at 45 °C (μg/cm² h)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	2.9	6.3	11.7	15.8	9.0	14.2	15.8	21.5
2	2.0	5.8	15.0	16.0	12.5	15.2	16.6	26.2
3	2.7	6.2	12.4	14.9	14.8	12.7	16.4	26.5
4	2.7	6.7	12.4	15.8	14.9	14.0	15.6	25.1
5	2.8	6.6	12.4	15.6	14.9	15.5	16.8	24.6
Mean	2.6	6.3	12.8	15.6	13.2	14.3	16.3	24.8
SEM	0.2	0.2	0.6	0.2	1.1	0.5	0.2	0.9

Table 4.20. Steady state flux of DEET in PEG through PDMS at 45 °C (μg/cm² h)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	6.5	11.6	29.9	69.8	7.3	19.7	39.1	75.7
2	6.4	14.4	33.3	64.8	8.1	18.8	40.8	90.0
3	5.2	14.1	30.6	93.4	8.8	20.1	44.6	84.2
4	5.8	12.5	30.5	85.4	8.6	20.7	45.7	83.2
5	5.3	12.9	30.1	83.2	8.3	22.7	44.7	81.2
Mean	5.9	13.1	30.9	79.3	8.2	20.4	43.0	82.9
SEM	0.3	0.5	0.6	5.3	0.3	0.7	4.03	2.3

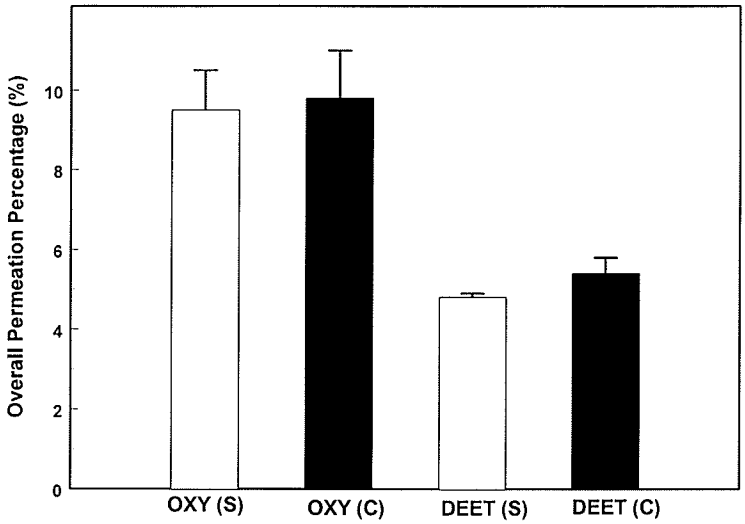


Figure 4.21. DEET and OXY (1%) in PG at 45 °C through PDMS

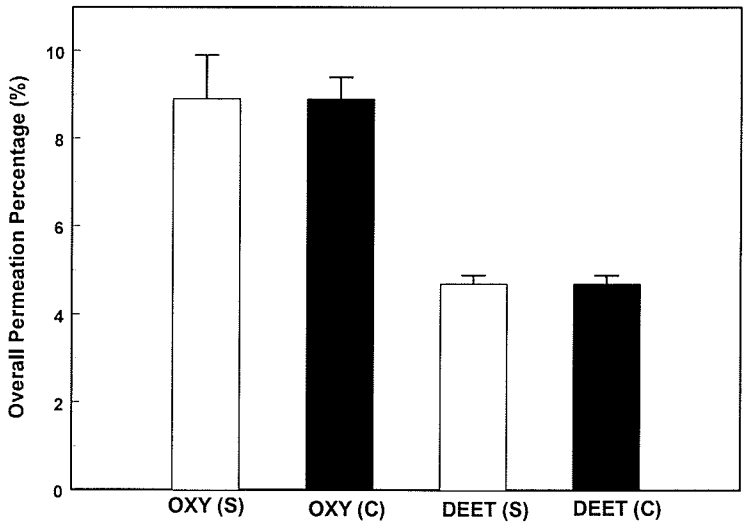


Figure 4.22. DEET and OXY (2.5%) in PG at 45 °C through PDMS

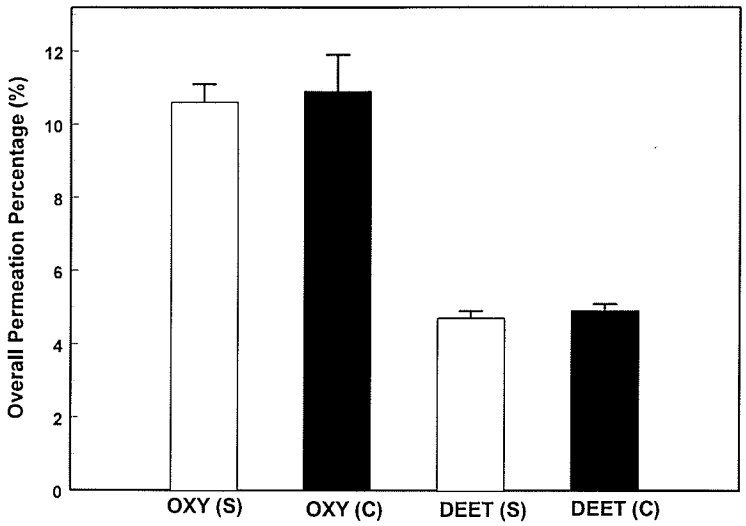


Figure 4.23. DEET and OXY (5%) in PG at 45 °C through PDMS

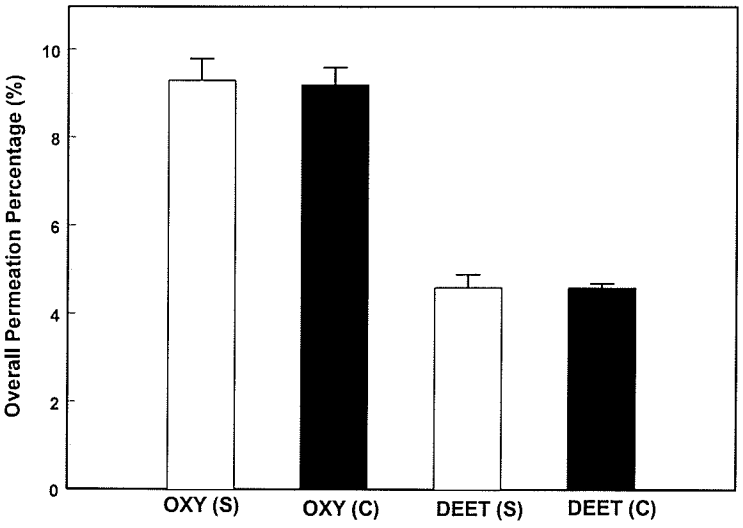


Figure 4.24. DEET and OXY (10%) in PG at 45 °C through PDMS

Table 4.21. Penetration of OXY in PG through PDMS at 45 °C (μg/cm²)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	74.8	306.6	575.1	875.4	80.6	172.2	478.6	924.5
2	83.7	176.3	599.4	911.1	147.7	229.9	412.5	826.2
3	95.8	236.6	470.4	961.3	86.1	246.8	504.7	847.4
4	88.8	174.9	471.2	790.6	87.9	231.6	643.6	1044.5
5	130.4	213.0	522.3	1109.8	90.1	225.8	679.9	947.6
Mean	94.7	221.5	527.7	929.7	98.5	221.3	543.9	918.0
SEM	9.6	24.3	26.4	53.0	12.4	12.8	50.7	38.9

Table 4.22. Penetration of DEET in PG through PDMS at 45 °C (μg/cm²)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	45.8	105.9	224.4	490.1	48.4	99.5	235.4	485.0
2	50.1	123.9	210.5	470.9	67.6	120.4	217.7	473.1
3	48.5	134.7	233.0	514.3	49.3	128.8	244.7	418.5
4	45.8	106.2	256.8	364.0	51.4	121.6	283.2	473.7
5	48.2	113.2	243.2	472.8	51.9	119.0	247.4	445.1
Mean	47.7	116.8	233.6	462.4	53.7	117.8	245.7	459.1
SEM	0.8	5.5	7.9	25.8	3.5	4.9	10.7	12.1

Table 4.23. Steady state flux of OXY in PG through PDMS at 45 °C (μg/cm² h)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	11.3	46.5	87.1	132.6	12.2	26.1	72.5	140.1
2	12.7	26.7	90.8	138.0	22.4	34.8	62.5	125.2
3	14.5	35.9	71.3	145.7	13.0	37.4	76.5	128.4
4	13.5	26.5	71.4	119.8	13.3	35.1	97.5	158.3
5	19.8	32.3	79.1	168.2	13.6	34.2	103.0	143.6
Mean	14.3	33.6	80.0	140.9	14.9	33.5	82.4	139.1
SEM	1.4	3.7	4.0	8.0	1.9	1.9	7.7	5.9

Table 4.24. Steady state flux of DEET in PG through PDMS at 45 °C (μg/cm² h)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	6.9	16.0	34.0	74.3	7.3	15.1	35.7	73.5
2	7.6	18.8	31.9	71.3	10.2	18.2	33.0	71.7
3	7.4	20.4	35.3	77.9	7.5	19.5	37.1	63.4
4	6.9	16.1	38.9	55.1	7.8	18.4	42.9	71.8
5	7.3	17.1	36.8	71.6	7.9	18.0	37.5	67.4
Mean	7.2	17.7	35.4	70.1	8.1	17.9	37.2	69.6
SEM	0.1	0.8	1.2	3.9	0.5	0.7	1.6	1.8

4.2.2. Permeation across Piglet Skin

Diffusion conditions using piglet skin were identical to those using PDMS membranes at 45 °C. Three vehicles were studied in the diffusion studies for their dissolving capacity and percutaneous characteristics. PEG and propylene glycol are commonly used in emulsion-based lotions as auxiliary agents. While most commercial sunscreen products are now labeled as "alcohol-free", ethanol and/or alcohol substitutes are still used in some insect repellent formulations either as solubilizing agents or for facilitating quick drying of the application through instant evaporation. *Figures 4.25-4.32* show the overall permeation percentage of DEET and oxybenzone after 6 hours in the three solvents. *Tables 4.25-4.36* show the overall permeation amount and the steady state flux of oxybenzone and DEET in three solvents across the piglet skin.

Synergistic permeation of both DEET and oxybenzone was observed when the compounds were present in combination. The average increment in permeability of DEET was 289% in propylene glycol, 243% in ethanol, and 112% in PEG, respectively. The average increment in permeability of oxybenzone was 139% in PEG, 120% in propylene glycol and 112% in ethanol, respectively. Compared to individual components, there were statistically significant increases in permeation of DEET in ethanol and propylene glycol when oxybenzone was simultaneously present. Permeation of both DEET and oxybenzone in ethanol and propylene glycol was also significantly higher than those in PEG. The impact of coexistence of DEET and oxybenzone in combination was more statistically significant on DEET than on oxybenzone. PEG appeared to be an inert vehicle that produced minimal percutaneous permeation of DEET and oxybenzone, whether or not the compounds were tested individually or in combination.

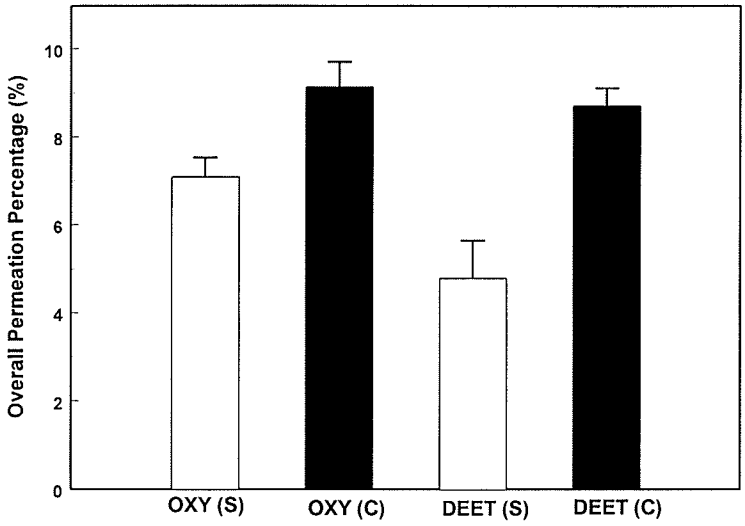


Figure 4.25. DEET and OXY (1%) in Ethanol at 45 °C through pigskin

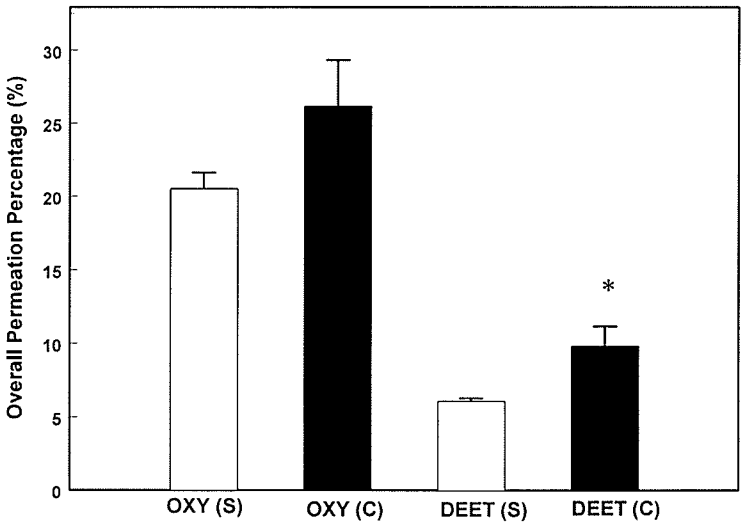


Figure 4.26. DEET and OXY (2.5%) in Ethanol at 45 °C through pigskin

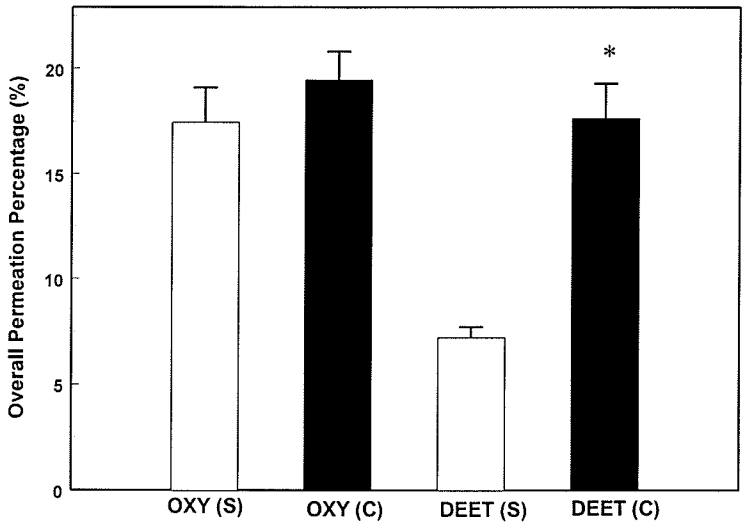


Figure 4.27. DEET and OXY (5%) in Ethanol at 45 °C through pigskin

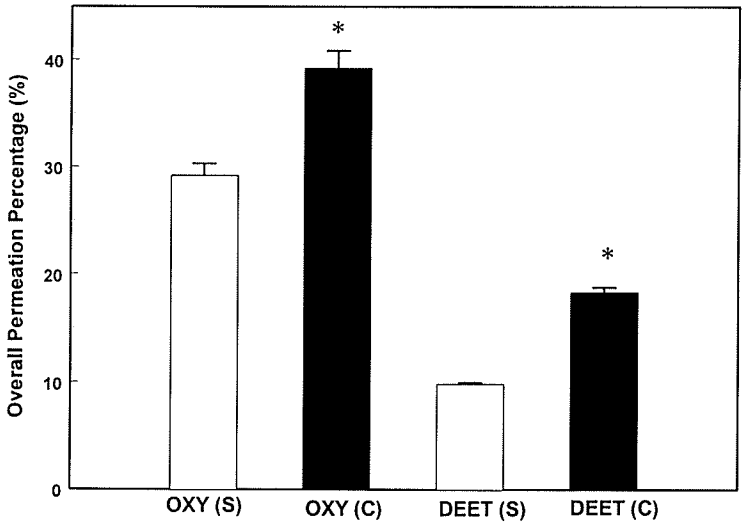


Figure 4.28. DEET and OXY (10%) in Ethanol at 45 °C through pigskin

Table 4.25. Penetration of OXY in Ethanol through pigskin at 45 °C ($\mu g/cm^2$)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	65.3	540.9	902.2	1598.3	84.7	768.4	1071.1	1875.4
2	73.8	474.5	774.5	1498.2	92.8	645.9	966.6	1982.4
3	75.1	523.9	968.6	1332.4	98.3	598.4	917.9	2183.9
4	69.3	512.4	845.3	1402.4	89.3	603.4	937.8	1799.3
Mean	70.9	512.9	872.6	1457.8	91.3	654.0	973.4	1960.2
SD	4.46	28.2	82.6	115.7	5.7	79.2	68.2	166.9

Table 4.26. Penetration of DEET in Ethanol through pigskin at 45 °C ($\mu g/cm^2$)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	50.3	156.4	398.5	476.3	87.9	218.0	540.9	768.4
2	39.8	154.8	347.1	483.4	82.5	292.3	474.5	645.9
3	58.9	152.4	341.7	510.4	92.3	247.3	523.9	598.4
4	42.3	144.3	356.3	492.3	85.4	223.4	512.4	603.4
Mean	47.8	152.0	360.9	490.6	87.0	245.2	881.5	914.2
SD	8.6	5.3	25.7	14.7	4.1	33.8	83.8	52.9

Table 4.27. Steady state flux of OXY in Ethanol through pigskin at 45 °C (μg/cm² h)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	15.3	62.3	75.3	93.4	31.2	72.3	87.3	120.0
2	25.6	58.2	78.2	98.7	22.3	85.2	94.5	127.3
3	20.2	65.9	63.2	94.3	29.3	88.4	88.3	116.4
4	18.3	66.3	84.2	101.3	27.9	79.4	96.9	118.3
Mean	19.8	63.1	75.2	96.9	27.6	81.3	91.7	120.5
SD	4.3	3.7	8.8	3.7	3.8	7.0	4.6	4.7

Table 4.28. Steady state flux of DEET in Ethanol through pigskin at 45 °C (μg/cm² h)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	13.2	56.5	64.2	89.7	24.3	43.2	59.2	128.3
2	11.7	57.3	67.8	83.9	29.7	48.7	61.2	112.0
3	19.8	46.0	72.3	92.3	34.2	52.2	55.3	118.3
4	17.4	49.3	79.3	91.2	36.9	57.2	53.0	116.5
Mean	15.5	52.2	70.9	89.2	31.2	50.3	57.1	118.7
SD	3.7	5.5	6.5	3.7	5.5	5.9	3.7	6.9

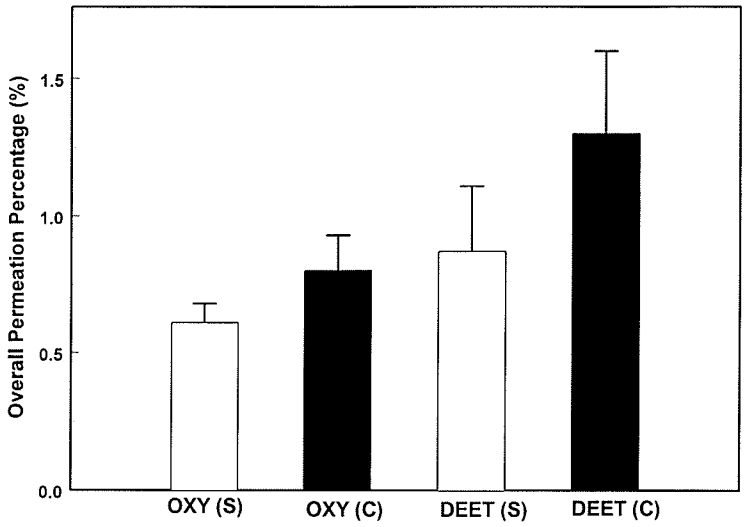


Figure 4.29. DEET and OXY (1%) in PEG at 45 °C through pigskin

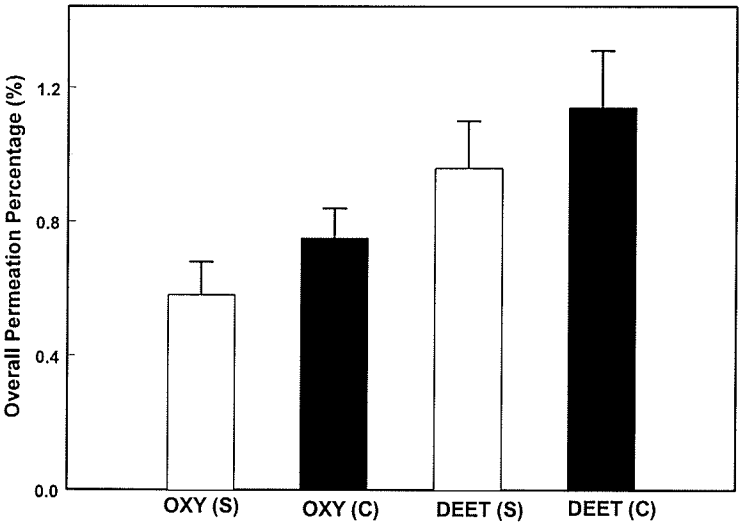


Figure 4.30. DEET and OXY (2.5%) in PEG at 45 °C through pigskin

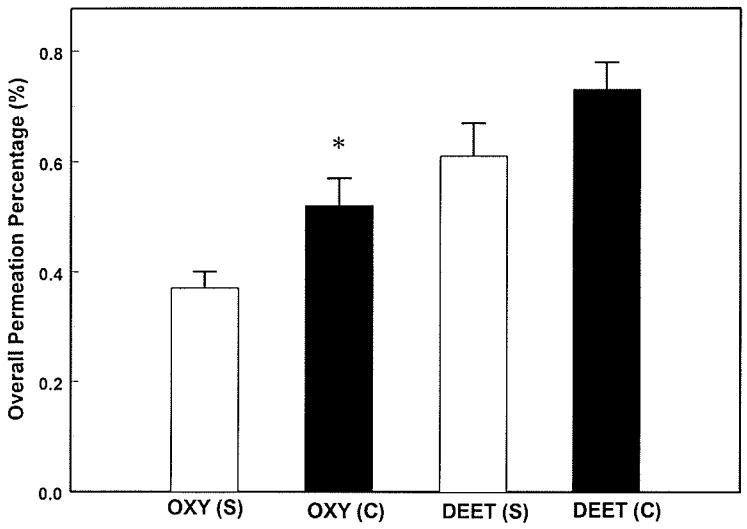


Figure 4.31. DEET and OXY (5%) in PEG at 45 °C through pigskin

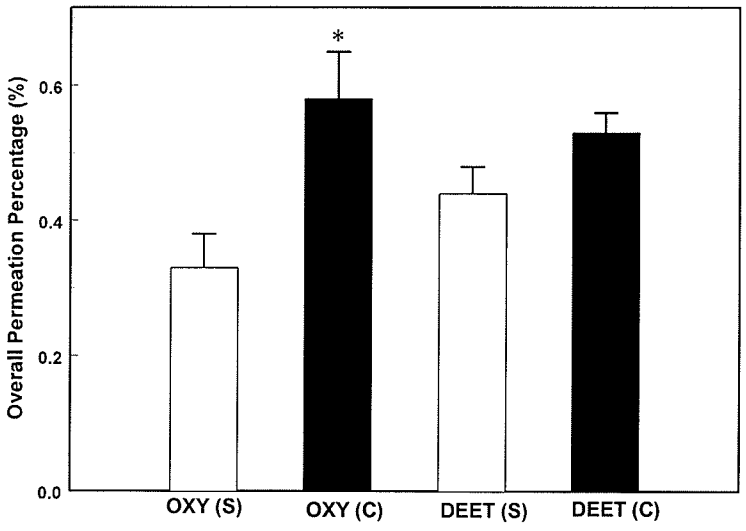


Figure 4.32. DEET and OXY (10%) in PEG at 45 °C through pigskin

Table 4.29. Penetration of OXY in PEG through pigskin at 45 °C ($\mu g/cm^2$)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	6.2	12.9	16.8	36.8	6.2	12.9	22.7	52.7
2	5.4	17.3	19.8	39.8	5.4	17.3	22.7	57.6
3	7.2	12.0	20.3	27.3	7.2	12.0	29.6	69.6
4	5.8	16.4	18.4	31.3	5.8	16.4	25.4	55.4
Mean	6.1	14.7	18.7	33.8	8.0	18.8	26.3	58.8
SD	0.7	2.5	1.5	5.5	1.3	2.4	2.9	7.4

Table 4.30. Penetration of DEET in PEG through pigskin at 45 °C ($\mu g/cm^2$)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	6.3	28.9	34.5	44.5	8.9	34.8	40.0	50.2
2	9.3	22.8	33.4	48.4	12.9	26.8	34.8	54.4
3	11.9	20.4	29.5	38.5	16.2	25.4	34.0	58.6
4	7.5	24.3	26.3	45.3	14.2	27.3	37.3	51.3
Mean	8.7	24.1	30.9	44.1	13.0	28.6	36.5	53.6
SD	2.4	3.5	3.7	4.1	3.0	4.2	2.7	3.7

Table 4.31. Steady state flux of OXY in PEG through pigskin at 45 °C (μg/cm² h)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	2.1	4.5	7.6	10.8	5.2	11.2	12.9	18.7
2	1.8	4.8	8.2	12.0	4.8	12.9	11.8	19.8
3	1.7	4.2	8.4	11.2	3.9	11.7	13.8	21.9
4	1.5	3.7	8.7	9.9	4.3	10.9	14.5	23.2
Mean	1.7	4.3	8.2	10.9	4.5	11.6	13.2	20.9
SD	0.2	0.4	0.5	0.8	0.5	0.8	1.1	2.0

Table 4.32. Steady state flux of DEET in PEG through pigskin at 45 °C (μg/cm² h)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	3.5	5.4	19.9	29.7	5.3	15.7	29.3	35.7
2	2.4	5.8	13.3	24.8	6.1	19.8	20.8	40.0
3	3.2	6.1	20.6	23.4	5.7	28.1	24.0	44.2
4	3.8	6.5	16.8	25.4	5.9	29.7	28.7	38.2
Mean	3.2	5.9	17.6	25.8	5.7	23.3	25.7	39.5
SD	0.6	0.4	3.3	2.7	0.3	6.6	3.1	3.5

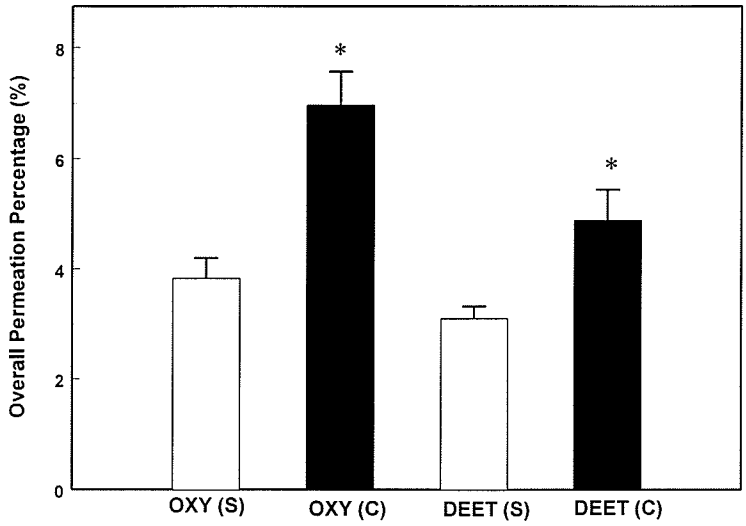


Figure 4.33. DEET and OXY (1%) in PG at 45 °C through pigskin

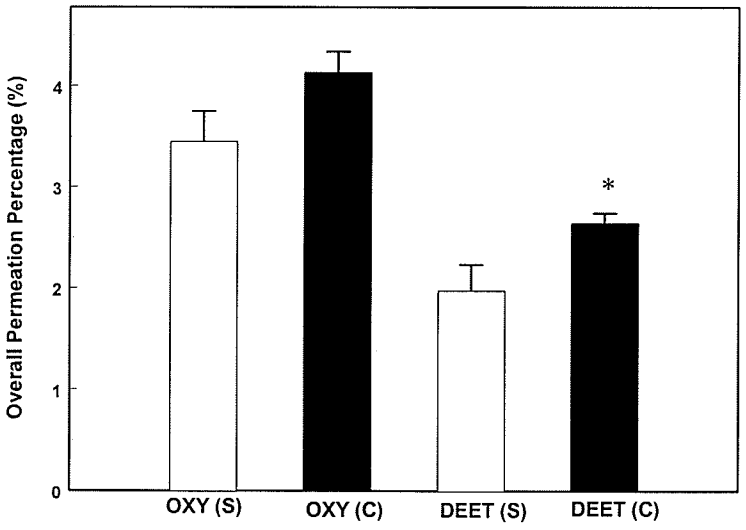


Figure 4.34. DEET and OXY (2.5%) in PG at 45 °C through pigskin

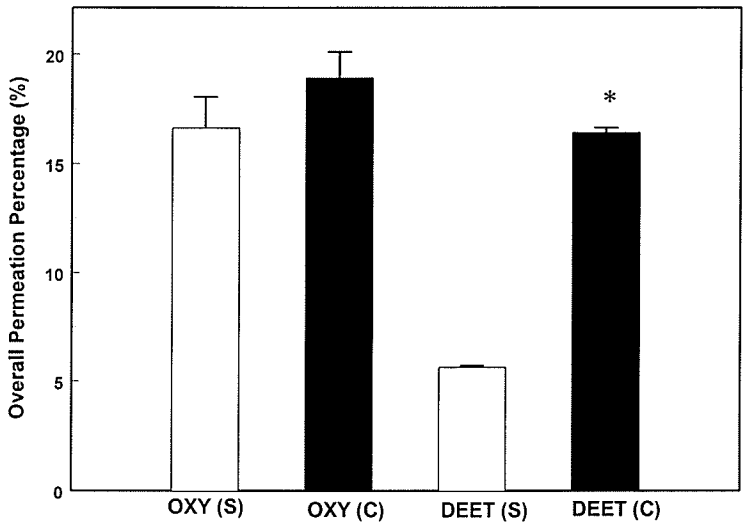


Figure 4.35. DEET and OXY (5%) in PG at 45 °C through pigskin

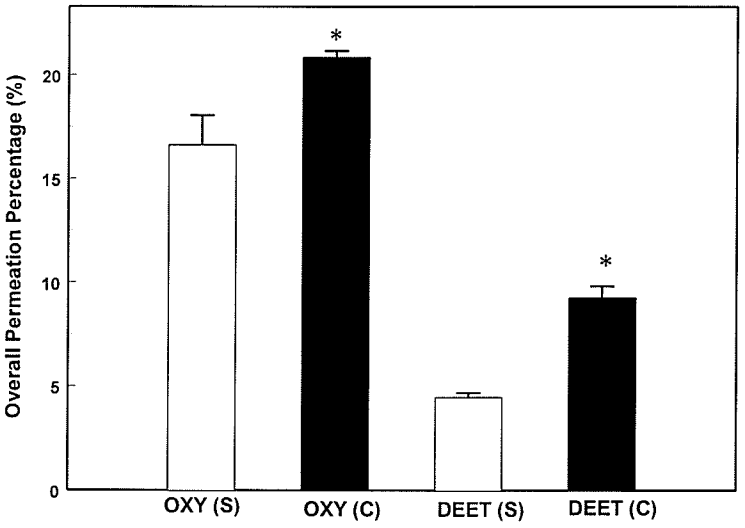


Figure 4.36. DEET and OXY (10%) in PG at 45 °C through pigskin

Table 4.33. Penetration of OXY in PG through pigskin at 45 °C ($\mu g/cm^2$)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	39.3	78.3	740.6	1634.4	62.3	98.2	1029.8	2094.3
2	42.9	87.3	912.1	1872.3	67.5	103.3	952.1	2113.9
3	36.4	96.3	745.6	1563.9	76.5	110.9	894.1	2093.4
4	34.4	83.2	835.5	1573.4	72.2	101.2	909.3	2036.3
Mean	38.3	86.2	808.4	1661.0	69.6	103.4	946.3	2084.5
SD	3.7	7.6	81.7	144.2	6.1	5.4	60.8	33.5

Table 4.34. Penetration of DEET in PG through pigskin at 45 °C ($\mu g/cm^2$)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	28.9	56.3	287.5	452.8	49.4	67.2	834.4	963.2
2	34.2	47.2	283.1	435.2	56.2	68.3	824.9	872.4
3	29.4	52.3	283.5	476.2	46.8	67.0	804.8	983.9
4	31.2	41.2	275.3	423.3	42.8	62.3	818.3	878.3
Mean	30.9	49.2	282.3	446.9	48.8	66.2	820.6	924.4
SD	2.3	6.5	5.0	23.0	5.6	2.6	12.4	57.3

Table 4.35. Steady state flux of OXY in PG through pigskin at 45 °C (μg/cm² h)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	4.3	6.5	28.9	52.9	8.2	16.5	42.5	90.1
2	4.7	6.7	32.4	58.4	12.3	14.8	76.7	105.8
3	4.5	6.9	33.7	65.9	9.7	17.4	69.5	118.7
4	4.8	6.8	38.6	69.0	10.2	15.8	77.5	127.0
Mean	4.5	6.7	33.4	61.5	10.1	16.1	66.5	110.4
SD	0.2	0.1	4.0	7.2	1.6	1.0	16.4	17.1

Table 4.36. Steady state flux of DEET in PG through pigskin at 45 °C (μg/cm² h)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	3.2	7.5	14.0	24.3	5.4	10.2	35.4	53.5
2	2.7	7.8	17.1	31.3	4.2	18.9	33.0	51.7
3	2.4	7.4	15.3	37.9	5.9	17.5	37.9	58.4
4	2.9	7.1	18.7	35.1	5.8	20.4	32.9	49.8
Mean	2.8	7.4	16.2	32.1	5.3	16.7	34.8	53.3
SD	0.3	0.2	2.0	5.8	0.7	4.5	2.3	3.6

4.2.3. Permeation through Human Skin

Diffusion conditions using human skin were identical to those using PDMS and piglet skin membranes at 45 °C. *Figures 4.37-4.48* show the overall permeation percentage of DEET and oxybenzone after 6 hours in the three solvents across the three membranes. *Tables 4.37-4.48* show the overall permeation amount and the steady state flux of oxybenzone and DEET in three solvents across the human skin.

The permeability results from the PDMS and piglet skin membrane in all the formulations were higher than those obtained from human skin. In comparison to the PDMS membrane and piglet skin, the values obtained in human skin were approximately 30% for oxybenzone and 90% for DEET in ethanol, 50% for oxybenzone and 40% for DEET in PEG and 50% for oxybenzone and 70% for DEET in propylene glycol, respectively. Although many artificial and animal skin models have been studied, human skin is still considered as the best membrane to study the permeation of drug candidates. The variation in human and animal skin can be attributed to the varying thickness and the lipid content in the membranes, on which the permeation depends, especially for the permeation enhancement behavior of compounds. Difference in permeation results between biological and artificial membranes may be attributed to variations in the thickness of the human skin, piglet skin and the PDMS membrane, and variable permeability of the test vehicles across the study membranes. Compared to lower concentrations there was a significant difference in the higher concentrations in all the formulations in all the three membranes.

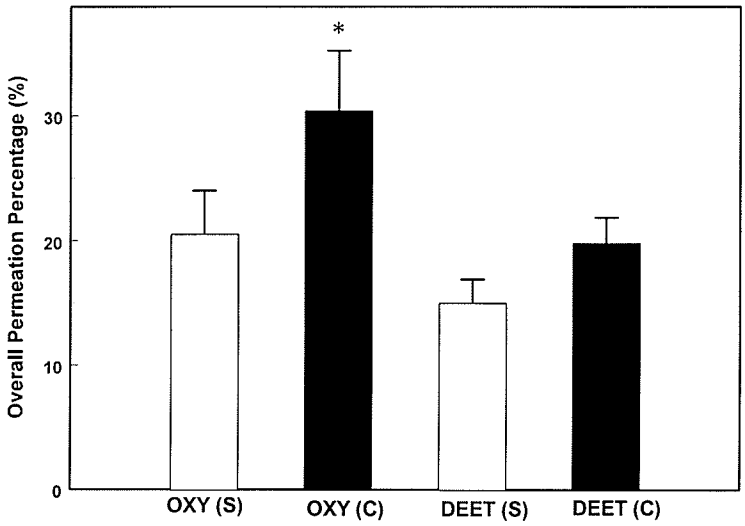


Figure 4.37. DEET and OXY (1%) in Ethanol at 45 °C through human skin

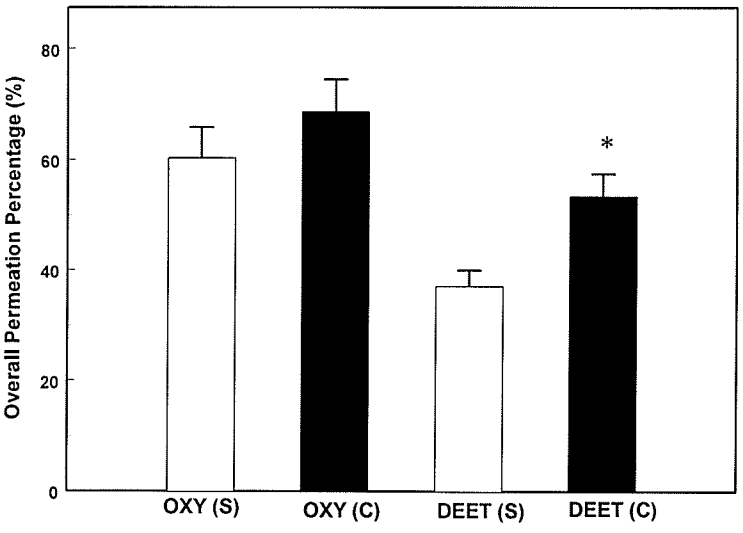


Figure 4.38. DEET and OXY (2.5%) in Ethanol at 45 °C through human skin

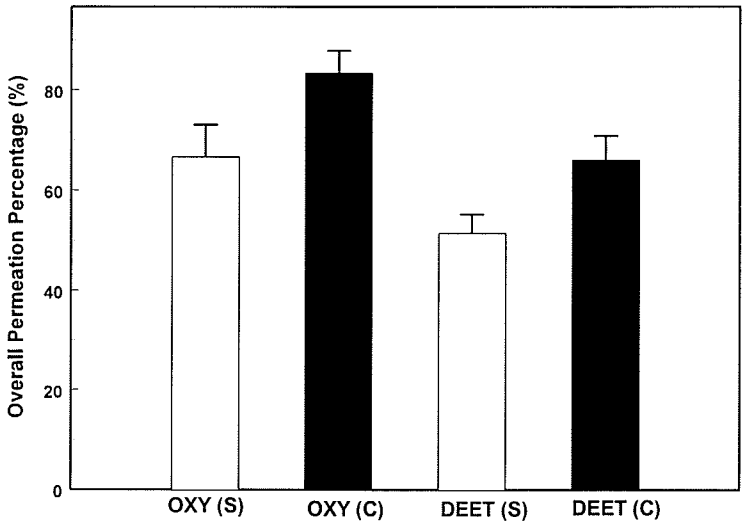


Figure 4.39. DEET and OXY (5%) in Ethanol at 45 °C through human skin

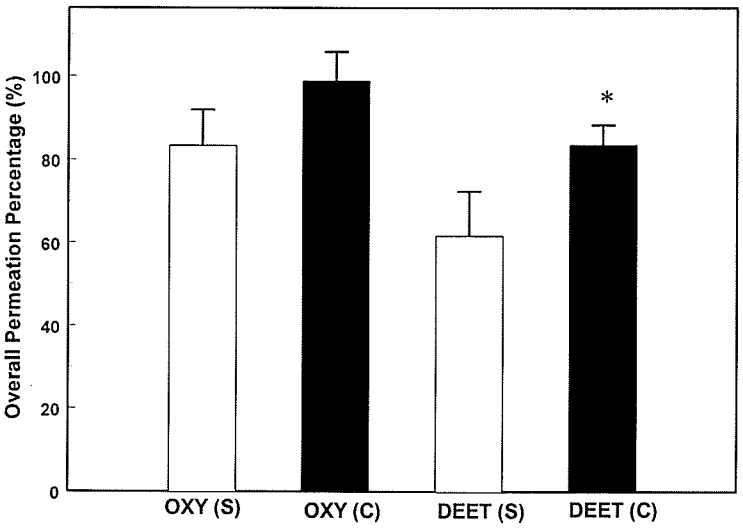


Figure 4.40. DEET and OXY (10%) in Ethanol at 45 °C through human skin

Table 4.37. Penetration of OXY in Ethanol through human skin at 45 °C (μg/cm²)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	18.9	52.4	67.3	87.4	32.3	65.4	89.3	98.3
2	17.3	62.3	62.4	92.3	36.3	75.4	78.3	102.4
3	20.3	65.3	75.4	81.3	27.3	71.2	82.3	105.3
4	25.4	61.3	61.3	72.3	25.6	62.3	83.4	88.9
Mean	20.5	60.3	66.6	83.3	25.6	68.6	83.3	98.7
SD	3.5	5.5	6.4	8.6	4.8	5.8	4.5	7.1

Table 4.38. Penetration of DEET in Ethanol through human skin at 45°C (μg/cm²)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	12.3	37.6	51.2	57.4	18.9	50.3	62.2	78.3
2	16.4	32.8	50.3	61.2	22.3	51.4	68.9	80.3
3	14.8	38.5	47.3	76.4	20.4	59.4	71.2	85.7
4	16.4	39.4	56.4	50.9	17.4	52.3	61.2	88.9
Mean	15.0	37.1	51.3	61.5	19.7	53.3	65.8	83.3
SD	1.9	2.9	3.7	10.8	2.0	4.1	4.9	4.8

Table 4.39. Steady state flux of OXY in Ethanol through human skin at 45 °C (μg/cm²h)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	7.3	10.6	25.0	33.5	11.5	22.2	37.3	52.3
2	6.2	18.8	28.1	48.1	15.3	25.5	38.5	57.1
3	8.2	15.0	23.7	44.8	19.6	28.3	38.3	66.4
4	6.7	16.3	34.5	41.4	17.2	18.4	46.7	78.3
Mean	7.1	15.2	27.8	42.0	15.9	23.6	40.3	63.5
SD	0.8	3.4	4.8	6.2	3.4	4.2	4.4	11.4

Table 4.40. Steady state flux of DEET in Ethanol through human skin at 45 °C (μg/cm² h)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	7.2	16.3	24.4	32.4	14.2	26.1	36.2	59.1
2	6.3	11.1	17.3	33.2	19.0	29.7	32.4	42.6
3	5.1	13.5	22.9	29.2	24.2	32.9	37.3	68.3
4	7.2	17.6	19.0	30.8	16.9	37.6	33.0	56.9
Mean	6.5	14.6	20.9	31.4	18.6	31.5	34.7	56.7
SD	0.9	2.9	3.3	1.7	4.2	4.8	2.3	10.6

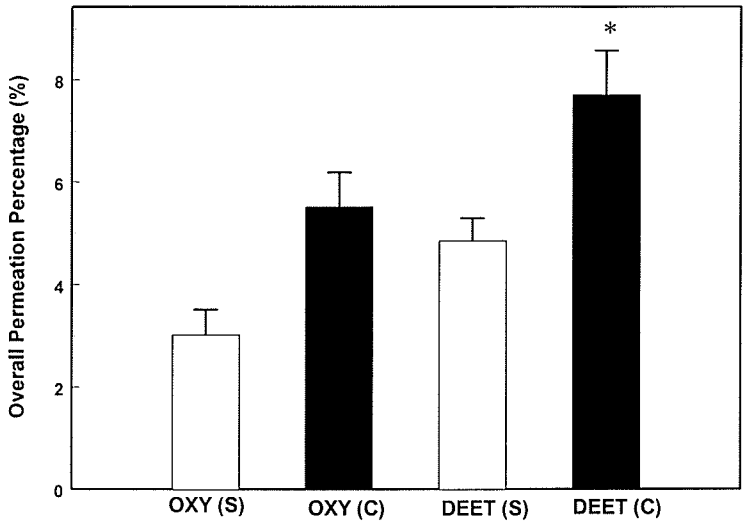


Figure 4.41. DEET and OXY (1%) in PEG at 45 °C through human skin

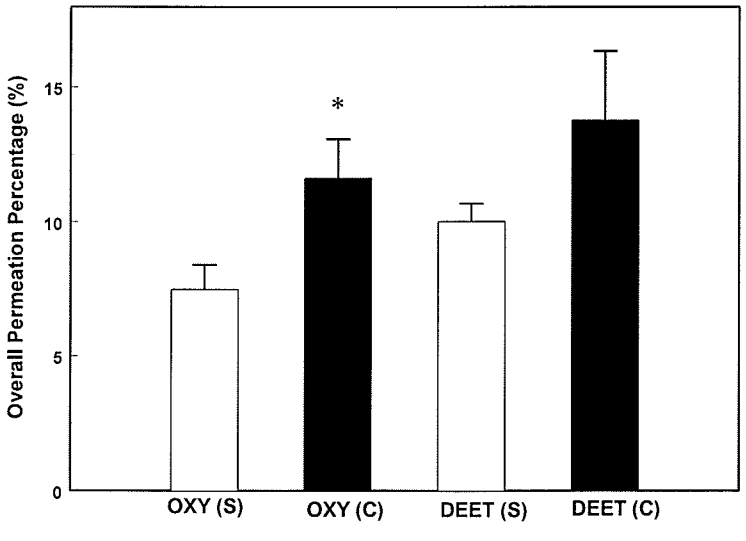


Figure 4.42. DEET and OXY (2.5%) in PEG at 45 °C through human skin

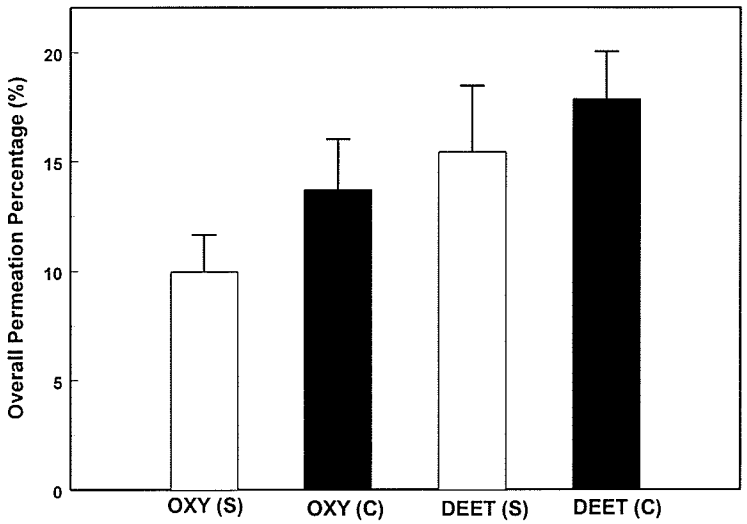


Figure 4.43. DEET and OXY (5%) in PEG at 45 °C through human skin

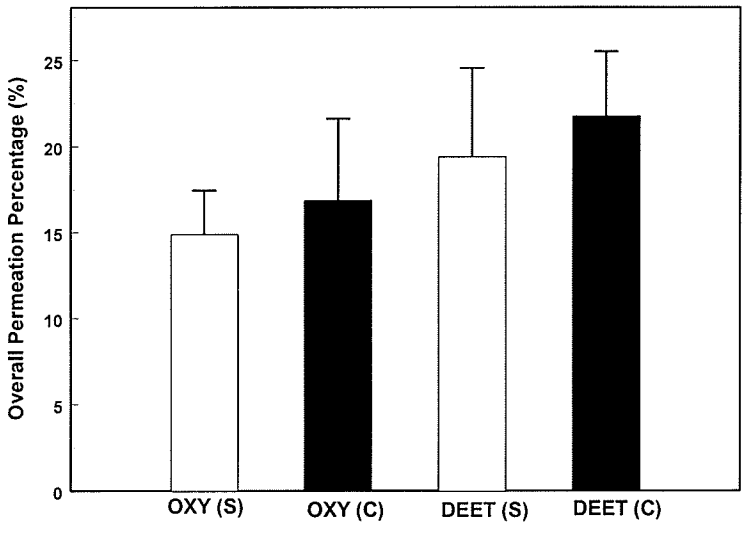


Figure 4.44. DEET and OXY (10%) in PEG at 45 °C through human skin

Table 4.41. Penetration of OXY in PEG through human skin at 45 °C (μg/cm²)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	2.3	7.3	9.8	18.3	6.4	13.3	15.4	19.3
2	3.5	8.7	8.3	13.4	5.4	12.2	12.2	22.3
3	3.0	6.5	12.3	15.3	4.7	10.9	15.9	13.3
4	3.2	7.3	9.3	12.4	5.4	9.9	11.2	12.4
Mean	3.0	7.4	9.9	14.8	5.5	11.6	13.7	16.8
SD	0.5	0.9	1.7	2.5	0.6	1.4	2.3	4.7

Table 4.42. Penetration of DEET in PEG through human skin at 45 °C (μg/cm²)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	4.3	10.1	14.5	13.4	7.8	13.6	18.9	18.9
2	5.3	9.8	12.7	17.4	8.9	11.5	20.3	20.3
3	5.1	9.3	14.7	25.4	7.2	17.4	16.7	27.3
4	4.7	10.9	19.8	21.2	6.9	12.4	15.4	20.3
Mean	4.8	10.0	15.4	19.3	7.7	13.7	17.8	21.7
SD	0.4	0.6	3.0	5.1	0.8	2.5	2.1	3.7

Table 4.43. Steady state flux of OXY in PEG through human skin at 45 °C (μg/cm² h)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	1.1	3.5	5.7	8.7	1.7	5.5	7.9	12.3
2	1.2	2.3	5.3	12.6	2.2	6.9	8.8	15.6
3	0.9	2.0	6.7	6.5	2.7	5.3	7.2	14.9
4	1.2	2.8	9.9	8.2	2.0	4.2	7.5	15.0
Mean	1.1	2.6	6.9	9.0	2.1	5.4	7.8	14.4
SD	0.1	0.6	2.0	2.5	0.4	1.1	0.7	1.4

Table 4.44. Steady state flux of DEET in PEG through human skin at 45 °C (μg/cm² h)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	2.8	3.1	4.9	6.1	3.3	5.0	8.9	19.5
2	1.9	3.6	4.3	6.8	4.2	5.8	7.8	14.6
3	2.0	3.9	5.2	7.2	2.8	6.1	9.1	14.2
4	2.1	3.5	4.5	6.0	4.9	5.7	6.9	18.9
Mean	2.2	3.5	4.7	6.5	3.8	5.6	8.1	16.8
SD	0.4	0.3	0.4	0.5	0.9	0.4	1.0	2.7

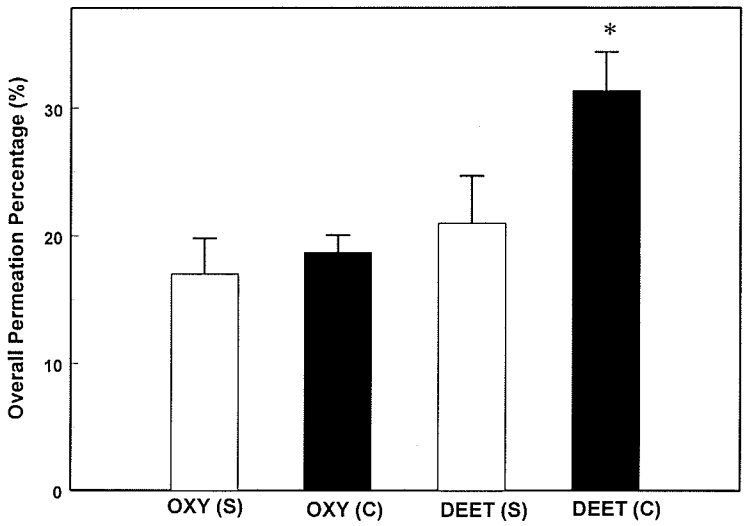


Figure 4.45. DEET and OXY (1%) in PG at 45 °C through human skin

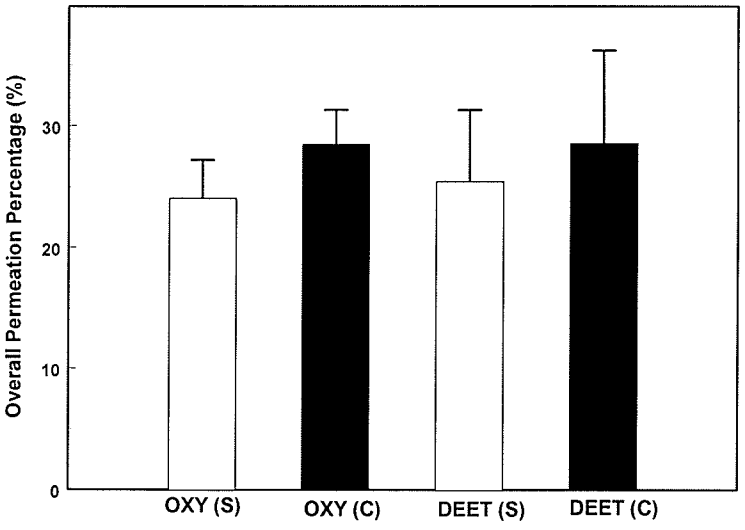


Figure 4.46. DEET and OXY (2.5%) in PG at 45 °C through human skin

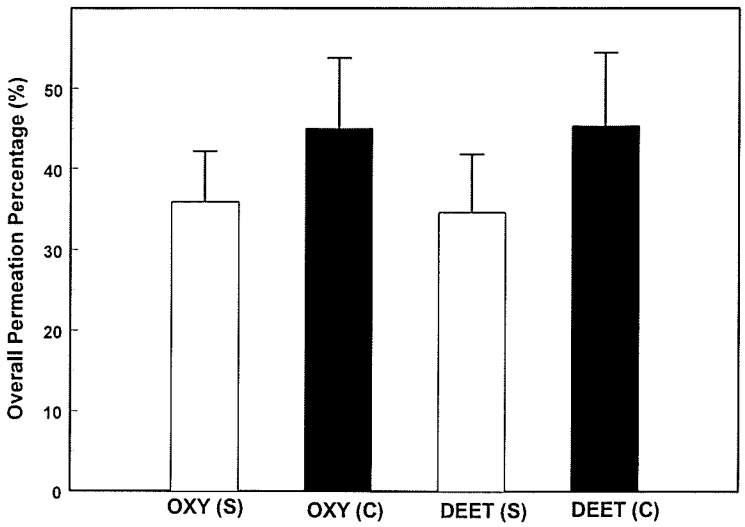


Figure 4.47. DEET and OXY (5%) in PG at 45 °C through human skin

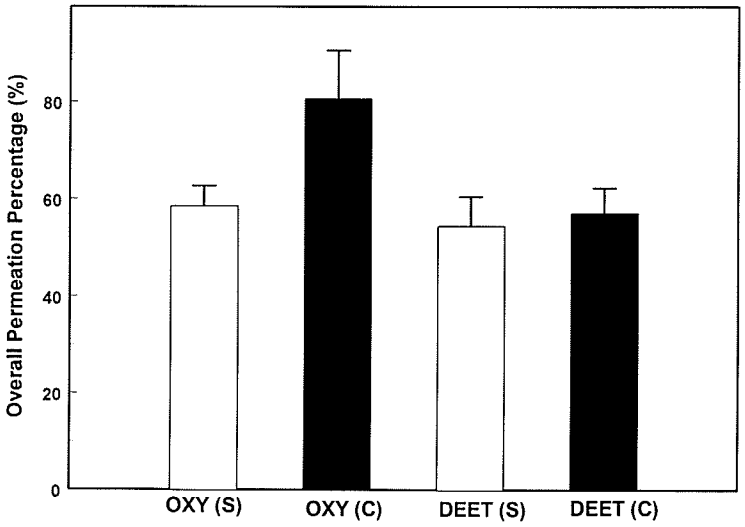


Figure 4.48. DEET and OXY (10%) in PG at 45 °C through human skin

Table 4.45. Penetration of OXY in PG through human skin at 45 °C ($\mu g/cm^2$)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	15.3	28.3	43.3	63.4	17.3	32.3	39.2	89.3
2	18.2	22.3	32.3	53.2	19.3	28.2	55.3	78.2
3	20.3	21.0	29.3	58.3	20.3	27.8	49.2	87.3
4	14.2	24.5	38.6	59.1	17.8	25.4	36.3	67.3
Mean	17.0	24.0	35.9	58.5	18.7	28.4	45.0	80.5
SD	2.7	3.1	6.2	4.1	1.3	2.8	8.8	10.0

Table 4.46. Penetration of DEET in PG through human skin at 45 °C ($\mu g/cm^2$)

No.	DEET Alone (mg)				DEET+ OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	17.4	17.3	31.3	47.0	32.2	37.2	58.9	63.4
2	19.2	27.3	32.5	52.3	29.4	31.2	40.3	58.9
3	21.2	25.8	45.3	56.8	28.4	19.2	42.2	52.3
4	26.0	31.3	29.3	61.0	35.3	26.2	39.8	53.4
Mean	20.9	25.3	34.5	54.3	31.3	28.5	45.5	57.2
SD	3.6	5.8	7.2	6.0	3.0	7.6	9.1	5.1

Table 4.47. Steady state flux of OXY in PG through human skin at 45 °C (μg/cm² h)

No.	OXY Alone (mg)				OXY + DEET (mg)			
	1	2.5	5	10	1	2.5	5	10
1	3.4	5.5	14.9	32.6	7.1	15.0	22.4	60.3
2	2.7	3.7	12.4	48.4	10.3	13.8	26.0	75.2
3	3.7	4.9	23.3	25.7	4.7	18.4	29.5	68.9
4	2.8	5.8	18.2	49.1	5.3	13.8	27.1	87.1
Mean	3.1	4.9	17.0	38.9	6.8	15.2	26.2	72.8
SD	0.4	0.9	4.7	11.6	2.5	2.1	2.9	11.2

Table 4.48. Steady state flux of DEET in PG through human skin at 45 °C (μg/cm² h)

No.	DEET Alone (mg)				DEET + OXY (mg)			
	1	2.5	5	10	1	2.5	5	10
1	2.5	6.5	12.3	34.2	4.4	11.4	25.4	63.5
2	1.9	8.6	15.8	21.0	5.8	12.8	31.0	51.0
3	1.5	6.3	17.9	27.4	6.9	14.0	32.9	88.3
4	3.2	5.8	28.7	30.1	6.8	26.4	22.9	79.6
Mean	2.2	6.8	18.6	28.1	5.9	16.1	28.0	70.6
SD	0.7	1.2	7.0	5.5	1.1	6.9	4.6	16.6

4.2.4. Solubility of DEET and Oxybenzone

Table 4.49 shows the solubility of DEET and oxybenzone in three dissolving media and the receptor fluid. Since solubility of a substance might influence its percutaneous characteristics across a membrane model, the solubility of both DEET and oxybenzone was investigated to check whether or not the solubility of an individual compound would be changed due to the presence of another compound. Compared to single compound in the same vehicle, there were no statistically different changes in solubility of DEET and oxybenzone in PEG and in the receptor fluid, when the two compounds were combined together. This suggested that there was no molecular interaction between DEET and oxybenzone in these two solvents. Solubility of DEET in propylene glycol was reduced when oxybenzone was added, but DEET did not affect the solubility of oxybenzone. Solubility profiles of DEET and oxybenzone were distinctly different among the four test solvents. Based on the solubility data obtained, it was concluded that "Sink condition" was maintained in all *in vitro* diffusion studies.

Table 4.49. Solubility of DEET and OXY (g/ml)

No.	DEET								OXY							
	Single				Combined				Single				Combined			
	PG	PEG	ET	RF	PG	PEG	ET	RF	PG	PEG	ET	RF	PG	PEG	ET	RF
1	0.5	3.5	1.6	0.1	0.2	3.4	2.1	0.01	0.3	1.3	0.3	0.002	0.6	1.2	0.5	0.002
2	0.6	3.7	1.6	0.1	0.2	3.7	2.3	0.01	0.5	1.5	0.2	0.002	0.5	1.4	0.7	0.002
3	0.4	4.3	1.6	0.1	0.2	3.6	1.9	0.01	0.4	1.4	0.2	0.002	0.4	1.4	0.5	0.002
4	0.5	3.8	1.6	0.1	0.2	3.7	2.3	0.01	0.4	1.8	0.3	0.002	0.5	1.2	0.6	0.002
Mean	0.5	3.5	1.6	0.1	0.2	3.6	2.2	0.01	0.4	1.5	0.3	0.002	0.5	1.3	0.6	0.002
SEM	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.1	0.0

PG = Propylene glycol; PEG = Polyethylene glycol; ET = Ethanol; RF = Receptor fluid

4.3. Discussion

4.3.1. Artificial PDMS Membrane

DEET and oxybenzone both increased in permeation when used concurrently in the diffusion studies. Permeability of DEET and oxybenzone across this artificial membrane model was also dependent on dissolving vehicles and application concentrations. Twist and Zatz reported that solvents such as water, PEG, and PG behaved as ideal vehicles across PDMS membrane, and that the permeation and diffusion flux of a solute from these vehicles were functions of permeant activity independent of the solvent [155]. PDMS was previously indicated as a suitable artificial membrane to study the transmembrane properties of various candidates [156]. However, it has not been widely used for penetration enhancement assessment. Furthermore, there are not many published studies that used identical experimental conditions such as solute concentration, formulation, and temperature to compare an artificial membrane to biological membranes. Commercially available repellent and sunscreen products are commonly composed of multiple active and non-medicinal ingredients to facilitate protection efficacy and improve formulation stability, elegancy and application convenience. By using artificial membranes for initial and individual evaluation of the transmembrane properties of certain active compounds and excipients, it is possible to reduce experimental cost associated with using a large quantity of animal or human skin specimens and minimize data variations frequently encountered with the use of biological membranes.

There was an increase in the steady-state diffusion flux of DEET and oxybenzone across PDMS membrane when the test concentrations were raised. Compared to single component, flux of DEET and oxybenzone in combined samples were higher in ethanol and PEG. Propylene glycol did not change the diffusion flux of DEET and oxybenzone. No definitive correlation was observed between overall permeation percentage and test concentration. The general tendency was that the permeation percentage decreased when concentration increased. High concentrations tend to result in presence of more solute molecules in the vehicle, which consequently leads to a reduction in the thermodynamic activity of the solute as well as availability of the solute from the vehicle to the diffusional process [157-159]. The recommendation of using sunscreen liberally with ample amount has its practical merits. A large amount of sunscreen to be spread over the surface of skin can not only effectively diffuse or absorb UV radiation, but also saturate the stratum corneum, preventing the active components from further diffusing and penetrating into deeper skin layers. If possible, it is always appropriate to wear protective clothing and apply insect repellents sparsely on the clothing. This is of clinical relevance to children and the elderly, as skin conditions as well as metabolic functions in these population groups are unique due to age factors. When repellent and sunscreens are applied concurrently, sunscreens should be applied first to block the surface of skin before the repellent is further used on the top of the sunscreens. This application practice can normally result in maximal protection effects for both products [160].

The effects of temperature on transmembrane permeation of DEET and oxybenzone were perhaps more complex in nature than those of preparation vehicles and application concentrations. High temperatures theoretically promote the diffusion process

of any molecules by increasing molecular movement, resulting in enhanced transmembrane permeability. Numerous studies have achieved improved percutaneous penetration of various compounds when they were applied at elevated temperatures [161,162]. In our PDMS study, experimental condition at 45 °C produced slightly higher permeation of the compounds than 37 °C, and the temperature appeared to influence oxybenzone more than DEET, especially when ethanol or PEG was used as the dissolving vehicle. However, analysis of the data between two temperatures did not reveal statistically significant differences in overall permeation percentage of DEET and oxybenzone. Clarys *et al* have recently concluded from an *in vivo* human study that temperature did not significantly alter the adsorption of sunscreen into the stratum corneum [161]. The study was conducted at 31 °C and 40 °C, and only penetration patterns of sunscreens into stratum corneum were measured through skin stripping. The authors did find the loss of sunscreens from the stratum corneum with elapse of the time, but did not provide an explanation. Insect repellents and sunscreens are usually applied to the exposed skin at elevated environmental temperature. Most users also take part in physical activities such as running, swimming and jogging after the application. There are some other physiological factors that could contribute to overall transdermal absorption of these preparations in association with elevated environmental and/or body temperatures, such as increased blood flow within the skin layers, sweating and skin hydration, the loss of applied doses through evaporation or washing, UV intensity and exposure time. All these factors have to be considered as a whole in order to realistically assess skin penetration and absorption of insect repellents and sunscreens. While *in vitro* diffusion studies can provide useful information to certain degrees, *in vivo* studies in

either animals or humans would be more appropriate for such transdermal absorption assessment.

4.3.2. Piglet Skin Model

In vitro diffusion studies using biological membranes were carried out at 45 °C, based on the fact that both insect repellents and sunscreens are applied more frequently during the summer months. Although the normal temperature on the surface of skin was generally known to be approximately 32 °C, exposure to the sunlight during hot summer days could significantly increase the surface skin temperatures. Assessing permeation of DEET and oxybenzone at elevated temperature conditions would therefore provide a more realistic simulation to real-life application conditions. Permeability of DEET and oxybenzone across piglet skin was clearly dependent on dissolving vehicles, which resulted in variable steady-state flux values from the experiments. This might be associated with the ability of a solvent to solublize the solutes as well as to interact with the skin membrane. A higher flux from a given vehicle most likely reflects an increased permeability of the solute across the barrier due to vehicle-membrane interaction [111]. Results from this membrane model demonstrated permeability in the ranking order of ethanol > propylene glycol > PEG, which were identical to those reported in previous studies in which the effect of vehicles on permeation of either DEET or sunscreen components was evaluated [111,163]. Increase in permeability when DEET and oxybenzone were concurrently present clearly suggested the potential for both compounds to act as permeation enhancers, which would result in transdermal absorption synergy, an undesirable property in "real-life" application situations. Ethanol and other

alcohol substitutes are known as percutaneous penetration enhancers to other chemicals; they are not appropriate vehicles for any insect repellent and sunscreen formulations. Vehicles such as PEG and propylene glycol might be suitable for topical repellent and sunscreen preparations, because both DEET and oxybenzone showed relatively low overall permeation percentage in these two vehicles [111]. Solubilizing capacity of PEG might also result in strong affinity to lipophilic compounds such as DEET and oxybenzone, rendering them incapable of penetrating across the membrane to a significant extent. Furthermore, high vehicle viscosity from PEG and propylene glycol could reduce permeation process of DEET. Studies have indicated greater penetration of sunscreen chemicals from emulsion-based formulations than from petrolatum-based preparations [164]. As a biological membrane, piglet skin seemed serving well for the *in vitro* diffusion experiments.

4.3.3. Human Skin Model

The stratum corneum represents the main barrier against skin permeation of drugs and other substances [165,166]. There are significant similarities between porcine and human skin in terms of transdermal properties as well as permeability of the membranes to a diverse range of compounds [167]. Although many studies have validated pig skin as a suitable skin substitute for *in vitro* assessment of percutaneous drug delivery, using human skin whenever possible has always been regarded essential and is more accurate and relevant for clinical significance.

Numerous studies have been performed to investigate transdermal permeation of DEET in human skin. Nevertheless, no study has been reported on the synergistic

permeation of DEET and oxybenzone through human skin. Ross and Shah reported that permeability and flux of DEET were dependent upon partition coefficient of the skin and the vehicle. While transdermal permeation of DEET depends on the vehicles in which it is incorporated, this permeation might also be influenced by some other interactions from components or additives present in the formulations. Transdermal penetration of both DEET and oxybenzone was higher when they were present in combination, which might suggest that both compounds acted as permeation enhancers to each other. The effect of permeation was higher for DEET than for oxybenzone. Pont *et al* concluded recently that sunscreens could increase permeation of 2,4-diphenoxyacetic acid, a common agricultural chemical [168,169]. Their results partially confirmed the synergistic phenomenon observed in our studies. Although the overall penetration amount of DEET and oxybenzone through human skin was lower than that obtained from other membrane models, the trend of percutaneous penetration was identical throughout the experiments. PEG remained to be the solvent in which least DEET penetrated across the skin. Ethanol and propylene glycol appeared to facilitate the permeation process, even when DEET or oxybenzone was present as a single ingredient in the formulation. Overall amount of permeation per vehicle followed the following trend, i.e., ethanol > propylene glycol > PEG. The greater skin uptake from ethanol and propylene glycol could attribute to either an enhanced thermodynamic activity on the surface of skin as a result of evaporation or a lower solubility of DEET and oxybenzone in the vehicles [170]. Gupta *et al* had compared the penetration characteristics of oxybenzone *in vitro*, and drawn a relationship between effects of vehicle affinity and lipophilicity of sunscreens [171]. Biphasic solvent systems are known to exert synergistic activity on percutaneous penetration and act as

penetration enhancers. Leichtnam *et al* recently demonstrated that using multiphasic solvents could increase drug penetration flux; a modest increase of penetration was achieved when a mixture of propylene glycol/ethanol (1:1) was used. Their studies also indicated a correlation between diffusion flux and solvent type [172,173]. In general, topical preparations are formulated with multiple excipients and additives, and solvents such as ethanol and propylene glycol are commonly used in commercial products to facilitate product manufacture and stability. The presence of these substances could hence inadvertently affect transdermal characterization of the active ingredients, which should certainly be carefully investigated and assessed.

4.3.4. Similarities and Differences

Three dissolving solvents and three membrane models were employed in the thesis to study penetration enhancement activity of DEET and oxybenzone *in vitro*. As an artificial membrane model, PDMS membrane has been frequently used for *in vitro* diffusion studies of various compounds. It is able to generate vital information on transmembrane penetration characteristics of diffusants, providing insight into effective drug delivery through the skin. For DEET and oxybenzone, permeation characteristics through PDMS membrane seemed correlative to those found in biological membranes, in terms of transmembrane permeation tendency; overall permeation of both DEET and oxybenzone was increased when they were present in combination. This was beneficial and useful in directing further experiments *in vitro* and *in vivo*. Differences in permeation percentages between artificial and biological membranes might be attributed to variations in thickness of testing models, as well as permeability of the test vehicles within the study

membranes. There was also a significant difference in permeation when temperature factor was counted in PDMS membrane, which was not observed with the biological membranes. It was fully understood that a significant difference between an artificial membrane and a biological membrane was the lack of lipid or keratin barrier in synthetic membrane, which could subsequently influence permeation of some testing compounds.

Percutaneous penetration of DEET and oxybenzone involved in the epidermis of both pig skin and human skin was perhaps much more complex than that in PDMS membrane. The lowest permeation percentages of the test compounds were observed in the human skin, which was not only expected but also well correlative to other similar studies [174]. This was largely attributed to differences in lipid contents and stratum corneum thickness between pig skin and human skin; both parameters were critical in determining the rate and extent of any transepidermal penetration. Similar to the PDMS membrane, pig skin provided a more easily available yet less expensive approach for systematic evaluation of percutaneous drug penetration and delivery *in vitro*; it was beneficial in developing and designing further research methodology. The *in vivo* study design in a piglet model was based on data obtained in this membrane model, which also allowed for *in vitro* and *in vivo* correlation analysis of the percutaneous characterization of DEET and oxybenzone.

The relationship between the molecular structure and the permeability has been previously described. Chemical structure of a penetrant dictates its thermodynamic activity within the skin, consequently resulting in variations in transdermal penetration. In addition, molecular polarity also determine whether or not a compound will achieve transdermal penetration through intercellular or intracellular lipid route in the stratum

corneum [175-177]. Both DEET and oxybenzone are highly non-polar compounds. The nature of the solutes and solvents will eventually affect partition coefficients of the test compounds [178]. Being more lipophilic in nature, oxybenzone should possess higher tendency of penetration than DEET; however, DEET demonstrated higher permeation than oxybenzone in our studies. This might have been due to interactions among solutes, vehicles and skin membranes. DEET showed higher solubility than oxybenzone in all three dissolving solutions, which would allow for more molecules available for diffusion across the membranes. This was further proven with the increase of solute concentrations in the experiments; as testing concentration increased, so did the penetration of both DEET and oxybenzone. On the other hand, solubility might also exert a negative effect on transdermal absorption; increased solubility would increase the affinity of the solute towards the solvent and thus less likely the partition into the skin [1]. This might be the case with PEG, in which DEET and oxybenzone demonstrated the lowest penetration in all three membrane models.

4.4. Conclusions

In vitro diffusion studies revealed an increased permeation of DEET and oxybenzone when they were used concurrently. This synergistic permeation was dependent on the dissolving vehicles and the application concentrations. Ethanol was not considered a satisfactory vehicle for insect repellents or sunscreens, because it enhanced the permeation of DEET and oxybenzone across the membranes. The permeability of DEET was also affected to a greater extent, than that of oxybenzone. Artificial PDMS membrane was found to be a suitable membrane substitute for preliminary evaluation of the transmembrane characterization of DEET and oxybenzone. Ranking order of permeation from PDMS membrane was identical to what was observed in the biological membranes. Even though the study was conducted under *in vitro* conditions, percutaneous patterns of this kind would be considered undesirable *in vivo*. As a result, concurrent application of DEET-based insect repellents and sunscreens should be further assessed *in vivo* in an animal model.

Chapter 5

In Vivo Assessment of DEET and Oxybenzone

5.1. Hypothesis

DEET has been marketed as one of the most effective and inexpensive broad-spectrum topical insect repellents for more than five decades. As long as it is applied appropriately according to manufacturers' recommendations, DEET is generally considered safe as a topical preparation. However, it has also been associated with variable adverse effects such as toxic encephalopathy, seizures, acute manic psychosis, cardiovascular toxicity, and dermatitis, along with a few cases of death, due to either ingestion or extensive penetration through the skin.

The increasing awareness of detrimental effects of carcinogen and photoaging of ultraviolet (UV) radiation has also led to an increase in use of sunscreens by the general public. As a result, active sunscreen ingredients are being added to a wider range of cosmetic products, including moisturizers and lipsticks. Adverse reactions to sunscreen compounds are well known [156,157]. They include allergic contact dermatitis, phototoxicity and photoallergy.

Since a synergistic transmembrane property had been observed between DEET and oxybenzone from all *in vitro* diffusion studies, it became obvious and logical that an *in vivo* study in an animal model would be the next step to prove the characteristics in living subjects. Therefore the objective of this *in vivo* experimentation was to assess the percutaneous penetration of DEET and oxybenzone from commercial products, with the study goal to determine concentrations of DEET and oxybenzone in plasma, urine and skin, and to correlate *in vivo* permeation characteristics with *in vitro* diffusion profiles.

As was proposed in the *in vitro* diffusion experiments, it was hypothesized that,

There would be an enhanced transdermal penetration of DEET and oxybenzone in an animal model when both DEET and oxybenzone were applied concurrently;

Transdermal penetration of DEET and oxybenzone could be correlated among plasma, urine and skin samples.

The general aims of the *in vivo* animal studies were,

To evaluate percutaneous penetration profiles of DEET and oxybenzone in a piglet model using three commercially available repellent and sunscreen preparations;

To measure and compare skin penetration, concentrations of DEET and oxybenzone in blood and urine after topical application of the commercial products;

To calculate pharmacokinetic properties of DEET and oxybenzone from topical application of the commercial preparations.

All test products were commercially available preparations that were purchased from a local pharmacy and used directly without further manipulation. The content of the active ingredients was 10% DEET and 5% oxybenzone, either individually as lotions of repellent and sunscreen or in combination as a compound lotion. The selection of lotion preparations for the *in vivo* animal study would allow for prolonged skin retention of the applied dose, subsequently ensuring the accuracy and reproducibility of the animal study results.

5.2. Results

Skin tape strips were used to study the penetration amount and diffusion kinetics of DEET and oxybenzone from the study formulations after topical application. The twelve tape strips collected at 2, 12 and 48 hours post application were further divided in four subgroups in order to clearly interpret percutaneous penetration kinetics of DEET and oxybenzone within different skin layers. The tape strips were divided as follows, Group 1: Tape No. 1, Group 2: Tape No. 2-5, Group 3: Tape No. 6-9, and Group 4: Tape No. 10-12.

5.2.1. Recovery of DEET and Oxybenzone (2 Hours)

A maximal amount of DEET and oxybenzone was recovered from skin tapes at 2 hours post application of the commercial formulations. While more amounts of DEET and oxybenzone were recovered overall from test Product 3, which was a combination application, there was clearly an increase in recovery amount in Group 2 in comparison to any other groups, indicating that both DEET and oxybenzone were largely located in the upper layers of stratum cornuem at 2 hours.

Figures 5.1-5.2 show the detailed recovery percentage of DEET and oxybenzone from the test preparations. Combined lotion of DEET and oxybenzone demonstrated significantly higher permeation of the two compounds in all four subgroups than its corresponding single-component counterparts. The greatest difference was observed in Group 2. *Tables 5.1-5.4* list individual recovery of DEET and oxybenzone from the three test products, respectively.

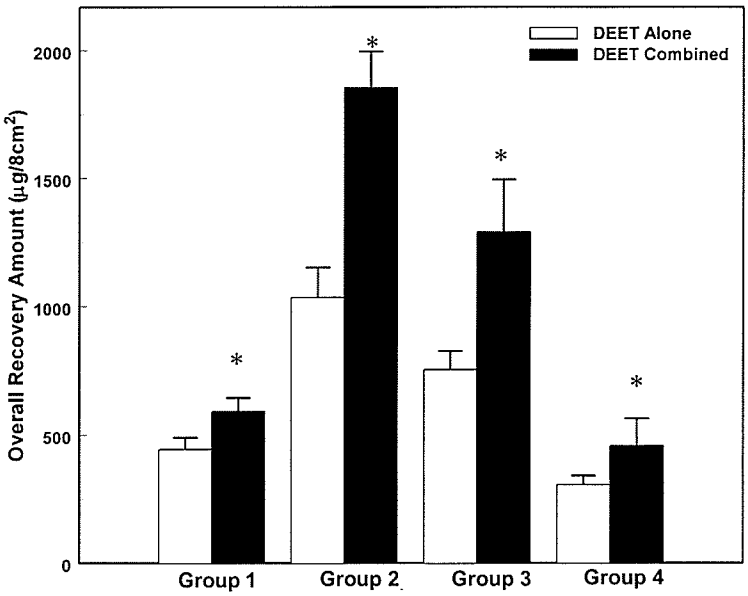


Figure 5.1. DEET in tape strips at 2 hours post application

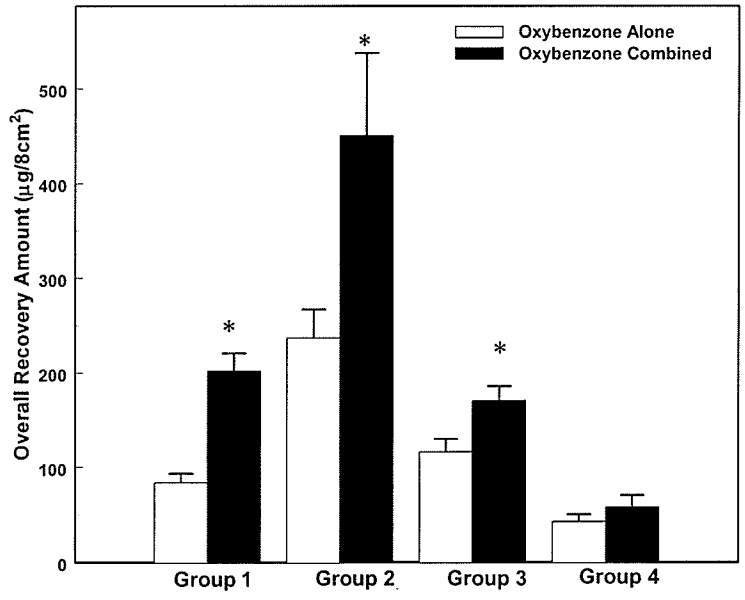


Figure 5.2. OXY in tape strips at 2 hours post application

Table 5.1. Recovery of DEET in tape strips at 2 hours post application from Product 1 ($\mu g/cm^2$)

Animal	Tape Strips											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	433.8	255.8	250.8	237.5	229.7	216.7	198.2	187.4	177.6	138.0	117.9	92.7
2	390.9	329.0	316.9	273.0	294.9	252.9	219.1	215.5	169.1	104.4	96.3	65.8
3	527.1	243.3	327.4	213.9	212.2	193.8	198.2	174.2	174.9	147.2	113.9	82.7
4	450.7	291.7	277.2	247.7	233.9	212.2	192.4	180.8	184.8	122.9	108.9	62.2
5	420.6	274.3	243.4	249.2	237.7	197.7	189.4	172.7	149.3	127.4	98.9	72.7
6	448.7	282.3	239.4	232.9	216.3	193.9	171.3	162.9	144.8	123.3	93.7	67.4
Mean	445.3	279.4	275.8	242.4	237.5	211.2	194.8	182.3	166.8	127.2	104.9	73.9
SD	45.6	30.0	38.3	19.6	29.7	22.5	15.5	18.2	16.1	14.6	10.0	11.6

Table 5.2. Recovery of OXY in tape strips at 2 hours post application from Product 2 (μg/cm²)

Animal	Tape Strips											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	92.6	78.4	64.5	52.3	43.1	36.8	34.3	28.6	25.4	22.9	13.0	11.9
2	92.1	73.7	68.3	36.9	29.8	29.4	32.9	13.4	17.4	16.9	7.0	7.0
3	89.4	83.8	78.1	49.8	42.8	36.2	32.9	28.9	24.3	20.0	14.3	12.4
4	84.3	79.3	77.3	67.4	48.7	36.4	31.2	27.4	23.9	15.3	12.7	13.3
5	77.4	72.9	67.2	48.9	40.2	35.7	31.5	26.4	21.3	20.1	12.4	10.1
6	67.2	63.9	60.2	52.4	42.4	35.7	31.2	29.3	27.3	19.3	15.0	12.3
Mean	83.8	75.3	69.2	51.3	41.2	35.0	32.3	25.7	23.3	19.1	12.4	11.2
SD	9.8	6.8	7.1	9.7	6.2	2.8	1.2	6.0	3.4	2.6	2.8	2.3

Table 5.3. Recovery of DEET in tape strips at 2 hours post application from Product 3 ($\mu g/cm^2$)

Animal	Tape Strips											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	629.5	570.9	457.3	418.9	353.6	325.0	291.9	294.4	249.4	108.5	145.8	131.6
2	580.9	504.5	511.7	463.8	420.8	492.2	414.7	389.4	279.6	238.3	152.6	143.6
3	677.7	627.6	472.7	402.3	397.8	324.4	299.3	288.7	244.8	104.4	139.2	129.3
4	562.7	489.2	492.3	427.3	392.7	310.7	274.3	256.0	232.7	107.9	127.4	107.2
5	522.4	489.9	512.2	471.7	417.8	391.7	407.2	389.4	279.6	212.8	177.7	143.6
6	574.0	474.3	470.2	450.7	442.4	372.9	360.7	307.2	270.5	243.9	187.4	147.8
Mean	591.2	526.0	486.1	439.1	404.2	369.5	341.3	320.8	259.4	169.3	155.0	133.8
SD	54.5	60.2	22.9	27.2	30.4	67.8	61.3	55.7	19.8	69.1	23.0	14.9

Table 5.4. Recovery of OXY in tape strips at 2 hours post application from Product 3 ($\mu g/cm^2$)

Animal	Tape Strips											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	175.5	141.1	80.2	78.8	54.6	50.0	45.1	33.9	35.8	32.3	26.9	19.8
2	229.0	137.4	95.4	70.8	71.4	46.8	47.1	40.8	27.9	22.2	20.1	18.3
3	212.7	149.4	95.4	79.3	84.8	48.9	47.1	41.2	23.5	21.3	19.4	18.3
4	207.6	177.4	137.4	92.3	77.4	57.3	48.9	40.3	27.4	19.3	16.3	15.0
5	199.3	177.4	142.2	122.7	93.9	62.2	47.9	37.4	32.3	20.6	15.4	14.4
6	187.4	174.4	148.9	122.7	98.9	62.3	49.7	39.2	30.1	22.9	14.4	10.3
Mean	201.9	159.5	116.6	94.4	80.2	54.6	47.6	38.8	29.5	23.1	18.7	16.0
SD	18.9	18.9	29.5	22.9	16.0	6.9	1.5	2.7	4.2	4.6	4.5	3.4

5.2.2. Recovery of DEET and Oxybenzone (12 Hours)

Recovery of DEET and oxybenzone in skin tape strips was greater from Group 2 and Group 3 than from Group 1 and Group 4. Again, greater amounts of DEET and oxybenzone were recovered from Product 3 than from Products 1 and 2. The recovery of DEET and oxybenzone at this collection interval increased in Group 3 in comparison to that at 2 hours, indicating that both DEET and oxybenzone were further diffusing into stratum cornuem. Data from Groups 1-3 also demonstrated that DEET and oxybenzone penetrated at a faster rate into deeper layers of the skin when they were combined, suggesting a synergistic penetration enhancement of DEET and oxybenzone.

Figures 5.3-5.4 show the detailed recovery percentage of DEET and oxybenzone from the test preparations 12 hours after the topical skin application. There was significantly higher penetration of DEET and oxybenzone from the combined lotion than their individual counterparts in Groups 1-3. Recovery percentages in Group 4 were however different from previous sampling interval; single-component lotions showed significantly higher permeation than their combined counterpart. The greatest difference was observed in Group 2, or the upper layers of stratum corneum. *Tables 5.5-5.8* list individual recovery amount of DEET and oxybenzone from the three test products, respectively.

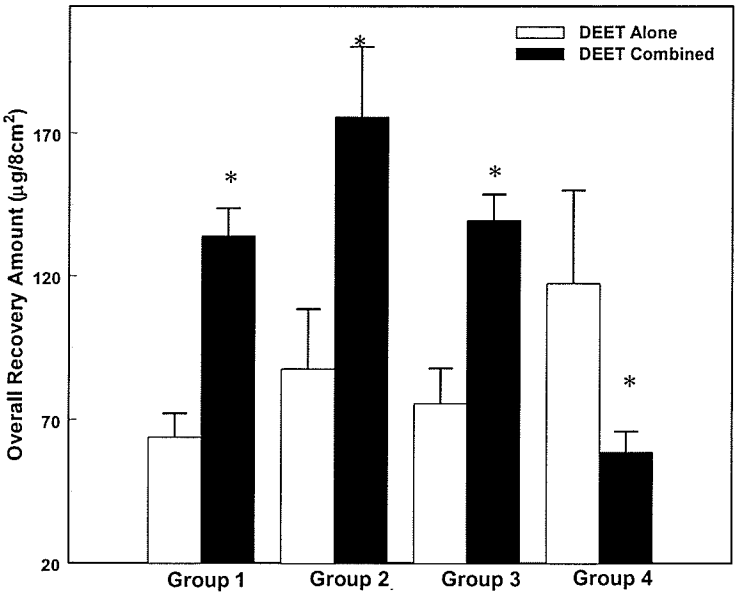


Figure 5.3. DEET in tape strips at 12 hours post application

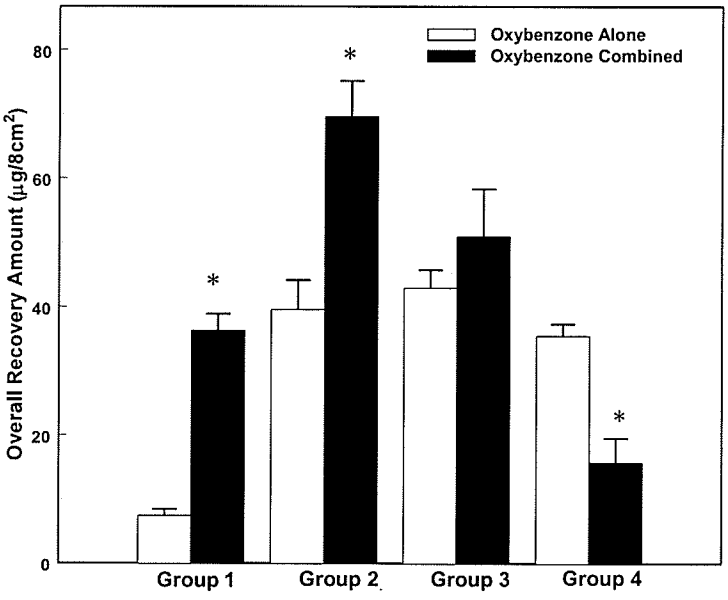


Figure 5.4. OXY in tape strips at 12 hours post application

Table 5.5. Recovery of DEET in tape strips at 12 hours post application from Product 1 ($\mu g/cm^2$)

Animal	Tape Strips											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	55.4	40.9	11.2	3.2	11.8	4.5	4.5	2.5	55.1	45.5	58.5	31.4
2	78.5	63.2	24.0	20.2	10.0	9.1	13.5	7.9	58.7	11.2	26.1	19.7
3	62.9	47.2	10.7	3.9	12.4	4.7	3.9	2.7	56.3	51.2	62.8	35.4
4	67.7	57.4	17.9	15.0	10.5	8.7	9.3	7.1	51.4	44.8	54.9	27.4
5	60.2	51.2	12.8	11.0	9.9	9.2	9.8	8.2	50.3	39.4	50.5	32.9
6	57.7	50.2	10.7	9.9	8.9	8.2	9.2	8.3	49.3	33.9	47.6	30.2
Mean	63.7	51.7	14.6	10.5	10.6	7.4	8.4	6.1	53.5	37.7	50.0	29.5
SD	8.4	7.8	5.3	6.5	1.2	2.1	3.6	2.7	3.7	14.2	12.9	5.5

Table 5.6. Recovery of OXY in tape strips at 12 hours post application from Product 2 ($\mu g/cm^2$)

Animal	Tape Strips											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	7.8	8.9	12.8	9.4	7.6	12.5	7.7	12.7	9.4	8.3	9.8	16.2
2	9.4	9.2	16.9	8.6	9.5	13.3	9.9	10.5	8.8	8.2	8.7	17.0
3	5.1	6.3	12.2	7.4	8.1	13.4	8.2	10.2	8.9	10.2	10.2	17.8
4	6.2	8.2	14.4	8.7	6.7	12.2	8.9	12.3	9.4	8.9	9.4	17.2
5	8.2	7.8	14.6	9.7	9.4	12.7	9.2	12.3	9.7	9.2	9.3	16.6
6	7.2	8.2	13.6	9.1	8.5	13.2	9.4	12.7	8.6	8.3	8.9	17.2
Mean	7.3	8.1	14.1	8.8	8.3	12.9	8.9	11.8	9.1	8.9	9.4	17.0
SD	1.5	1.0	1.6	0.8	1.0	0.4	0.8	1.1	0.4	0.7	0.5	0.5

Table 5.7. Recovery of DEET in tape strips at 12 hours post application from Product 3 ($\mu g/cm^2$)

Animal	Tape Strips											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	124.0	147.8	20.4	16.6	14.8	12.1	6.0	9.8	105.8	8.3	6.6	42.2
2	148.9	137.4	21.5	17.9	13.9	9.9	5.1	9.4	112.7	10.6	8.2	32.9
3	142.8	107.9	26.4	21.2	18.2	12.4	7.2	13.2	105.3	7.9	6.7	41.3
4	127.2	100.7	20.4	20.7	14.8	11.0	6.2	12.4	105.4	9.8	7.7	39.4
5	132.4	107.8	20.6	20.1	13.9	11.3	7.2	12.7	110.3	10.1	7.9	44.8
6	127.4	112.2	22.2	20.0	14.2	10.3	8.9	13.4	117.8	10.2	7.3	49.5
Mean	133.8	119.0	21.9	19.4	15.0	11.1	6.8	11.8	109.5	9.5	7.4	41.7
SD	8.4	7.8	5.3	6.5	1.2	2.1	3.6	2.7	3.7	14.2	12.9	5.5

Table 5.8. Recovery of OXY in tape strips at 12 hours post application from Product 3 ($\mu g/cm^2$)

Animal	Tape Strips											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	32.2	22.6	21.0	18.8	7.5	5.0	34.5	7.1	4.7	5.5	5.3	4.5
2	34.0	21.7	23.4	14.8	10.7	12.9	31.1	8.6	4.1	4.5	8.7	3.6
3	39.2	22.9	20.4	19.7	7.7	6.0	27.4	6.4	5.7	6.2	1.3	4.7
4	37.4	24.3	21.9	14.3	9.3	7.4	30.5	8.9	5.4	5.9	8.0	3.7
5	37.5	22.9	21.3	14.4	9.5	6.7	28.9	8.4	5.0	5.2	6.2	4.9
6	37.1	22.9	20.6	14.8	9.1	6.2	27.4	9.9	5.7	4.8	5.6	4.7
Mean	36.2	22.9	21.4	16.1	9.0	7.4	30.0	8.2	5.1	5.3	5.8	4.3
SD	1.5	1.0	1.6	0.8	1.0	0.4	0.8	1.1	0.4	0.7	0.5	0.5

5.2.3. Recovery of DEET and Oxybenzone (48 Hours)

Figures 5.5-5.6 show the detailed recovery percentage of DEET and oxybenzone from the test preparations 48 hours after the topical skin application. Even though the total recovery amount of both compounds was much lower than that at 2 and 12 hours, Product 3 still showed higher recovery percentages of DEET and oxybenzone in some sub-groups. Significant increase of penetration was observed in Groups 1, 2, and 4 for both DEET and oxybenzone. The greatest difference between compound lotion and single-component lotion was found in Group 4 for DEET but Group 2 for oxybenzone, indicating that DEET permeated the skin much deeper at a faster penetration rate while oxybenzone might form a reservoir with stratum corneum. *Tables 5.9-5.12* list individual recovery amount of DEET and oxybenzone from the three test products, respectively.

Tables 5.13-5.15 detail the skin recovery of DEET and oxybenzone from the three test preparations at 2, 12 and 48 hours after topical application in piglets. The total recovery of DEET from Product 1 was 47.4%, 6.4% and 1.2% at 2, 12 and 48 hours, respectively. The total recovery of oxybenzone from Product 2 was 18.0%, 4.7% and 3.7% at 2, 12 and 48 hours, respectively. For Product 3, the total recovery of DEET and oxybenzone was 78.7%/33.0%, 9.5%/6.4% and 2.5%/6.4% at 2, 12 and 48 hours, respectively.

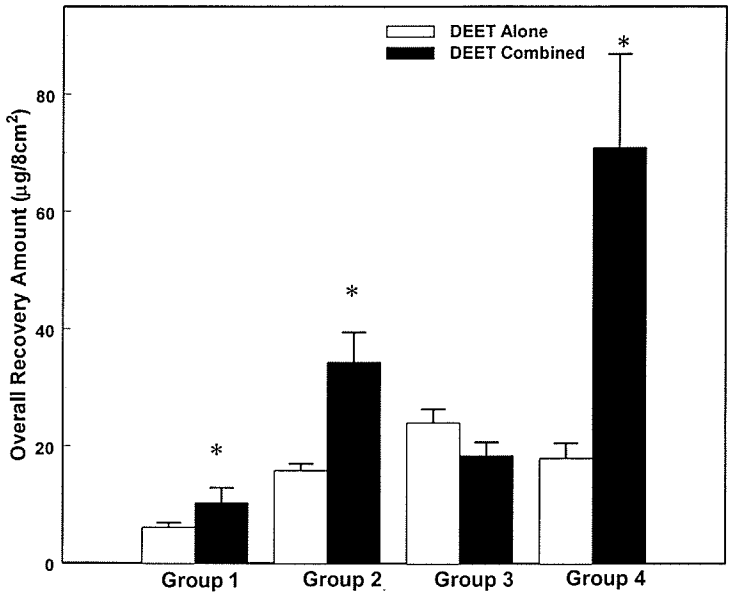


Figure 5.5. DEET in tape strips at 48 hours post application

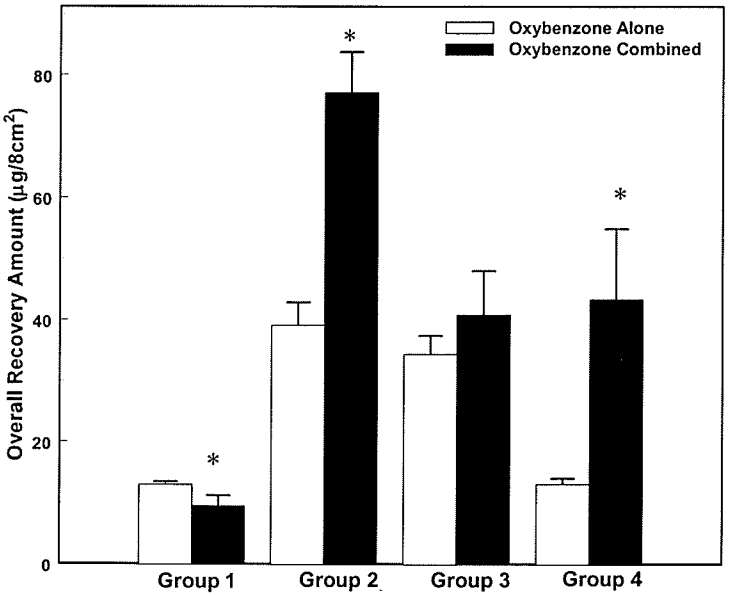


Figure 5.6. OXY in tape strips at 48 hours post application

Table 5.9. Recovery of DEET in tape strips at 48 hours post application from Product 1 (μg/cm²)

Animal	Tape Strips											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	7.2	4.7	4.3	3.9	3.2	2.9	7.5	12.5	3.7	2.6	5.7	8.2
2	5.7	4.3	4.0	3.5	4.1	2.8	5.0	10.1	3.0	2.4	5.8	6.2
3	5.1	4.1	3.9	3.2	2.9	2.6	5.2	10.1	3.6	3.9	7.9	9.7
4	5.5	4.3	4.1	3.7	3.9	2.8	6.3	11.2	3.7	3.7	6.2	8.7
5	6.2	4.2	4.1	3.5	4.3	2.8	6.5	11.5	3.9	3.2	6.5	9.3
6	6.6	4.3	3.9	3.9	3.6	3.1	6.7	11.7	3.7	3.2	6.2	7.5
Mean	6.0	4.3	4.0	3.6	3.7	2.8	6.2	11.2	3.6	3.2	6.4	8.3
SD	0.7	0.2	0.1	0.2	0.5	0.1	0.9	0.9	0.3	0.5	0.7	1.2

Table 5.10. Recovery of OXY in tape strips at 48 hours post application from Product 2 ($\mu g/cm^2$)

Animal	Tape Strips											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	13.6	8.6	18.7	6.5	4.4	4.8	11.5	13.4	4.3	5.2	4.3	3.6
2	13.2	9.1	16.0	8.3	3.2	4.4	14.5	13.2	3.1	4.0	3.9	3.9
3	13.2	8.9	19.7	8.2	2.6	3.7	10.8	13.7	3.8	4.6	4.3	3.9
4	12.7	10.2	17.7	8.5	2.8	4.9	11.2	14.9	4.6	5.4	3.9	4.1
5	12.2	10.4	17.0	8.9	3.1	4.7	12.3	13.3	3.9	4.9	4.3	3.9
6	12.3	10.7	17.4	8.5	4.1	3.8	11.7	13.9	4.7	5.5	4.4	3.6
Mean	12.9	9.6	17.7	8.1	3.4	4.4	12.0	13.7	4.1	4.9	4.2	3.8
SD	0.5	0.8	1.3	0.8	0.7	0.4	1.3	0.6	0.6	0.5	0.2	0.1

Table 5.11. Recovery of DEET in tape strips at 48 hours post application from Product 3 (μg/cm²)

Animal	Tape Strips											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	12.0	13.2	5.2	2.1	13.5	5.7	4.7	5.7	3.9	5.2	4.6	49.3
2	5.6	9.3	2.9	3.3	11.4	5.6	4.2	5.9	3.8	4.7	3.9	40.1
3	12.8	15.1	5.3	2.1	13.5	4.7	5.4	3.7	2.4	10.2	19.6	62.9
4	9.3	14.3	6.2	4.2	12.5	5.1	4.4	5.6	2.9	7.9	12.7	52.7
5	9.7	13.9	5.9	3.9	11.7	4.9	4.9	4.7	3.2	10.7	14.4	51.0
6	11.8	12.7	6.2	4.1	12.4	5.7	4.7	4.3	2.9	10.1	13.3	50.9
Mean	10.2	13.1	5.3	3.3	12.5	5.3	4.7	5.0	3.2	8.1	11.4	51.2
SD	2.6	2.0	1.2	0.9	0.8	0.4	0.4	0.8	0.5	2.6	6.0	7.3

Table 5.12. Recovery of OXY in tape strips at 48 hours post application from Product 3 ($\mu g/cm^2$)

Animal	Tape Strips											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	12.8	12.7	10.7	42.7	10.5	7.7	10.4	12.5	12.3	10.3	10.2	19.0
2	8.7	9.3	16.5	46.7	10.0	8.5	11.8	14.9	6.3	5.1	11.3	10.4
3	7.8	10.2	10.4	43.9	10.3	8.9	2.8	12.9	12.6	19.4	16.8	22.8
4	8.9	10.5	12.9	39.2	10.7	8.4	11.2	13.2	9.4	13.9	13.2	14.4
5	9.9	10.7	12.4	43.2	10.9	7.8	9.9	12.4	10.2	12.8	14.3	16.6
6	8.2	10.4	12.7	43.4	10.5	7.4	10.5	11.7	9.4	14.3	16.2	17.8
Mean	9.4	10.6	12.6	43.2	10.5	8.1	9.4	12.9	10.0	12.6	13.7	16.8
SD	1.8	1.1	2.1	2.4	0.3	0.5	3.3	1.0	2.2	4.7	2.6	4.2

Table 5.13. Recovery percentage of DEET and OXY at 2 hours (%)

Group	DEET		OXY	
	Single	Combined	Single	Combined
1	8.3 ± 0.3	11.1 ± 0.4	3.1 ± 0.2	7.6 ± 0.3
2	19.4 ± 0.7	34.8 ± 0.4	8.9 ± 0.4	16.9 ± 1.6
3	14.2 ± 0.5	24.2 ± 1.5	4.4 ± 0.2	6.4 ± 0.1
4	5.7 ± 0.3	8.6 ± 0.6	1.6 ± 0.1	2.2 ± 0.2

Table 5.14. Recovery percentage of DEET and OXY at 12 hours (%)

Group	DEET		OXY	
	Single	Combined	Single	Combined
1	1.2 ± 0.1	2.5 ± 0.1	0.3 ± 0.0	1.4 ± 0.0
2	1.6 ± 0.1	3.3 ± 0.1	1.5 ± 0.1	2.6 ± 0.0
3	1.4 ± 0.1	2.6 ± 0.0	1.6 ± 0.0	1.9 ± 0.1
4	2.2 ± 0.2	1.1 ± 0.0	1.3 ± 0.0	0.6 ± 0.0

Table 5.15. Recovery percentage of DEET and OXY at 48 hours (%)

Group	DEET		OXY	
	Single	Combined	Single	Combined
1	0.1 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
2	0.3 ± 0.0	0.6 ± 0.0	1.5 ± 0.0	2.9 ± 0.0
3	0.4 ± 0.0	0.3 ± 0.0	1.3 ± 0.0	1.5 ± 0.0
4	0.3 ± 0.0	1.3 ± 0.1	0.5 ± 0.0	1.6 ± 0.2

5.2.4. Concentrations of DEET and Oxybenzone in Plasma

DEET and oxybenzone were detected in the plasma till the end of the study at 48 hours. There was a statistically significant increase in the amount of penetration from the combined lotion of repellent and sunscreens (Product 3) as compared to its single-component formulations (Product 1 and Product 2). *Figures 5.7-5.8* show the concentration-time curves of DEET and oxybenzone, respectively. Concentration of DEET and oxybenzone peaked at 2 hours after the topical application. Increment of DEET absorption from Product 3 in comparison to Product 1 was found to be 11%, 43% and 9% at 2, 12 and 48 hours; increment of oxybenzone absorption from Product 3 in comparison to Product 2 was found to be 64%, 33% and 35% at 2, 12 and 48 hours. *Tables 5.16-5.19* list plasma concentrations of DEET and oxybenzone from individual animals in the study.

Tables 5.20-5.21 list the pharmacokinetic parameters of DEET and oxybenzone from the test preparations, respectively. The data also demonstrated synergistic percutaneous penetration and systemic absorption of both DEET and oxybenzone when the two ingredients were applied concurrently. This phenomenon that was previously found in the *in vitro* diffusion studies was further observed in the animal model.

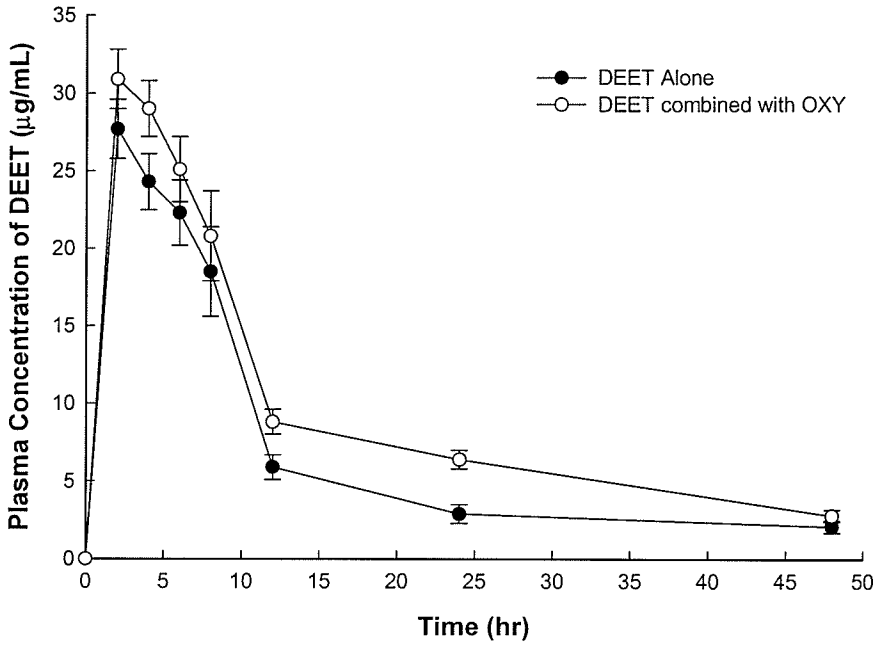


Figure 5.7. Plasma concentration-time curves of DEET

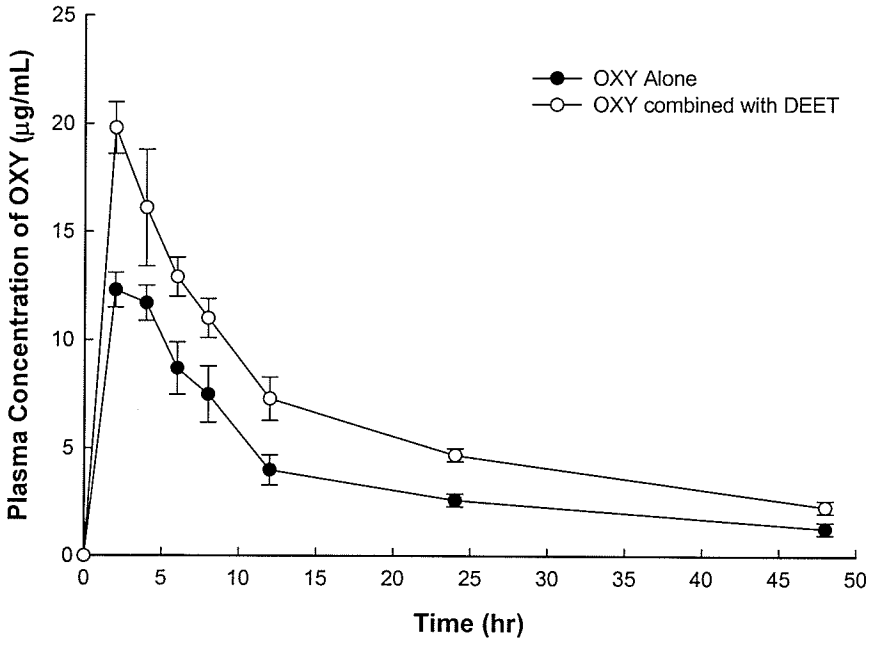


Figure 5.8. Plasma concentration-time curves of OXY

Table 5.16. Concentration of DEET in plasma from Product 1 (μg/mL)

Sample	Time (hour)						
	2	4	6	8	12	24	48
1	26.9	25.9	21.4	18.2	6.2	3.6	2.2
2	25.7	22.8	22.6	17.9	6.5	2.1	2.7
3	29.7	25.1	23.5	18.1	5.2	3.5	1.9
4	28.4	23.6	22.1	19.0	6.7	3.0	2.1
5	27.8	23.0	22.5	19.8	5.1	2.1	2.1
6	27.4	25.4	22.0	18.0	5.7	3.1	1.4
Mean	27.7	24.3	22.3	18.5	5.9	2.9	2.1
SD	1.3	1.3	0.7	0.7	0.6	0.6	0.4

Table 5.17. Concentration of OXY in plasma from Product 2 (μg/mL)

Sample	Time (hour)						
	2	4	6	8	12	24	48
1	12.1	10.6	7.0	7.4	4.8	2.7	1.1
2	13.0	12.5	9.0	7.2	4.7	2.9	1.0
3	11.0	11.4	8.0	5.5	3.6	1.9	0.9
4	11.8	11.0	8.0	7.1	4.2	2.8	1.4
5	12.4	12.4	9.4	8.4	3.1	2.4	1.5
6	13.5	12.4	10.4	9.4	3.4	2.9	1.8
Mean	12.3	11.7	8.7	7.5	4.0	2.6	1.3
SD	0.8	0.8	1.2	1.3	0.7	0.3	0.3

Table 5.18. Concentration of DEET in plasma from Product 3 (μg/mL)

Sample	Time (hour)						
	2	4	6	8	12	24	48
1	29.9	27.8	22.5	18.5	8.4	6.8	2.7
2	28.8	27.71	23.0	20.2	7.8	6.3	2.8
3	32.8	30.6	26.7	18.8	9.4	7.2	1.9
4	32.1	28.4	25.4	19.7	8.4	6.1	2.9
5	28.9	27.4	24.9	21.0	8.9	5.4	2.9
6	33.2	31.9	28.1	26.5	10.1	6.7	3.2
Mean	30.9	29.0	25.1	20.8	8.8	6.4	2.8
SD	1.9	1.8	2.1	2.9	0.8	0.6	0.4

Table 5.19. Concentration of OXY in plasma from Product 3 (μg/mL)

Sample	Time (hour)						
	2	4	6	8	12	24	48
1	20.3	14.6	13.2	9.8	6.5	4.2	2.3
2	19.5	16.4	12.6	10.4	7.0	5.0	2.0
3	18.8	14.0	12.9	11.2	6.0	4.3	2.0
4	18.9	15.1	12.7	11.5	7.0	4.8	2.9
5	22.1	21.4	14.5	12.6	8.5	4.7	2.2
6	19.4	14.7	11.5	10.7	8.7	4.9	2.2
Mean	19.8	16.1	12.9	11.0	7.3	4.7	2.3
SD	1.2	2.7	0.9	0.9	1.0	0.3	0.3

Table 5.20. Pharmacokinetic parameters of DEET from Products 1 and 3

Animal	Parameters							
	AUC_{0-48} (μg hr/L)	AUC_{0-48} (μg hr/L)#	C_{max} (μg/mL)	C_{max} (μg/mL)#	T_{max} (hr)	T_{max} (hr)#	MRT (hr)	MRT (hr)#
1	431.6	440.6	26.9	29.9	2.0	2.0	13.0	14.5
2	369.8	431.3	25.8	28.8	2.0	2.0	12.8	14.4
3	417.1	466.6	29.7	32.8	2.0	2.0	12.3	13.5
4	413.3	445.3	28.4	32.1	2.0	2.0	12.4	14.1
5	420.2	431.2	27.8	28.9	2.0	2.0	11.9	14.0
6	363.5	508.8	27.4	33.2	2.0	2.0	11.6	14.0
Mean	331.1	454.0	27.7	30.9	2.0	2.0	12.3	14.1
SD	5.4	12.1	0.5	0.8	0.0	0.0	0.2	0.1

#: Product 3

Table 5.21. Pharmacokinetic parameters of OXY from Products 2 and 3

Animal	Parameters							
	AUC_{0-48} (μg hr/L)	AUC_{0-48} (μg hr/L)#	C_{max} (μg/mL)	C_{max} (μg/mL)#	T_{max} (hr)	T_{max} (hr)#	MRT (hr)	MRT (hr)#
1	195.7	283.3	13.0	20.3	2.0	2.0	14.3	15.6
2	183.2	299.5	12.4	19.5	4.0	2.0	15.0	15.4
3	187.1	276.9	11.8	18.8	2.0	2.0	15.5	15.3
4	205.1	308.4	13.5	18.9	2.0	2.0	15.6	16.6
5	184.5	335.9	12.1	22.1	2.0	2.0	14.6	14.7
6	154.5	309.9	11.4	19.4	4.0	2.0	13.8	15.6
Mean	185.0	302.3	12.4	19.8	2.6	2.0	14.8	15.5
SD	6.9	8.6	0.3	0.5	0.4	0.0	0.2	0.2

#: Product 3

5.2.5. Quantification of DEET and Oxybenzone in Urine

While both DEET and oxybenzone were detected in plasma samples, only oxybenzone was quantified in urine samples collected at the end of study at 48 hours. Concentration of DEET in all urine samples was below the detection limit of the HPLC assay, indicating that DEET was either eliminated by the body or distributed into various tissues within 48 hours after a topical skin application.

The major metabolites of DEET and oxybenzone were, however, detected at the end of 48 hours. *Tables 5.22-5.23* list detailed concentrations of compounds that were measured with a simultaneous HPLC assay. The major metabolites were found to be 2,4-dihydroxybenzophenone (DHB) and 2,2'-dihyroxy-4-methoxybenzophenone (DHMB) for oxybenzone and N-ethyl m-toluamide (ET) for DEET, respectively. The increment of oxybenzone recovery in urine from Product 3 was found to be 27% in comparison to Product 2, which was statistically significant. No other compounds were found to be statistically significant in presence in urine samples at the end of the study.

Table 5.22. Concentrations of DEET, OXY and their primary metabolites found in urine at the end of the study from Product 1 and Product 2 (μg/mL)

Sample	Analytes in Urine						
	DEET	**OXY**	**2'DMB**	**THB**	**DHB**	**ET**	**DHMB**
1	ND	1.0	ND	0.4	3.4	4.9	9.8
2	ND	0.8	ND	0.6	4.0	4.4	9.4
3	ND	1.1	ND	0.7	4.0	4.9	10.2
4	ND	0.7	ND	0.5	3.2	4.0	8.6
5	ND	1.1	ND	0.8	2.9	3.9	11.0
6	ND	1.0	ND	0.4	3.0	4.5	10.1
Mean	-	0.9	-	0.6	3.4	4.4	9.9
SD	-	0.1	-	0.1	0.4	0.4	0.7

Table 5.23. Concentrations of DEET, OXY and their primary metabolites found in urine at the end of study from Product 3 (μg/mL)

Sample	Analytes in Urine						
	DEET	**OXY**	**2'DMB**	**THB**	**DHB**	**ET**	**DHMB**
1	ND	1.2	ND	0.4	5.2	5.0	12.5
2	ND	1.3	ND	0.5	4.0	5.4	13.2
3	ND	1.4	ND	0.9	4.9	5.9	14.0
4	ND	1.1	ND	0.6	4.3	5.3	11.1
5	ND	1.4	ND	0.7	3.9	5.0	12.8
6	ND	1.0	ND	0.9	4.2	4.8	10.0
Mean	-	1.2	-	0.7	4.4	5.2	12.3
SD	-	0.1	-	0.1	0.4	0.3	1.3

5.3. Discussion

5.3.1. Animal Model

The stratum corneum is the main barrier and rate-limiting step in percutaneous penetration and absorption. Most of lipid molecules such as DEET and oxybenzone achieve skin penetration through the intercellular laminea of stratum corneum [1]. There is very limited transdermal penetration of substances through skin appendages such as sweat glands and hair follicles. Six classes of ceramides have been identified in the human stratum corneum. While numerous animal models have been used to investigate transdermal penetration of medications, pig model as well as skin samples from pigs have been proven to be the most comparable type to humans. Gray *et al* conducted an extensive study to assess lipid composition of epidermis cells isolated from humans, pigs and rats [158]. The nonpolar lipids of epidermal cells in human and pig were similar, with sterols, triglycerides and free fatty acids as the primary components. However, only sterols and triglycerides were found as major components in rat epidermal cells. It was also reported that wax esters was another type of major lipid class in rat epidermal cells, which was detected in neither human nor pig epidermal cells. In addition, rat epidermis contained three to six times the amounts of nonpolar lipids as a percentage of dry cell weight, even though its composition of phospholipids was similar to that of human and pig epidermis. Montonga *et al* cited some of the striking resemblances between human skin and pig skin, which included a thick epidermis and dermis, and a large presence of elastic fibers [159]. The dissimilarities between humans and pigs in terms of

percutaneous penetration and absorption may include a lack of sebaceous glands and a poorly vascularized dermis in pigs.

Skin specimens from both piglets and humans were used for *in vitro* diffusion characterization of DEET and oxybenzone previously. Considering the fact that it was impractical to carry out an *in vivo* study in humans to evaluate percutaneous absorption and pharmacokinetics of DEET and oxybenzone, piglets was subsequently the most appropriate substitute for this thesis. The experiments also demonstrated study feasibility in animal handling, sampling convenience and study cost, all of which were important and critical parameters for the success of a pharmacokinetic study *in vivo*.

5.3.2. Tape Strips

Stratum corneum, the outermost layer of the skin, can be removed sequentially by repeated applications of appropriate adhesive tape; this procedure is commonly known as tape stripping of stratum corneum [160,161]. With this approach, layers of corneocytes are removed and characterization of the revealed stratum corneum is then investigated [48,162,163]. Transport across stratum corneum is believed to be the rate-determining step in skin diffusion and penetration. Tape stripping allows not only investigation of barrier functions of the skin layers, but also determination of drug concentration profiles within the stratum corneum [164].

Chemical substances existing in the exterior environment are able to partially or fully breach the exposed skin surface of an individual; these compounds may also interact with dermal macromolecules, being metabolized, transported, and distributed to other target sites in the body [165]. Studies using tape stripping will therefore help explain the

rate and extent of penetration of topically applied compounds. It is also useful in correlating systemic absorption, distribution and elimination of various drugs in pharmacokinetic, pharmacodynamic and toxicological investigation.

Dividing tape strips into different groups aids in the evaluation of drug movement within the stratum corneum per each depth of the skin. Jacobi *et al* attempted to establish a relationship between the amount of stratum corneum removed and the compound recovered from tape strips in porcine [166]. While drug recovery from the skin was highly dependent on the sensitivity of analytical methods to be employed, it was concluded that drug diffusion within the stratum corneum could be satisfactorily characterized using up to twenty tape strips.

Twelve tape strips were collected from the piglets in this study to assess skin penetration and absorption. These tapes were further divided into four subgroups for proper evaluation of percutaneous permeation of DEET and oxybenzone. After 2 hours of the topical skin application, both DEET and oxybenzone increased their penetration when used in combination. However, the rate of penetration between DEET and oxybenzone was different; DEET permeated at a faster rate than oxybenzone, so that the recovery amount of DEET in Group 3 was much higher than Group 1 *(Figure 5.1)*. Oxybenzone showed the highest recovery in Group 2, but the recovery in Group 1 and Group 3 was comparable *(Figure 5.2)*. This penetration pattern was also observed with Product 1 and Product 2, in which only DEET or oxybenzone was present as a single active ingredient. The overall percentage of penetration was different for DEET and oxybenzone; DEET permeated transdermally much more than oxybenzone. It has been known that both DEET and oxybenzone act as percutaneous penetration enhancers; from this study it was

evident that enhancement of DEET was influenced more than that of oxybenzone when two compounds were used simultaneously, which might have resulted from their own individual enhancement effect and/or the combined enhancement effects of both components.

As the time elapsed, not only did the penetration rate of DEET and oxybenzone change, but the overall penetration amount also disappeared. This indicated a partition of DEET and oxybenzone within the skin layers and a systemic absorption of the compounds by the body. For the combined lotion (Product 3), DEET remained mainly in the stratum corneum at 12 hours after the application *(Figure 5.3)*, but moved much deeper into the skin at 48 hours as the last collection point *(Figure 5.4)*. Oxybenzone, on the other hand, remained generally within the stratum corneum, with a possible formation of a concentrated depot *(Figures 5.4 & 5.6)*. For single DEET lotion (Product 1), the movement of DEET within the skin layers was almost complete by 12 hours after one single topical application, suggesting a faster absorption and metabolism of the compound. The diffusion of oxybenzone within the skin from single sunscreen lotion (Product 2) was slower, with similar overall recovery percentage for both 12 hours and 48 hours, further confirming the formation of an oxybenzone depot within the skin layers. This might also indicate that transdermal penetration and systemic absorption of sunscreen oxybenzone would last longer but at a steadier rate.

It has been postulated that percutaneous penetration takes place by both intracellular routes and lateral diffusion once the dosage form is applied to the surface of skin [167]. Lipophilic molecules tend to penetrate at a slower pace than hydrophilic molecules. The lipophilicity of oxybenzone is lager than that of DEET, which explained

why DEET demonstrated a faster percutaneous penetration than oxybenzone. In addition, percutaneous diffusion and penetration of any substance also depend on the vehicles in which the molecules are incorporated. To make the substance readily available for diffusion on the surface of skin, the molecules have to leave the vehicles with ease. The effects of vehicles and/or formulations on percutaneous permeation of DEET and oxybenzone were not studied in this animal study. However, we did carry out a series *in vitro* diffusion experiments to evaluate vehicle factors, and found that emulsion-based lotions facilitated percutaneous permeation of DEET and oxybenzone [133].

Topical skin application of insect repellents and sunscreens is not intended for the active ingredients such as DEET and oxybenzone to penetrate into deeper layers of the skin. Once a compound passes the stratum corneum, it will be systemically absorbed into the general circulation. Previous studies have proven systemic absorption of both DEET and oxybenzone from topical skin applications [103,109]. However, concurrent use of both DEET-based repellents and sunscreens has never been investigated. This would be the first time in this thesis to prove that DEET and oxybenzone demonstrate a synergistic percutaneous enhancement to each other. Tape stripping seemed to be a satisfactory approach for quantitative assessment of DEET and oxybenzone penetration into the stratum corneum after topical skin application.

5.3.3. Plasma Concentrations

Systemic absorption of DEET and oxybenzone had been known. In this study, blood samples were collected from study animals for 48 hours post topical application of the formulations. Both DEET and oxybenzone were detected throughout the study from

all three test products; however, no metabolites were found in the plasma. DEET and oxybenzone from Product 3 demonstrated higher plasma concentrations than its counterparts from Products 1 and 2.

It is well known that transdermal penetration of drugs occurs via diffusion through intact epidermis and/or through skin appendages. The barrier function of mammalian skin is principally attributed to the intercellular lipid bilayers of the outer 10-20 μm of epidermis, stratum corneum. DEET and oxybenzone are both highly lipophilic molecules, which exhibit high permeability across the skin layers. Foldvari discussed several chemical penetration enhancers and possible mechanisms of permeation of lipid-mediated drug delivery [168]. Transdermal penetration enhancement can take place by modifying barrier properties of stratum corneum or altering intercellular lipids within stratum corneum, which are the rate-limiting step in drug diffusion through the skin. The enhancers can either act alone or by a combination of mechanisms, which is still waiting for further elucidation. Although percutaneous penetration mechanism from concurrent use of DEET and oxybenzone was not explored in the thesis, it was believed that they likely followed similar penetration kinetics as the rest of lipophilic molecules.

Maximum plasma concentration of DEET and oxybenzone was observed at 2 hours after the application *(Figures 5.7 & 5.8)*, which was in agreement with the percutaneous characteristics of most lipophilic compounds. This permeation pattern was also correlative to drug contents found in the skin tape strips at 2 hours, indicating a rapid absorption and distribution of both DEET and oxybenzone. Product 3 achieved higher plasma concentrations of DEET and oxybenzone than Product 1 and Product 2, demonstrating a percutaneous enhancement effect from the two components. This

observation also coincided with skin tape strips; recovery amount of DEET and oxybenzone from Products 1 and 2 at 12 and 48 hours was higher than that from Product 3. It appeared that DEET and oxybenzone in single-component lotions took longer to reach skin equilibrium through the tortuous route of stratum corneum than DEET and oxybenzone in combined lotion, where coexistence of the compounds facilitated both skin permeation and systemic absorption.

Pharmacokinetic parameters obtained from this study, in particular maximum plasma concentration (C_{max}) and area under the plasma-time curve (AUC_{0-48}), were in agreement to those previously published for individual DEET or sunscreen preparations [104,169]. Peak DEET absorption had been found to be around 1-2 hours after skin application, with an approximately plasma concentration of 20 μg/ml [170,171]. Permeation of oxybenzone was slower than DEET, because it was able to form a depot within the epidermis and continuously diffuse through the skin into the general blood circulation.

The statistically higher plasma concentrations of DEET and oxybenzone from a combined product were a concern in terms of systemic absorption of insect repellents and sunscreens. Toxicological properties of DEET and oxybenzone are both incomplete and inconclusive. It appears that concurrent use of DEET-based repellent and sunscreen preparations would become more prevalent with an increased awareness of West Nile virus and skin cancer. Both DEET and oxybenzone possess a greater percutaneous efficacy when applied topically to the surface of the skin. Repeated skin application of the compounds could inadvertently saturate the skin and increase the cumulative amount of percutaneous penetration and systemic absorption *in vivo*. Although percutaneous

penetration in humans is known to be lower than that in piglets, it is undesirable to have any topical compounds in the systemic circulation. Toxicokinetic profiles can be of great use in formulation development, especially with compounds such as DEET and oxybenzone. Findings from this *in vivo* study indicated a need to reformulate repellent and sunscreen preparations to reduce overall percutaneous absorption of the active ingredients, or modify application approach to optimize the use of both products.

5.3.4. Urine Metabolites

DEET and oxybenzone are known to be metabolized and eliminated via the urine. Qui *et al* studied the pharmacokinetics of DEET in beagle dogs [104]; the volume of distribution, systemic clearance, mean residence time and terminal elimination half-life after an intravenous administration were evaluated as 6.2 l/kg, 2.7 l/h/kg, 2.3 h and 2.6 h, respectively. The authors also reported that parameters from a dermal application were comparable to those of an intravenous administration. Metabolism of DEET in rats was studied by Selim *et al*; no DEET was detected in the urine after a dermal application, and the metabolites were identified as inactive [110]. Schoenig *et al* found from another study that no parent compound was present in the urine after rats were treated with ^{14}C-labeled DEET in oral and dermal administrations [111].

The results obtained from this study were similar to what has been reported. Even though DEET was detected in plasma at 48 hours after the topical application, DEET concentration in urine was below the quantification limit of the HPLC assay. This might be attributed to quick metabolism and excretion of DEET *in vivo*. No previous studies have evaluated metabolism and elimination of DEET and oxybenzone in the combined

dermal application. Extensive extravascular distribution of DEET was evident in this study; the mean residence time was higher than those previously published, which could have resulted from either the concurrent use of both compounds, or effects from the preparations. It was interesting to note that no difference was observed regarding DEET metabolites between Product 1 and Product 3. The explanation could again be attributed to a rapid DEET elimination *in vivo*. Elimination of DEET in humans was reported to be fast and complete; bioaccumulation in humans is therefore unlikely.

Oxybenzone is known to be metabolized to at least 3 compounds *in vivo*; urine is also known as the primary route of excretion for oxybenzone and its metabolites. Okereke *et al* had reported that a majority of the applied dose was recovered during 0-24 hours after a topical application. Oxybenzone undergoes rapid absorption and extensive distribution, which follows by a biphasic elimination. This may suggest distribution of the compound to other compartments in the body [79].

Similar absorption patters of oxybenzone through the skin were observed from this study. The maximum plasma concentration (C_{max}) of oxybenzone was significantly increased when combination product (Product 3) was used, indicating an enhanced percutaneous absorption with the help of DEET. The elimination pattern of oxybenzone was clearer than that of DEET; oxybenzone exhibited more distribution but slower elimination than DEET. This could explain the reason why oxybenzone was still detectable in the urine at 48 hours post the application. This finding confirms previous studies performed on human volunteers by some other investigators [103,124]. Considerable amount of work is still underway, and more results are needed to

understand the penetration enhancement effect of oxybenzone when combined with other compounds.

5.3.5. In Vitro and In Vivo Correlation

Establishing a definitive relationship between data obtained from *in vitro* experiments and *in vivo* tests allows for not only a better understanding and interpretation of the study results, but also a more effective direction to further experimentation and study designs. *In vitro* and *in vivo* correlation of drug administration by oral drug administration has seen certain degrees of success for tablets and capsules. *In vitro* dissolution testing has been recognized as one of the well-defined experiments to correlate drug bioavailability *in vivo*. Correlation of transdermal drug applications was also attempted with numerous compounds; however, limited success has been achieved with this route of administration because of complexity of drug diffusion across the skin as well as variations in experimental settings. It was well understood that different experimental conditions or parameters could yield variable results of data correlation. Since enhancement in percutaneous penetration of DEET and oxybenzone across piglet skin was observed both *in vitro* and *in vivo*, when the two components were present simultaneously, results obtained were analyzed for plausible data correlation and interpretation.

Three different solvents were used in the *in vitro* diffusion experiments to assess percutaneous characterization of DEET and oxybenzone; three commercially available repellent and sunscreen products were tested in piglets for their *in vivo* percutaneous permeation. The increases in percutaneous penetration in comparison to single-

component preparations were then plotted to check for *in vitro-in vivo* data correlation. *Figures 5.9-5.12* show the correlation of DEET between *in vivo* application and *in vitro* diffusion at four different concentrations, respectively. The increase in percutaneous penetration of DEET was more significant under *in vitro* experimental conditions than *in vivo* setting in living animals. In addition, the overall patterns of correlation were similar; PEG was the solvent that produced the smallest percutaneous permeation of DEET across piglet skin, while ethanol was the solvent that produced the largest percutaneous permeation of DEET across piglet skin. Permeation of DEET was also dependent on concentration used. Lower concentration of DEET appeared to result in larger penetration increase; with the increase of concentration used, permeation changes decreased for both PEG and propylene glycol. Ethanol was the only solvent that demonstrated enhanced permeation of DEET at all four concentrations, indicating the undesirable characteristics of this solvent as a percutaneous absorption enhancer. Excipients or additives present in the commercial products used for *in vivo* testing were unknown. However, *in vitro* diffusion studies previously conducted in our laboratory indicated a dependence of permeation of DEET and oxybenzone on formulation type [133]. The permeation increase of DEET from *in vivo* studies was the combined effects of DEET, oxybenzone and excipients in the preparation.

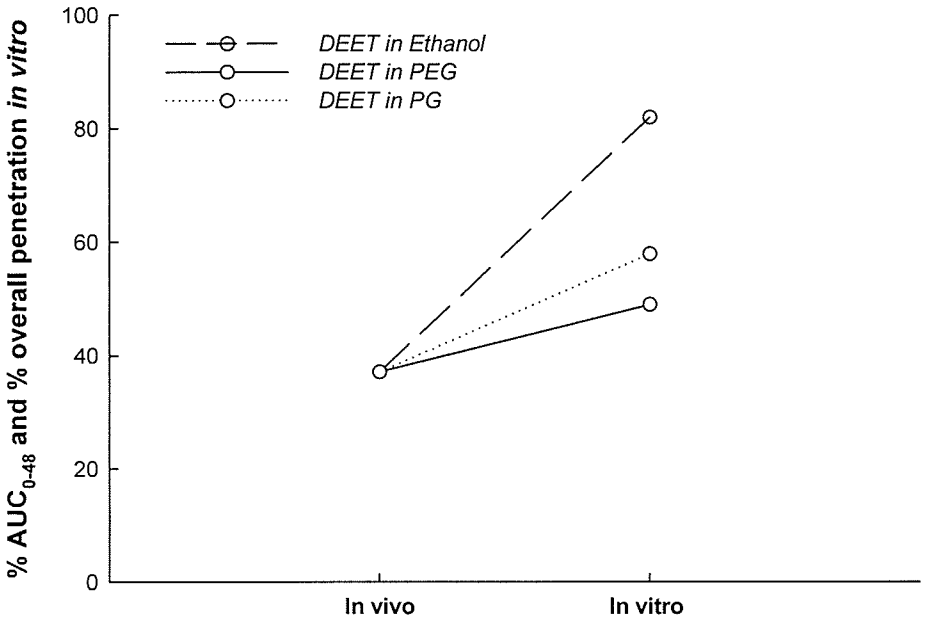


Figure 5.9. In vivo and in vitro correlation of DEET (1.0 mg/ml in vitro)

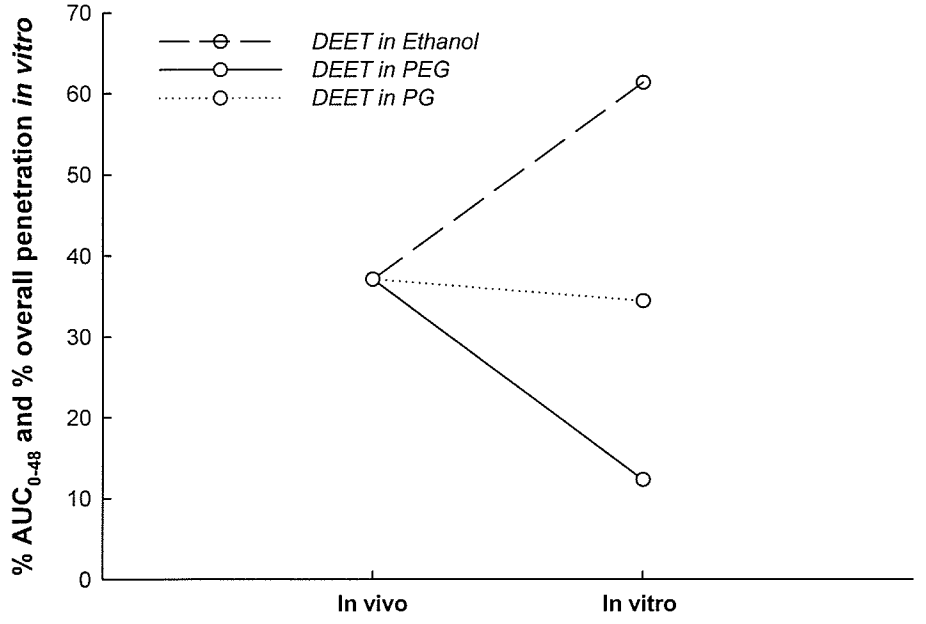


Figure 5.10. In vivo and in vitro correlation of DEET (2.5 mg/ml in vitro)

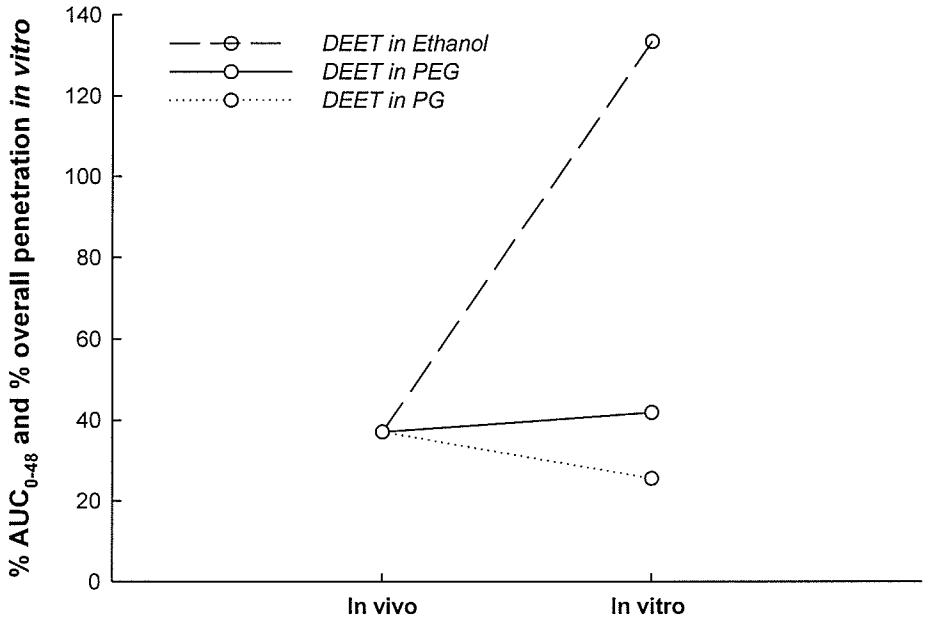


Figure 5.11. In vivo and in vitro correlation of DEET (5.0 mg/ml in vitro)

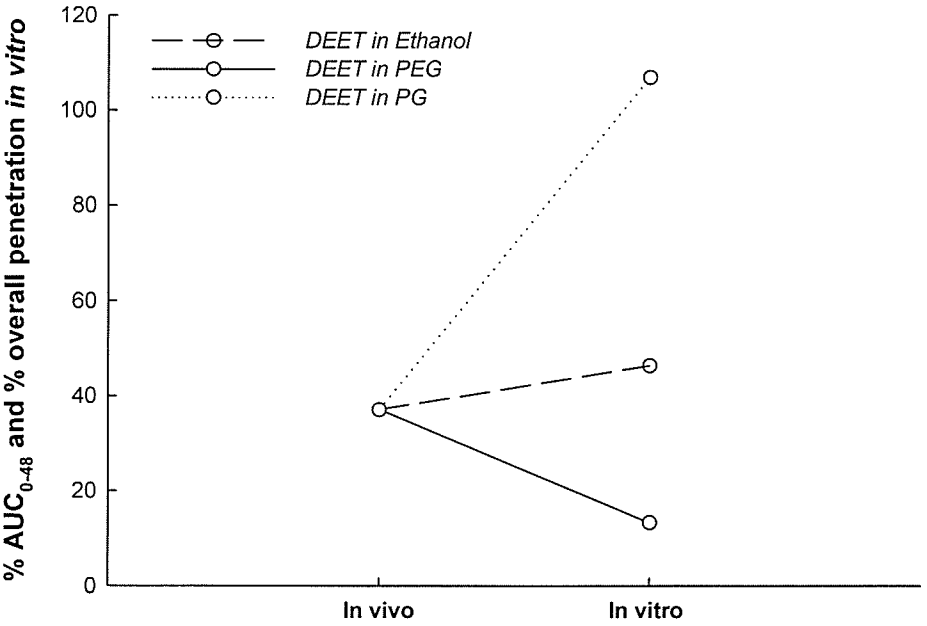


Figure 5.12. In vivo and in vitro correlation of DEET (10.0 mg/ml in vitro)

In vitro and *in vivo* correlation of oxybenzone did not generate similar results as DEET. The overall plot patterns demonstrated variations among the four concentrations used. *Figures 5.13-5.16* show the correlation of oxybenzone between *in vivo* application and *in vitro* diffusion using the same data treatment schemes, respectively. The increase in percutaneous penetration of oxybenzone was more statistically significant in living animals than under *in vitro* experimental conditions. PEG was found to be the solvent that could increase percutaneous absorption of oxybenzone at higher application concentrations, with the presence of DEET. Ethanol did not actually increase the permeation of oxybenzone. The contribution factor from application concentration to percutaneous permeation of oxybenzone was not as obvious as what had been observed with DEET; this might have been attributed to differences in the solubility and partition coefficients between the two compounds. DEET possessed a higher solubility in all study solvents than oxybenzone, which could contribute to faster and enhanced percutaneous penetration. In addition, oxybenzone demonstrated a stronger affinity to skin than DEET, which could facilitate prolonged retention of the compound within the skin layers without penetration. Again, permeation increase of oxybenzone from *in vivo* studies was the combined effects of oxybenzone, DEET and excipients in the preparation.

The effects of the blood circulation and skin metabolism on percutaneous penetration of DEET and oxybenzone in living animals were not investigated and correlated, because these two parameters that contribute to the overall systemic absorption were missing during *in vitro* diffusion. While this correlation of *in vitro* and *in vivo* data was a good practice, more systematic data analysis is required to create a feasible correlation model that could be used for all transdermal drug delivery systems.

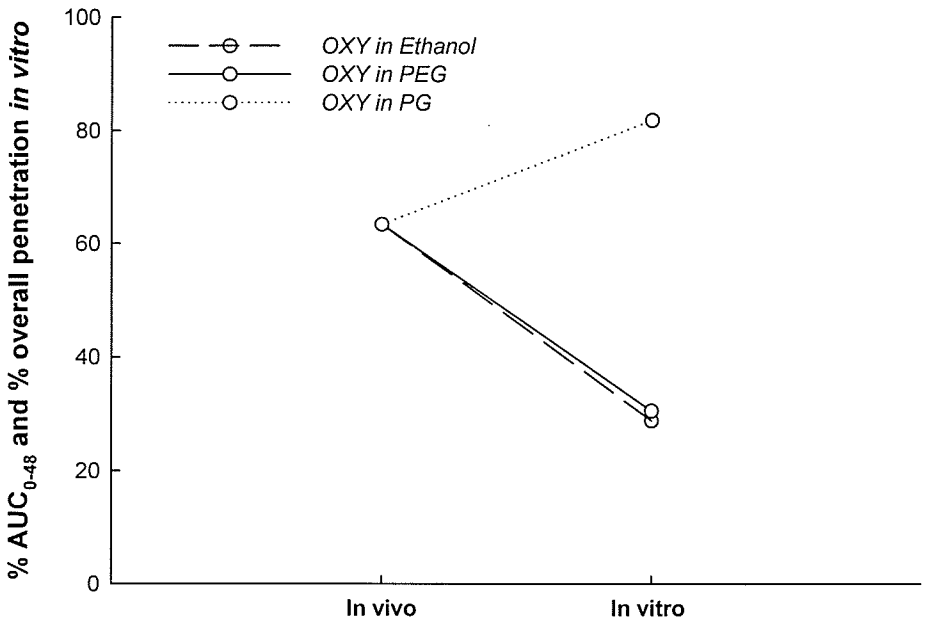


Figure 5.13. In vivo and in vitro correlation of OXY (1.0 mg/ml)

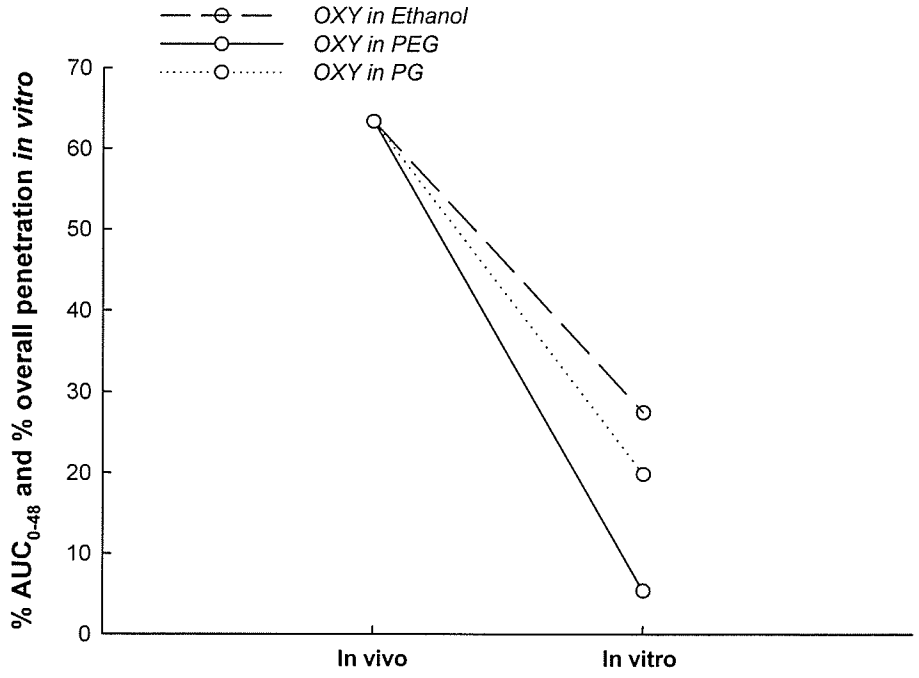


Figure 5.14. In vivo and in vitro correlation of OXY (2.5 mg/ml)

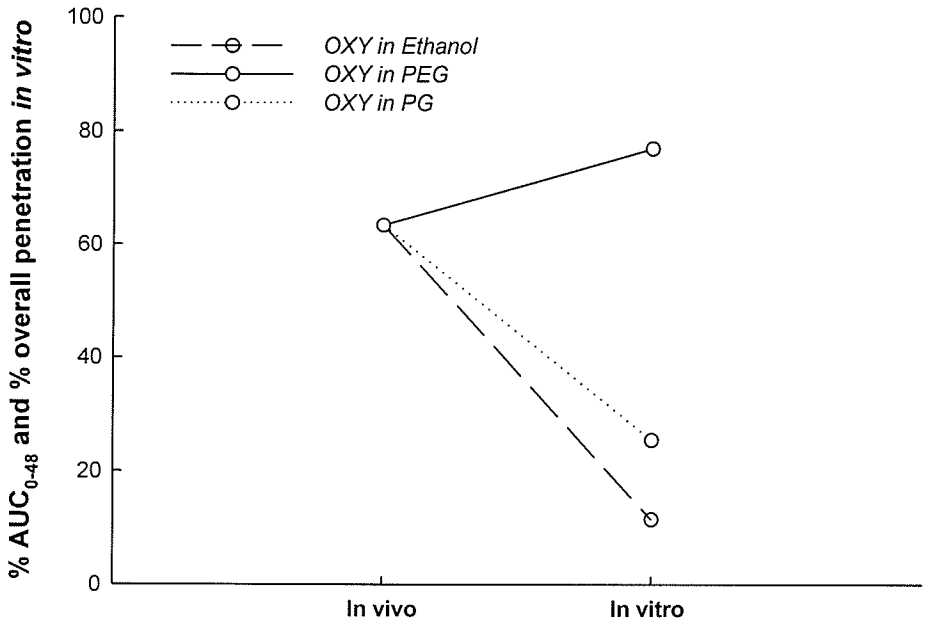


Figure 5.15. In vivo and in vitro correlation of OXY (5.0 mg/ml)

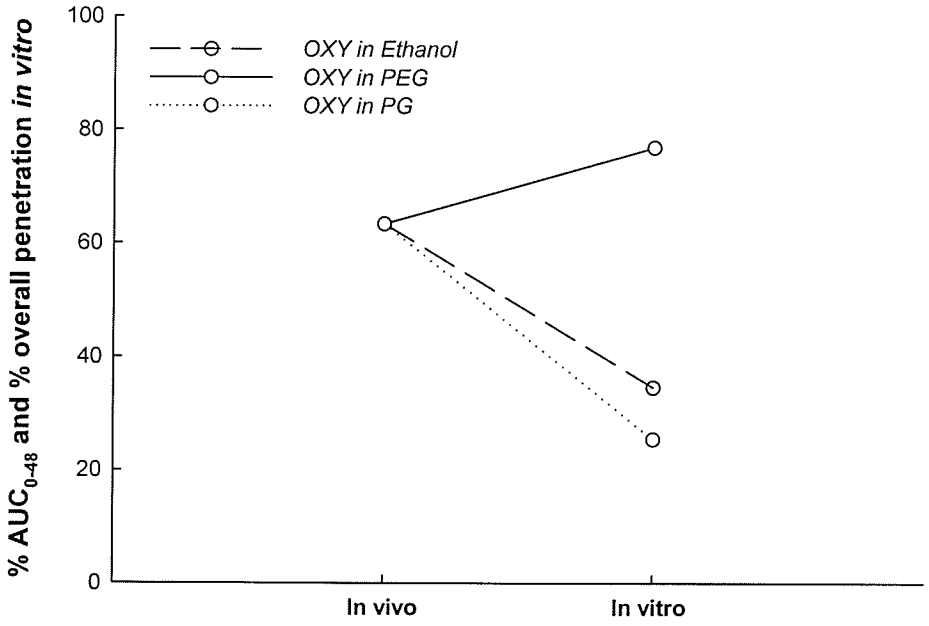


Figure 5.16. In vivo and in vitro correlation of OXY (10.0 mg/ml)

5.4. Conclusions

Appreciable amounts of DEET and oxybenzone were shown to penetrate across the skin after topical application of repellent and sunscreen lotions in an animal model. The increases in the rate and extent of percutaneous penetration and systemic absorption were consistent for skin tape strips, plasma and urine samples. Similar to what had been observed *in vitro* with the three membrane models, concurrent use of DEET and oxybenzone resulted in synergistic permeation of the two compounds *in vivo*. Both DEET and oxybenzone demonstrated a fast percutaneous penetration and systemic absorption; however, the metabolism and elimination of DEET was faster than that of oxybenzone, due possibly to a prolonged retention of oxybenzone within the skin. Correlation between the *in vitro* diffusion and *in vivo* penetration was performed by plotting the relationship between the AUC values and the overall *in vitro* penetration; the results obtained provided some insights into the influences of the application preparation, excipients and additives on the overall penetration of DEET and oxybenzone through the pig skin.

Chapter 6

Conclusions

Percutaneous penetration and systemic absorption of chemical compounds, specifically therapeutically active molecules, have been used for effective drug administration for decades, By applying this unique and innovative route of drug administration, it is possible for pharmaceutical investigators as well as medical researchers to accomplish desirable outcomes for the control and management of diseases. Percutaneous enhancement is one of the approaches commonly used to improve transdermal permeation of those compounds that otherwise would have difficulty in diffusing across the skin on their own capability. Using appropriately, permeation enhancement can achieve desirable efficacy for special purposes; however, it could also result in undesirable adverse effects, such as what we had observed with the concurrent use of DEET-based insect repellent and sunscreens in this thesis.

DEET and oxybenzone were able to generate synergistic transdermal permeation to each other when they were simultaneously used. This transmembrane characterization was proved both *in vitro* with diffusion studies and *in vivo* in a living animal model. Furthermore, permeation of DEET and oxybenzone was influenced by numerous parameters, including preparation materials, application amount, membrane models and experimental conditions. While the two test compounds acted as permeation enhancers, studies revealed that oxybenzone influenced the permeation of DEET more than DEET did to oxybenzone. Both DEET and oxybenzone were absorbed systemically through the skin after topical application in piglets. Percutaneous absorption and metabolism of DEET was fast and complete; no DEET or its metabolites were detectable in the urine 48 hours after one singly skin application. Oxybenzone on the other hand was capable of delivering percutaneous absorption at a slower rate from a skin depot; its principal

metabolites were also found in the urine at 48 hours. Concurrent use of commercially available repellent and sunscreen preparation produced significantly higher blood concentrations of DEET and oxybenzone than its single-component counterparts in piglets, which indicated a potential for increased exposure to topically-intended chemicals such as DEET and oxybenzone. This was also the first time that these unique percutaneous permeation characteristics were observed and reported, both *in vitro* and *in vivo*.

With the beginning of a new 21st century, the standards of living and quality of life have been significantly improved around the globe. The general public has also been continuously seeking not only for healthier life styles through various activities but also for better self-prevention and protection against all diseases. Skin cancer and mosquito-borne diseases are two types of primary health threats that exert serious impacts on those who enjoy active and dynamic life styles outdoors. Insect repellents and sunscreens have been the well-accepted and extensively-used consumer care products for decades; they will continue to remain as one of the most effective and economic healthcare products in the future.

User guidelines concerning the application of insect repellents and sunscreens are much less stringently regulated than those of pharmaceutical products. They also subject to continuous revisions in accordance to latest findings in pharmaceutical research and development; both environmental changes and migration of diseases around the world also prompt updates of guidelines for all consumer care products. With the findings obtained from this thesis, it appears that concurrent use of DEET-based insect repellent and sunscreen preparations is not a desirable approach for the protection against

mosquito-borne diseases and skin cancer. Moreover, systematical investigations should be carried out to reevaluate efficacy and safety of concurrent use of topical skin care products and/or pharmaceutical dosage forms. Results from research in these fields can not only contribute to a better application outcome of all skin care products by the general public, but also advance our knowledge in understanding skin as one of the administration sites for novel and noninvasive drug delivery.

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