Pharmacokinetic and Toxicological Characterization of Repellent DEET and Sunscreen Oxybenzone

By

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THIS THESIS IS DEDICATED TO

MY PARENTS

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DISCLAIMER

The presentation of this thesis is in the form of a sandwich thesis. Daryl Fediuk is the first author of Chapter 2 and 3, which are multi-authored manuscripts and have been published in the *International Journal of Toxicology* and *Biopharmaceutics and Drug Disposition*, respectively. Daryl Fediuk is also the first author of Chapter 4, which is a multi-authored manuscript and has been submitted for publication to the *International Journal of Toxicology*.

For Chapter 2 data, Joshua Raizman performed all cellular studies in rat primary cortical neurons and astrocytes in the laboratory of Dr. Fiona Parkinson. The animal study and sample collection was performed by Daryl Fediuk, with assistance from Congcong Xie, Robert Hardy, and the Department of Zoology at the University of Manitoba. Behavioral testing was performed by Daryl Fediuk with assistance from Tao Wang, Robert Hardy, and Congcong Xie. Data analysis was completed by Daryl Fediuk.

For Chapter 3 data, the animal study, the intravenous administration and the topical application were performed by Daryl Fediuk with technical assistance from Tao Wang, Heather Simpson, Susan Blair and Teri Whittington. Sample collection after intravenous and topical dosing was done by Daryl Fediuk and Dr. Xiaochen Gu. Concentration measurement was completed by Daryl Fediuk. For the cell culture assay, Yufei Chen assisted in the initial growth, maintenance and seeding of rat hepatoma cell line 1548 onto 96-well plates in the laboratory of Dr. Frank Burczynski. Removal and addition of cell media containing DEET and oxybenzone at pre-determined concentrations was done by Daryl Fediuk. Both Daryl Fediuk and Yufei Chen were involved in the addition of Cell Proliferation Reagent WST-1 (water-soluble tetrazolium salt) and the utilization of ELx 808 Ultra Microplate Reader for absorbance measurement. Data analysis was completed by Daryl Fediuk.

For Chapter 4 data, concentration measurement of all metabolites was done by Daryl Fediuk. Yufei Chen and Daryl Fediuk had similar roles in the rat hepatoma cell line 1548 study performed with DEET and oxybenzone metabolites as was done previously with the two parent compounds. Data analysis was completed by Daryl Fediuk.

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.

Daryl Fediuk

April 2012

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ABSTRACT

Insect repellent *N,N*-diethyl-*m*-toluamide (DEET) and sunscreen oxybenzone are commonly incorporated into commercially available repellent and sunscreen preparations. Both compounds have demonstrated an increased percutaneous permeation and systemic disposition after concurrent application *in vitro* and *in vivo*. The permeation enhancement between DEET and oxybenzone not only compromises their respective protective efficacy against biting insects and UV radiation, but also potentiates toxicological properties in susceptible subjects. The pharmacokinetic and toxicological profiles from concurrent use of DEET and oxybenzone were evaluated and compared in this thesis.

DEET and oxybenzone were administered by intravenous and topical routes in rats, either alone and/or in combination, to compare the pharmacokinetics of parent compounds and their primary metabolites *in vivo*. To evaluate toxicological characteristics, rat primary cortical neurons and astrocytes, and rat hepatoma 1548 cells were exposed to DEET, oxybenzone and their metabolites *in vitro*, and cell viability was analyzed. Various behavioral testing protocols were also performed to assess arousal, locomotion, habituation, and motor coordination of rats over a 30-day study period.

Concurrent topical application of DEET and oxybenzone enhanced the disposition of DEET and its metabolites in rats, but did not consistently affect the distribution of oxybenzone and its metabolites. The disappearance of DEET from skin application site was accelerated; its apparent elimination half-life was decreased while its plasma and tissue concentrations were predominantly increased. Cellular toxicity occurred at 1 µg/ml for neurons and 7-day exposure for both astrocytes and neurons. Viability of hepatoma cells was also reduced when treated with DEET, oxybenzone and their metabolites, either alone or in combination, most notably after 72 hours of exposure. However, no overt signs of toxicity were observed from behavioral testing in rats after a 30-day topical study.

The pharmacokinetic data obtained was beneficial in understanding and elucidating absorption and biodistribution of DEET and oxybenzone *in vivo*. The toxicological data suggested that the risk for increasing adverse effects from concurrent skin application of repellents and sunscreens would be low and marginal in healthy individuals. Nevertheless, further studies should be carried out to assess the long-term health impact of these compounds in susceptible subjects, especially at higher application doses.

Chapter 1

Introduction

1.1. Introduction to Percutaneous Drug Absorption

For centuries, people have applied chemical substances to the surface of the skin, for a variety of reasons. These topical preparations, in the form of lotions, sprays, gels, ointments, suspensions or pastes, have been applied for both non-medicinal and/or medicinal intentions. Non-medicated substances are applied to the skin for an assortment of purposes, ranging from traditional cultural skin markings, to fragrances and body sprays, and to cosmetic and consumer-care skin products. Alternatively, when active pharmaceutical ingredients (APIs) are present in a product, the objective becomes to establish a pharmacodynamic response on the skin, where the pharmacologically active components will produce a local effect around the site of application. Xeroderma, eczema, acne, and pruritus are skin conditions that require direct application of medications on or near the superficial surface of the affected skin. Transdermal drug delivery is an approach to deliver drugs across the skin membrane, to produce a systemic pharmacodynamic response achieved through drug diffusion and permeation, which also bypasses the hepatic first-pass metabolism. At present, novel transdermal drug delivery systems have been successfully utilized in numerous forms, e.g., a topical patch for smoking cessation, an iontophoresis system for plantar fasciitis, and microneedles for vaccine administration. Consequently, these innovative techniques have allowed humans to penetrate the natural protective barrier of the skin to attain desired therapeutic effects. Scientists have gradually realized the importance of skin as a route of drug administration for many active ingredients. As a result, a significant amount of time and resources is being invested in order to further understand percutaneous mechanisms as a novel treatment strategy for a variety of medical conditions.

1.2. Percutaneous Drug Absorption

1.2.1. Human Skin

Skin is the largest organ of the human body, which possesses a unique and complex structure that forms an effective physical barrier between the underlying organs and the external environment. The skin is involved in a variety of biological and physiological functions that are essential for human survival. One of the most obvious is the natural defense mechanism that the skin provides, shielding all internal organs from invasion of foreign pathogens, toxins, and radiation [1]. Acting as a sensory monitor, the skin also detects various external stimuli such as heat, cold, pain and pressure, while communicating with internal systems including the endocrine system, nervous system, and immune system [2]. Other functions of human skin include temperature regulation, vitamin D synthesis, lipid regeneration and storage, and substance metabolism and disposition.

Human skin is composed of several anatomically distinct layers that include: the non-viable epidermis (stratum corneum), the viable epidermis (stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale), and the dermis. A cross-section of the skin is shown in **Figure 1.1**. Anchoring the skin to the muscle tissue and bone below lies the subcutis or hypodermis. The thickness of the subcutis varies, depending on the individual subject or the particular area of the body. The subcutis consists primarily of fat deposits (adipose tissue), which works as an insulator to conserve heat and as a protective layer for all internal organs of the body [3].

Directly above the subcutis is the dermis, a 1-2 μm thick structure containing capillaries, lymph vessels, sebaceous glands, sweat glands, hair follicles and nerves.

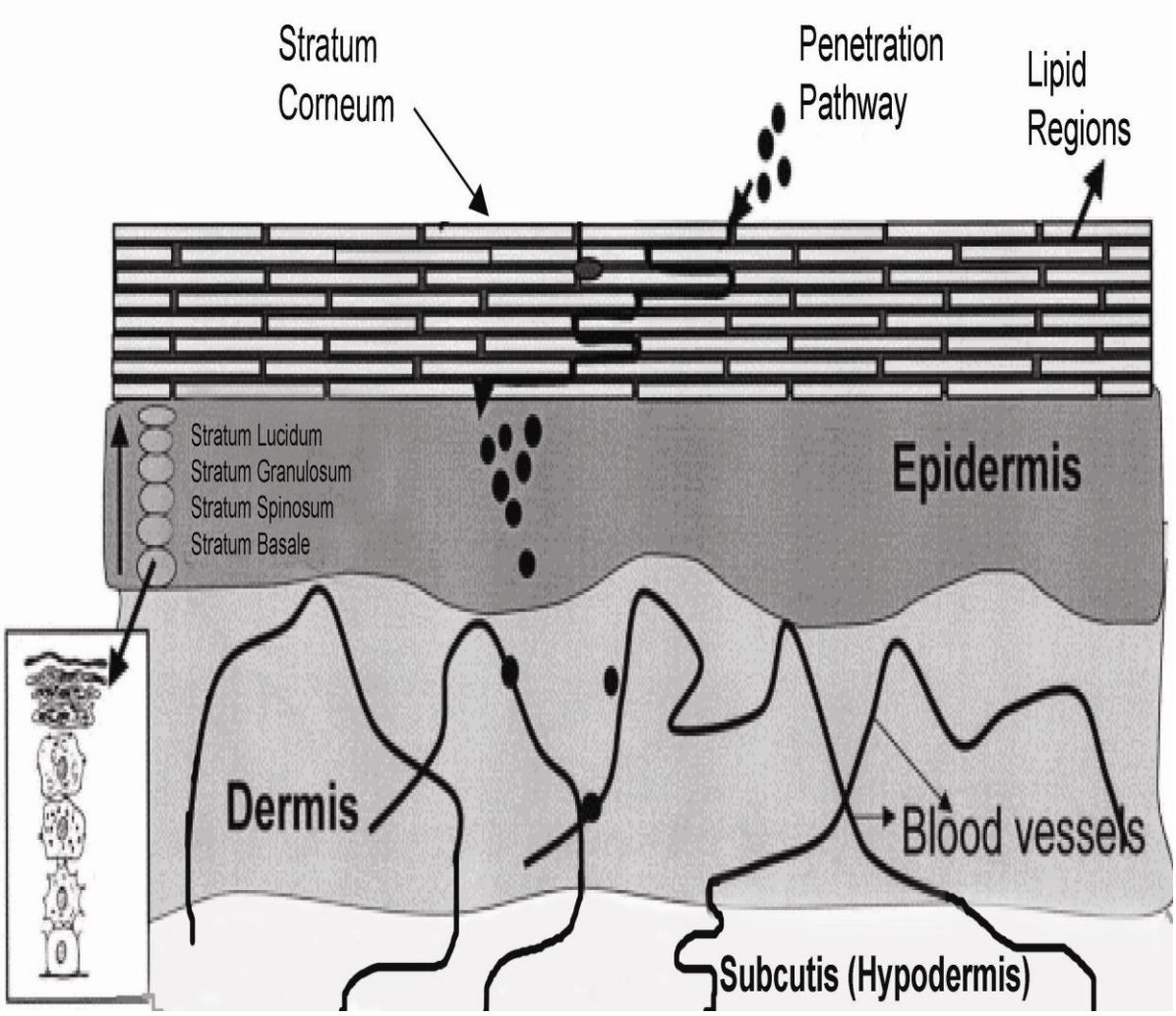

Figure 1.1. The differentiation of skin [4].

There are two distinct sub-layers of the dermis, i.e., the upper papillary layer and lower reticular layer, which are rich in two types of extracellular matrices, collagen and elastin. Collagen provides the dermis with mechanical support while elastin is responsible for tissue elasticity and stretch-ability [5].

Above the dermis is the viable epidermis, which is approximately 50-100 μm in thickness and consists of four separate layers of highly differentiated cells: stratum basale, stratum spinosum, stratum granulosum, and stratum lucidum. The stratum basale

is a proliferative layer; cuboidal-shaped cells (or basal keratinocyte cells) are active and prolific from the continuous basal lamina, differentiating and migrating towards the skin surface [4]. Other cells found in the stratum basale include melanocytes (pigment cells), Merkel cells (sensory receptors), and Langerhans cells (involved in immune responses). As basal keratinocyte cells continue to divide and progress upward (see **Figure 1.1** inset), they begin to form the stratum spinosum, where the cuboidal cellular shape begins to flatten into the more mature keratinocyte, or squamous cell. In the stratum spinosum, prickle cells (or desmosomes) and tonofilaments interconnect the surrounding keratinocytes with their spiny outcroppings, creating a specialized cell-cell junction. As keratinocytes continue progression upwards, they synthesize an abundance of lipids, eventually taking the form of keratohyaline granules, and growing into the next layer, stratum granulosum. The stratum granulosum helps form a watertight barrier in the skin to prevent excessive fluid loss. Cell mitosis begins to decline in the stratum granulosum, and these dying cells start to lose their nuclei. Rising above to the top of the viable epidermis is the stratum lucidum, which contains cells filled with the pre-keratin protein eleidin, and is responsible for the very thick skin that is located on the palms of hands and soles of feet [5]. Keratinocytes continue their differentiation upwards from the stratum lucidum to the stratum corneum, where keratin-filled cells are formed with help from the protein filaggrin. This cellular layer is also known as corneocytes or horny cells.

The uppermost layer of the skin is known as non-viable epidermis, or stratum corneum. The stratum corneum is only 10-20 μm in thickness, but it is a highly impermeable barrier to a majority of chemical substances. There are three principal

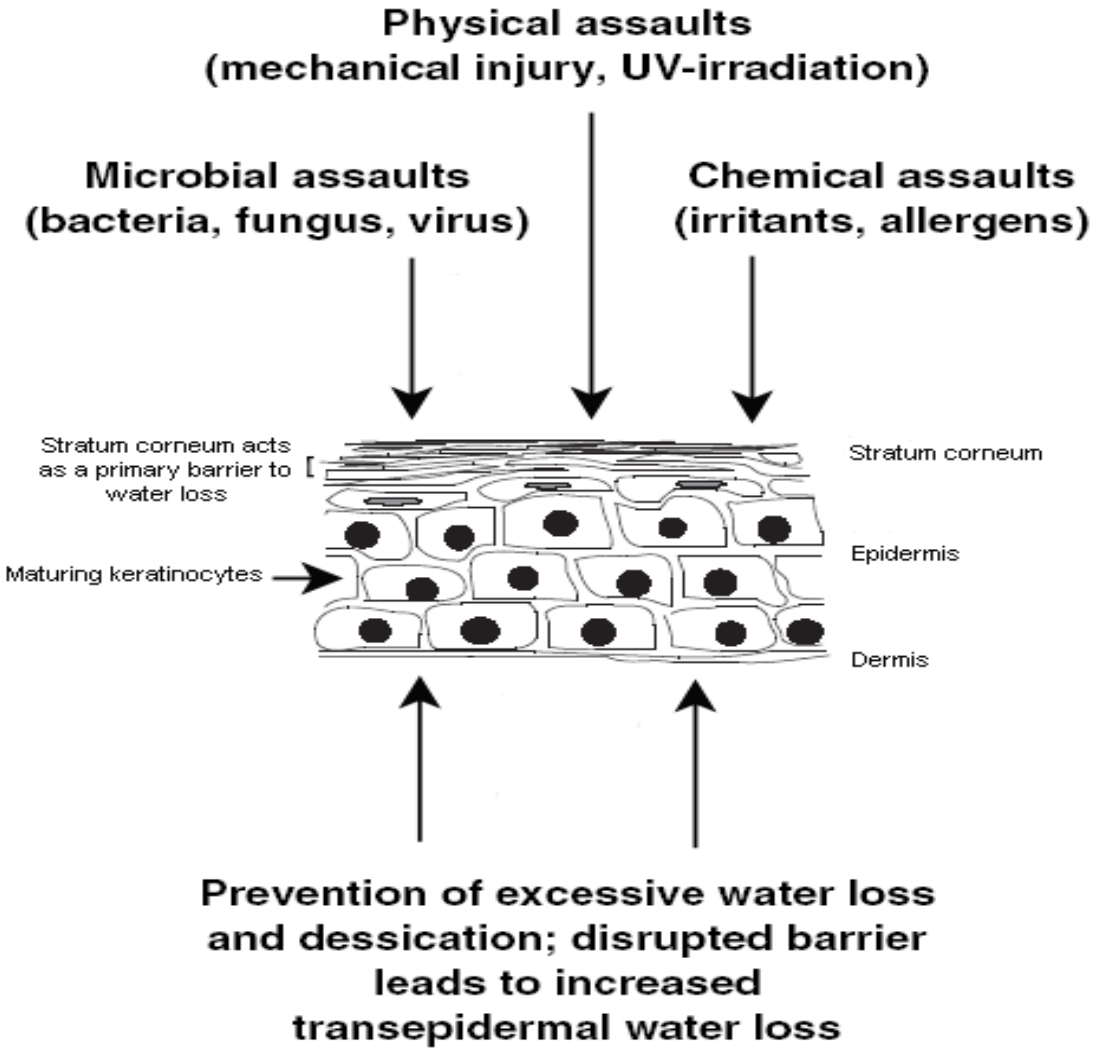

Figure 1.2. Function of the stratum corneum [6].

components that make up the stratum corneum which include: ceramides 1-6, fatty acids, and cholesterol. Ceramides are the largest group of lipids in the stratum corneum, representing approximately 50% of the total lipid weight. Cholesterol accounts for approximately 25-30% of the total stratum corneum lipids while free fatty acids (consisting mainly of straight-chained, chemically saturated fatty acids with 14-28 carbon

atoms), make up to about 15% [5]. The stratum corneum is arranged in approximately 15 layers of flattened, hexagonal corneocytes on top of one another, which are sometimes described as a heterogeneous "Brick and Mortar" model, corneocytes being the bricks and the intercellular lipids being the mortar [7]. The stratum corneum has lower water content (15-20%) than the viable epidermis (70%). Together with the lipid bilayers around the corneocytes, this exceptional structural matrix not only creates a major barrier to percutaneous penetration of foreign chemical compounds but also prevents internal water from evaporating [8]. A final step in the differentiation process involves the continuous desquamation of the corneocytes from the horny layer, which further strengthens the barrier properties of the stratum corneum against xenobiotics, radiation and microbes (as shown in **Figure 1.2**).

1.2.2. Skin Appendages

There are four primary skin appendages: hair follicles and their associated sebaceous glands, eccrine/apocrine sweat glands, and fingernails and toenails. Hair follicles vary considerably in shape and size. Rapidly differentiating matrix cells lead to production of the hair shafts, where melanocytes at the base of hair shaft also produce pigment [9]. Hair follicles and their connected sebaceous glands exist throughout human skin, with the exception of palms and soles. Human sebaceous glands are responsible for the production and secretion of sebum, a mixture of nonpolar lipids [10]. Sebum is capable of protecting the skin by exhibiting antimicrobial activity with both pro- and anti-inflammatory actions. Eccrine sweat glands are responsible for the thermoregulation of the skin where sweat is released from the coiled gland of the eccrine unit. Sometimes,

emotional stress and stimuli may also lead to sweat secretion. Apocrine glands excrete a more viscous but smaller quantity of sweat than eccrine glands. Sweat from apocrine glands consists of sialomucin, a substance that produces a faint odor after interaction with microbes on the skin surface. As a result, apocrine glands are also categorized as scent glands [11]. Both fingernails and toenails contain keratin from the keratinization process; they possess physical protective functions for fingers and toes. Even though skin appendages play an important role in skin anatomy and physiology, their overall involvement in percutaneous drug permeation is marginal because of their relative small proportion of the entire skin surface.

1.2.3. Percutaneous Permeation

An important determinant governing the rate and extent of percutaneous absorption is the relative solubility of a compound in water and oil (octanol). This solubility ratio is known as partition coefficient. Therefore, partitioning into the stratum corneum by an active pharmaceutical ingredient (API) often dictates its overall permeation into the skin layers. However, before an API can reach the skin surface, it has to diffuse out of the formulation in which it was applied to the skin. Its solubility and diffusivity characteristics may also influence its permeation through the lipids of the stratum corneum [12].

Figure 1.3 shows three different penetration routes by an API across the stratum corneum, dependent upon the nature of the substance. The transcellular route takes a direct path across corneocytes and intervening lipids, the intercellular lipid route proceeds with a tortuous path between corneocytes, and the transappendageal route utilizes hair

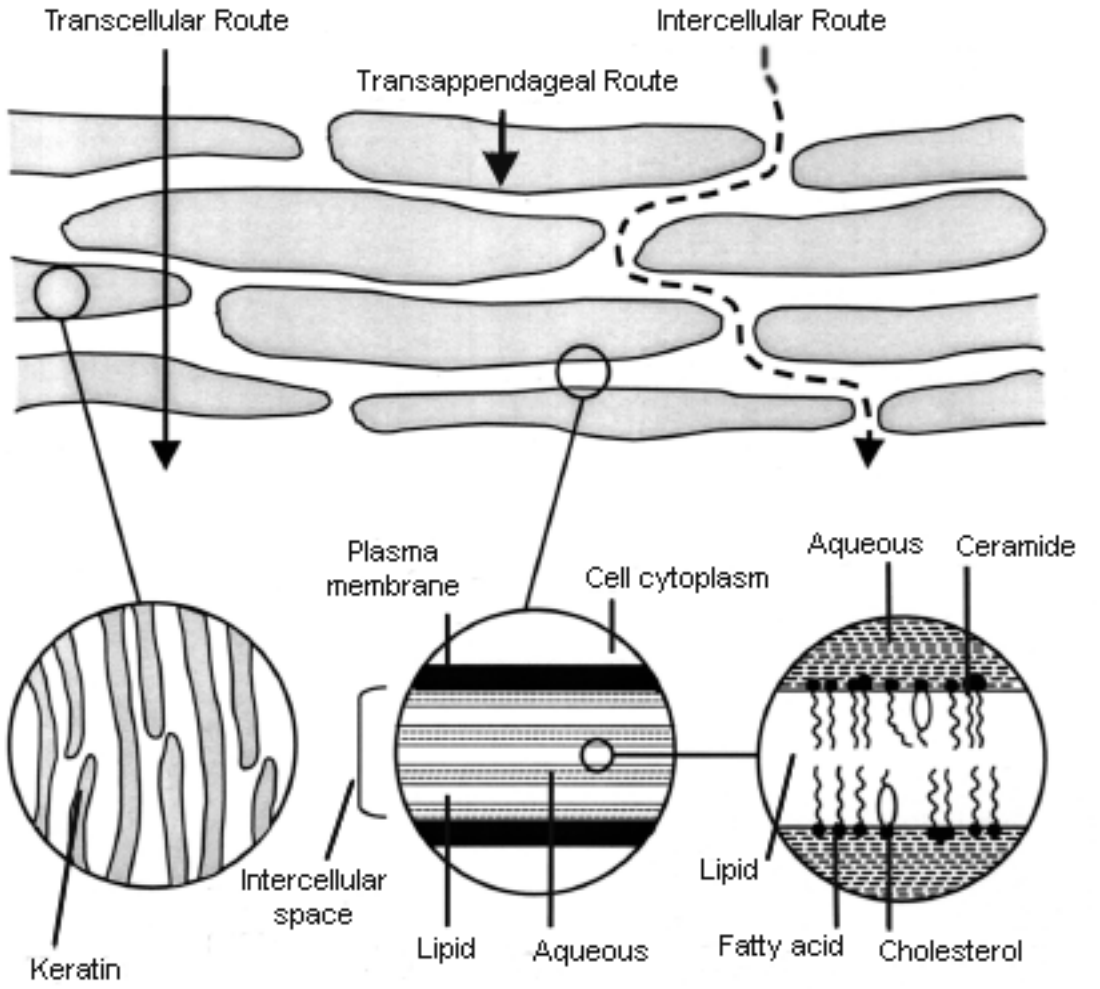

Figure 1.3. Penetration route through stratum corneum [7].

follicles and sweat glands as a shunt pathway through the intact epidermis. Lipophilic compounds, with larger partition coefficients (octanol/water log K >2), have a tendency to penetrate the similarly lipophilic intercellular spaces, therefore taking the intercellular route. Hydrophilic compounds may penetrate the stratum corneum both transcellularly and through hair follicles and sweat glands. Nevertheless, it is also possible for a lipophilic compound to penetrate in transcellular and transappendageal mechanisms.

Because skin appendages occupy only 0.1% of the total human skin surface, contribution from transappendageal pathway is usually negligible [7].

Another factor that may influence drug permeation is the formation of a reservoir or depot in the stratum corneum, the keratin spaces, the follicular openings and/or the skin surface folds [13]. Once an API has diffused through the stratum corneum, it will undergo a second partitioning as it passes from lipid-rich regions of the horny layer to the largely aqueous environment of the viable epidermis [12]. Transepidermal transport involves APIs permeating intracellularly, through the cells, or intercellularly, in between the epidermal keratinocytes. However, this tortuous intercellular route is generally considered to be the principal barrier to most drug permeation [14].

1.2.4. Penetration Enhancement

Certain chemical substances possess an innate ability to enhance permeation of other compounds through the resistant barrier of the stratum corneum. In transdermal drug delivery, this enhancement property is preferred because it facilitates maximal percutaneous penetration and optimal systemic absorption. However, when a compound is intended only for local topical effect, penetration enhancement would be unnecessary and totally unacceptable. Ethanol, for example, has been recognized as a penetration enhancer, which is attributed to two main effects, a "push effect" that increases or optimizes the thermodynamic activity across the skin due to evaporation of ethanol, and a "pull effect" that facilitates drug molecular permeation by reducing the barrier property of the stratum corneum [15]. The "pull effect" is initiated when ethanol increases the rotational freedom of lipid acyl chains, thereby increasing fluidity of the stratum

corneum, disrupting the lipid bilayer structure, and resulting in a reversible decrease of barrier function. Other possible mechanisms of penetration enhancement include increasing molecular diffusivity within the skin, modifying the partition coefficient of a chemical compound, promoting fast drug release from a preparation, and increasing hydration of the stratum corneum. Water can sometimes act as a penetration enhancer, where the stratum corneum is hydrated to make skin cells swell, thereby opening up the horny layer, and allowing increased drug flow through these newly exposed pores.

1.2.5. Fick's Law of Diffusion

Most drug absorption through the skin is governed by passive diffusion. Fick's Law of Diffusion describes this process mathematically and hence is a fundamental concept for understanding topical drug permeation through the stratum corneum. A modified Fick's Law of Diffusion is expressed in the following equation [3]:

$$Q = \frac{P}{MW \cdot \Delta X} \cdot \Delta C \cdot A$$

Where Q is the net rate of diffusion

P is the permeability of the stratum corneum to the permeant

MW is the molecular weight of the permeant

ΔX is the thickness of the stratum corneum (or membrane)

ΔC is the concentration gradient across the membrane

A is the surface area of skin available for application

The above equation illustrates the important relationship among drug diffusion, concentration utilized, and application surface. Drug transport across the stratum corneum is directly proportional to P, ΔC and A. An increase in the permeability of the membrane (P) will lead to an increased net rate of diffusion (Q). An increase in the magnitude of the concentration gradient (ΔC) will lead to a faster net rate of diffusion (Q). An increase in the surface area of the membrane through which diffusion is taking place (A) will lead to an increased net rate of diffusion (Q). Drug transport across the stratum corneum is inversely proportional to MW and ΔX. An increase in the molecular weight of the substance permeating (MW) will lead to a decreased net rate of diffusion (Q). Likewise, an increase in membrane thickness (ΔX) will lead to a decrease in net rate of diffusion (Q).

1.3. Insect Repellents

The use of insect repellents dates back to ancient times, when human civilization would ward off biting insects by smoke, tars and plant oils. Before the introduction of *N*,*N*-diethyl-*m*-toluamide (*N*,*N*-diethyl-3-methylbenzamide or DEET), several active insect repellents had been utilized to protect humans from mosquitoes, flies, fleas, and ticks. Discovered in 1901, citronella was one of the most widely used repellents in the 1940s. However, the repellency efficacy of citronella lasts for only 20-30 minutes [16]. Dialkyl phthalate was one type of earlier, synthetic insect repellents discovered in 1988, but its use was discontinued due to known toxicity [17]. Indalone was also used as an insect repellent, even though it possessed low volatility and repellent efficacy. Rutgers 612, containing 2-ethyl-1,3-hexanediol, was tested for its repellent activity one time; the

compound was known to cause adverse effects such as mild erythema and possible teratogenicity. Therefore DEET has remained as the most effective and preferable insect repellent since it was put into civil usage in 1956.

1.3.1. Insect-transmitted Diseases

Insect-transmitted diseases remain a major source of morbidity and mortality worldwide; in particular, they are a greater health threat in tropical and subtropical climates, and in developing countries. Mosquitoes are responsible for transmitting various diseases to more than 700 million people around the world each year. Malaria, which is caused by the parasite *Plasmodium* and spread by the *Anopheles* mosquito, was responsible for 655,000 deaths in 2010, with approximately 1 child dying every minute in Africa [18]. In addition, arboviruses transmitted by mosquitoes can lead to sporadic outbreaks of eastern equine encephalitis, western equine encephalitis, St. Louis encephalitis, La Crosse encephalitis, and West Nile Virus [19].

West Nile Virus (WNV) was first identified in 1937 in the blood of a woman with a febrile illness in the West Nile Region of Uganda [20]. The virus had become endemic throughout Africa, the Middle East, West and Central Asia, and the Mediterranean in the past; however, the majority of early epidemics occurred in rural populations with few cases of severe neurological disease [21, 22]. WNV is a member of the Flavivirus genus of the family Flaviviridae, which contains approximately 70 members and is transmitted by either mosquitoes or ticks [23]. WNV first appeared in North America during summer 1999 when an outbreak in the New York City area led to encephalitis in 62 patients and a total of 7 deaths. Since then WNV has spread across the North American continent to reach all

forty-eight continental United States, 7 Canadian provinces, Mexico, the Caribbean islands, and Colombia. Primary species involved in WNV transmission in the United States and Canada include *Culex pipiens*, *Culex quiquefasciatus*, *Culex tarsalis*, *Culex restuans*, *Culex salinarius*, and *Culex nigripalpus.* WNV has emerged as the most common cause of epidemic meningoencephalitis in North America and the leading cause of arboviral encephalitis in the United States [20].

According to reports from the US Centers for Disease Control and Prevention (CDC), more than one million people have been infected with WNV in the United States, with severity ranging from asymptomatic to neuroinvasive diseases. The symptoms of overt disease occur in approximately 20% of individuals, which tends to appear after an initial incubation period of 3-14 days [24]. In general, milder symptoms associated with WNV infection would include fever, headache, lymphadenopathy, malaise, and a short-lived truncal rash. The less common neurological presentations may manifest as high fever, neck stiffness, pain with eye movement, disorientation, stupor, tremors, seizures, paralysis, and even coma. The incidence of CNS/neuroinvasive disease seems to increase with age, in immunocompromised individuals, and in male patients. Furthermore, WNV infection may be more prevalent in patients with hypertension, cerebrovascular disease, and diabetes [25-28]. No effective vaccine or treatment protocol exists for WNV infections at present. Consequently, successful prevention of WNV infection will demand multiple levels of intervention, including proactive mosquito control programs, avoidance of outdoor exposure in infested areas, and use of insect repellent products whenever necessary [29].

1.3.2. DEET

The insect repellent DEET was developed by the US Department of Agriculture in 1946, used initially by the US Army for military purposes, and then gradually came into use for civilians and the general population. Approximately one-third of the US population uses topical DEET products each year, and billions of DEET doses have been applied worldwide over the last five decades [30]. **Figure 1.4** shows the chemical structure of DEET ($C_{12}H_{17}NO$).

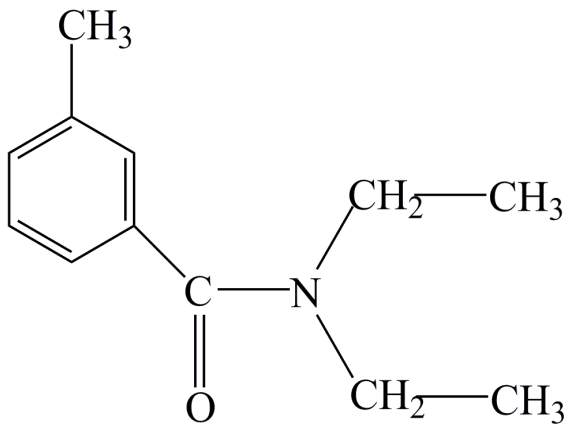

Figure 1.4. Chemical structure of DEET.

Formulations containing 6.65% and 23.8% of DEET demonstrated effective repellency for up to 112 and 302 minutes, respectively [19]. The higher the DEET concentration, the longer the duration of protection. However, this relationship does plateau at higher concentrations. The repellency characteristics are not only based on the physical/chemical properties of DEET, as listed in **Table 1.1**, but are also dependent on

Table 1.1. Physical and chemical properties of DEET [31].

Property	Value/Description
Molecular Weight	191.26 g/mol
Physical State	Colorless to off-white, amber-like liquid
EPA Toxicity Classification	Class III
Specific Gravity	0.992 – 0.999 at 25°C
Solubility	Practically insoluble at 25°C
Boiling Point	111°C at 1 atm
Melting Point	-45°C
Flash Point	155°C
Vapor Pressure	1.67×10^{-3} mm Hg at 25°C
Odor	Aromatic
Viscosity	13.3cps at 30°C

other environmental factors such as air temperature, wind speed, clothing abrasion, loss from sweating or washing, mosquito density, and percutaneous absorption [32-34]. For example, high temperature and strong wind conditions would promote excessive evaporation of the active ingredient, and a large mosquito population would require more repellent dose to be applied.

Although DEET has been extensively utilized for over five decades, the repellent mechanism of DEET against mosquitoes has not been fully elucidated. It was originally postulated that mosquitoes use a combination of visual, thermal and olfactory stimuli to detect their target. However, evidence has suggested that human-mosquito attraction is largely odor-mediated; the human body releases numerous metabolic byproducts such as carbon dioxide, lactic acid, and 1-octen-3-ol which act as strong mosquito attractants. DEET was shown to block detection of biting insects to lactic acid and 1-octen-3-ol from

a distance of at least 38 cm, by shutting down their receptors that work in tandem with a smell coreceptor called Or83b, and thus inhibiting the electrophysiological activity of olfactory sensory neurons on the antennae of *Aedes aegyptii* [35]. More recent evidence has demonstrated that mosquitoes are strongly repelled by the smell of DEET. Behavioral bioassays showed that specific olfactory receptor neurons (ORN) on the antenna of male and female *Culex quinquefasciatus* detect DEET in a dose-dependent manner and thereby avoid this topical repellent [36]. Subsequently, carbon dioxide, lactic acid, and 1-octen-3-ol do not appear to be involved in DEET repellency. It was hypothesized that other insect repellents might possess similar repellent mechanism against mosquitoes. Evidence has also suggested that affinity to *Anopheles gambiae* Odorant Binding Protein-1 (OBP-1) by DEET facilitates supreme repellent efficacy of this compound against mosquitoes [37].

1.3.3. Percutaneous Absorption of DEET

Percutaneous absorption and systemic disposition of DEET is an undesirable characteristic because DEET exerts its protective effects on the surface of skin. Systemic disposition of this active repellent compound is neither productive nor necessary. Permeation of DEET through the skin has been documented for several decades. Earlier studies in animal models indicated that 10-60% of DEET dose was percutaneously absorbed [30, 38-40]. Dermal absorption of DEET through human skin was reported as 5-17% of the applied dose [38, 41, 42]. Despite continued efforts in optimizing DEET preparations to reduce overall skin permeation, systemic absorption of DEET following topical application in humans ranged between 5-59% of the dose applied [38, 41-43]. The permeation

was also directly proportional to concentration used such that high test concentrations would lead to an increase in skin permeation of the compound.

Synergistic permeation of DEET and sunscreen oxybenzone has been reported from concurrent use of both components. This was first observed *in vitro* using pig skin and artificial PDMS membrane; DEET permeability increased by 107-289% in propylene glycol, 207-243% in ethanol, and 112-124% in PEG-400 when comparing the combined formulation (DEET and oxybenzone) to single DEET formulation [44]. A DEET permeation study across three artificial membranes, low-density polyethylene, low fouling composite and mixed cellulose esters, indicated a synergistic increase by up to 500% when both repellent and oxybenzone-based sunscreen formulations were applied simultaneously [45]. Lipophilic and hydrophilic characteristics of the membranes utilized were important determinants of DEET permeation, as was the physical and chemical properties of the numerous solvents and additives that made up the repellent and/or sunscreen formulation. Further study found that accumulated percutaneous permeation of DEET was 0.5-25.7% across human skin *in vitro* [46]. Concurrent use of repellent spray and sunscreen lotion increased DEET permeation across human skin *in vitro*, most notably after pre-mixing the two preparations. Penetration of DEET from a 7% (w/w) commercial spray and a 7.5% commercial lotion together with a 5% commercial oxybenzone lotion was 1640% and 282% higher than applying DEET preparations alone [47]. Placing repellent spray on top of sunscreen lotion with no mixing seemed to be the best approach to diminish DEET from penetrating through the skin. In addition, recovery of DEET within the skin also indicated a higher DEET content from a combined preparation than from a single-component counterpart [48]. Although combined repellent

and sunscreen products produced up to 79% higher permeation than the repellent lotion and were consequently withdrawn from the Canadian market in 2003 by Health Canada, applying the individual counterparts simultaneously led to even greater penetration. In an animal model, DEET was detected in plasma 48 hours post topical skin application; statistically significant increases in AUC by 37% and C_{max} by 12% were observed from combined repellent/sunscreen preparation versus single-component preparation [49].

Metabolism of DEET had been studied in rats. It was postulated that DEET achieved similar metabolic pathways in rats and humans [40]. Two major metabolic pathways of DEET have been elucidated. The first pathway involves oxidative hydroxylation of the aromatic methyl group in the meta position producing *N*,*N*-diethyl-*m*-hydroxymethylbenzamide (DHMB), while the second pathway relates to dealkylation of an *N*-ethyl group leading to *N*-ethyl-m-toluamide (ET) [50]. These metabolic pathways are depicted in **Figure 1.5**.

Even though DEET is primarily eliminated through the kidney, little or no unmetabolized DEET is excreted in the urine from rats and humans [40, 42, 51]. However, reports have also indicated significant amounts of unmetabolized DEET in urine from human studies [52, 53]. The discrepancy between the observations may be attributed to differences in study dosing such that higher dermal doses (145 mg to 15 g per subject) resulted in unmetabolized DEET while smaller dermal doses (12-15 mg per subject) yielded DEET metabolites [42, 52-54]. Concurrent topical application of DEET and oxybenzone also resulted in increased concentration of DEET metabolites (DHMB and ET) in urine in piglets [49]. Rats excreted 3-7% of the administered DEET dose as metabolites via feces after oral and dermal administrations, but humans apparently

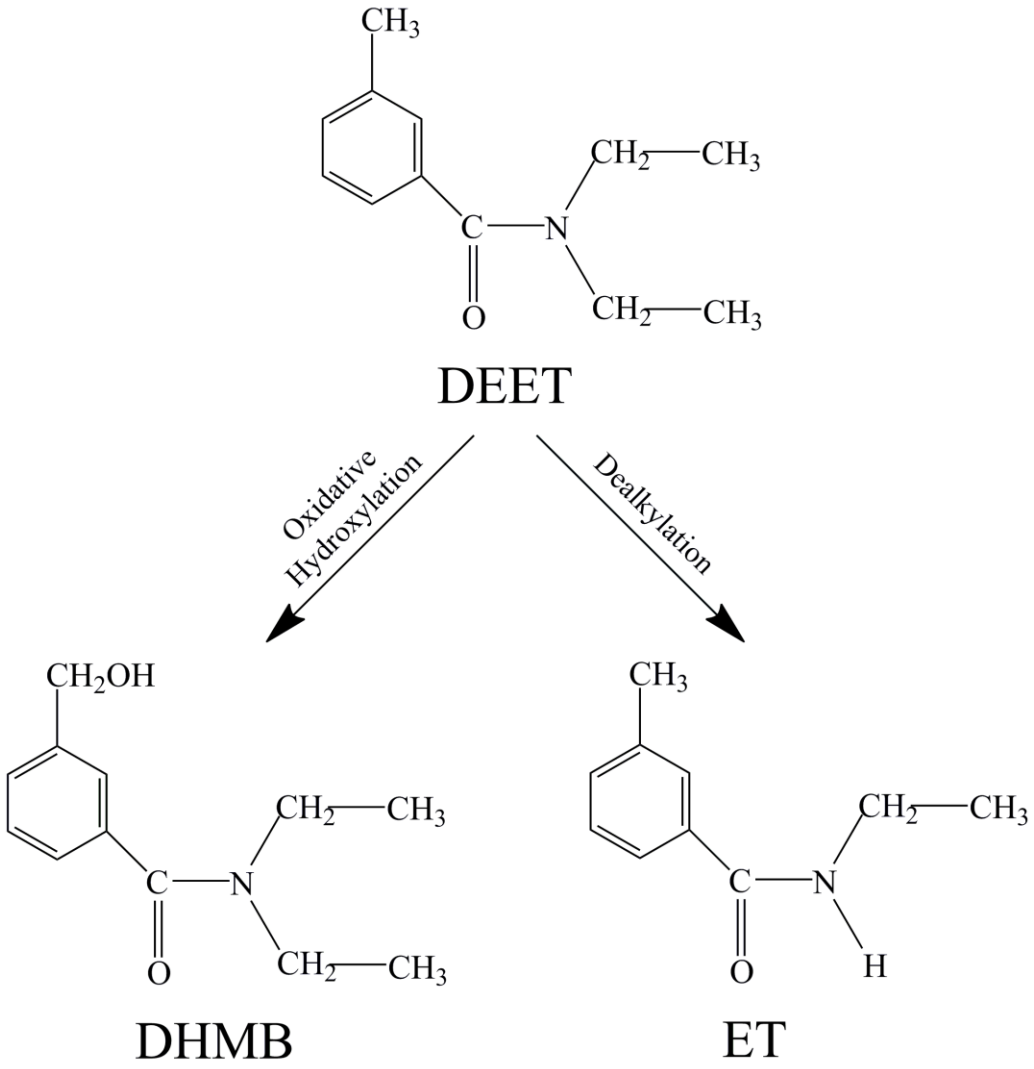

Figure 1.5. Proposed metabolic pathways for DEET in rats; DEET – *N*,*N*-diethyl-*m*-toluamide, DHMB – *N*,*N*-diethyl-*m*-hydroxymethyl-benzamide, ET – *N*-ethyl-*m*-toluamide.

excreted DEET metabolites exclusively in urine [40, 51].

1.3.4. Adverse Effects of DEET

The toxicological profiles of DEET *in vivo* have been investigated over the years since its debut. Specifically, studies were focused on its effects on endocrine, urinary, reproductive, respiratory, cardiovascular, and neurological systems. The research

outcomes have not always been consistent and conclusive; occasionally there were even contradictory findings, which raised concerns about the safety of this common insect repellent. With the recent advancement in instrumentation and molecular biology, more studies might be needed to better understand its pharmacological and toxicological characteristics in humans.

The most sensitive indicator for toxicity in any animal model has been reduced body-weight gain, regardless of animal species and/or route of exposure [55]. For DEET, the lowest no-observed-adverse-effect-levels (NOAEL) ranged from 300 to 500 mg/kg bw/day for a 90-day dermal study where reduced terminal body weights of 10% or less were noted in comparison to controls [51]. Subchronic oral and dermal dosing of DEET resulted in kidney abnormalities in rats, including kidney lesions, hyaline droplet formation and granular cast accumulation in the renal tubules as well as chronic inflammation and tubular epithelial regeneration [51]. However, dermal dosing at 100-1000 mg/kg did not induce visible adverse toxicity to the reproductive system in rats over two generations [56].

Changes in respiratory activity following DEET exposure have been reported in animals. Respiratory failure usually preceded cardiac failure following lethal oral doses of DEET in rats [57]. High oral dosing of DEET ranging 2.5-4 g/kg led to progressive respiratory depression or distress following by death in rats [58, 59]. DEET also caused changes in respiration rhythm that were manifested as "gasping" responses in rats, and immediate rapid and shallow breathing in hens [55, 60]. It was postulated that these responses might have resulted from DEET-induced disorganization of central respiratory control mechanisms [61].

One of the common DEET adverse effects has been related to its interaction with the CNS. Neurotoxic potential from DEET usage had been specifically cited in "Gulf War Syndrome", in which neurological fatigue was a primary symptom. A 30-day, dermal treatment with DEET (40mg/kg), permethrin (0.13mg/kg) and malathion (44.4mg/kg), alone or in combination, demonstrated significant sensorimotor impairment in rats, which was reflected in inclined plane performance, forepaw grip time, beam-walk score, and beam-walk time [62, 63]. Significant neuronal degeneration in the dentate gyrus, the midbrain, the CA1 and CA3 regions of the hippocampal formation, the striatum pyramidal, the granule cell layer, and the dentate hilus was observed, with the midbrain having the highest levels of degeneration. Similarly, a 60-day, dermal study using DEET (40mg/kg) alone or in combination with permethrin also found significant neuronal degeneration in several areas of the brain in rats including the motor cerebral cortex, the dentate gyrus, hippocampal cells, and the Purkinje cell layer of the cerebellum [64]. When physical stress was combined with low dermal doses of pyridostigmine bromide, DEET and permethrin, significant brain injury was induced in rats [65, 66]. Since all above studies had come from the same research team, questions were raised regarding the study methodology and conclusion [67]. The NOAEL of DEET in acute and multigeneration rat studies was reported to be 200 mg/kg/day and 100 mg/kg/day, respectively [68]. Signs of toxicity at near lethal dosing included ataxia, tremors, prostration, lack of balance, convulsions, chromodacryorrhea, and lethargy; nevertheless, no neuropathology was observed at nonlethal doses [51]. The cause of these discrepancies in all clinical, functional, and histopathological findings in animal models has yet to be explored and elucidated [29].

There have been numerous speculations on possible neurotoxic mechanisms from DEET use. Theories had related the toxicity to acetylcholinesterase activity, increased cortical M2 muscarinic ACh receptor binding, demyelination in cerebellar roof nuclei, spongiform myelinopathy, neuronal degeneration, and/or cell death [58, 62, 69-74]. Small increases in the frequency of enlarged axons in the sciatic nerve as well as slight inhibition of butyrylcholinesterase (BuChE) activity were also reported [55]. Another hypothesis indicated that DEET might produce free radicals, leading to increased 8-hydroxy-2-deoxyguanosine through excessive generation of reactive oxygen species (ROS) or by decreased repair efficiency of the oxidative damage [75]. Primary efflux transport proteins such as organic anion transporting polypeptide transporters, organic cation transporters, *p*-glycoprotein, and multidrug resistance associated protein in the CNS, may also play a role in affecting DEET brain disposition and/or toxicity [76]. In spite of extensive public use and numerous experiments, the true mechanisms of neurotoxicity potentiated by DEET have so far remained unclear.

Clinically DEET has been responsible for various adverse effects in humans including cutaneous or allergic reaction, hypotension, headache, daytime sleepiness, lethargy, ataxia, impaired cognitive function, depressed respiratory function, disorientation, seizure, acute manic psychosis, toxic encephalopathy and even death [60, 77-82]. Rare and nonspecific incidents of DEET toxicity have been thoroughly documented in the literature. A healthy 30-yr-old male developed symptoms of psychomotor hyperactivity, rapid and pressured speech, tangentiality, flight of ideas, and grandiose delusions after applying a DEET-based repellent daily for two weeks followed by a stay in a sauna for up to 2 hours; the patient was treated successfully by administration of

haloperidol for 6 days [77, 83]. A 61-yr-old healthy female developed severe hypotension, nausea, vomiting, and diarrhea after she applied DEET repellent with sunscreen while working outdoors, which later resolved spontaneously [84]. A healthy 42-yr-old woman developed pruritus followed by anaphylaxis, by simply touching a person who had just applied a 52% DEET spray product [78]. Approximately 90% of individuals who had suffered toxic encephalopathy from using DEET-based repellents were children aged 8 years or younger [85-90]. Respiratory distress, seizure and coma were also the most common symptoms in this patient group [83, 90, 91]. Higher DEET concentrations (>20%) were used in approximately 45% of the incident reports, with exposure time ranging between several hours to 3 months. At present, over 90% of commercially available repellent products contain DEET concentrations at 10% or below.

From 1961 to 2002, six cases of intentional ingestion of DEET led to three cases of death; an additional five cases of death associated with dermal DEET exposure were reported [81, 92]. A 17-month-old girl died of acute encephalopathy after having received frequent skin application of a DEET-containing lotion for 3 weeks [93]. A 6-yr-old girl applied a 15% DEET spray to extensive skin areas on at least 10 occasions, and subsequently died 8 days after she was admitted into the hospital [94]. A 5-yr-old girl died 24 days after she had utilized a 10% DEET spray daily for 3 months; her death was partially attributed to brain edema with intensive congestion of the meninges [95]. Exposure to DEET during pregnancy did not induce changes in survival, growth, and neurological development in infants from birth to one year of age [96, 97]. Since a majority of severe adverse effects from DEET use had occurred to children, regulatory bodies and health authorities recommend cautious use of DEET in young children at low concentrations.

Based on published data in the past 50 years and the extent of DEET usage by the general public, this insect repellent should be considered effective and safe in terms of repellency efficacy and adverse effects. One of the most recent incident reports would have been the so-called "Gulf War Syndrome", in which veterans from the Persian Gulf War (1990-91) experienced severe neurological side effects after excessive or repeated exposure to DEET and other insecticides. No conclusive finding has been obtained so far to explain this unique medical condition. Pharmacogeneticists have also attempted to link increased DEET sensitivity to deficiencies in ornithine transcarbamylase (OTC) in young children, but this mechanism needs to be further evaluated. Health Canada decided in 2003 to cease manufacturing and marketing preparations containing greater than 30% DEET, and to limit products designated for children use to less than 10% DEET, in order to minimize unnecessary daily dermal exposure. Further evaluation of this common insect repellent, together with some other consumer-care products such as sunscreens, can certainly add useful and beneficial information to existing data, and provide comprehensive user guidelines for the safe and effective application of these products by the general public.

1.4. Sunscreens

Human civilization has utilized tars, clays, oils, herbs and plant extracts to preserve skin surface and prevent the harmful effects of ultraviolet (UV) radiation from the sun for centuries. Experimentation with modern sunscreens started in the early 1900's, when several preparations containing active sun-blocking ingredients such as phenyl salicylate, quinine sulfate, aesculin, and *para*-amino-benzoic acid were tested for

their sun-blocking efficacy [98, 99]. Eugène Schueller, who founded the company known today as L'Oréal Group, was credited with developing modern sunscreen products when he prepared a formulation containing benzyl salicylate [100]. Titanium dioxide and zinc oxide were also evaluated for their protective effects in absorbing UV radiation in the 1940s [101]. By the late 1970s and early 1980s, sunscreen products had been developed to offer a wide range of protection across the UV spectrum. At present, active sunscreen ingredients are incorporated in many common cosmetics and over-the-counter consumer care products; sunscreens are also recommended by medical professionals and healthcare authorities for regular daily applications to reduce skin aging in children and adults [102-104]. **Table 1.2** lists some common sunscreen compounds that have been approved for civil use by the US FDA. Even though sunscreen use has been increased over the years owing to an extensive awareness of the possible relationship between excessive sun exposure and skin aging, the incidents of skin cancer continue to rise significantly around the world [105].

Sunlight is categorized into three major wavelength spectra, ultraviolet light, visible light, and infrared light [106]. Among them ultraviolet light (UV) can be further divided into three types of radiation according to their wavelengths, UVA consisting of UVA-I (340-400nm) and UVA-II (320-340 nm), UVB (280-320nm), and UVC (100-280nm) [107]. UVC is completely absorbed by the ozone layer in the atmosphere, hence its impact on human skin is negligible. UVA and UVB are not completely absorbed by the atmosphere; approximately 95% of UVA and 1-10% of UVB will reach the surface of the earth, and exert a collective damaging effect on human skin [108, 109]. Since UVA has the longest wavelength, it is capable of penetrating deeper into human skin layers. Consequently, UVA not only plays an important role in skin tanning, but is also

Table 1.2. Current FDA approved sunscreen ingredients [110].

Drug Name	Concentration, %	Absorbance
Aminobenzoic acid	Up to 15	UVB
Avobenzone	2-3	UVA-I
Cinoxate	Up to 3	UV-B
Dioxybenzone	Up to 3	UVB, UVA-II
Ecamsule	2	UVA-II
Ensulizole	Up to 4	UVB
Homosalate	Up to 15	UVB
Meradimate	Up to 5	UVA-II
Octocrylene	Up to 10	UVB
Octinoxate	Up to 7.5	UVB
Octisalate	Up to 5	UVB
Oxybenzone	Up to 6	UVB, UVA-II
Padimate O	Up to 8	UVB
Sulisobenzone	Up to 10	UVB, UVA-II
Titanium dioxide	2 to 25	Physical
Trolamine salicylate	Up to 12	UVB
Zinc oxide	2 to 20	Physical

considered a significant contributor to skin aging and skin damage when human skin is under prolonged exposure to sunlight. Similarly, the higher energy of UVB rays also causes increased carcinogenicity, skin aging and skin damage.

The protection efficacy of a sunscreen preparation is measured by the Sun Protection Factor (SPF), a value assigned to the product in accordance to its ability to block UVB. SPF of a sunscreen preparation is obtained by calculating the ratio between

the Minimum Erythema Dose (MED) of the sunscreen-treated skin and the Minimum Erythema Dose (MED) of the unprotected skin, as described in the following equation[111]:

$$\text{SPF} = \frac{\text{MED (sunscreen treated skin)}}{\text{MED (unprotected skin)}}$$

Based on the theory of SPF, a sunscreen preparation with an SPF of 15 would protect the skin from UVB radiation 15 times longer than if the skin is left unprotected. However, as SPF is an artificial value exclusively designated for sunscreen products, differences in sun-blocking efficacy do exist between individual users and geographic locations. While an SPF 15 sunscreen blocks approximately 95% of UVB, an SPF 30 sunscreen will only offer an additional 3% of protection [112]. In general, dermatologists recommend that sunscreen application be generous and frequent, by utilizing sunscreens with a minimum of SPF 15, and applying at least once every two hours or more for outdoor activities, especially during the summer months.

1.4.1. Skin Cancer

Skin cancer is the most common type of cancer in light skinned populations around the world today. It is estimated that two to three million cases of skin cancers occur worldwide each year [113]. There are two primary types of skin cancers, melanoma and non-melanoma skin cancers (NSMCs). NMSCs can be further categorized into basal cell carcinoma and squamous cell carcinoma.

Basal cell carcinoma accounts for approximately 85% of all skin cancers diagnosed. This type of skin cancer rarely metastasizes to other organs; therefore it is

responsible for less than 0.1% of skin cancer deaths [114]. Basal cell carcinoma occurs primarily on skin regions that are regularly exposed to sunlight. The appearance of a basal cell carcinoma may vary upon presentation, ranging from a small, flat, fleshy pink area to a raised pink or red dome-shaped lesion with a pearly or milky white border. Known risk factors for basal cell carcinomas are extensive, which may include age, light-colored skin type, ultraviolet radiation or other radiation exposure, gene mutations, and immunosuppression. Prognosis for this locally destructive type of skin malignancy is excellent, with an estimated 5% 5-year recurrence rate depending on metastasis [115]. Treatment of basal cell carcinoma includes curettage and desiccation, surgical excision, radiation therapy, cryosurgery, Mohs micrographic surgery and topical creams such as 5-fluorouracil and imiquimod.

Squamous cell carcinoma accounts for approximately 10-15% of all skin cancers diagnosed, but it possesses a higher risk of metastasis (~10-fold) than basal cell carcinoma [116]. In its early stages, squamous cell carcinoma may present as actinic keratosis, where initially rough, scaly, and disproportionate papules may transform into thicker, wart-like growths that are tender and may bleed. A history of sun exposure and light-colored skin are significant predisposing factors to squamous cell carcinoma, even more so than basal cell carcinoma [117]. Other etiologic agents and treatments are similar to basal cell carcinoma, but the risk of mortality is higher with this form of NMSC.

Melanoma accounts for approximately 1-5% of all skin cancers, and results from malignant melanocytes, the pigment-making cells in human skin. With more advanced cases of melanoma, the cancer is able to spread rapidly to the lymph system, and eventually metastasize to internal organs, leading to death. Melanoma is responsible for

the majority of skin cancer related mortalities, accounting for approximately 75% of all skin cancer deaths [118]. Melanoma skin tumors present as dark or black areas that quickly advance in size. Public health guidelines have developed an acronym to help self-diagnose melanoma, i.e., A, Asymmetry, B, Border irregularity, C, Color has several shades or is different from other moles, D, Diameter is larger than a pencil eraser, and E, Evolving. Melanoma can occur on any surface of the skin, but has a greater tendency to form on the trunk of men and the lower legs of women [119]. Melanoma is the most aggressive form of skin cancer, but it is also curable with early detection and prompt treatment.

Skin cancer occurrence rate has been steadily increasing over the years in the world. One of the primary risk factors of skin cancers, excessive exposure to UV radiation, is actually avoidable by appropriate protection by the general public. Firstly, educational programs such as proper sun-protection and skin cancer awareness procedures are practical and beneficial in reducing overall skin cancer incidents in general [120]. Secondly, self-protection approaches can be exercised regularly to preserve skin health, for example, minimizing sun exposure during peak hours, wearing protective clothing and sunglasses, avoiding indoor sunlamps and tanning beds, and applying a broad-spectrum sunscreen preparation [121]. The use of sunscreens is a very practical, economical and acceptable way of minimizing skin cancer incidence for the general public in long-term skin care and protection.

1.4.2. Oxybenzone

Benzophenones are a group of UV-absorbing agents that have been used in sunscreen preparations for decades. In addition, benzophenones are also used in various materials to reduce sunlight-related discoloration and/or deterioration [122]. There are 12 types of benzophenones available, designated from benzophenone-1 through benzophenone-12, which are all derived from the primary parent compound 2-hydroxybenzophenone [123]. Oxybenzone, which is also known as benzophenone-3 or 2-hydroxy-4-methoxybenzophenone, is the most frequently used benzophenone in topical sunscreen products.

Figure 1.6 shows the chemical structure of oxybenzone. **Table 1.3** lists the physical and chemical properties of oxybenzone. Oxybenzone is essentially a monomethoxylated derivative of the parent compound benzophenone. The presence of two aromatic rings in the structure results in a large degree of lipophilicity. Subsequently, oxybenzone is miscible with many organic solvents such as ethyl alcohol, isopropyl alcohol, methanol, mineral oil, and propylene glycol. In addition, oxybenzone is soluble in other cosmetic excipients such as oils and emulsifiers. At present, the recommended maximal amount of oxybenzone in cosmetic and sunscreen preparations is 6% (w/w). Oxybenzone is used in 20-30% of commercial sunscreen preparations, especially in those with a SPF greater than 8 [103, 124, 125]. Oxybenzone is also found in a variety of cosmetic products such as dyes, hair sprays, skin lotions and lipsticks, as a photostabilizer at a concentration of 0.05-0.5% [123]. As a broad-spectrum sunscreen compound, oxybenzone absorbs a wide range of UV light, with absorption peaking in UVB region and extending into UVA region as well.

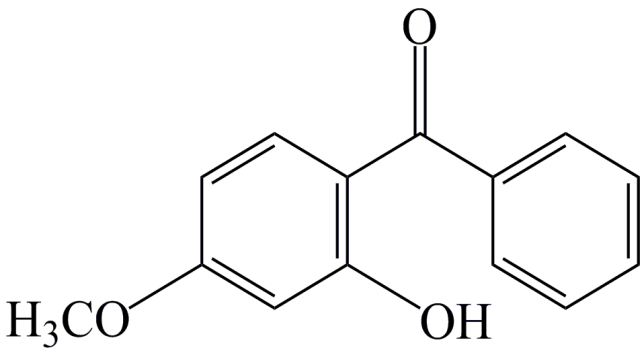

Figure 1.6. Chemical structure of oxybenzone

Oxybenzone is a chemical sunscreen that is capable of absorbing certain wavelengths of ultraviolet radiation before they enter the nonviable and viable layers of the skin. By absorbing this radiation energy, electrons in low energy orbitals are excited to previously unoccupied, higher energy orbitals [126]. Oxybenzone will then undergo a rapid vibrational relaxation, which involves releasing the absorbed energy thermally and returning back to its ground state afterwards [127-130]. This cycle of energy absorption and release thereby effectively shields the skin from damaging ultraviolet radiation [131]. On the contrary, physical sunscreens such as titanium dioxide and zinc oxide produce a protective physical barrier that absorbs, scatters, and reflects the ultraviolet radiation. In general, chemical sunscreens are more commonly utilized in commercial sunscreen preparations than physical sunscreens due to their esthetic acceptability. A commercial product also contains multiple active sun-blocking ingredients, in order to absorb and reflect a majority of ultraviolet radiation and achieve a broad-spectrum SPF protection.

1.4.3. Percutaneous Absorption of Oxybenzone

The role of sun protection from a sunscreen preparation relies on its residence

Table 1.3. Physical and chemical properties of oxybenzone [132].

Property	Description
Molecular Weight	228.25 g/mol
Physical State	White yellowish, cream colored powder
Specific Gravity	1.32 at 25°C
Solubility	Nearly insoluble at 25°C
Boiling Point	150 - 160°C (5 mmHg)
Melting Point	63 - 65°C
Flash Point	216°C
Vapor Pressure	Not determined
Odor	Almost odorless or faint characteristic
Stability	Stable under normal conditions

near the surface of the stratum corneum, from where active sunscreen compounds absorb and/or reflect the harmful effects of UV radiation from the sunlight. Any percutaneous absorption and subsequent systemic disposition of the sunscreen substances *in vivo* is neither productive nor desirable. Similar to the insect repellent DEET, oxybenzone and many other sunscreen compounds are capable of penetrating through the epidermis and entering the general circulation. Effects from systemic disposition of sunscreen compounds have not been well studied in the past.

Various studies had been carried out *in vitro* and *in vivo* to evaluate percutaneous absorption of active sunscreen ingredients across human skin. Oxybenzone was found to possess variable permeation properties from different experiments. One *in vitro* study showed 10% of oxybenzone penetration across human epidermis [133], while another found 1.16% of overall dermal absorption of oxybenzone with 5.8% of the applied dose

deposited in the stratum corneum [134]. A study in human subjects using a commercial sunscreen product demonstrated that 1-2% of the oxybenzone dose applied was absorbed over a 10-hour period [135]. Studies had also indicated that formulation types (e.g., o/w emulsion, w/o emulsion, gels, oils, creams) would ultimately influence the absorption rate and amount of oxybenzone *in vivo* [136-138]. A recent study conducted by the US CDC revealed that among 2517 urine samples collected as part of the 2003-2004 National Health and Nutrition Examination Survey, 96.8% of the samples contained oxybenzone with concentrations ranging between 0.4-21,700 µg/L [139]. In addition, oxybenzone was found to enhance the percutaneous permeation of other substances such as the sunscreen octyl methoxycinnamate [140] and herbicide 2,4-dichlorophenoxyacetic acid [141].

Synergistic permeation between oxybenzone and insect repellent DEET from concurrent use has been reported in our laboratory from a series of studies. This profile was first observed *in vitro* using pigskin and the artificial membrane polydimethylsiloxane (PDMS); permeability of oxybenzone was enhanced by 139-254% in polyethylene glycol, 105-120% in propylene glycol, and 112-154% in ethanol, respectively [44]. Premixing sunscreen lotion with either repellent spray or repellent lotion also yielded up to 1825% increases in oxybenzone permeation compared to the control [45, 46]. Commercial sunscreen lotions produced 189-296% more oxybenzone permeation when they were used together with repellent sprays or lotions, or when combination sunscreen/repellent products were utilized, in comparison to individual products [47]. Overall permeation percentage of oxybenzone across human skin after 6 hours rangedfrom 0.3% to 1.6% for single and combined applications, respectively [46]. In an animal study, permeation of oxybenzone from concurrent use exceeded single

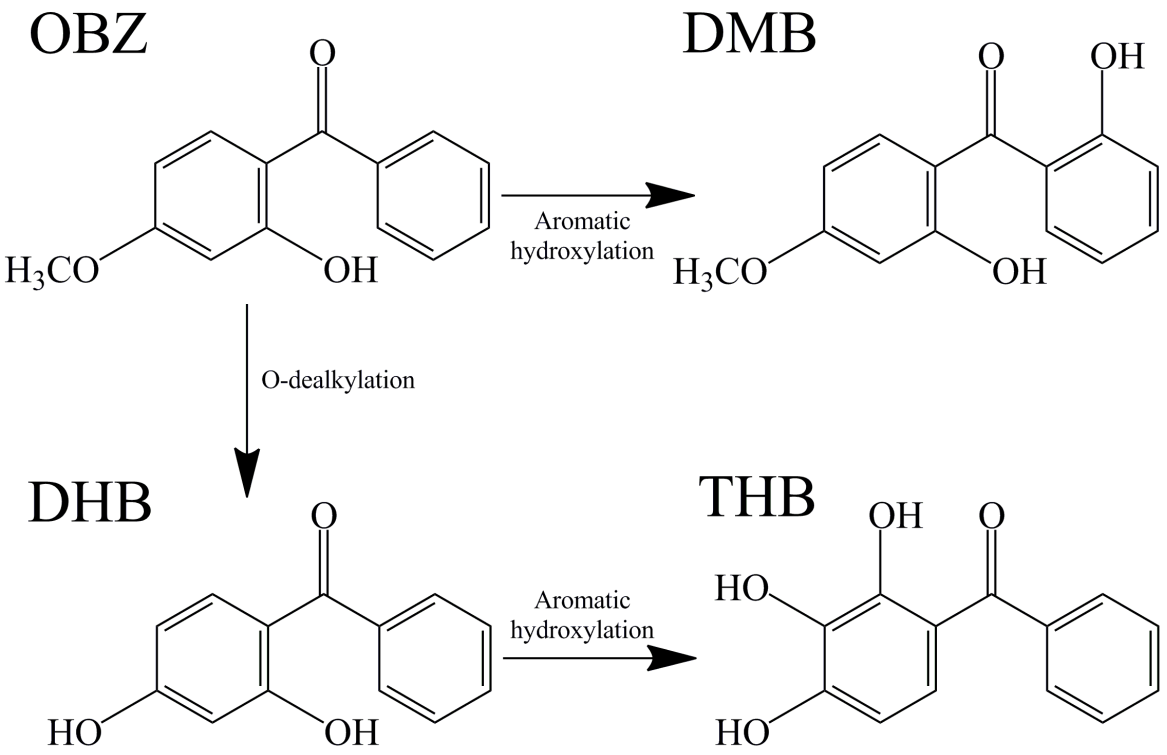

Figure 1.7. Proposed metabolic pathway of oxybenzone in rats; OBZ: oxybenzone; DMB: 2,2´-dihydroxy-4-methoxy-benzophenone; DHB: 2,4-dihydroxybenzophenone; THB: 2,3,4-trihydroxybenzophenone.

preparation by 77-231%; AUC and C_{max} values were 63% and 60% higher than those of individual preparation, respectively [48, 49]. It was obvious that oxybenzone was capable of enhancing transdermal permeation of DEET while its permeation was also increased at the same time.

Metabolism and excretion of oxybenzone had been studied in various animal models. **Figure 1.7** depicts the two primary metabolic pathways of oxybenzone. The first pathway involves aromatic hydroxylation at the *ortho*-position on ring B of oxybenzone nucleus, producing *2,2´*-dihydroxy-*4*-methoxy-benzophenone (DMB). The second pathway involves *O*-dealkylation of the methoxy side chain at *para*-position on

oxybenzone nucleus, producing *2,4*-dihydroxybenzophenone (DHB), followed by secondary aromatic hydroxylation of ring A at the *meta*-position of DHB producing *2,3,4*-trihydroxybenzophenone (THB) [123, 142]. Excretion studies had indicated that urine was the major route of excretion for oxybenzone, followed by excretion in feces [48, 143]. A recent study demonstrated that 1.2-8.7% of oxybenzone applied was detected in human urine for up to five days after the dermal application [144].

1.4.4. Adverse Effects of Oxybenzone

Many researchers have attempted to characterize potential toxicity from topical application of sunscreen oxybenzone over the years. Oxybenzone has been included in FDA's original monograph proposing conditions for safety, efficacy, and labeling for sunscreen products since 1978; these conditions were again confirmed for topical use in humans in 1983 [145]; the compound was proposed to be a safe and effective active sun-blocking agent [103, 146]. As a result, data on pharmacological and toxicological properties of oxybenzone has been relatively scarce.

A few toxicity studies using animal models have indicated potential for oxybenzone to induce adverse effects, most notably at higher doses. First signs and symptoms of adverse effects from oral administration (no-observable-adverse-effects-levels or NOAEL) were observed at 200 mg/kg – 1247 mg/kg with the range resulting from alterations in exposure time or animal species; variations in liver and kidney weights were initially detected after extended durations of exposure in rats and mice [147, 148]. After topical application, increased liver and kidney weights occurred when rats

received an acute dermal dose of oxybenzone 120 mg/kg; similar effects were noted when oxybenzone 364 mg/kg was topically applied in mice for 90 days [147].

Like other commonly utilized topical products, application of oxybenzone-based sunscreens may yield potential skin reactions resulting in localized rash, irritation, and inflammation or in more severe circumstances, skin carcinogenicity. *In vitro* and *in vivo* investigations into potential phototoxicity and bacterial or mammalian mutagenicity of oxybenzone did not reveal any damaging effects [147, 149-156]. Reactive oxygen species in the skin may be enhanced with oxybenzone use, progressing to potential cell damage, and skin carcinogenicity [157-160]. Clinical studies investigating allergic contact dermatitis from oxybenzone use in a patient population and other photosensitization reactions have been reported in the literature [145, 161-171].

Certain studies have indicated potential for oxybenzone and its metabolites to induce hormone-related or reproductive adverse effects under acute or prolonged use conditions. It was shown that oxybenzone and metabolites possess a certain structural resemblance to steroid hormones and thus may potentiate steroid-like effects and general endocrine disruption [172-177]; increase of uterine weight had been observed in rats when they were exposed to oxybenzone at oral doses of >1500 mg/kg/day [178]. Additive estrogenic effects of oxybenzone with other topical sunscreens may also occur [179]. Animal reproductive, prenatal and maternal developmental toxicity was observed at doses from 828-16,238 mg/kg, with the range depending on mode of administration and duration of exposure [147, 149, 180]. A single-blinded clinical study involving 15 young males (aged 23-29) and 17 postmenopausal females who applied a full-body topical application of oxybenzone 10% (w/w) cream at a dose of 2mg/cm^2 found that within the first four

hours after application, males demonstrated slightly lower levels of estradiol and testosterone, while inhibin B was slightly increased; females showed a slight decrease in testosterone within the first 24 hours [181]. Infants and children may be more susceptible to hormonal changes from chemical sunscreen exposure due to less developed elimination systems as well as larger surface areas per body weight; consequently, higher maternal oxybenzone concentrations were associated with a decrease in birth weight among girls and an increase in birth weight among boys [182].

Given the overall size of the population utilizing oxybenzone-based sunscreen products and the scarcity of published toxicity data from over thirty years of public use, oxybenzone may be regarded as safe in terms of protection efficacy and adverse effects. To date, however, evaluations by federal agencies have not entirely considered recent concerns regarding potential toxicological effects resulting from skin permeation and systemic absorption of oxybenzone. Safe plasma levels of oxybenzone have not been established in the human body. Effects of oxybenzone in children also require more attention; chemical exposure in the early years of life may lead to future medical conditions [181, 183]. Oxybenzone has an ability to be absorbed transdermally, and can also act as a penetration enhancer to other topical compounds. Therefore, further investigation and continued follow-up by health authorities on oxybenzone, together with some other extensively utilized over-the-counter products such as insect repellents, will assist in formulating a thorough understanding of current data, and will lead to the development of comprehensive regulations for the risk-free application of topical sunscreens.

1.5. Hypotheses and Objectives

Mosquitoes are the prominent vectors responsible for transmitting human diseases such as malaria and West Nile encephalitis. The safety of the general public would be compromised if inadequate precautions were not taken for outdoor activities, in particular, for those working in forest, agricultural, military, and postal posts and for those traveling in insect-infested regions. Proper epidemic surveillance, efficient insect control, and timely treatment procedures can all help minimize the overall health risk of these diseases upon humans. Nevertheless, the most effective and practical preventive approach for the mass is still the application of topical insect repellents.

While sunlight is beneficial and essential with aiding in Vitamin D synthesis in the body, ultraviolet radiation from the sun can induce erythema (sunburn), skin aging, photodermatoses, actinic keratosis, and skin cancer in humans. There are numerous practical ways of reducing overexposure to UV radiation for the general public such as wearing protective clothing, limiting direct sunlight contact, staying indoors between noon and early afternoon, and avoiding sunbathing altogether. Similarly, the frequent and ample application of sunscreen products could be equally regarded as an essential and economical form of protection against skin aging and skin cancer for humans.

Insect repellents and sunscreens were infrequently applied concurrently in the past, since mosquitoes and other biting insects were likely more aggressive before dawn and after dusk when sunlight was not yet strong enough for sunscreen use. With the rapid climate changes and extensive public awareness of health threats from both biting insects and UV radiation from the sun, however, the general public has started to alter their attitude and approach towards regular application of these two consumer care products in

recent years. Consequently, concurrent use of topical repellent and sunscreen products has become one of the widely practiced routines for many in developed countries such as Canada and the US. Although repellents and sunscreens are designed to exert their protective effects on the surface of the skin, synergistic penetration of both DEET and oxybenzone through stratum corneum and systemic disposition of the compounds have been reported. In addition, there have been inconclusive results on pharmacokinetic and toxicological properties of the two substances *in vivo*. Therefore, the focus of this thesis was based on the following two hypotheses, each having multiple research objectives to characterize the pharmacokinetics and potential adverse effects from concurrent application of insect repellent DEET and sunscreen oxybenzone.

Hypothesis 1: The pharmacokinetic properties of insect repellent DEET and sunscreen oxybenzone are altered after short-term and long-term concurrent application of both ingredients in an animal model.

Previous studies have demonstrated percutaneous permeation enhancement and subsequent systemic absorption after concurrent application of DEET and oxybenzone, both *in vitro* and *in vivo*. These short-term studies had not investigated disposition of DEET and oxybenzone metabolites in blood and tissue specimens. In order to validate and evaluate existing toxicological data of the two compounds, a 24-hour intravenous administration, a 24-hour topical application, and a 30-day topical application of the substances were carried out in Sprague Dawley rats. To the best of our knowledge, there are no similar studies that had explored the pharmacokinetic parameters of DEET and oxybenzone, alone or in combination, in a rat animal model previously. The study objectives were: 1) To assess and compare the pharmacokinetic parameters of DEET and

oxybenzone after a single 24-hour topical application, a 30-day once-daily repeated topical skin application, and an intravenous administration in a rat model; 2) To assess and compare concentrations of DEET and its metabolites (DHMB and ET), and oxybenzone and its metabolites (DHB, DMB, and THB) in the skin, plasma, brain, liver, kidney, urine and feces after a single 24-hour topical application, a 30-day once-daily repeated topical skin application, and an intravenous administration in a rat model.

Hypothesis 2: Increased toxicity results from a 24-hour and a 30-day once-daily repeated topical skin concurrent application of insect repellent DEET and sunscreen oxybenzone.

One of the primary adverse effects of DEET reported in the literature was neurotoxicity. Although oxybenzone had not demonstrated any direct neurotoxic effect, synergistic percutaneous enhancement from concurrent application of DEET and oxybenzone might lead to elevated DEET concentrations in plasma and brain, thereby potentiating neurological toxicity in susceptible subjects. In addition, DEET and oxybenzone had shown potential for hepatic toxicity *in vitro* and *in vivo*. No previous study had fully elucidated the mechanisms by which DEET produced toxicity, while potential mechanisms of toxicity from oxybenzone were still under investigation. Additionally, there was a lack of toxicological data for the metabolites of DEET and oxybenzone in the literature. Hence, various behavioral and cellular tests were carried out to achieve the following research objectives: 1) To assess the cellular viability of rat primary astrocytes and neurons using an MTT assay after exposure to DEET and oxybenzone, either alone or in combination, at various concentrations and duration; 2) To assess the cellular viability of rat 1548 hepatoma cells using a WST-1 assay after

exposure to DEET and its metabolites (DHMB and ET), and oxybenzone and its metabolites (DHB, DMB, and THB), either alone or in combination, at various concentrations and duration; 3) To assess the arousal (open field test), locomotion (open field and ladder test), habituation (open field test), and motor coordination (open field test and ladder test) after a 30-day, once-daily, repeated topical skin application in Sprague Dawley rats.

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Chapter 2

Tissue Deposition of the Insect Repellent DEET and the Sunscreen Oxybenzone from Repeated Topical Skin Applications in Rats

2.1. Abstract

Insect repellent DEET and sunscreen oxybenzone are capable of enhancing skin permeation of each other when applied simultaneously. We carried out a cellular study in rat astrocytes and neurons to assess cell toxicity of DEET and oxybenzone, and a 30-day study in Sprague-Dawley rats to characterize skin permeation and tissue disposition of the compounds. Cellular toxicity occurred at 1 μg/ml for neurons and 7-day treatment for both astrocytes and neurons. DEET and oxybenzone permeated across the skin to accumulate in blood, liver and brain after repeated topical applications. DEET disappeared from the application site faster than oxybenzone. Combined application enhanced the disposition of DEET in liver. No overt sign of behavioral toxicity was observed from several behavioral testing protocols. It was concluded that despite measurable disposition of the study compounds *in vivo*, there was no evidence of neurotoxicological deficits from repeated topical applications of DEET, oxybenzone or both.

2.2. Introduction

Application of insect repellents and sunscreens has been integrated into summer daily life for the general public in the developed countries, due primarily to the conscious awareness of the health threats from vector-borne diseases and skin cancers induced by sunlight radiation. Repellent and sunscreen products are the most practical, cost-effective, and well-accepted choice of defense, and a variety of preparations including sprays, lotions, aerosols, and cloth wipes have been commercially available as specialty products for decades. Concurrent application of repellent and sunscreen preparations has become prevalent in North America since 1999 when mosquito-transmitted West Nile virus first arrived in the continent.

Numerous active repellent and sunscreen ingredients are utilized for commercial civil use. DEET (N,N-diethyl-*m*-toluamide) and oxybenzone are two principal repellent and sunscreen components, respectively. Their repellency and UV-blockage efficacy have been adequately investigated and documented [1, 2]. With the approach to concurrent application, the latest investigations have been focused on the percutaneous interactions between active repellent and sunscreen compounds [3-5].

As topically applied preparations, protection efficacy of the repellent and sunscreen products relies solely on their presence and retention on the skin surface after each application. Transdermal permeation and systemic absorption of the active ingredients are considered neither desirable nor productive. Nevertheless, both DEET and oxybenzone are known to be capable of permeating across stratum corneum and reaching the general circulation after topical skin applications [6, 7]. Moreover, laboratory tests demonstrated permeation synergy between DEET and oxybenzone when both

compounds were applied simultaneously [4, 5]. The rate and extent of permeation varies, depending on application dose, preparation type, and application method.

Previous studies were carried out primarily to assess pharmacokinetics and toxicology of DEET and oxybenzone from individual applications [8-10], as concurrent application of repellents and sunscreens was not common. There were studies evaluating concurrent use of DEET or oxybenzone in association with several chemicals that were specifically designed for military or farming purposes [11, 12]. Loss of sun protection factor (SPF) has also been reported from mixing DEET-based repellents and sunscreens [13, 14].

Both repellents and sunscreens are designed for regular daily application over the summer months; it is not uncommon for military personnel, field workers, and farmers to apply both products for an extended period of time while working or training outdoors. Inappropriate use of these chemicals could potentiate unwanted adverse effects in susceptible subjects. In particular, enhanced systemic permeation and absorption of DEET and oxybenzone from repeated concurrent applications may be of health concerns and potential adverse effects should be subsequently elucidated and eliminated. Therefore, the primary objective of this study was to assess the disposition of DEET and oxybenzone from prolonged exposure at cellular levels and repeated skin applications in an animal model, respectively. Since DEET had demonstrated potential neurological toxicity in several earlier studies [11,15-17], we focused our experiments on cellular viability of rat astrocytes and neurons *in vitro* and tissue retention of the test compounds *in vivo*. Various behavioral testing protocols were also utilized to assess whether or not noticeable toxicological evidence would be observed from concurrent use of DEET and oxybenzone.

2.3. Materials and Methods

2.3.1. Materials and Reagents

Pure DEET and oxybenzone standards were purchased from Fluka Chemika GmbH (Buchs, Switzerland) and Riedel-de Haën GmbH (Seelze, Germany), respectively. They were accurately weighed and dissolved in 70% ethanol at a concentration of 100 mg/ml of DEET and 12.5 mg/ml of oxybenzone, either individually or in combination. The prepared test solutions were tightly packed in amber glass bottles and stored in a refrigerator for the duration of the study.

To carry out cellular studies, Neurobasal media, Dulbecco's modified Eagle medium-F12 (DMEM-F12), B-27 supplement, fetal bovine serum (FBS), L-glutamine, and antibiotic/antimycotic were purchased from Invitrogen (Burlington, Ontario, Canada). Rabbit anti-cow glial fibrillary acidic protein (GFAP) was obtained from Dako Diagnostics Canada (Mississauga, Ontario, Canada). Fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit and Texas red-conjugated sheep anti-mouse antibodies were supplied by Jackson Immunoresearch Laboratories (West Grove, Pennsylvania, USA). Anti-β-tubulin III came from Promega (Nepean, Ontario, Canada), and four-chamber culture slides were purchased from BD Bioscience (Mississauga, Ontario, Canada). [^{3}H]Adenine was purchased from Perkin Elmer (Boston, Massachusetts, USA), and Silica Gel TLC plates were obtained from Fisher Scientific (Whitby, Ontario, Canada).

To carry out drug extraction and analysis, acetonitrile, methanol, and sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Glacial acetic acid was obtained from Mallinckrodt Specialty Chemical Company (Paris,

Kentucky, USA). Ammonium acetate was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wisconsin, USA). Nitric acid was purchased from LabChem Inc. (Pittsburgh, Pennsylvania, USA). All solvents were HPLC-grade and other chemicals were AC-grade. Deionized water was obtained from a Milli-Q® Pure Water System (Nepean, Ontario, Canada) in the laboratory.

For negative and positive control studies in rats, 0.9% sodium chloride injection USP was obtained from Astra Zeneca Inc. (Mississauga, Ontario, Canada), and acrylamide standard was purchased from Sigma-Aldrich Corporation (St. Louis, Missouri, USA). The saline solution was used directly; sterile acrylamide solution (6.25 mg/ml) was prepared using deionized water and sealed in ampoules prior to the study.

2.3.2. Cellular Study

Rat primary cortical neurons and primary cortical astrocytes were cultured from E17 and E19 rat fetuses respectively according to procedures described previously [18]. The neurons and astrocytes were cultured on 24 well plates and were generally ≥ 95% and ≥ 98% pure, respectively.

The cultures were treated with DEET, oxybenzone or DEET/oxybenzone combination at various concentrations (0.1 µg/ml, 1 µg/ml, 10 µg/ml) for different time durations (24 h, 48 h, 7 d). After the treatment, cells were washed and incubated with respective media for 24 hours. The cell viability was then analyzed using standard MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay by adding MTT at a final concentration of 0.25 mg/ml into wells containing media and cells. With this assay, yellow MTT is metabolized by mitochondria of living cells and membrane impermeable

purple formazan crystals accumulate. After 4 hour treatment, the media/MTT supernatant was aspirated followed by solubilization of the crystals with a mixture of 2-propanol and 0.2 M HCl (7.3:1). Absorbance, which was proportional to the number of surviving cells, was read at 570 nM in triplicates in a 96-well scanning spectrophotometer. The experiments were performed in triplicates on a minimum of 3 independent cultures.

2.3.3. Animal Study

The Animal Use Protocol was approved by the University of Manitoba Animal Use Protocol Management and Review Committee, and conducted according to the current guidelines published by the Canadian Council for Animal Care (CCAC).

Sixty Sprague-Dawley rats were used in the study, which were randomly divided into six groups of ten animals (five males and five females in each group). The 8-week old rats (mean weight for males 268 g and mean weight for females 256 g) were obtained from the Central Animal Care Services, University of Manitoba. They were housed individually in holding cages and provided food and water *ad libitum*. The holding room was programmed to a 12-hour reversed light cycle in order to facilitate behavioral experiments that were performed on the animals during the study period.

Prior to topical skin application of the study preparations, an area of approximately 4 cm^2 (2×2 cm) on the back of the study animals was shaven using an electric clipper. This area remained relatively hair-free by regular shaving in order to facilitate topical applications and skin observations over the 30-day study period.

The three study doses were: Group 1, 40 mg/kg (2500 $\mu g/cm^2$) of DEET; Group 2, 5 mg/kg (312.5 $\mu g/cm^2$) of oxybenzone; Group 3, 40 mg/kg (2500 $\mu g/cm^2$) of DEET and

5 mg/kg (312.5 µg/cm^2) of oxybenzone. The weight of the study animals was recorded periodically over the study duration and doses were adjusted accordingly. Briefly, approximately 100 µl of the test solution was measured and applied onto the skin surface using a pipette. The solution was carefully spread over the shaven skin area with a disposable pipette tip and allowed for ambient evaporation before the study rats were returned to their holding cages. The upper dorsal end was selected for dose application because it was extremely difficult for the study animals to reach this area of the body. This application was repeated daily for a 30-day period. The applications were performed by the animal facility staff and blinded to the investigators involved in all other testing. Use of animal care staff ensured the consistency of the topical applications.

Two negative control study groups received topical applications of 0.9% saline (Group 4) and 70% ethanol solution (testing vehicle, Group 5) for 30 days. The positive control study group received acrylamide at a dose of 25 mg/kg, by intraperitoneal injection, for 10 days (Group 6). Behavioral testing was performed in all study groups to examine potential toxicity from topical application of DEET and oxybenzone.

2.3.4. Sample Collection

After the last topical application of the study dosing on day 30, 300 µl of blood samples were collected from the saphenous vein of the rats using Microvette® capillary collection tubes (Sarstedt AG & Co., Nümbrecht, Germany) at time 2, 6, 8 and 24 hours. The plasma was separated by centrifugation of the samples at 13,000 g for 30 minutes and stored in labeled polypropylene tubes at –20 °C until drug analysis.

Skin tape stripping was also performed to evaluate retention and penetration of DEET and oxybenzone after euthanasia of the animals. Before skin stripping, the application surface was swabbed using 400 µl of acetonitrile to collect extra dose that was still left behind. Upon complete drying of the skin, 12 pieces of D-Squame® stripping disks (CuDerm Corporation, Dallas, Texas, USA) were consecutively applied to the site, gently pressed for 10 seconds, and then peeled off. The collected tape strips were placed individually in labeled polypropylene tubes, and stored at −20 °C until drug analysis.

The liver and brain of the study animals were also collected after the euthanasia. They were first perfused with saline to remove blood, then harvested, dried, weighed, and stored in labeled polypropylene tubes at −80 °C until drug analysis.

Concentrations of DEET and oxybenzone in all collected samples were measured using an HPLC assay developed and validated in our laboratory [19]. The method was able to simultaneously quantify the compounds using photodiode array detection. For drug extraction, an automatic solid-phase extraction method was developed using a Zymark Rapidtrace® SPE Workstation (Caliper Life Sciences, Hopkinton, Massachusetts, USA). Briefly, the separation was completed on a Waters® Oasis® MAX 3cc (60 mg) extraction cartridge, by using acetonitrile, 0.03 M ammonium acetate (pH 4.5) and water as preconditioning and washing solvents. 300 µl of methanol was used as the final solvent to elute the cartridge; the eluent was vortexed for 15 seconds, transferred to an HPLC vial and 50 µl of the sample injected. 50 µl of plasma samples were used for drug extraction. For skin swipes and tape strips, they were dissolved in 1.5 ml of acetonitrile and extracted. Liver and brain samples were first homogenized in acetonitrile using an

electric homogenizer (Biospec Products, Bartlesville, OK). A portion of the homogenate was further subjected to solid-phase extraction, and 50 µl of the eluent was injected to the HPLC system for drug measurement.

2.3.5. Behavioral Testing

Various behavioral testing protocols were employed to assess the arousal (open field test), locomotion (open field and ladder test), habituation (open field test) and motor coordination (open field test and ladder test) of the animals over the study duration [20]. It was hypothesized that systemic exposure of the study substances, particularly repellent DEET, could induce behavioral changes due to its reported effect on the neurological system. It was anticipated that the behavioral testing could identify changes among the different study groups.

The ladder rung walking test evaluated the ability of study animals crossing a narrow passage of ladder rungs [21]. The device was composed of a 1m-long Plexiglass alley equipped with irregularly spaced stainless steel rungs. The ladder was elevated 100 cm above the ground, with a goal box located at one end of the track. The progress of the animals was videotaped so that the time used by the animals to cross the entire ladder as well as the miss or slip of the rungs by the animals could be accurately scored and compared.

The open field testing assessed the mobility and agility of the study animals over the course of the experiment [22]. The apparatus was composed of a wooden square box (1×1 m) with walls of 30 cm; the whole floor area was further divided into 16 equally sized squares. Each animal was placed in the center of the apparatus to begin the

experiment, and the movement was videotaped for 4 minutes. The study recording was also scored and analyzed afterwards.

All behavioral testing was performed in a quiet dark room under red lights, separated from the animal holding room. Each rat was tested individually for these experiments. The study animals were trained in the ladder test prior to each study, and baseline was recorded. The positive control study group was tested on day 10, when clear signs of adverse effects were observed. All other study groups were tested 4 hours after the dosing on day 29 in order to assess behavioral changes from the topical applications.

2.3.6. Data Analysis

Amounts of DEET and oxybenzone were calculated from the average HPLC calibration curve. Total recovery of the compounds in collected samples was obtained where applicable. Drug concentrations among biological samples and between study groups were correlated. In addition, results from behavioral testing were also scored and compiled for data analysis.

Plasma concentrations of DEET and oxybenzone were subjected to non-compartmental pharmacokinetic simulation using WinNonlin® software (Version 5.0.1, Pharsight Corporation, Mountain View, California, USA) to calculate apparent elimination half-life. The following statistical analyses of the data were also conducted, a) a one-way ANOVA followed by the Tukey's test for all behavioral results among the six study groups and all cellular results among the study groups (PC-SAS® 8.02, SAS Institute Inc., Cary, North Carolina, USA); b) skin distribution parameters, liver and brain concentrations, and total recoveries were calculated as a percentage of the final dose, and

then compared between single and combined applications using the Mann-Whitney *U* Test (PC-SAS® 8.02, SAS Institute Inc., Cary, North Carolina, USA). Normally distributed data was expressed in Mean±SEM while non-parametric data was represented as Median±SD. Differences were considered statistically significant at $p < 0.05$.

2.4. Results

2.4.1. Cell Viability

Figure 2.1 shows the cellular viability results from rat astrocytes and neurons after the cultures were exposed to DEET, oxybenzone or a combination of the two compounds at three concentration levels for three time intervals. No significant differences in astrocyte viability were observed for 24-hour or 48-hour exposure to the test substances in comparison to the control group. However, astrocyte viability was significantly reduced by 25% after the cultures were exposed to both DEET and oxybenzone at 10 µg/ml for 7 days.

Similarly, no significant differences in neuron viability were observed for 24-hour or 48- hour exposure to the test substances in comparison to the control group. However, treating the neurons for 7 days with 1 µg/ml and 10 µg/ml of DEET decreased the cellular survival by 34% and 49% respectively. A 36% decrease in neuron viability was also noted when the cultures were exposed to 10 µg/ml of oxybenzone for 7 days. Combined use of DEET and oxybenzone did not affect the viability of neurons.

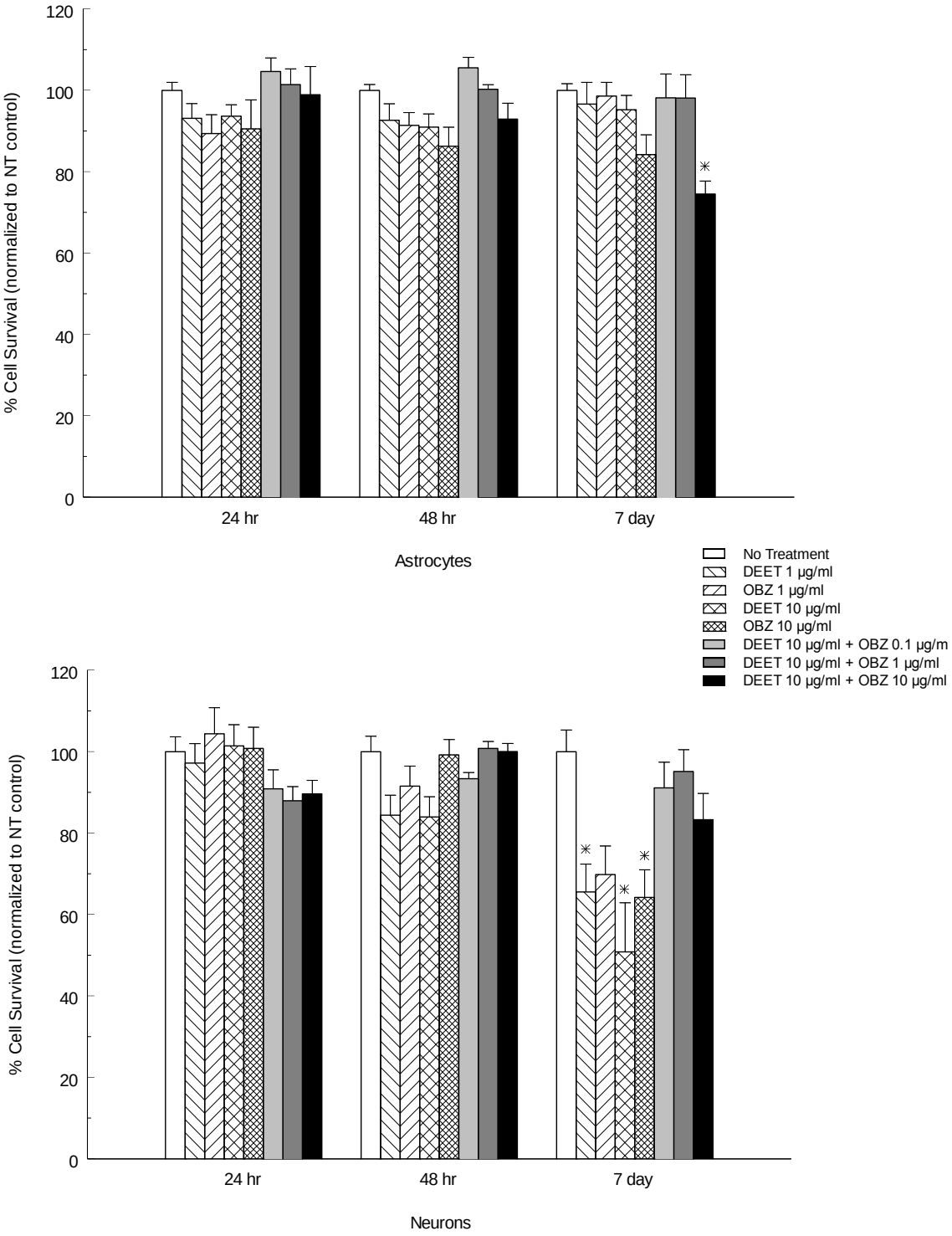

Figure 2.1. Results of cellular viability of astrocytes and neurons exposed to DEET and oxybenzone (* significant difference from NT control, $p < 0.05$, n = 4-8, mean±SEM).

Table 2.1. Concentration (μg/cm^2) and total recovery (%) of DEET and oxybenzone from skin application site.

Sampling Type	DEET		Oxybenzone	
	Group 1	Group 3	Group 2	Group 3
Skin Swipe	950.6±586.8	761.8±398.1	192.1±102.9	161.7±96.1
Tape Strips	2.8±1.4	2.7±0.6	4.9±5.1	7.0±8.2
Recovery	38.1±23.5	30.6±15.9	63.0±34.6	54.0±33.4

Median±SD, n = 10

2.4.2. Recovery of DEET and Oxybenzone from the Skin

DEET and oxybenzone were recovered from the site of skin application in two ways. Skin swipes were taken 24 hours after the final application to collect the extra dosing that was still left after a 30-day topical use, while skin tape strips recovered the application dose that permeated into skin layers. **Table 2.1** lists the concentration and last dosing recovery of DEET and oxybenzone from the topical application site.

Total recovery of DEET was 25% lower in Group 3 (combined DEET and oxybenzone) than Group 1 (DEET), and that of oxybenzone was 17% lower in Group 3 than Group 2 (oxybenzone). Although no statistically significant difference was observed between the two application groups for either DEET or oxybenzone, recovery data indicated that the combined application disappeared more quickly from the surface of the skin than the single application. This skin disposition profile might also have been expedited by the solvent, as ethanol is a quickly-dispatching vehicle that facilitates permeation and evaporation of DEET after topical skin application. Oxybenzone tends to form a skin deposition due to its higher lipophilicity; this property had been observed in our previous studies [5].

Table 2.2. Concentration (ng/g) and total recovery (‰) of DEET and oxybenzone in the liver and brain.

Sampling Type	DEET		Oxybenzone	
	Group 1	Group 3	Group 2	Group 3
Liver	95.9±209.3	350.8±155.9*	108.1±567.6	58.0±105.3
Brain	7.6±5.9	8.5±7.2	34.1±5.6	15.4±8.1†
Recovery	0.14±0.28	0.54±0.37*	1.27±4.45	0.78±1.80

* significant difference from Group 1 ($p < 0.05$); † significant difference from Group 2 ($p < 0.05$); Median±SD, n = 10

2.4.3. Concentrations of DEET and Oxybenzone in Plasma and Tissues

Both DEET and oxybenzone were detected in plasma. **Figure 2.2** shows the concentrations of DEET and oxybenzone over a period of 24 hours after the last skin application. Concurrent application of DEET and oxybenzone appeared to elevate the levels of DEET (20-66%) but not oxybenzone. Concentration of oxybenzone at 2 hours was approximately twice that of DEET. DEET and oxybenzone were still detectable in plasma 24 hours after the skin application.

DEET and oxybenzone were also detected in both liver and brain samples. **Table 2.2** lists the concentration and recovery of the compounds. Concurrent application of DEET and oxybenzone produced significantly higher deposition of DEET in liver than application of DEET alone, although disposition of DEET in brain was much lower than that in liver, the prime metabolism site of the body. Concurrent application of DEET and oxybenzone did not produce enhanced disposition of oxybenzone in liver and brain; the only difference found was lower oxybenzone concentration in brain (121% lower than single application).

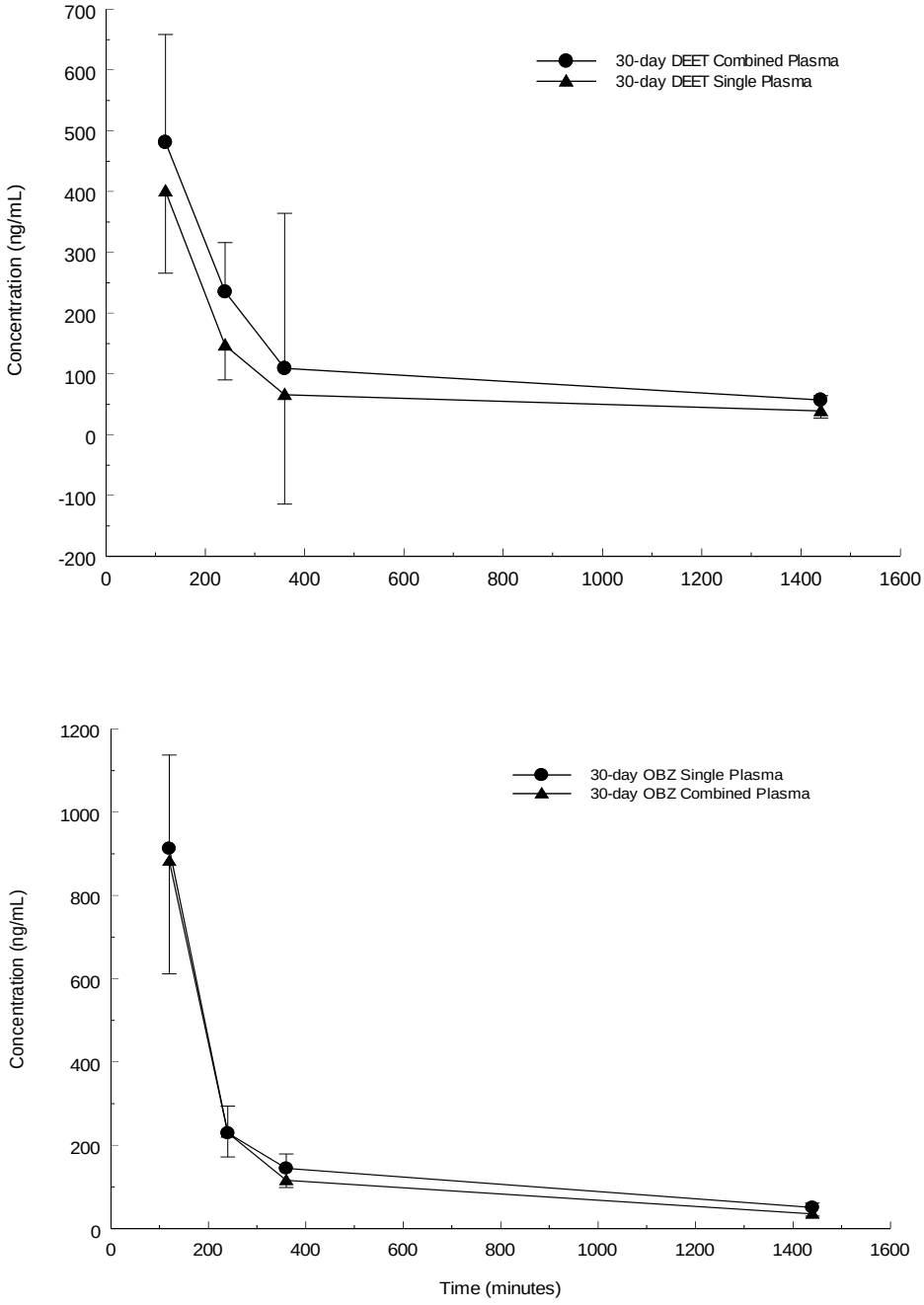

Figure 2.2. Plasma concentrations of DEET and oxybenzone after last topical skin application on day 30 (n = 10, mean±SEM).

2.4.4. Behavioral Testing Results

As expected, the positive control of acrylamide resulted in visible impairment of the study animals after a 10-day injection period. There was a significant difference detected between this positive control group and negative control group (saline solution, topical application) or vehicle control group (70% ethanol solution, topical application) for all study groups. No significant differences were observed among rats when DEET, oxybenzone and combined DEET/oxybenzone were topically applied to the animals for a 30 day period. **Figures 2.3 and 2.4** show the results of ladder test and open field test for all study groups, respectively.

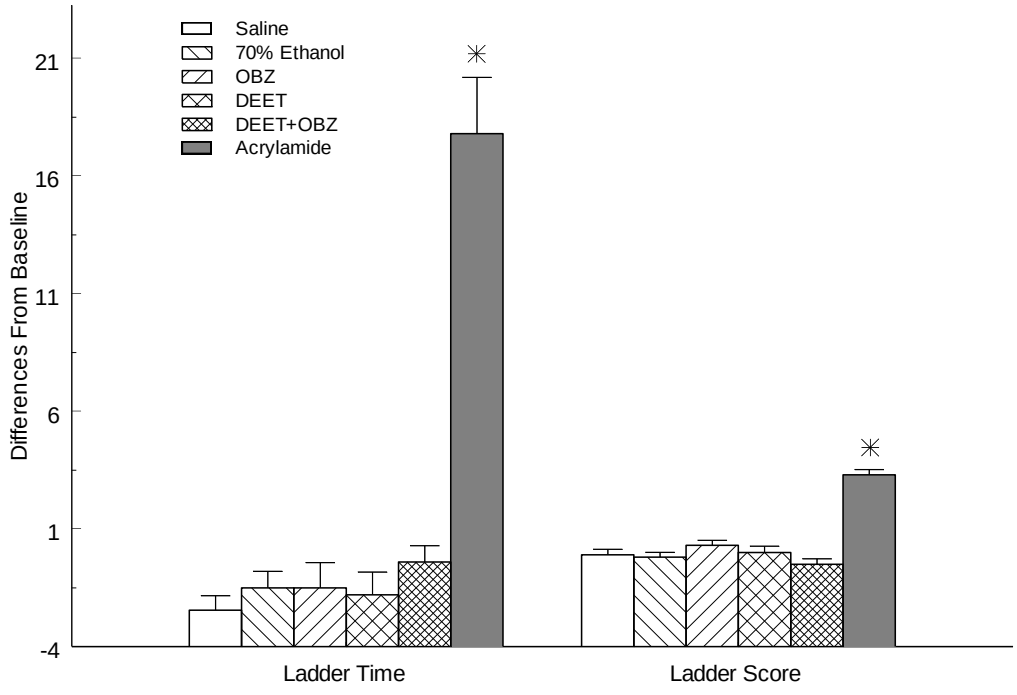

Figure 2.3. Results of ladder test from 6 study groups on day 29 (* significant difference from saline, vehicle, OBZ, DEET and combined groups, $p < 0.05$, n = 10, mean±SEM).

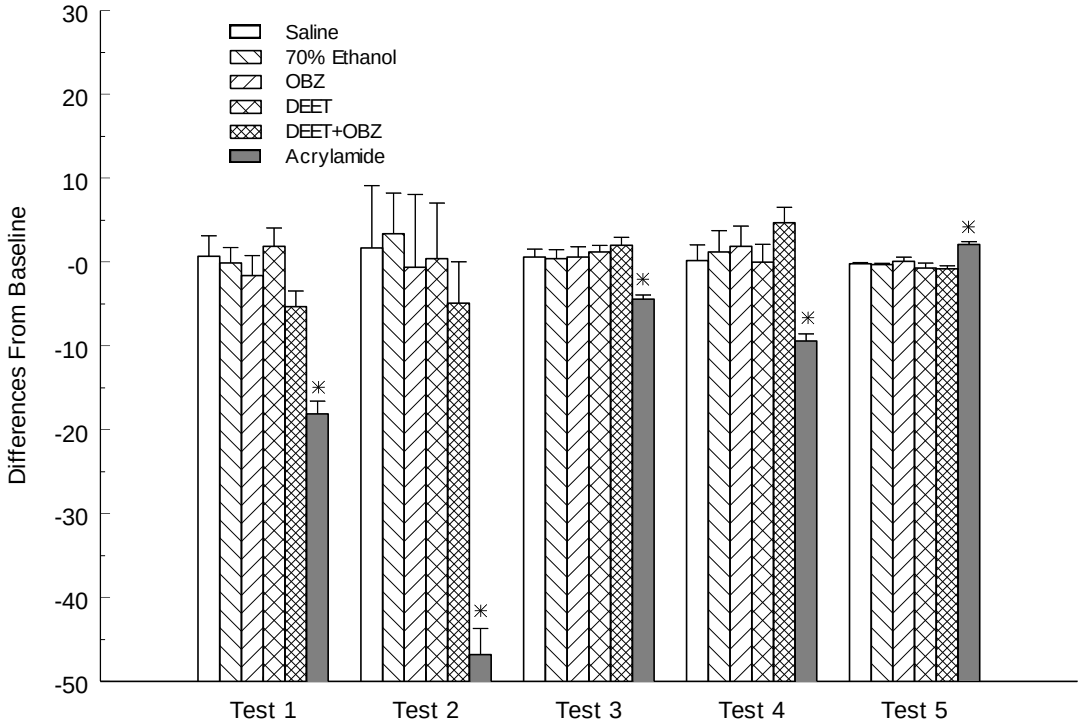

Figure 2.4. Results of open field test from 6 study groups on day 29. Test 1: Number of Rearings; Test 2: Total Number of Squares Entered; Test 3: Number of Times Entered Center Squares; Test 4: Number of Center Squares Entered; Test 5: Number of Fecal Boli. (* significant difference from saline, vehicle, OBZ, DEET and combined groups, $p < 0.05$, n = 10, mean±SEM).

In the ladder test, slips were defined as a total miss or a deep slip of the paws of the study animal while passing the ladder corridor [21]. Rats that did not finish (DNF) the ladder test were automatically assigned a slip number 4 and a time of 30 seconds, and differences between study day 29 and baseline (day 0) were calculated. A negative score indicated an increase in moving ability and a positive score a reduction in moving ability.

In the open field test, total and center squares entered were measured, as well as the number of times the animals entered the central 4 squares, number of fecal boli and

rearings. Habituation is defined as a form of simple, nonassociative learning by a subject in which the magnitude of the response to a specific stimulus decreases with repeated exposure to that stimulus; a reduction in the number of rearings may indicate either a weakness in hind limbs of the subjects or habituation of the testing environment [23, 24].

No significant changes were recorded from the study groups on the testing day. Study animals from the three treatment groups as well as the negative/vehicle control groups passed the 29 day study period without significant adverse effects. Further systemic studies are therefore needed to refine behavioral testing criteria and observe for subtle changes possibly associated with the study compounds.

2.5. Discussion

In general, preparations intended for topical skin application should exert optimal efficacy either on the skin surface or within the top skin layers, without substantial percutaneous systemic permeation and absorption. This characteristic is of particular importance to specialty products like repellents and sunscreens, because they are designed to protect the skin from externally damaging factors such as biting insects and UV radiation. Systemic exposure of the body to DEET and oxybenzone is detrimental and counterproductive from clinical perspectives. Repellents and sunscreens are largely applied at the discretion of individual users, without medically-recommended or accepted doses. Moreover, repeated applications of repellents and sunscreens are commonplace during summer months when mosquito infestation and UV radiation are high. Any inadvertent exposure of the body to an increased level of DEET and oxybenzone could lead to potential toxicity of the substances in susceptible subjects.

It is well known that neurons have a remarkable ability to transmit rapid electrical signals in the form of action potentials [25]. However, in the past few years, the emergence of astrocytes as the main neural cell type responsible for the maintenance of brain homeostasis has become evident due to their cooperation with neurons on several levels, including neurotransmitter trafficking and recycling, ion homeostasis, energy metabolism, and defense against oxidative stress [26]. Disposition of DEET and oxybenzone in astrocytes and neurons could result in severe neurological effects by disrupting this brain homeostasis. Thus, neurocellular experiments were an essential component of this study, as it could provide a better understanding of the underlying mechanisms causing potential behavioral deficits in the study animals from repeated skin applications.

Although the mammalian brain has a very high metabolic rate, neurons are by nature extremely sensitive to small modifications to their microenvironment [26]. However, the viability of rat astrocytes and neurons was affected only after a prolonged exposure to the test compounds in this study. Astrocytes were sensitive to high concentrations of DEET and oxybenzone in combination, while neurons were more sensitive to individual applications. Past studies looking at DEET alone reported significant neuronal degeneration in the motor cerebral cortex, the dentate gyrus, hippocampal cells, and the Purkinje cell layer of the cerebellum, but those studies did not find any significant behavioral differences between DEET and control animals [15]. Based on results obtained from our study, further cellular studies might be beneficial to clarify the inconsistencies in the literature regarding the toxic effects of insect repellent DEET [27].

As one of the largest organs in surface area and body weight, skin has long been viewed as a passive physical barrier between human body and external environment.

Stratum corneum is the outmost skin barrier that restricts the penetration of a majority of foreign substances. Systemic percutaneous absorption will take place only when the barrier function of stratum corneum is compromised. There are various mechanisms by which chemical substances permeate across stratum corneum. Passive diffusion driven by a high concentration gradient is one of the primary permeation mechanisms for drug compounds to overcome the skin barrier and reach the systemic circulation. For lipophilic molecules like DEET and oxybenzone, direct penetration through the intercellular laminea of stratum corneum should also allow for ready availability of the compounds for systemic absorption within the skin [28].

Both DEET and oxybenzone were recovered in appreciable amounts from the application site in the study animals. Recovery of oxybenzone was higher than that of DEET, attributing partially to ready absorption/evaporation of DEET and prolonged retention of oxybenzone at ambient temperature. 70% Ethanol was used in this study as a vehicle to dissolve both test compounds and to facilitate easy skin applications. Recent studies have demonstrated that evaporation of DEET was 47-49% and 33-40% for microcapsules and ethanol solution, respectively [29]. In addition, DEET evaporates from the skin surface at a rate proportional to its localized concentration in the skin [30]. Results obtained from this study were consistent with those previously reported. In view of the significant recovery of DEET and oxybenzone by skin swabs, it is therefore important for individuals to clean skin thoroughly of repellent and sunscreen products once indoors. This will significantly minimize prolonged transdermal exposure of the active ingredients, particularly beneficial to frequent and heavy users of the preparations.

Skin tape stripping was utilized to assess movement of DEET/oxybenzone within the skin. Other studies have indicated no DEET accumulation in the skin [10, 31], which was similar to what was found in this study. In comparison to a previous study using piglets, recovery of DEET and oxybenzone by tape stripping was much lower from this study [5]. This discrepancy might be partially attributed to the lotion products used in piglets where preparations prolonged the retention of test compounds in the skin. In addition, anatomical differences between rat skin and piglet skin might have also played a role in skin retention of DEET and oxybenzone. Sprague-Dawley rats have a thickness of 14.7±2.2 μm and 4.3±1.2 μm for the epidermis and stratum corneum respectively, while piglets have a thickness of 36.9±7.4 μm and 3.5±0.9 μm for the epidermis and stratum corneum respectively [32].

Quantifiable amounts of DEET and oxybenzone were detected from plasma samples in the study. This indicated ready systemic absorption of the compounds from the skin applications. Both DEET and oxybenzone primarily undergo metabolism in the liver and are subsequently excreted in urine. This metabolism is relatively quick as previous studies have shown; elimination half-life of DEET was reported to be approximately 2.5 hours in beagle dogs and cattle [7, 33]. In our previous study with piglets, the half life of DEET and oxybenzone was 7.3 hours and 8.0 hours, respectively [5]. Apparent elimination half-life from this study was estimated 9.1±2.1 hours for DEET and 7.9±1.7 hours for oxybenzone. Both DEET and oxybenzone were measurable 24 hours after the skin application. This might have resulted from two factors, i.e., topical skin applications and a 30-day study period. Dermal application tends to prolong the absorption phase of applied chemicals across the skin, which subsequently influenced the

disposition of the compounds *in vivo*. Higher amounts of oxybenzone were detected in the tape strips; therefore, as has been reported, it is probable that oxybenzone was forming a skin depot that continued to supply the compound into the general circulation [5,9,28]. This characteristic should be further assessed in order to provide guidelines for the safe and effective use of repellents and sunscreens for long-term skin applications.

Similarly, both DEET and oxybenzone were detected in the two vital organs from the study. Tissue levels of DEET and oxybenzone tend to be higher in the liver, the prime metabolism site of the body, which had also been proved in previous studies [8, 9, 34]. The differences observed between combined application of DEET with oxybenzone and application of DEET alone might be detrimental from a clinical viewpoint, as oxybenzone appeared to slightly enhance systemic accumulation and disposition of DEET. This could result in undesirable adverse effects of the repellents in susceptible subjects, from prolonged application of the two preparations. Disposition of oxybenzone in liver and brain was quite different from that of DEET, in which concurrent application with DEET did not apparently enhance its percutaneous absorption. In our previous studies with the compounds, it was found that concurrent application affected DEET more than oxybenzone [5]. In this study, concentration of oxybenzone present in brain was higher than DEET. This might be attributed to different lipophilicity attributes of the two compounds. Partition coefficients of DEET and oxybenzone in octanol and water (log P octanol/water) are 2.0 and 3.8, respectively [35, 36]. High concentrations of oxybenzone had also been detected from fat tissues after topical administration [37]. Based on disposition results obtained from this study, further studies should be carried out to investigate the distribution of the compounds in other tissues or organs.

The primary objective of the animal study was to evaluate whether or not concurrent application of DEET and oxybenzone would induce observable adverse effects in the study animals. Neurological toxicity of DEET in combination with permethrin and/or malathion/pyridostigmine bromide from repeated applications had been documented in previous studies [11, 15-17, 38]. Oxybenzone, on the other hand, was only related to minor side effects such as skin allergy and contact dermatitis [39]. However, oxybenzone possesses structural resemblance to steroid hormones, and it demonstrated disruptive potential to the general endocrine system [40]. Carcinogenicity of oxybenzone was also studied, but results were inconclusive [41]. In this study, experiments were focused on behavioral abnormalities, which were considered directly correlative of neurological toxicity from DEET and potentially oxybenzone, as shown by neurocellular data.

Animal behavioral responses have been shown to provide integrative markers of neurotoxicity reflecting biochemical, physiological, and neuropathological reactions to toxicants [42]. Previous research has indicated that animals treated with DEET 40 mg/kg alone exhibited significant sensorimotor impairment compared to control, which was reflected in inclined plane performance, forepaw grip time, beam-walk scores, and beam-walk time when assessed after thirty days of daily exposure [11]. However, no significant behavioral changes were observed in this study when DEET and/or oxybenzone were applied topically for 30 days. Any small differences seen among the six treatment groups were likely attributed to acute increases in emotionality and/or unfamiliarity with the testing protocols. In addition, to minimize the risk of type II statistical error, male and female rats were grouped together to create a sufficient power index. Furthermore, this study demonstrated that the chemical concentrations found in the biological samples did

not produce any changes in animal behavior thus indicating that concurrent use of DEET and oxybenzone at 40 mg/kg and 5 mg/kg did not produce any overt signs of toxicity. Behavioral testing is one of the assessment methods for neurological toxicity, but it does sometimes yield variable results when replicated in different laboratories; the degree of variability may also increase with experimental parameters such as laboratory environment, apparatus setting, testing time and evaluation criteria [43]. Studies at higher doses or in susceptible animal models might therefore be needed in future experiments.

2.6. Conclusion

In conclusion, exposure of rat astrocytes and neurons to the insect repellent DEET and the sunscreen oxybenzone led to reduced astrocyte viability from combined application, while neurons were affected only by DEET or oxybenzone used alone. DEET and oxybenzone were detected to penetrate across the skin after a 30-day topical application in a rat animal model. Systemic absorption of the compounds was correlative among skin, plasma and tissue samples. Both DEET and oxybenzone demonstrated a fast transdermal penetration, but no significant differences were observed between single and combined applications. Concurrent application of DEET and oxybenzone enhanced the concentration of DEET in the liver, but it reduced the brain disposition of oxybenzone. Behavioral testing did not find overt signs of toxicity that had been reported from previous studies, from either single or combined application of the study substances. Insect repellents and sunscreens are specialty consumer care products widely utilized by the general public for summer outdoor activities. Future studies will evaluate kinetic profiles of concurrent application as well as the potential for hepatic toxicity due to the

accumulation of the parent compounds and/or primary metabolites. Formulation development will also be optimized to minimize overall skin permeation of the active ingredients; this will be particularly beneficial to those special workers and outdoor enthusiasts that utilize repellents and sunscreens regularly for extended periods of time.

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Chapter 3

Tissue Disposition of the Insect Repellent DEET and the Sunscreen Oxybenzone Following Intravenous and Topical Administration in Rats

3.1. Abstract

Insect repellent DEET and sunscreen oxybenzone (OBZ) have been shown to produce synergistic permeation enhancement when applied concurrently *in vitro* and *in vivo*. The disposition of both compounds following intravenous administration (2 mg/kg of DEET or OBZ) and topical skin application (100 mg/kg of DEET and 40 mg/kg of OBZ) was determined in male Sprague-Dawley rats. Pharmacokinetic analysis was also conducted using compartmental and non-compartmental methods. A two-compartment model was deemed the best fit for intravenous administration. DEET and oxybenzone permeated across the skin to accumulate in blood, liver, and kidney following topical skin application. Combined use of DEET and oxybenzone accelerated the disappearance of both compounds from the application site, increased their disposition in the liver, and significantly decreased the apparent elimination half-lives of both compounds ($p<0.05$). Hepatoma cell studies revealed toxicity from exposure to all treatment concentrations, most notably at 72 hours. Although DEET and oxybenzone were capable of mutually enhancing their percutaneous permeation and systemic disposition from topical skin application, there was no evidence of increased hepatotoxic deficits from concurrent application.

3.2. Introduction

Topical application of insect repellents and sunscreens has become an integral part of summer daily life due primarily to the awareness of health threats from vector-borne diseases and sun-exposure safety. Topical repellents and sunscreens are the most practical, cost-effective, and universally accepted products of choice. A variety of preparations including sprays, lotions, aerosols, and cloth wipes have been available as non-prescription specialty products for decades. Concurrent skin application of repellent and sunscreen preparations has been prevalent in North America since 1999 when mosquito-transmitted West Nile virus was first identified in the continent.

There are numerous active repellent and sunscreen ingredients utilized for commercial civil use; N,N-diethyl-*m*-toluamide (DEET) and oxybenzone (OBZ) are the two principal repellent and sunscreen components, respectively. Their repellency and UV-blockage efficacy have been adequately investigated and documented [1, 2]. Interactions between active repellent and sunscreen compounds, as well as subsequent percutaneous disposition of these substances after topical application, have also been studied and reported [3-5].

Designed as topical preparations, insect repellents and sunscreens exert their protection efficacy on the skin surface after each application. Percutaneous permeation and systemic absorption of the active ingredients are considered neither desirable nor productive. Nevertheless, both DEET and oxybenzone are known to be capable of permeating through the stratum corneum and reaching the general circulation after topical skin applications [6, 7]. Laboratory tests have demonstrated permeation synergy between DEET and oxybenzone when both compounds were applied simultaneously [4, 5]. The rate

and extent of permeation varies, depending on application dose, formulation type, and application method.

Previous studies were carried out primarily to assess pharmacokinetics and toxicology of DEET and oxybenzone from application of individual compound [8-10], as concurrent skin application of repellents and sunscreens was not common. There were studies evaluating concurrent use of DEET or oxybenzone in association with several other chemicals that were specifically utilized for military or farming purposes [11, 12]. Loss of sun protection factor (SPF) has also been reported from mixing DEET-based repellents and commercial sunscreens [13, 14]. The primary purpose of this study was to evaluate the pharmacokinetics and tissue disposition of DEET and oxybenzone from intravenous and topical administration in rats. In addition, we carried out *in vitro* hepatoma cell experiments to assess potential hepatotoxicity resulting from the enhanced disposition of DEET and oxybenzone in the liver.

3.3. Materials and Methods

3.3.1. Materials and Reagents

Pure DEET and oxybenzone standards were purchased from Fluka Chemika GmbH (Buchs, Switzerland) and Riedel-de Haën GmbH (Seelze, Germany), respectively. Sodium chloride injection (0.9%, USP) was obtained from Astra Zeneca Inc. (Mississauga, Ontario, Canada); anhydrous ethyl alcohol was obtained from Commercial Alcohols Inc. (Brampton, Ontario, Canada) and Emulphor® (ethoxylated castor oil) was purchased from Nihon Emulsion Co. Ltd. (Tokyo, Japan).

Drug extraction and analysis used acetonitrile, methanol, and sodium hydroxide that were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Glacial acetic acid was obtained from Mallinckrodt Specialty Chemical Company (Paris, Kentucky, USA). Ammonium acetate was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wisconsin, USA). Nitric acid was purchased from LabChem Inc. (Pittsburgh, Pennsylvania, USA). All solvents were HPLC-grade and other chemicals were AC-grade. Deionized water was obtained from a Milli-Q® Pure Water System (Nepean, Ontario, Canada) in the laboratory.

Cell viability studies utilized HyClone classical liquid media Minimum Essential Medium with Earle's Balanced Salts (MEM/EBSS), which was purchased from Thermo Fisher Scientific (Nepean, Ontario, Canada). Fetal bovine serum, pyruvate, L-glutamine, penicillin, streptomycin, and ethylenediaminetetraacetic acid (EDTA) trypsin were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Rat hepatoma cell line 1548 was purchased from American Type Culture Collection (ATCC, Rockville, Maryland, USA). Cell Proliferation Reagent WST-1 was obtained from Roche (Laval, Quebec, Canada). Cell culture plates were purchased from Sarstedt (96-well, Cat# 83.1835, Montreal, Quebec, Canada).

3.3.2. Animal Study Model

The Animal Use Protocol was approved by the University of Manitoba Fort Garry Campus Animal Use Protocol Management and Review Committee, and conducted according to the current guidelines published by the Canadian Council for Animal Care (CCAC). Twenty seven male Sprague-Dawley rats were used in the study, which were

randomly divided into five study groups, i.e., two groups for intravenous application (n=6) and three groups for topical application (n=5).

Rats (average body weight of 250 g) were obtained from the Central Animal Care Services, University of Manitoba. Animals were checked by a University veterinarian for general health conditions upon arrival at the Department of Zoology Animal Holding Facility. They were housed individually in holding cages and provided food and water *ad libitum*. A 5-day quarantine period was provided to the study animals, allowing them to adapt to the environment before the actual experiment commenced.

3.3.3. Intravenous and Topical Administration

For intravenous administration, solutions of DEET (0.3%, w/v) and oxybenzone (0.1%, w/v) were prepared using a mixture of Emulphor:ethanol:water (2:3:5, v/v/v). The study preparation was injected into tail vein of the animals at 2 mg/kg of DEET (Group 1) or 2 mg/kg of oxybenzone (Group 2)[7, 15-17].

For topical skin application, DEET and oxybenzone were weighed and dissolved in anhydrous ethanol at a concentration of 120 mg/ml DEET and 48 mg/ml oxybenzone, either individually (Single, S) or in combination (Combined, C). Prior to administration, an area of approximately 25 cm^2 (5×5 cm) on the back of the study animals was shaved using an electric clipper. The three study doses were 100 mg/kg of DEET (Group 3), 40 mg/kg of oxybenzone (Group 4), and combined 100 mg/kg of DEET and 40 mg/kg of oxybenzone (Group 5), respectively. These topical doses were selected based on previous literature [9, 18-20], and were considerably larger than intravenous doses to ensure adequate tissue detection of the study compounds. A total of 250 µl of test solution was measured

and applied onto the skin surface using a pipette. The solution was carefully spread over the shaven skin area with a disposable pipette tip, and sufficient time for the ambient evaporation of the solvent was allowed before the study rats were returned to their holding cages. The application procedures were performed by an animal care technician in order to ensure the consistency of the drug administration and the accuracy of the study results.

3.3.4. Sample Collection After Intravenous and Topical Dosing

After intravenous injection or topical dosing, 150 µl blood samples were collected from the saphenous vein of the study animals using heparinized Microvette® capillary collection tubes (Sarstedt AG & Co., Nümbrecht, Germany) at 30, 60, 90, 120, 150, 180, 240, 360, 480, 600, and 1400 minutes. A bolus of 10 ml saline was injected subcutaneously after the 120-minute point to compensate for the loss of blood volume. Plasma was separated by centrifugation of the samples immediately at 13,000 g for 30 minutes, and stored in labeled polypropylene tubes at –20 °C until drug analysis.

After topical application, skin tape stripping was performed at 7 hours and 24 hours to evaluate percutaneous penetration of DEET and oxybenzone into various skin layers. Before skin stripping, the application surface was swabbed once using cotton swabs saturated with 400 µl of acetonitrile. Upon complete drying of the skin, 4 (at 7 hours) and 12 (at 24 hours) pieces of D-Squame® stripping disks (CuDerm Corporation, Dallas, Texas, USA) were consecutively applied to the site, gently pressed for 10 seconds, and then peeled off. Different application sites were used for each skin stripping in order to realistically assess the dermal penetration of DEET and oxybenzone from the

applied preparation. The collected tape strips were placed individually in labeled polypropylene tubes, and stored at –20 °C until drug analysis.

Liver and kidney specimens were also collected after study animals were euthanized at the end of the study. They were rinsed with saline solution to remove blood, dried, weighed, and stored in labeled polypropylene tubes at –80 °C until drug analysis.

3.3.5. Concentration Measurement

Concentrations of DEET and oxybenzone were measured using an HPLC assay developed and validated in our laboratory [21]. The method was capable of quantifying the compounds simultaneously using photodiode array detection.

To extract DEET and oxybenzone from plasma, an automatic solid-phase extraction method was developed using a Zymark Rapidtrace® SPE Workstation (Caliper Life Sciences, Hopkinton, Massachusetts, USA) [17]. Briefly, the separation was completed on Waters® Oasis® MAX 3cc (60 mg) extraction cartridges, by using acetonitrile, 0.03 M ammonium acetate (pH 4.5) and water as preconditioning and washing solvents; 50 µl plasma was used for drug extraction, and 300 µl methanol as the final elute solvent. The eluent was vortexed for 15 seconds, transferred to an HPLC vial and 50 µl of the sample injected. Skin swipes and tape strips were dissolved in 1.5 ml of acetonitrile and extracted. Liver and kidney samples were first homogenized in acetonitrile using an electronic homogenizer (Biospec Products, Bartlesville, Oklahoma, USA). A portion of the homogenate was further subjected to solid-phase extraction, and 50 µl of the eluent was injected to the HPLC system for drug measurement.

3.3.6. Cell Culture Assay

Rat hepatoma cell line 1548 was grown in MEM/EBSS supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10% fetal bovine serum, 4 mM L-glutamine, and 0.011% sodium pyruvate in a humidified, 37 °C incubator under an atmosphere of 95% air and 5% CO_2. Cells were maintained in a 75 cm^2 culture flask (Corning, Acton, Massachusetts, USA).

Cells were trypsinized from the 75 cm^2 culture flask and seeded at a density of 3,000 cells per well in 96-well plates. Cells were allowed to attach to the plates in an incubator containing 5% CO_2 at 37 °C for 12 hours. Following attachment, the medium in each well was removed and replaced with 100 µl fresh medium containing DEET and oxybenzone at pre-determined concentrations of 0.1 µg/ml, 1 µg/ml, and 10 µg/ml. Cells were then incubated in their respective media for 24, 48, and 72 hours. In order to maintain DEET and oxybenzone concentrations and to ensure sufficient exposure time, medium in each well was renewed every 24 hours by adding 50 µl of fresh medium containing the same concentrations of DEET and/or oxybenzone. After each interval of exposure time, Cell Proliferation Reagent WST-1 was added to the wells to achieve a 1:10 dilution of the WST-1 reagent. Plates were gently shaken for 5 minutes and then cultured cells were incubated in the presence of WST-1 reagent for 2 hours according to manufacturer's recommendation. The absorbance of each well was measured by using an ELx 808 Ultra Microplate Reader (BIO-TEK Instrument, Winooski, Vermont, USA) at 450 nm. Wells with only the medium were utilized as the background control, while wells containing normal medium with the same amount of cells (3,000 per well) were used as the treatment control.

3.3.7. Data Analysis

Amounts of DEET and oxybenzone in biological specimens were calculated using an average HPLC calibration curve [21]. Plasma concentrations of DEET and oxybenzone from topical administration were subjected to non-compartmental analysis using WinNonlin® software (Version 5.0.1, Pharsight Corporation, Mountain View, California, USA) for determination of pharmacokinetic parameters [22, 23]. Linear pharmacokinetics were assumed as reported in previous studies on DEET and oxybenzone [5, 7, 8, 15, 16]. In addition, data from intravenous administration was fit to a two-compartmental IV bolus model, with no lag time, $1/(Y_{hat} \cdot Y_{hat})$ weighting, and first-order elimination ($C_p = Ae^{-\alpha t} + Be^{-\beta t}$) [22, 23]. Following topical application, peak plasma concentration (C_{max}) and time to the peak concentration (t_{max}) were determined directly from the plasma-time profiles [22]. Mean absorption time (MAT) was calculated from the following equation, $MAT = MRT_{Topical} - MRT_{IV}$. Absolute availability (F) was calculated by comparing the respective area under the curve (AUCs) after topical and intravenous administration, $([AUC]_{Topical}/dose_{Topical})/([AUC]_{IV}/dose_{IV})$ [16].

Skin distribution parameters, urine and fecal concentrations of DEET and oxybenzone were compared between single (Group 3 or Group 4) and combined (Group 5) applications using the Mann-Whitney *U* Test (PC-SAS® 8.02, SAS Institute Inc., Cary, North Carolina, USA). Unabsorbed DEET and oxybenzone on the surface of the stratum corneum at 7 and 24 hours after the topical application was calculated as a percentage of the final dose. In order to characterize the percutaneous penetration rate and extent within the stratum corneum after the topical application, tape strips at 24 hours were further divided into 4 subgroups, i.e., tape 1 (stratum corneum surface), tapes 2-5 (upper stratum

corneum), tapes 6-9 (lower stratum corneum), and tapes 10-12 (viable epidermis), and drug recovery of those subgroups was obtained as a percentage of the final dose. Concentrations of DEET and oxybenzone in liver and kidney, and total tissue recoveries were calculated as a percentage of the final dose. The data was compared between single and combined topical applications along with intravenous administration using the Kruskal Wallis Test followed by a post-hoc analysis using Mann-Whitney U Test. For hepatocellular data, a one-way ANOVA (PC-SAS® 8.02, SAS Institute Inc., Cary, North Carolina, USA) followed by the Tukey's test was conducted to determine the differences among the 5 study groups at each time interval and drug concentration. Non-parametric tests were utilized when variance ratios were unequal [24]. Normally distributed data was expressed in mean ± SEM while non-parametric data were represented as median ± SD. Differences were considered statistically significant at $p<0.05$.

3.4. Results and Discussion

3.4.1. Pharmacokinetic Parameters of DEET and Oxybenzone

Figures 3.1 and **3.2** show the comparative plasma concentration-time curves following the two administration routes, and the plasma levels of DEET and oxybenzone remained detectable 24 hours post-dose. Plasma concentrations of DEET and oxybenzone decreased exponentially after the intravenous dosing, with an elimination half-life of 102.7 min for DEET and 122.4 min for oxybenzone, respectively. Plasma concentrations of DEET and oxybenzone also rose rapidly following dermal application. The combined formulation (Group 5) produced significantly higher C_{max} values for both DEET (33% increase) and oxybenzone (67% increase) in comparison to its individual counterpart,

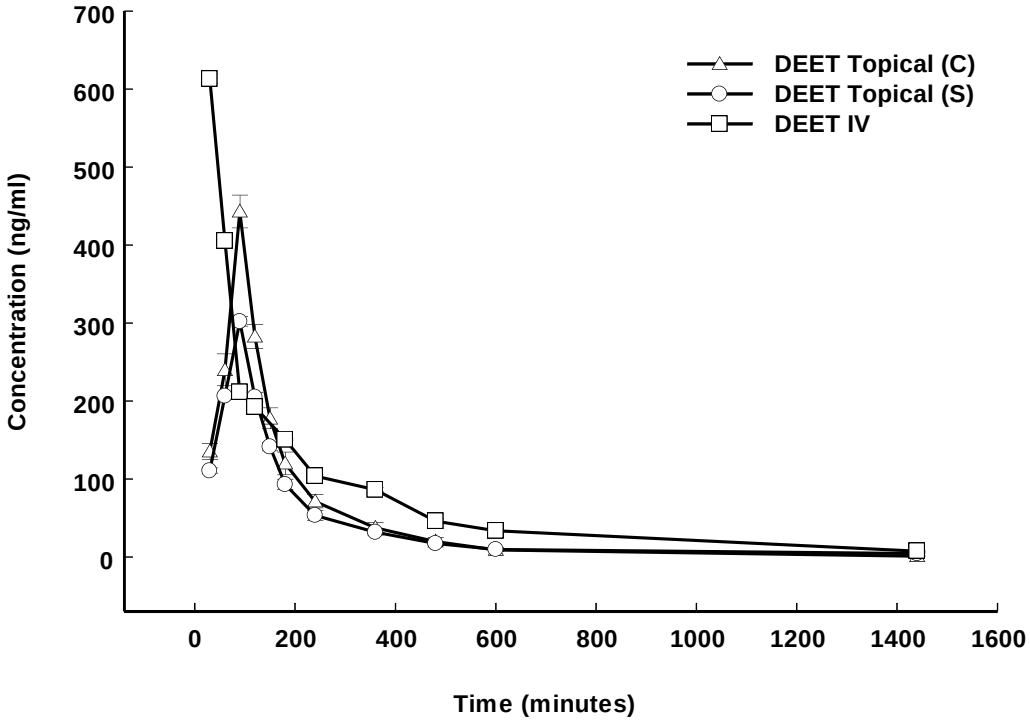

Figure 3.1. Comparative plasma concentration-time curves of DEET following an intravenous administration (n = 5, Mean±SEM) and a 24-hour topical application (n = 6, Mean±SEM; C: combined application, S: single application) in rats.

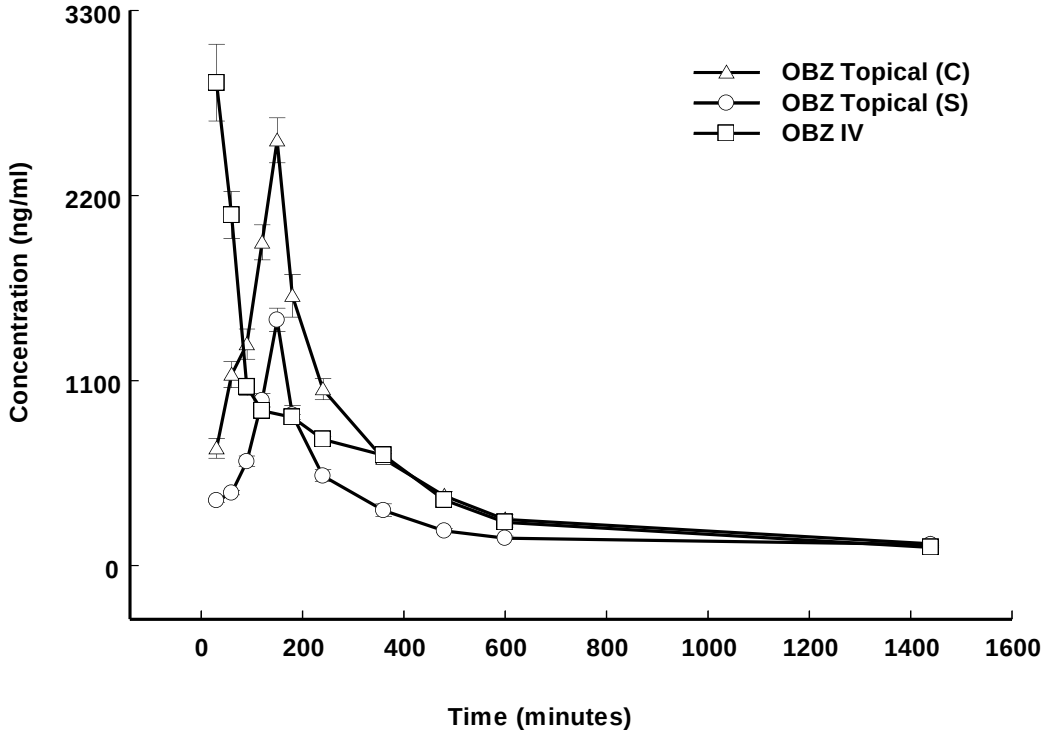

Figure 3.2. Comparative plasma concentration-time curves of oxybenzone following an intravenous administration (n = 5, Mean±SEM) and a 24-hour topical application (n = 6, Mean±SEM; C: combined application, S: single application) in rats.

Table 3.1. Pharmacokinetic parameters of DEET and oxybenzone following an intravenous administration in rats (n = 6, Mean±SEM)

PK Parameter	Group 1	Group 2
Dose (mg/kg)	2	2
α (1/min×10^{-3})	19.0±4.3	18.7±8.1
β (1/min×10^{-3})	1.9±0.4	1.4±0.3
$t_{1/2\alpha}$ (min)	38.6±7.6	48.3±19.0
$t_{1/2\beta}$ (min)	342.1±28.5	450.0±62.9
$t_{1/2k_e}$ (min)	102.7±20.8	122.4±36.9
k_e (1/min×10^{-3})	9.6±1.4	6.1±1.7
MRT (min)	128.9±15.0	243.9±28.6
AUC (µg/ml·min)	66.9±5.3	411.5±58.5
Cl (L/hr/kg)	1.1±0.07	0.1±0.01
Vd_{ss} (L/kg)	5.6±0.6	1.4±0.3

Group 1: 2 mg/kg DEET, Group 2: 2 mg/kg Oxybenzone

Group 3 and Group 4. This is a commonly observed phenomenon as many compounds may initially decline rapidly soon after administration, and then decline moderately as some of the dosing may have initially gone into tissues and then gradually distribute back into the plasma [25].

The primary pharmacokinetic parameters of DEET and oxybenzone from intravenous administration are shown in **Table 3.1**. Higher systemic clearance was observed for DEET than for oxybenzone. At identical dosing, the area under the plasma concentration-time curve (AUC) for oxybenzone was 6-fold higher than that for DEET. The combination of a short elimination half-life and a high systemic clearance suggested that DEET was rapidly removed from the general circulation after administration. The

Table 3.2. Pharmacokinetic parameters of DEET and oxybenzone after a topical skin application (n = 5, Mean±SEM)

PK Parameter	DEET		OBZ	
	Group 3	Group 5	Group 4	Group 5
Dose (mg/kg)	100	100	40	40
C_{max} (µg/ml)	0.3±0.01	0.4±0.02*	1.5±0.07	2.5±0.13*
T_{max} (min)	90	90	150	150
$t_{1/2}$ (min)	361.0±22.5	202.4±19.6*	741.6±73.4	449.3±18.3*
AUC (µg/ml·min)	50.8±1.9	62.2±4.8	400.8±14.8	707.3±22.0*
MRT (min)	249.6±11.2	194.8±15.2	452.6±11.6	381.0±9.1
MAT (min)	120.7±26.3	65.9±30.2	208.7±40.2	137.1±37.8
k_a (1/min×10^{-3})	8.6±2.8	15.6±11.2	3.6±2.1	7.5±4.6
k_e (1/min×10^{-3})	1.9±0.1	3.6±0.4*	1.0±0.1	1.6±0.1*
F (%)	1.5±0.1	1.9±0.2	5.3±0.2	9.4±0.3*

Group 3: 100 mg/kg DEET, Group 4: 40 mg/kg OBZ, Group 5: 100 mg/kg DEET/40 mg/kg OBZ
*significant difference from Group 3 or Group 4 ($p < 0.05$)

volume of distribution demonstrated that both test compounds were also subjected to substantial extravascular distribution *in vivo*.

The primary pharmacokinetic parameters of DEET and oxybenzone following topical application are shown in **Table 3.2**. DEET reached maximal plasma concentrations more rapidly than oxybenzone. The combined application resulted in a shorter $t_{1/2}$, MAT, and MRT of DEET by 44%, 45%, and 22%, and those of oxybenzone by 39%, 34%, and 16% in comparison to individual applications. The AUC of oxybenzone was significantly greater than that of DEET (p<0.05), even though the dose of oxybenzone was smaller than that of DEET. A statistically significant increase was

found in AUC of oxybenzone (76%) but not in AUC of DEET (22%) when the two compounds were applied concurrently.

For dermal application, drug absorption phase is generally prolonged across the skin due to a slow diffusion process, which may subsequently influence the systemic disposition of the compounds. It has been reported that single application of DEET or oxybenzone was capable of forming a skin depot that continuously supplied the topically applied compounds into the general circulation [5, 9, 26]. The permeability of DEET and oxybenzone across the skin is also dependent upon the solubility of both compounds in the preparation and in the skin [27]. Combined application seemed to significantly increase the solubility of DEET and oxybenzone in ethanol, which subsequently enhanced the overall permeation percentage and steady-state flux, leading to an increase in the rate of absorption and elimination and decrease in the half-life for both compounds [27, 28]. In addition, percutaneous absorption and disposition is a complex process driven by a variety of enzymes and transporters for which significant inter-species and intra-species differences exist [29]. Characteristics from combined use of DEET and oxybenzone should hence be further assessed in order to provide appropriate guidelines for the safe and effective use of repellents and sunscreens in long-term skin applications.

The relative bioavailability from the topical application was approximately 2% for DEET and 5-9% for oxybenzone, respectively. Even though applied DEET dose primarily remained on the skin surface, dermal evaporation might be partially responsible for low DEET bioavailability from dermal application. It has been reported that DEET evaporation accounted for 33-49% of the applied dose in a microencapsulation and 95% ethanol formulation [30]. In addition, the rate of DEET evaporation was also proportional to

its local concentration on the skin [31]. Since absolute ethanol was used as the solvent for skin application, skin evaporation of volatile DEET was likely a contributing factor to the low percutaneous permeation of the substance. Under real-life conditions, low percutaneous permeation plus adequate skin evaporation is desirable to repellent efficacy. However, ambient evaporation greater than the minimum effective evaporation rate may also account for DEET loss from the skin surface [32].

3.4.2. Tissue Disposition of DEET and Oxybenzone

DEET and oxybenzone were detected on the surface of the skin as well as within the skin layers after topical application, and combined use of DEET and oxybenzone appeared to facilitate skin permeation and disposition. **Table 3.3** shows the recovery of the compounds from skin after topical application. Combined presence of DEET and oxybenzone produced lower skin recovery of the substances than their individual counterparts at 7 hours and 24 hours. Twelve tape strips also collected 50% more DEET and 17% more oxybenzone from Group 5, indicating an enhanced passage of the chemicals through the stratum corneum.

The percutaneous permeation of DEET and oxybenzone after topical application was further characterized by dividing the tape strips into 4 subgroups. Each subgroup indicated an approximation of the different layers of the stratum corneum and epidermis, i.e., subgroup 1, stratum corneum surface, subgroup 2, upper stratum corneum, subgroup 3, lower stratum corneum, and subgroup 4, viable epidermis surface [5]. **Figure 3.3** shows the recovery of DEET and oxybenzone from each subgroup at 24 hours. Both compounds demonstrated similar skin disposition patterns, with the majority of each compound

Table 3.3. Total recovery of DEET and oxybenzone from the application site after a topical skin application (n = 5, Median±SD)

Parameter	DEET		OBZ	
	Group 3	Group 5	Group 4	Group 5
% Unabsorbed (7 hr)	67.4±1.5	64.2±1.6*	82.3±4.3	73.7±1.3*
% Unabsorbed (24 hr)	32.1±0.7	31.5±0.5*	68.1±10.7	54.9±3.0
Tape Strips (%)	0.2±0.01	0.3±0.01*	1.8±0.07	2.1±0.02*

Group 3: 100 mg/kg DEET, Group 4: 40 mg/kg OBZ, Group 5: 100 mg/kg DEET/ 40 mg/kg OBZ
*significant difference from Group 3 or Group 4 ($p < 0.05$)

recovered in the vicinity of the viable epidermis. Combined application led to greater recovery for DEET (48-138% increment) and oxybenzone (12-55% increment) in all layers of the skin.

Table 3.4 shows the concentrations of DEET and oxybenzone in liver and kidney from both intravenous and topical administration. Considerable tissue distribution of the compounds indicated that the parent compounds had not been fully metabolized after 24 hours. Disposition of oxybenzone in both liver and kidney was larger than that of DEET, possibly due to its lower clearance value and prolonged absorption from the skin depot. Liver concentrations of DEET and oxybenzone from combined topical application were significantly greater than those observed from intravenous and single topical administration, which was consistent with previous studies indicating an enhancement of mutual permeation from concurrent application of the two test compounds [5, 17]. Although this study did not elucidate where DEET or oxybenzone was metabolized, the metabolism most likely occurred in the liver, as reports indicated that DEET was oxidized by the cytochrome P-450 family of enzymes in rodent liver microsomes [33].

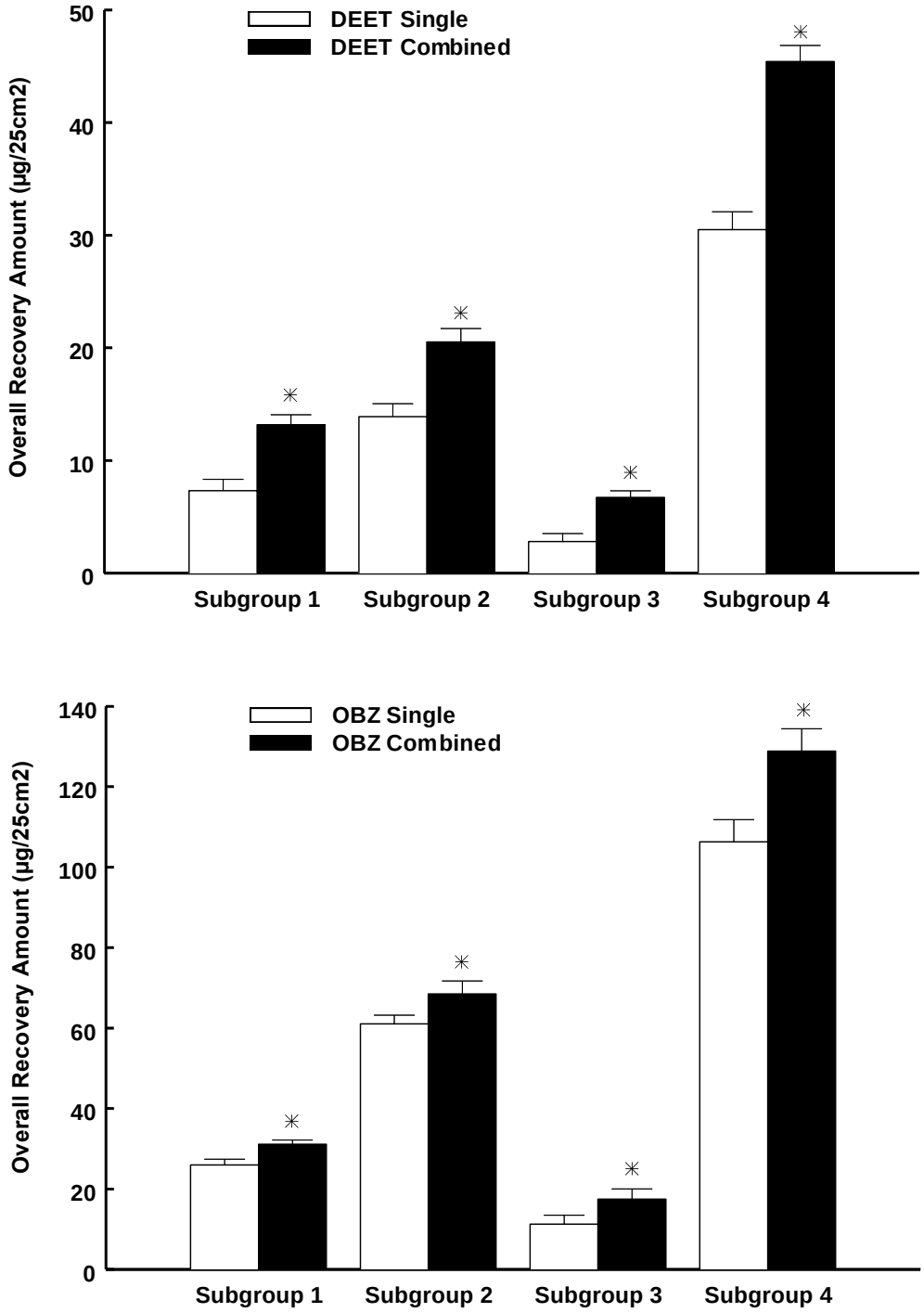

Figure 3.3. Concentrations of DEET and oxybenzone in different subgroups of skin tape strips at 24 hours after topical skin application, Subgroup 1: Tape Strip 1, Subgroup 2: Tape Strip 2-5, Subgroup 3: Tape Strip 6-9, Subgroup 4: Tape Strip 10-12 (* significant difference from single product, $p < 0.05$, n = 5, Median±SD).

Table 3.4. Concentration and total recovery of DEET and oxybenzone in tissues following intravenous and topical administrations (n = 5-6, Median±SD)

Sampling Type	DEET			OBZ		
	Group 1	Group 3	Group 5	Group 2	Group 4	Group 5
Liver (ng/g)	20.2±2.4	14.6±6.5	34.2±6.1*	34.6±3.8	38.8±5.0	63.1±9.4*
Kidney (ng/g)	11.2±1.2	12.2±4.1	7.9±3.5	62.6±2.9	66.2±15.5	90.1±7.5^{+}
Recovery (‰×10^{-2})	57.8±8.5	0.9±0.3†	1.9±0.4*	105.6±10.1	6.3±1.0†	10.7±1.3*

Group 1: 2 mg/kg DEET, IV, Group 2: 2 mg/kg OBZ, IV, Group 3: 100 mg/kg DEET, topical, Group 4: 40 mg/kg OBZ, topical, Group 5: 100 mg/kg DEET/40 mg/kg OBZ, topical

* significant difference from Groups 1/3 or Groups 2/4 ($p < 0.05$)

† significant difference from Group 1 or Group 2 ($p < 0.05$)

$^{+}$ significant differences from Group 2 ($p < 0.05$)

While DEET was still detectable in the plasma 24 hours after topical application, DEET concentrations in urine and fecal samples collected at the conclusion of the study were below the limit of quantification. This may be attributed to quick metabolism and excretion of DEET *in vivo* [5]. However, oxybenzone was detected in both urine and feces (0.8±0.06 μg/ml and 2.0±0.5 μg/g from Group 5, 0.5±0.03 μg/ml and 1.2±0.4 μg/g from Group 4, respectively). This might indicate a prolonged permeation and retention from a skin depot of the substance formed *in vivo*, in particular from concurrent application of the two test compounds. This was also consistent with skin recovery of the substances, as a higher amount of oxybenzone remained at the application site than DEET.

The existence of a distribution phase for both intravenous DEET and oxybenzone data indicated a biexponential decline, and therefore a two-compartment model was utilized. This model was chosen on the basis of goodness-of-fit criteria known as Akaike information criteria (AIC) and the correlation coefficient. A greater correlation coefficient and a lower AIC value indicated a better statistical fit and thus described the

pharmacokinetic data the most adequately [34, 35]. Based on two-compartmental modeling, DEET and oxybenzone did not rapidly equilibrate throughout the entire body, but instead they distributed quickly and uniformly into plasma, extracellular fluid, and highly perfused tissues such as the liver and kidney. Both compounds were eliminated from the central compartment by hepatic metabolism followed by renal excretion [25]. Concurrent topical application enhanced the disposition of DEET and oxybenzone in the liver, which may indicate the potential for hepatic toxicity under long-term exposure [36]. No DEET was detected in urine and feces 24 hours after a single topical dosing, which was similar to previous results in various species [5, 9, 37, 38]. DEET and oxybenzone are lipophilic compounds, and their partition coefficient is 2.0 and 3.8, respectively [39, 40]. Extensive systemic distribution of both substances may subsequently result in slow equilibration into the fatty tissues of the peripheral compartment, which should be further evaluated for potential toxicological properties.

3.4.3. Cell Viability Study

Impairment of mitochondrial function is implicated in the etiology of drug-induced liver injury and other adverse drug reactions [41]. A wide range of mechanisms can be involved, such as impairment of the electron transport chain and uncoupling of electron transport from ATP synthesis, which subsequently result in a decrease in the membrane potential ($\Delta\Psi m$) and inhibition of ATP production [41]. In this study, we investigated the cellular viability by determining the number of viable cells through the level of activity of mitochondrial dehydrogenase. Mitochondrial respiration is a useful, preliminary screening parameter for evaluating *in vitro* hepatotoxicity, since alterations in

mitochondrial respiration can be detected in the early stages of cell exposure and thus well reflects the toxicological changes occurring inside the cells [42]. **Figure 3.4** shows the cellular viability results from the 1548 hepatoma cell line following exposure to DEET, oxybenzone, and a combination of the two compounds at different concentrations and exposure intervals. No significant differences in hepatoma cell viability were observed at 0.1 and 1 µg/ml concentrations after 24 and 48 hours of exposure in comparison to the control groups. However, treating the hepatoma cells for 72 hours resulted in significant reduction in cell viability in all treatment groups, except for oxybenzone at 0.1 µg/ml. Evidence of cellular toxicity was observed when the test concentration was increased to 10 µg/ml. Exposure to DEET significantly reduced the cell viability starting at 24 hours; exposure to oxybenzone and DEET/oxybenzone combination also significantly reduced the cell viability at 48 and 72 hours of exposure.

The cellular study demonstrated that DEET was capable of inducing cell toxicity at all test concentrations. Exposure to both DEET and oxybenzone for an extended period of time was also of concern, since that significantly reduced the cellular viability, but not more than their single counterparts. Previous studies have indicated that high doses of DEET alone induced adenylate kinase and caspase-3/7 release, both markers of cytotoxicity [36], and that DEET exposure led to induction of CYP2Bs in mice [33]. High doses of oxybenzone also increased liver weight in second-generation mice [36]. In addition, once-daily, repeated skin application of DEET and oxybenzone demonstrated increased disposition of the substances in the liver [17]. Results from this study clearly supported the current consumer health recommendation that the lowest possible

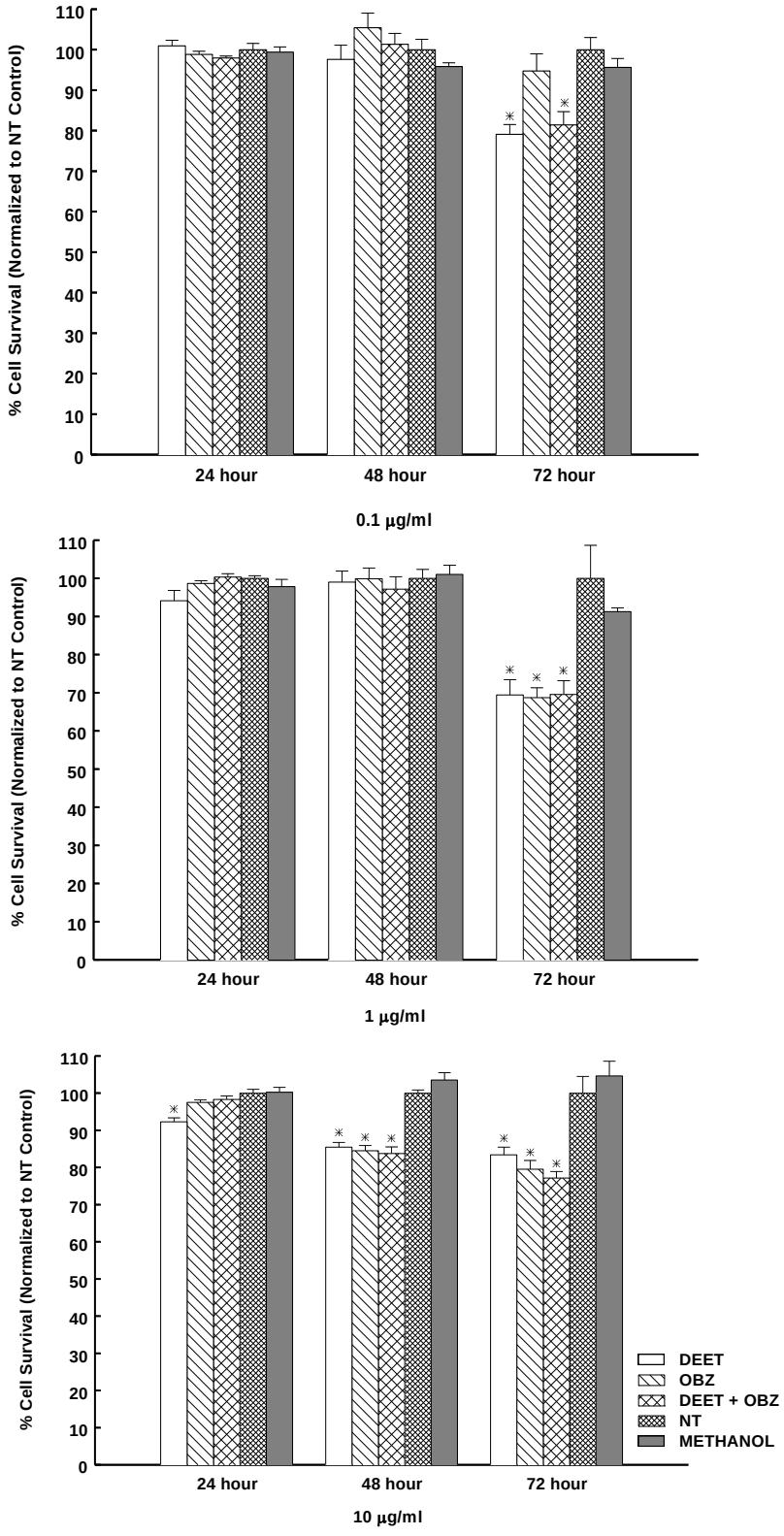

Figure 3.4. Cellular viability of rat hepatoma cell line 1548 exposed to DEET and oxybenzone at different concentrations and intervals (* significant difference from No Treatment (NT) control, $p < 0.05$, $n = 6$, Mean±SEM).

concentration of DEET be utilized in topical insect repellent preparations. As for long-term topical skin application of repellents and sunscreens, further systematic investigation is still required to fully understand its possible interactions and minimize their subsequent health impact on human consumers.

3.5. Conclusion

In summary, DEET and oxybenzone were extensively distributed and quickly eliminated in Sprague-Dawley rats following an intravenous administration. A two-compartment model was used to describe the pharmacokinetics of DEET and oxybenzone after intravenous administration *in vivo*. Following a topical skin application, metabolism and elimination of DEET were faster than those of oxybenzone, due partially to a prolonged retention of the more lipophilic oxybenzone within the skin. Topical application also demonstrated significant differences between single component and combination component for DEET and oxybenzone. Systemic permeation and absorption of the compounds were correlative among skin, plasma, tissue, urine and fecal samples. Despite the differences observed between the pharmacokinetic parameters of combined- and single-product applications, the physiological origin of these differences has yet to be clearly ascertained. While concurrent application of DEET and OBZ appeared to enhance biodistribution of the two compounds *in vivo*, no additional hepatotoxic effects related to this synergistic interaction were noted. Formulation modification will be the focus of future studies, where percutaneous permeation of the active ingredients will be optimally minimized or eradicated while still achieving their desired protection efficacy on the surface of the skin.

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Chapter 4

Metabolic Disposition of the Insect Repellent DEET and the Sunscreen Oxybenzone Following Intravenous and Skin Administration in Rats

4.1. Abstract

Insect repellent DEET and sunscreen oxybenzone have shown a synergistic percutaneous enhancement when applied concurrently. Both compounds are extensively metabolized *in vivo* into a series of potentially toxic metabolites: two metabolites of DEET, *N*,*N*-diethyl-*m*-hydroxymethylbenzamide (DHMB) and *N*-ethyl-*m*-toluamide (ET), and three metabolites of oxybenzone, 2,4-dihydroxybenzophenone (DHB), 2,2´-dihydroxy-4-methoxybenzophenone (DMB) and 2,3,4-trihydroxy-benzophenone (THB). In this study, the metabolites were extensively distributed following intravenous and topical skin administration of DEET and oxybenzone in rats. Combined application enhanced the disposition of all DEET metabolites in the liver, but did not consistently affect the distribution of oxybenzone metabolites. DHMB appeared to be the major metabolite for DEET, while THB and its precursor DHB were the main metabolites for oxybenzone. Repeated once-daily topical application for 30 days led to higher concentrations of oxybenzone metabolites in the liver. Hepatoma cell studies revealed toxicity from all metabolites as single and combined treatments, most notably at 72 hours. Increased accumulation of DHMB and ET in the liver together with their cytotoxic potential at achievable plasma concentrations indicated that simultaneous exposure to DEET and oxybenzone might have the potential to precipitate adverse effects in a rat animal model.

4.2. Introduction

Topical insect repellent and sunscreen products have become an integrated part of summer routines for the general public in developed countries, due primarily to a conscious awareness of the health threats from vector-borne diseases and nonessential sun exposure. These consumer-care products are the most practical, cost-effective, and well-accepted choice of defense, and there are a variety of preparations commercially available to consumers including sprays, lotions, aerosols, and cloth wipes. Concurrent skin application of sunscreen preparations along with insect repellents has been prevalent in North America since 1999 when mosquito-transmitted West Nile virus was first detected in the continent.

Numerous chemical repellent and sunscreen ingredients are utilized for commercial civil use; DEET (N,N-diethyl-*m*-toluamide) and oxybenzone are two principal repellent and sunscreen substances, respectively. Their repellency and UV-blockage efficacy have been investigated and documented [1, 2]. Interactions between active repellent and sunscreen compounds, as well as subsequent percutaneous disposition of these substances after topical skin application, have also been studied and reported [3-5].

Designated as topical products, repellents and sunscreens remain on the skin surface to achieve their protection efficacy. Percutaneous permeation and systemic absorption of the active ingredients are considered neither desirable nor productive. However, DEET and oxybenzone have been shown to permeate across the stratum corneum into the general circulation after topical skin application [6, 7]. *In vitro* studies have demonstrated permeation synergy between DEET and oxybenzone when both substances were applied simultaneously [4, 5]. The dose applied, formulation type, and

application method all influenced the rate and extent of permeation, as well as the interaction between the two active compounds.

The pharmacokinetics and toxicology of DEET and oxybenzone applied alone have been reported in some previous studies [8-10]. Since concurrent skin application of repellents and sunscreens was rare in the past, studies were only carried out to assess concurrent use of DEET or oxybenzone in association with several specialty chemical substances pertinent to military and/or farming usages [11, 12]. In addition, loss of Sun Protection Factor (SPF) of the sunscreens has been reported from mixing DEET-based repellents and commercial sunscreen products [13, 14]. With increasing administration of DEET-based repellents together with oxybenzone-containing sunscreens, it has become critical and essential to understand *in vivo* disposition profiles of the two substances. No studies have indicated whether the documented adverse effects of DEET and oxybenzone resulted from the parent compounds or their subsequent metabolites. Therefore, the objective of this study was to determine the metabolite disposition of DEET and oxybenzone following intravenous and topical skin administration in a rat model. *In vitro* hepatoma cell experiments were also performed to assess potential toxicity resulting from enhanced disposition of these metabolites in the liver.

4.3. Materials and Methods

4.3.1. Materials and Reagents

Sodium Chloride Injection USP (0.9%) was obtained from Astra Zeneca Inc. (Mississauga, Ontario, Canada) and used directly for intravenous injection. Anhydrous ethyl alcohol was purchased from Commercial Alcohols Inc. (Brampton, Ontario,

Canada) and Emulphor® (ethoxylated castor oil) was purchased from Nihon Emulsion Co. Ltd (Tokyo, Japan). Pure DEET and oxybenzone standards were obtained from Fluka Chemika GmbH (Buchs, Switzerland) and Riedel-de Haën GmbH (Seelze, Germany), respectively. Various formulations were prepared for the animal experiments without further purification.

For drug extraction and analysis, acetonitrile, methanol and sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Glacial acetic acid was obtained from Mallinckrodt Specialty Chemical Company (Paris, Kentucky, USA). Ammonium acetate was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wisconsin, USA). Nitric acid was purchased from LabChem Inc. (Pittsburgh, Pennsylvania, USA). All solvents were HPLC-grade and other chemicals were AC-grade. Deionized water was obtained from a Milli-Q® Pure Water System (Nepean, Ontario, Canada).

For studies of cell viability, HyClone classical liquid media Minimum Essential Medium with Earle's Balanced Salts (MEM/EBSS) was purchased from Thermo Fisher Scientific (Nepean, Ontario, Canada). Fetal bovine serum, pyruvate, L-glutamine, penicillin, streptomycin, and ethylenediaminetetraacetic acid (EDTA) trypsin were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Rat hepatoma cell line 1548 was obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA). Cell Proliferation Reagent WST-1 was obtained from Roche (Laval, Quebec, Canada). Cell culture plates were purchased from Sarstedt (96-well, Cat# 83.1835, Montreal, Quebec, Canada).

4.3.2. Animal Model

The Animal Use Protocol was approved by the University of Manitoba Fort Garry Campus Animal Use Protocol Management and Review Committee, and research was conducted according to the current guidelines published by the Canadian Council for Animal Care (CCAC). Fifty-seven Sprague-Dawley rats were used in the study, which were randomly divided into eight study groups, i.e., two groups for intravenous administration (n=6, 24 hours), three groups for single topical application (n=5, 24 hours), and three groups for once-daily repeated application (n=10, 30 days).

Rats (average body weight of 250 g) were obtained from the Central Animal Care Services, University of Manitoba. Animals were checked by a University veterinarian for general health conditions upon arrival at the Department of Zoology Animal Holding Facility. They were housed individually in holding cages and provided food and water *ad libitum*. A 5-day quarantine period was provided to the study animals, allowing them to adapt to the environment before the actual experiment commenced.

4.3.3. Intravenous and Topical Study Samples

For intravenous administration, solutions of DEET (0.3%, w/v) and oxybenzone (0.1%, w/v) were prepared using a mixture of Emulphor:ethanol:water (2:3:5, v/v/v) [15]. The study preparation was injected into the tail vein of the animals at 2 mg/kg DEET (Group 1) and 2 mg/kg oxybenzone (Group 2) [16].

For single, 24-hour topical skin administration, DEET and oxybenzone standards were weighed and dissolved in anhydrous ethanol at a concentration of 120 mg/ml DEET and 48 mg/ml oxybenzone, either individually or in combination. Prior to administration,

an area of 25 cm^2 (5×5 cm) on the back of each study animal was shaved using an electric clipper. The three study doses were 100 mg/kg DEET (Group 3), 40 mg/kg oxybenzone (Group 4), and combined 100 mg/kg DEET/40 mg/kg oxybenzone (Group 5) [16]. A total of 250 µl of test solution was measured and applied onto the skin surface using a pipette. The solution was carefully spread over the shaven skin area with a disposable pipette tip, and sufficient time for ambient evaporation of the solvent was allowed before the study rats were returned to their holding cages. The application was performed by an animal care technician in order to ensure the consistency of the drug administration and the accuracy of the study results.

For once-daily, 30-day repeated topical skin administration, DEET and oxybenzone standards were weighed and dissolved in 70% ethanol at a concentration of 100 mg/ml DEET and 12.5 mg/ml oxybenzone, either individually or in combination. The test samples were transferred to amber glass bottles and stored in a refrigerator for the duration of the experiment. Prior to skin application, an area of 4 cm^2 (2×2 cm) on the upper dorsal end of the study animals was shaved using an electric clipper. This area remained hair-free by regular shaving in order to facilitate skin applications and observations over the 30-day study period. The upper dorsal surface was selected for dose application because it was extremely difficult for the study animals to reach this area of the body. The three study doses were 40 mg/kg DEET (Group 6), 5 mg/kg oxybenzone (Group 7), and combined 40 mg/kg DEET/5 mg/kg oxybenzone (Group 8) [17]. Similar application procedures were utilized as those of single skin application, and the weight of the study animals was measured periodically over the study duration so that the study doses were adjusted accordingly.

4.3.4. Animal Study Sample Collection

Blood samples (150 µl) were collected from the saphenous vein of the study animals using Microvette® capillary collection tubes (Sarstedt AG & Co., Nümbrecht, Germany) at 30, 60, 90, 120, 150, 180, 240, 360, 480, 600, and 1400 minutes after intravenous injection and 24-hour single skin dosing. A bolus of 10 ml saline was injected subcutaneously after the 120-minute sample collection to compensate for the loss of blood volume. Plasma was separated by centrifugation of the samples at 13,000 g for 30 minutes, and stored in labeled polypropylene tubes at –20 °C until drug analysis. For 30-day repeated skin application, a 300 µl blood sample was collected from the saphenous vein of the rats using Microvette® capillary collection tubes at 2, 4, 6, and 24 hours after the last dosing on day 30. The plasma was separated and stored in labeled polypropylene tubes at –20 °C until drug analysis.

For topical skin studies, skin tape stripping was performed to evaluate percutaneous penetration of DEET and oxybenzone into various skin layers as well as the formation of metabolites. Before skin stripping, the application surface was swabbed once using cotton swabs saturated with 400 µl of acetonitrile. Upon complete drying of the skin, D-Squame® stripping disks (CuDerm Corporation, Dallas, Texas, USA) were consecutively applied to the site, gently pressed for 10 seconds, and then peeled off. For 24-hour study, 4 stripping disks were used at 7 hours on one particular area of the application site and 12 stripping disks were collected at 24 hours from a different area of the application site. For 30-day study, 12 stripping disks were used at 24 hours after the last dosing. All tape strips were placed individually in labeled polypropylene tubes, and stored at –20 °C until drug analysis.

Liver, brain and kidney specimens were also collected after study animals were euthanized at the end of the study. They were rinsed with saline solution to remove blood, air-dried, weighed, and stored in labeled polypropylene tubes at –80 °C until drug analysis.

Urine and feces samples were collected during the single dose 24-hour topical study and were stored in labeled polypropylene tubes at –20 °C until drug analysis.

4.3.5. Concentration Measurement

Concentrations of primary metabolites of DEET and oxybenzone, including N,N-diethyl-*m*-hydroxymethylbenzamide (DHMB), N-ethyl-*m*-toluamide (ET), 2,4-dihydroxybenzophenone (DHB), 2,2-dihydroxy-4-methoxybenzophenone (DMB), and 2,3,4-trihydroxybenzophenone (THB), were measured using an HPLC assay developed and validated in our laboratory [18]. The method was capable of quantifying the compounds simultaneously using photodiode array detection.

To extract the metabolites from the biological samples, an automatic solid-phase extraction method was developed using a Zymark Rapidtrace® SPE Workstation (Caliper Life Sciences, Hopkinton, Massachusetts, USA) [17]. Briefly, the separation was completed on a Waters® Oasis® MAX 3cc (60 mg) extraction cartridge, by using acetonitrile, 0.03 M ammonium acetate (pH 4.5) and water as preconditioning and washing solvents; 50 µl of plasma and urine samples were used for drug extraction, and 300 µl of methanol as the final elution solvent. The eluent was vortexed for 15 seconds, transferred to an HPLC vial and 50 µl of the sample injected. Skin tape strips were dissolved in 1.5 ml of acetonitrile and extracted. Liver, kidney, brain and feces samples were first homogenized in

acetonitrile using an electronic homogenizer (Biospec Products, Bartlesville, Oklahoma, USA). A portion of the homogenate was then subjected to solid-phase extraction, and 50 µl of the eluent was injected to the HPLC system for drug measurement.

4.3.6. Cell Culture Assay

Rat hepatoma cell line 1548 was grown in MEM/EBSS supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10% fetal bovine serum, 4 mM L-Glutamine, and 0.011% sodium pyruvate in a humidified, 37 °C incubator under an atmosphere of 95% air and 5% CO_2. Cells were maintained in a 75 cm^2 culture flask (Corning, Acton, Massachusetts, USA).

Cells were then trypsinized from 75 cm^2 culture flask and seeded at a density of 3,000 cells per well in 96-well plates. Cells were allowed to attach to the plates in an incubator containing 5% CO_2 at 37 °C for 12 hours. Following attachment, the medium in each well was removed and replaced with 100 µl fresh medium containing DHMB, ET, DHB, DMB, and THB at pre-determined concentrations of 0.1 µg/ml, 1 µg/ml, and 10 µg/ml. Cells were then incubated in their respective media for 24, 48, and 72 hours. In order to maintain sufficient concentration and exposure time, medium in each well was renewed every 24 hours by adding 50 µl of fresh medium containing the same concentrations of DHMB, ET, DHB, DMB, and THB. To evaluate the effect of test substances on cell proliferation, Cell Proliferation Reagent WST-1 (1:10 dilution) was added into wells after 24, 48 or 72 hours of exposure. Plates were gently shaken for 5 minutes and then cultured cells were incubated in the presence of WST-1 reagent for 2 hours according to manufacturer's recommendation. The absorbance of each well was

measured using an ELx 808 Ultra Microplate Reader (BIO-TEK Instrument, Winooski, Vermont, USA) at 450 nm. Wells with only the medium were utilized as the background control, while wells containing normal medium with the same amount of cells (3,000 per well) were used as the no treatment (NT) control. An additional control with 1% methanol was used because all metabolites required 1% methanol to dissolve into medium.

4.3.7. Data Analysis

Amounts of all metabolites in samples were calculated from the average HPLC calibration curve. Plasma concentrations of DHMB, ET, DHB, DMB and THB were averaged and plotted on concentration-versus-time graphs. Metabolite concentrations and total recovery (measured as a percentage of the final dose) in liver, kidney, and brain were also calculated for single and combined topical skin applications along with intravenous administration, and were compared using the Kruskal Wallis Test (PC-SAS® 8.02, SAS Institute Inc., Cary, North Carolina, USA) followed by a post-hoc analysis using Mann-Whitney U Test (PC-SAS® 8.02, SAS Institute Inc., Cary, North Carolina, USA). Metabolite concentrations in urine and feces were compared between single and combined skin applications using the Mann-Whitney *U* Test. For hepatocellular data, a one-way ANOVA (PC-SAS® 8.02, SAS Institute Inc., Cary, North Carolina, USA) followed by the Tukey's test was conducted on each metabolite to determine the differences among the 8 study groups at each time interval and drug concentration. Area-under-the-curve (AUC) was calculated using the trapezoidal rule method and compared between single and combined applications using the Mann-Whitney U Test. Non-

parametric tests were utilized when variance ratios were unequal. Normally distributed data was expressed as mean±SEM while non-parametric data was represented as median±SD. Differences were considered statistically significant at $p<0.05$.

4.4. Results and Discussion

Metabolism is one of the important functions to remove foreign substances from the body. While a majority of metabolites are considered weakly active or inactive by-products of a parent compound, certain metabolites may indeed either possess pharmacologic properties that correlate to the parent compound and thus augment its effect, or are toxic compounds that result in adverse effects and toxicity [19]. In addition, disposition of a parent compound may be affected by its metabolites due to competition for plasma and tissue binding sites during the course of body metabolism [19]. It is therefore critical to understand and elucidate the metabolic pathway for drug compounds, not only to help optimize the therapeutic outcomes but also to minimize potential for side effects and toxicity of the metabolites [20]. Both DEET and oxybenzone are metabolized to numerous compounds *in vivo* that have not been adequately studied in the past. With a synergistic percutaneous permeation of the two substances from a concurrent topical skin application, it is important to characterize the disposition of these metabolites and their relationship to the parent compounds.

4.4.1. Plasma Concentrations

Figure 4.1 shows the plasma-time course of the formation of DEET and oxybenzone metabolites after a single intravenous administration of DEET (2 mg/kg) and

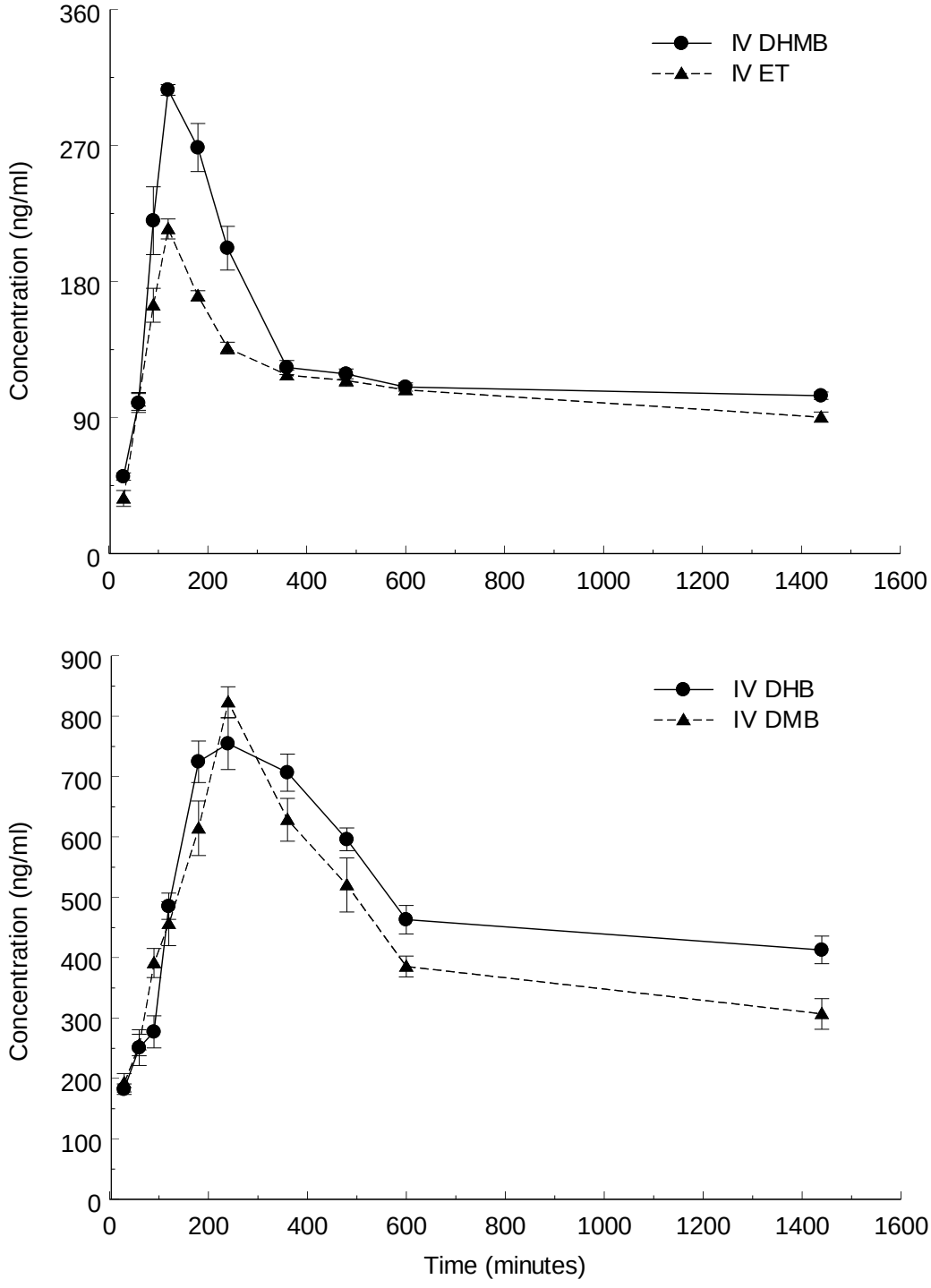

Figure 4.1. Plasma concentrations of metabolites of DEET (top) and oxybenzone (bottom) after an intravenous injection of 2 mg/kg DEET and 2 mg/kg oxybenzone (n = 6, Mean ± SEM).

oxybenzone (2 mg/kg). The metabolites of DEET, DHMB (formed via the oxidative hydroxylation of the ring methyl in the meta-position) and ET (formed via N-deethylation), were detected in plasma 30 minutes post-administration. Similarly, the metabolites of oxybenzone, DHB (formed via O-dealkylation of the methoxy side chain at para-position on the oxybenzone nucleus) and DMB (formed via aromatic hydroxylation at the ortho-position on ring B of the oxybenzone nucleus), were also detected in plasma at the same time. This indicated a rapid metabolism of the two parent compounds in rats. The peak concentrations of DEET metabolites were observed within two hours of intravenous administration; the peak levels of DHMB in plasma were 14% higher ($p<0.05$) than those of ET. Both metabolites were still detectable in plasma at the end of the study. For oxybenzone metabolites, the peak concentrations were observed within four hours of the administration; DHB was the predominant metabolite in plasma with a 15% higher AUC ($p<0.05$) than DMB. The two metabolites were still detectable in plasma 24 hours post-administration of the parent compound.

Figure 4.2 shows the plasma-time curves for the appearance of DEET and oxybenzone metabolites in plasma after a single topical skin application. Concurrent application of DEET and oxybenzone resulted in elevated metabolite concentrations for both substances compared to administration of DEET and oxybenzone alone. For DEET metabolites, AUC levels of DHMB and ET were significantly increased by 23% and 17% ($p<0.05$) respectively following DEET and oxybenzone application, in comparison to that from single skin application. For oxybenzone metabolites, AUC levels of DHB and DMB following DEET and oxybenzone application were significantly increased by 28% and 16% ($p<0.05$) respectively, in comparison to that from single skin application. Metabolite

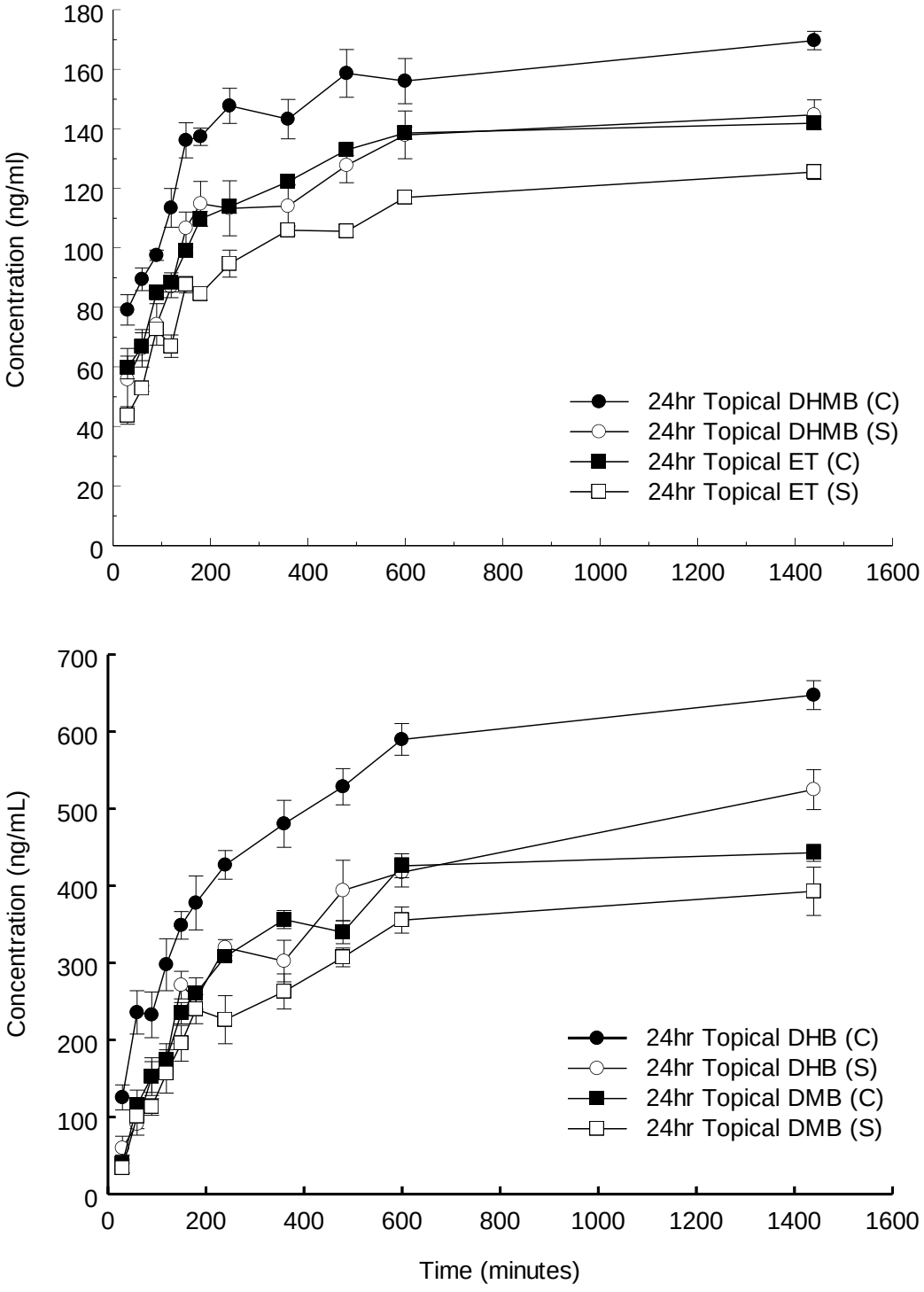

Figure 4.2. Plasma concentrations of metabolites of DEET (top) and oxybenzone (bottom) after a 24-hour topical application (n=5, Mean ± SEM; C – DEET and oxybenzone combined application; S – DEET or oxybenzone single application).

concentrations were still detected in plasma 24 hours post-administration from all application procedures.

Figure 4.3 shows the plasma-time curves in the formation of DEET and oxybenzone metabolites after repeated topical skin application once-daily for 30 days. Concurrent use of DEET and oxybenzone led to a higher DHMB level (30% increase) than individual administration, but ET levels were not different whether DEET was applied alone or with oxybenzone. For oxybenzone metabolites, no difference in AUC of the two metabolites was observed between single and co-application with DEET. Like all other studies, these metabolites were still detectable in plasma 24 hours after the skin applications.

It was evident that both DEET and oxybenzone were rapidly metabolized in the liver after intravenous and skin applications. The prompt appearance of metabolites in plasma from skin applications may indicate that metabolism had taken place while the parent compounds were being absorbed across the skin layers [8]. Skin has been proven to be a major organ for various enzyme activities [21]. Nevertheless, no quantitative concentrations of these metabolites were detected in skin tape strips, indicating minimal metabolism of DEET and oxybenzone in the stratum corneum and upper epidermis, which was similar to results reported in a previous study [5]. Several minor metabolites were also recorded in humans from subsequent oxidation, hydroxylation, and glucuronidation reactions (e.g., N,N-diethyl-m-carboxylbenzamide) [22-24]. Additional studies may be beneficial in clarifying the similarities and differences regarding metabolism of DEET and oxybenzone in rats and humans.

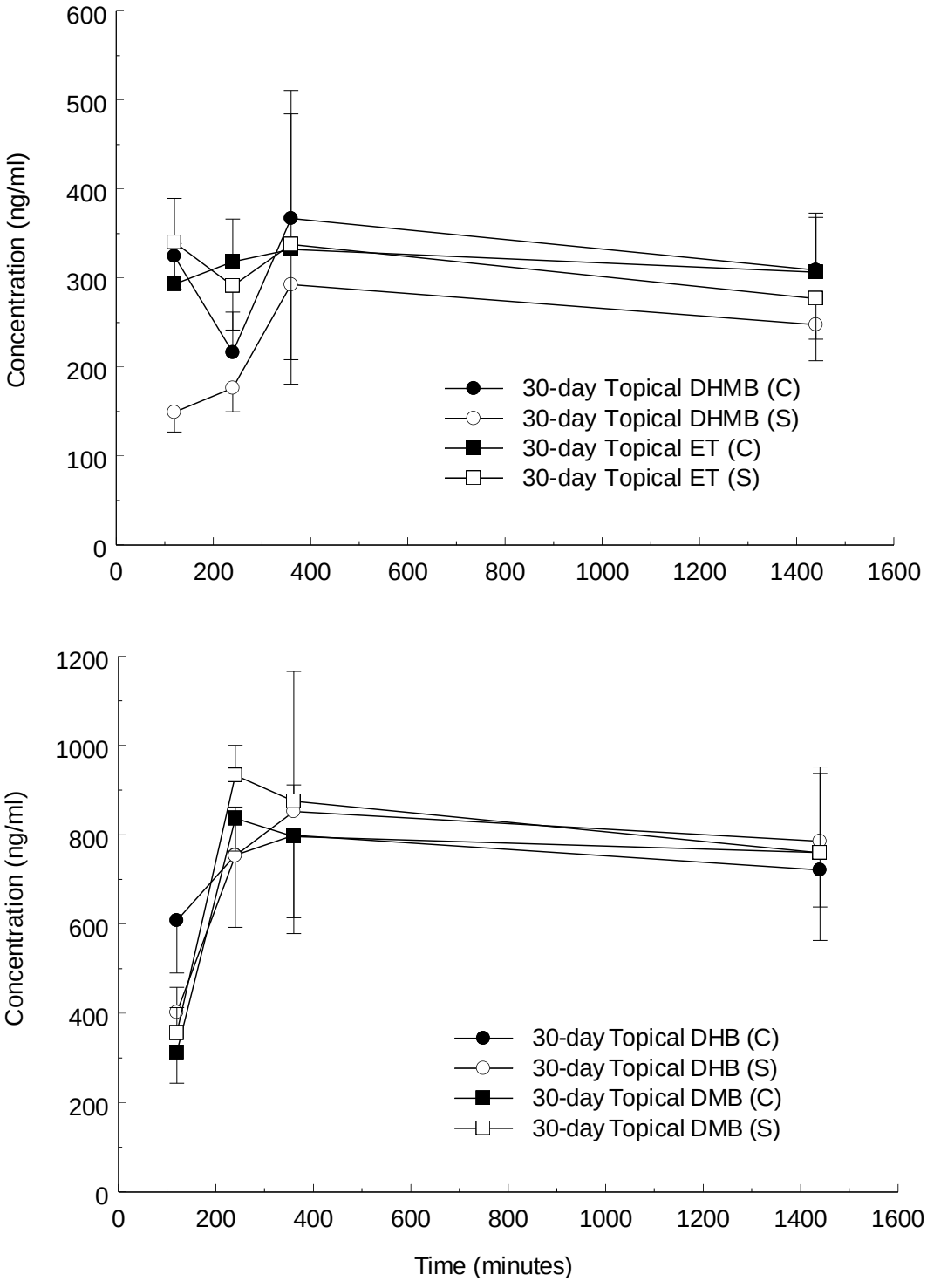

Figure 4.3. Plasma concentrations of metabolites of DEET (top) and oxybenzone (bottom) after the last dose of a 30-day repeated topical application (n = 10, Mean ± SEM; C – DEET and oxybenzone combined application; S – DEET or oxybenzone single application).

The presence of one aromatic ring in DEET and two aromatic rings in oxybenzone leads to favorable lipophilicity for percutaneous transport; their partition coefficients in octanol and water are 2.0 and 3.8, respectively [25, 26]. Topical skin application also tends to supply the general circulation with a continuous, slow delivery of the parent compounds, DEET and oxybenzone, and prolongs their absorption phase, which can subsequently influence *in vivo* disposition. In comparison, concentrations of the metabolites decreased much more slowly than the parent compounds over time [17]. The long-term impact of these substances may require further investigation [27].

4.4.2. Tissue Dispositions

Table 4.1 lists the total recovery of DEET and oxybenzone metabolites in liver from the three administration methods. Two DEET metabolites, DHMB and ET, were detected in all tissue samples collected, but DHMB appeared to be the dominant metabolite in liver after application of DEET to skin. Co-application of the two parent compounds together seemed to enhance the disposition of DHMB and ET after topical skin application. For single-dosing study, DHMB and ET concentration in liver was significantly increased by 60% and 37% respectively when concurrent use was compared to individual use of the compounds. For 30-day repeated-dosing study, DHMB and ET concentration in liver was significantly increased by 3% and 39% respectively from concurrent use. In addition, although ET concentration was larger than that of DHMB by a range of 8-27% in both single-dosing studies, DHMB concentration exceeded ET by a range of 104-175% in 30-day repeated-dosing study.

Table 4.1. Total recovery (‰ x 10^{-2}) of metabolites of DEET (DHMB, ET) and oxybenzone (DHB, DMB and THB) from liver following intravenous administration (Group 1/2), single skin application (Groups 3/4/5), and repeated skin application (Groups 6/7/8).

Metabolite	Application Type							
	Group 1 S	Group 2 S	Group 3 S	Group 4 S	Group 5 C	Group 6 S	Group 7 S	Group 8 C
DHMB	76.7±14.7[a]	---	1.5±0.1[b]	---	2.4±0.1[c]	69.3±11.6[a]	---	71.4±22.5[a]
ET	96.5±18.3[d]	---	1.9±0.1[b]	---	2.6±0.1[c]	25.2±13.5[e]	---	35.0±13.6[e]
DHB	---	27.8±2.8[f]	---	6.2±0.5[g]	7.6±0.4[h]	---	97.9±34.1[i]	79.7±26.8[i]
DMB	---	19.4±0.8[f]	---	4.0±0.5[j]	4.3±0.3[j]	---	118.1±55.1[i]	92.3±50.0[i]
THB	---	101.8±8.1[f]	---	30.2±2.4[g]	37.6±0.9[h]	---	321.7±81.8[i]	273.0±103.8[i]

S – DEET or oxybenzone single application, C – DEET and oxybenzone combined application
[a]significant difference from Group 3 and 5
[b]significant difference from Group 1, 5, 6 and 8
[c]significant difference from Group 1, 3, 6 and 8
[d]significant difference from Group 3, 5, 6 and 8
[e]significant difference from Group 1, 3 and 5
[f]significant difference from Group 4, 5, 7 and 8
[g]significant difference from Group 2, 5, 7 and 8
[h]significant difference from Group 2, 4, 7 and 8
[i]significant difference from Group 2, 4 and 5
[j]significant difference from Group 2, 7 and 8
($p < 0.05$, Median ± SD, n = 5-10)

Three oxybenzone metabolites were detected in the tissue samples collected. For single-dosing study, concurrent skin application of DEET and oxybenzone resulted in increased concentrations of metabolites in comparison to single skin application; i.e., 23% for DHB, 7.5% for DMB, and 25% for THB, respectively. For 30-day repeated-dosing study, concurrent application of the two substances led to a slight decrease in concentration in comparison to single application; i.e., 19% for DHB, 22% for DMB, and 15% for THB. THB is a metabolite formed from secondary aromatic hydroxylation of ring A at the meta-position of DHB. This compound was not detected in plasma samples, but it was the primary oxybenzone metabolite found in the liver with an amount

exceeding that of DHB and DMB. THB was 266-425% more than DHB/DMB after intravenous administration, 387-774% more than DHB/DMB after 24-hour single-dosing, and 172-243% more than DHB/DMB after 30-day repeated-dosing, respectively.

No comparable tissue disposition data is available from a human study. Nevertheless, it appeared that metabolism of DEET and oxybenzone from dermal administration in rats was qualitatively similar to their metabolism in humans after dermal exposure [10, 23]. Several case reports have described young girls developing encephalopathy after DEET exposure [28-32]. In addition, intra-individual variations in metabolism and the correlation between adverse risk and dermal DEET application have not been fully studied [24, 33]. The enhanced disposition of DEET metabolites from concurrent skin application raised concerns for these DEET metabolites that may exert pharmacological and/or toxicological effects from prolonged exposure to DEET and oxybenzone [9, 24]. Kidney and brain samples collected from this study also contained all DEET metabolites; ET was the predominant metabolite in these organs. Liver is a prime metabolism site of the body so it was expected that there would be a higher concentration of both parent compound and metabolites in this tissue [10]. The 30-day repeated-dosing study demonstrated increased potential for DHMB and ET accumulation, as a lower study dosage (versus 24-hour topical application), similar plasma profiles (versus intravenous and 24-hour topical administration) and a less penetrating formulation (70% ethanol for 30-day repeated-topical application versus 100% ethanol for 24-hour topical application) led to increased concentrations. Potential for undesirable side effects from chronic exposure to repellents in susceptible subjects should be further investigated.

Previous studies have indicated that liver contains the highest concentrations of oxybenzone metabolites per gram of tissue, followed by kidney [8]. Higher concentrations of these metabolites in liver was attributed to a high content of cytochrome P-450, which may explain why increased THB amounts were detected in the liver, as THB is derived from DHB through an aromatic hydroxylation reaction mediated by the cytochrome P-450 enzyme system [8]. All three metabolites were also detected in kidney and brain samples, with THB being the predominant metabolite. Transport proteins are responsible for transferring endogenous compounds (bile acids) and xenobiotics (toxins) within the body, and are therefore an important determinant of metabolite disposition and subsequent excretion [34]. Differences observed between combined and single topical application were considered detrimental from a clinical viewpoint, since oxybenzone and its metabolites enhanced the accumulation and disposition of DEET and its metabolites.

Urine and feces samples were collected from the 24-hour single-dosing study. DHMB was the major urine metabolite of DEET with combined application (6.3±0.1 µg/ml) significantly exceeding single application (5.9±0.1 µg/ml, $p<0.05$). DHMB and ET were also detected in feces; their concentrations were 1.9±0.4 ng/g (DHMB) and 0.6±0.1 ng/g (ET) in combined use and 1.8±0.5 ng/g (DHMB) and 0.5±0.1 ng/g (ET) in single use, respectively. This suggested that rats utilized both urinary and enterohepatic routes for eliminating DEET from the system as previously reported [10]. Past studies suggested that oxybenzone was excreted via urine and feces following oral administration; DHB was a major metabolite excreted in the urine [5, 35]. This study led to similar findings, in which DHB was the predominant metabolite in urine (2.0±0.1 µg/ml for combined use and 1.8±0.1 µg/ml for single use, $p<0.05$) and feces (1.7±0.1 ng/g for

combined use and 1.4±0.1 ng/g for single use, $p<0.05$). No THB was detected in the feces. The data suggested that rats utilized both urinary and enterohepatic routes for eliminating oxybenzone and its metabolites.

4.4.3. Cellular Viability

Figure 4.4 shows the cellular viability of rat hepatoma cells after the cultures were exposed to DHMB, ET, DHB, DMB and THB at concentrations of 0.1 µg/ml, 1 µg/ml, and 10 µg/ml for 48 and 72 hours. Colorimetric assays are used extensively in cell proliferation and cytotoxicity analysis, enzyme analysis and bacteriological screening [36]. The water-soluble-tetrazolium-1 (WST-1) test investigated the metabolic activity of mitochondria as an indication of the vital status of cells [37]. While no significant decrease in cellular viability was observed after 24 hour exposure at all concentrations (data not shown), reduced cellular viability was noted when exposure time to all metabolites was increased.

For DEET metabolites, DHMB reduced cellular viability by 30-44% for all three treatment concentrations after 72 hours. ET reduced cellular viability by 10% and 9% at 1 and 10 µg/ml, respectively, after a 48-hour exposure; a decrease of 28% and 34% respectively after a 72-hour exposure was also observed. For oxybenzone metabolites, DHB reduced cellular viability by 25% at 10 µg/ml and 48 hours; a reduction of 35% after 72 hours of exposure was also observed. DMB reduced cellular viability by 40% at 10 µg/ml and 72 hours. THB also reduced cellular viability by 38% and 43% at 1 and 10 µg/ml respectively after a 72-hour exposure. Combined exposure to all test metabolites reduced cellular viability by 22% and 48% at 1 and 10 µg/ml respectively after 72 hours

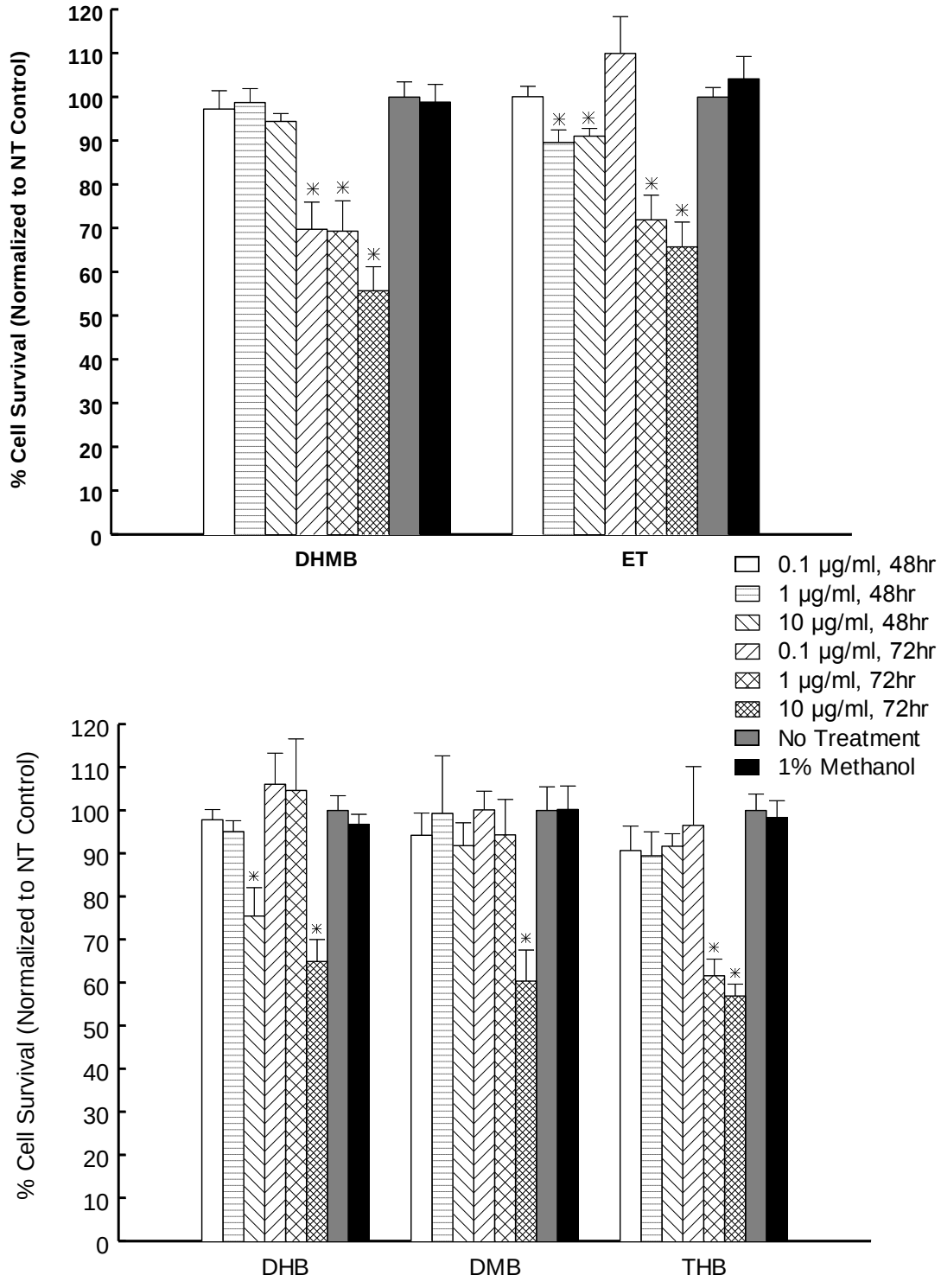

Figure 4.4. Cellular viability of rat hepatoma cell line 1548 exposed to metabolites of DEET and oxybenzone at different concentrations and durations (* significant difference from No Treatment control, $p < 0.05$, $n = 6$, Mean ± SEM).

(data not shown). Overall, DEET metabolites affected the viability of hepatoma cells more than oxybenzone metabolites, and THB appeared to be the most hepatotoxic oxybenzone metabolite.

Initiation of programmed cell death (apoptosis) is demonstrated by the release of mitochondrial cytochrome c activity, activation of caspases, elevation of 8-hydroxy-2-deoxyguanosine level, increased levels of 3-nitro-tyrosine, and alterations of p53 gene expression [38]. Previous studies found that concurrent application of DEET and permethrin in rats induced urinary excretion of 3-nitrotyrosine and 8-hydroxy-2´-deoxyguanosine, markers of DNA damage and oxidative stress, and mitochondrial cytochrome c release [39]. Another study using human hepatocytes confirmed that DEET was a mild inducer of adenylate kinase and caspase-3/7, both indicators of apoptotic cell death [40]. Therefore, DEET was capable of inducing apoptotic cell death in hepatocytes in humans and animals. Little information is available for DEET metabolites. Recent investigation indicated significant interindividual variability in CYP450 isoform activity in human microsomes relevant to DEET metabolism [24, 33]. Individuals with high activity of CYP2B6 and CYP1A2 activity produced the highest DHMB level, whereas individuals with high activity of CYP3A4 or CYP2C19 had the highest ET level. Our study is the first of its kind to observe suppression of cellular viability by ET and DHMB after 48 and 72 hours of exposure. Plasma concentrations of DEET metabolites also reached 0.1 µg/ml from *in vivo* animal studies, which may indicate that cell proliferation could be compromised over extended exposure to the compound.

Cellular viability was suppressed after concurrent administration of all metabolites including DHB, DMB, and THB at 1 µg/ml. Plasma concentrations of these

metabolites approached this range after intravenous administration and 30-day repeated topical application, which may indicate that *in vivo* cell proliferation could be affected. No toxicological mechanisms have been clearly defined for oxybenzone or its metabolites. Cases of allergic contact cheilitis, general endocrine disruption, birth weight variations, and even possible carcinogenicity have been reported with oxybenzone use [41-45]. Although the WST-1 assay measures mitochondrial activity, reduction of WST-1 may also be associated with superoxide, occurring in either the extracellular environment or the plasma membrane [36]. Depletion of NADH or other mechanisms that involve superoxide may potentially interfere with the reduction process of WST-1. Consequently, different cell types that possess variable extracellular superoxide contents could interfere in the reduction capacity of each cell type to different degrees [36]. Hepatocellular experiments conducted in this study did assist in providing a better understanding of the underlying mechanisms causing hepatotoxic deficits from short-term and long-term *in vitro* exposure of these test metabolites, but further analysis and studies with additional cell lines would be beneficial in formulating more conclusions regarding their toxicokinetic effects.

4.5. Conclusion

In conclusion, the intravenous and topical application studies demonstrated significant pharmacokinetic differences between administration or application of single and combined preparations for both DEET and oxybenzone. Concurrent application of DEET and oxybenzone enhanced the disposition of all DEET metabolites *in vivo*, but this effect was less apparent for oxybenzone metabolites. Plasma concentrations of all

metabolites remained detectable for 24 hours after topical application, which supported the presence of a skin depot by the parent compounds. Increased accumulation was observed for DHB, DMB and THB after once-daily repeated topical applications for 30 days. Cytotoxic effects were observed from all metabolites, occurring as early as 48 hours after exposure for ET and DHB. Due to the potential for cytotoxic effects as a result of exposure to DEET, oxybenzone, and their metabolites, either alone or in combination, consumers must exercise caution when applying these widely available over-the-counter topical skin products. DEET and oxybenzone are intended for remaining on the surface of the skin, and thus other studies are in progress to develop new formulations of the insect repellent and sunscreen, aiming at decreasing their overall percutaneous penetration and further evaluating their concurrent toxicological profiles.

4.6. References

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Chapter 5

Conclusion

5.1. Summary

Consumer-care products such as insect repellents and sunscreens are designed and intended for topical skin application. DEET exerts its protective repellency against insects by forming a vapor layer on the surface of the skin, subsequently deterring biting insects from direct contact with the skin surface. Oxybenzone and other sun-blocking agents absorb or reflect ultraviolet sunlight from the skin surface; they are effective only on the exterior of stratum corneum. Therefore, percutaneous permeation of DEET and oxybenzone will not only diminish the shielding protection of the insect repellent and sunscreen, but will also increase systemic disposition of the compounds in the body that is neither beneficial nor useful. In addition, concurrent application of DEET and oxybenzone is capable of enhancing mutual percutaneous permeation of the two substances across the skin membrane, which could result in increased risk for systemic adverse effects or even severe toxicity in susceptible subjects.

All commercially available insect repellent and sunscreen preparations have demonstrated a tendency to permeate across skin and achieve systemic disposition to a certain extent after topical skin application. This percutaneous characterization has not been well understood and systematically investigated, which certainly increases the complexity of using these consumer-care products for daily applications.

The primary objective of this thesis was to study the pharmacokinetic and toxicological characteristics of the insect repellent DEET and the sunscreen oxybenzone in an animal model, with the hypothesis that concurrent application of both DEET and oxybenzone would lead to an increase in systemic disposition of the substances and consequently toxicological effects from using the two compounds at the same time.

5.1.1. Methodology Summary

In this thesis, several key experimental components have been explored regarding concurrent application of DEET and oxybenzone, including single-dosing skin application, 30-day repeated skin application, intravenous administration, cellular studies utilizing rat primary cortical neurons and astrocytes and rat hepatoma 1548 cells, and behavioral testing of rats. To assess topical skin application, DEET and oxybenzone were applied to the skin, either alone or in combination, to the study animals. Blood, urine, fecal and skin samples were collected at pre-defined intervals. Liver, kidney and brain specimens were also harvested at the conclusion of the experiment. For intravenous administration, DEET or oxybenzone was injected into the tail vein of the study animals, and similar procedures were utilized to collect all biological samples. In all studies, concentrations of DEET, oxybenzone and their metabolites in collected samples were measured using a validated HPLC assay. To assess potential neurological toxicity, rat primary cortical neurons and astrocytes were treated with DEET, oxybenzone or DEET/oxybenzone at various concentrations and time durations. Cell viability was analyzed using the standard MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. To assess potential hepatotoxicity, rat hepatoma 1548 cells were treated with DEET, oxybenzone, and their metabolites at different concentrations and exposure intervals. Cell viability was determined using the standard WST-1 (water-soluble tetrazolium salt) assay. In addition, behavioral testing protocols were utilized to assess the arousal (open field test), locomotion (open field and ladder test), habituation (open field test), and motor coordination (open field test and ladder test) of rats over a 30-day

study period. The results were compared to those of two negative control groups and one positive control group.

5.1.2. DEET and Metabolites Results Summary

Plasma concentrations of DEET rose rapidly following single topical skin dosing in rats, indicating DEET's capability for a prompt percutaneous permeation. Combined application of DEET and oxybenzone produced a significantly higher C_{max} value as well as a shorter apparent $t_{1/2}$, MAT and MRT for DEET than its individual counterpart. Evidence of flip-flop kinetics was noted when comparing the topical application to the intravenous administration. The relative bioavailability of DEET from single-dosing topical application was approximately 2%. No statistically significant difference was observed in AUC whether DEET was applied individually or concurrently with oxybenzone. Skin recovery of DEET from concurrent use was lower than that collected at 7 hours and 24 hours from single DEET use, which indicated a faster disappearance of the compound from the skin when oxybenzone was simultaneously present. Combined application also led to an overall greater permeation and recovery of DEET in all skin layers, with the majority recovered in the vicinity of viable epidermis. Considerable tissue distribution of DEET from topical skin application suggested that DEET was not fully metabolized after 24 hours. Liver concentration of DEET from combined application was significantly higher than that observed from single-dosing and intravenous administration. While DEET was still detectable in the plasma 24 hours post-dosing, DEET concentrations in urine and fecal samples collected at the study conclusion were below the limit of quantification, which might indicate rapid metabolism and

excretion of DEET *in vivo*. In addition, co-application of DEET and oxybenzone reduced DEET concentration in the kidney.

In 30-day, once-daily, repeated topical study, DEET was detectable in the plasma 24 hours after the last skin application. Concurrent use of DEET and oxybenzone elevated the DEET level in plasma in comparison to single skin use. Skin recovery from concurrent application was lower than single DEET use, indicating quick disappearance of DEET from the application site. DEET was found in liver and brain samples at the conclusion of the study; concurrent application of DEET and oxybenzone led to significantly higher DEET disposition in liver than its single counterpart but no difference in brain samples was observed between the two application approaches. The synergistic enhancement between DEET and oxybenzone was also observed in this study; the level of enhancement was however smaller than what was reported in earlier studies. This discrepancy might be primarily attributed to the complexity of *in vivo* testing in animal models. Use of *in vitro* diffusion testing as a screening tool had simplified the skin permeation pathway by directly focusing on the chemical interactions between the two lipophilic compounds without taking consideration of any other influencing factors. *In vivo* testing in an animal model, on the other hand, would involve numerous biological and physiological parameters such as drug transporters, skin metabolism, and possible skin depot formation that were devoid in an *in vitro* experimental setting. This repeated topical application study did not reveal substantial neurotoxicity of DEET from exposure to DEET and oxybenzone, even though disposition of the test substances was increased in both liver and brain.

The pharmacokinetics of DEET has been previously published, but this was the first study to document pharmacokinetic properties of DEET in a rat model. Plasma concentration of DEET decreased rapidly after the intravenous administration, indicating a biexponential decline of DEET *in vivo*. Fitting the data to a two-compartment model implied that DEET did not promptly equilibrate throughout the entire body initially, but instead distributed quickly and uniformly into plasma, extracellular fluid and highly perfused tissues such as the liver and kidney. The higher volume of distribution suggested that DEET was subjected to substantial extravascular distribution *in vivo*. Elimination from the central compartment by hepatic metabolism was extensive as indicated by a short elimination half-life; similarly, a high systemic clearance suggested rapid renal excretion of the compound.

As to DEET metabolism *in vivo*, DHMB and ET were detected in plasma 30 minutes post intravenous injection, indicating a rapid metabolism of the parent compound in rats. The peak metabolite concentrations were observed within two hours of the administration, and both metabolites were still detectable in plasma after 24 hours. DHMB appeared to be the predominant metabolite in the plasma. For single-dosing topical application, concurrent application of DEET and oxybenzone resulted in significantly higher AUC levels for both DHMB and ET than those from single skin application. Repeated skin application for 30 days also revealed higher DHMB level from concurrent application, but ET level was not significantly different between the two application approaches. It was evident that DEET was rapidly metabolized in the plasma after intravenous and topical administrations.

DHMB and ET were detected in all tissue samples collected from the experiments, but DHMB was the predominant metabolite in liver. Co-application of DEET and oxybenzone enhanced the liver concentrations of DHMB and ET after both application approaches (24-hour and 30-day). DHMB and ET were also detected in kidney and brain samples; ET was the predominant metabolite in these tissues. The 30-day repeated-dosing study demonstrated increased potential for DHMB and ET accumulation, even though the application dose was lower than that used for single-dosing application. The higher concentration of DEET metabolites from concurrent skin application could be of concern since they might exert potential toxicological and/or pharmacological effects after prolonged exposure to DEET or DEET/oxybenzone combination.

5.1.3. Oxybenzone and Metabolites Results Summary

Plasma concentration of oxybenzone rose rapidly following single dermal application with the combined formulation producing a significantly higher C_{max} and AUC, as well as a shorter apparent $t_{1/2}$, MAT and MRT for oxybenzone than the single oxybenzone application. Evidence of flip-flop kinetics was noted when comparing the topical application to the intravenous administration. The relative bioavailability of oxybenzone from skin absorption ranged between 5-9% of the applied dose. Concurrent application led to increased permeation and recovery of oxybenzone in all skin layers, with the majority of oxybenzone recovered in the vicinity of viable epidermis. Consequently, lower skin surface recovery of oxybenzone was found from concurrent application than single application. Considerable tissue distribution of oxybenzone

indicated that the parent compound had not been fully metabolized within 24 hours. Liver concentration of oxybenzone from combined topical application was significantly greater than that observed from intravenous administration and single oxybenzone application. Unlike DEET, oxybenzone was detected in liver, urine and feces, which might suggest a prolonged percutaneous permeation and skin retention from an oxybenzone depot *in vivo*; this was more profound from concurrent application of the two test compounds.

In 30-day, once-daily, repeated application study, oxybenzone remained detectable in plasma 24 hours after the last skin application; it was also detected in brain and liver specimens. Skin recovery for oxybenzone was lower from concurrent application than from application alone, indicating a faster disappearance of oxybenzone from the application site when two compounds were simultaneously present. Nevertheless, concurrent application did not significantly elevate the plasma level or tissue deposition of oxybenzone; in fact, deposition was lower in concurrent use than in application alone. Previous *in vitro* diffusion studies demonstrated that oxybenzone influenced the overall permeation of DEET more than DEET influenced oxybenzone. Oxybenzone tends to form a skin depot due to its high lipophilicity, which may explain this disposition difference.

Similar to DEET, plasma concentration of oxybenzone decreased rapidly at a biexponential rate after intravenous administration. Oxybenzone did not equilibrate throughout the entire body at first; it distributed quickly and uniformly into plasma, extracellular fluid and highly perfused tissues such as the liver and kidney. In comparison to its DEET counterpart, AUC for oxybenzone was 6-fold higher than that for DEET. Oxybenzone also demonstrated lower volume of distribution, lower systemic clearance

and longer elimination half-life than DEET, which suggested that oxybenzone was not as extensively distributed or quickly eliminated as DEET. No pharmacokinetic data of oxybenzone had been previously reported in a rat model.

Metabolites of oxybenzone DHB and DMB were detected in the plasma 30 minutes post intravenous injection, indicating a rapid metabolism of the parent compound in rats. Peak concentrations of DHB and DMB were observed within four hours of the administration and both metabolites were still detectable in the plasma after 24 hours. DHB was the predominant metabolite in the plasma. Concurrent application of DEET and oxybenzone resulted in significantly higher AUC levels for DHB and DMB than application of oxybenzone alone in the single-dosing study. However, repeated skin application for 30 days did not lead to differences in plasma oxybenzone metabolite concentrations between the two application approaches.

THB, a third oxybenzone metabolite, was the primary compound found in the liver after intravenous administration, single-dosing topical application and repeated-dosing topical application, even though it was not detected in all plasma samples. For the 24-hour topical skin study, concurrent application of DEET and oxybenzone resulted in increased liver concentrations of three metabolites in comparison to oxybenone application alone. In 30-day, repeated-dosing study, concurrent application led to a slight decrease in oxybenzone metabolite liver concentrations in comparison to oxybenzone application alone. All three metabolites were also detected in kidney and brain samples, with THB being the predominant metabolite. Urine and feces specimens collected from the single-dosing study displayed elevated concentrations of metabolites after combined application; DHB was the predominant metabolite while no THB was detected.

Discrepancies in skin and tissue recovery between the single and repeated dosing studies might have been partially attributed to skin depot formation, small formulation difference, and variations in application amount and/or surface area.

5.1.4. Cellular Studies Summary

No significant difference in astrocyte viability was observed after 24-hour or 48-hour exposure to DEET or oxybenzone at various concentrations, either alone or in combination, in comparison to the no-treatment control group. However, astrocyte viability was significantly reduced after the cultures were exposed to both DEET and oxybenzone at 10 µg/ml for 7 days. Similarly, no significant difference in neuronal viability was observed after 24-hour or 48-hour exposure to DEET or oxybenzone at various concentrations, either alone or in combination, in comparison to the no-treatment control group. Treating the neurons for 7 days with 10 µg/ml of DEET or oxybenzone alone significantly decreased the neuronal viability, but combined use of DEET and oxybenzone did not significantly affect the viability of neurons. Overall, neurocellular assessment was an essential part of this project as the investigation provided a better understanding of potential neurological effects from using DEET and oxybenzone; no similar study had been previously performed or documented in the literature.

Hepatotoxicity is often associated with drug bioaccumulation and adverse drug effects *in vivo*, which is one of the most common criteria in drug use safety and post-marketing surveillance. The risk of liver injury may increase remarkably when multiple drug substances are utilized concurrently. *In vitro* hepatocellular studies are capable of providing useful, preliminary screening insight into characterizing potential liver damage

or toxicity, both from a parent compound and its respective metabolites. It was found from hepatoma cellular experiments that DEET was capable of inducing cell toxicity at all test concentrations, but not at all exposure times. Exposure to both DEET and oxybenzone for an extended period of time may also be of concern, because this significantly reduced the cellular viability, but no more than their single counterparts under the conditions tested.

For primary metabolites of DEET and oxybenzone, no significant decrease in rat hepatoma cell viability was observed after 24 hour exposure; however, cellular viability was reduced when exposure time was increased in all metabolites. Overall, metabolites of DEET affected the hepatoma cell viability more than those of oxybenzone, and THB exerted the most hepatotoxic potential of all oxybenzone metabolites. This study was the first to indicate suppression of cellular viability by ET and DHMB after 48 and 72 hours of exposure. Enhanced cellular toxicity occurred at correlative concentrations of DHB, DMB and THB that would be achieved systemically from intravenous administration and topical skin application of DEET and oxybenzone.

5.1.5. Behavioral Testing Summary

Behavioral testing was performed in the 30-day, once-daily, repeated skin application study to screen for possible neurological abnormality from using repellent DEET and sunscreen oxybenzone. This was a secondary experimental component in the original study design that actually consumed a significant amount of resource and time for execution and subsequent data analysis. Study animals from the 3 treatment groups (DEET, oxybenzone, and combined DEET/oxybenzone), as well as the negative and

vehicle control groups, all passed the behavioral testing without visible adverse effects or incidents. In comparison, study animals in the positive control group demonstrated significant behavioral impairment after a 10-day injection period of acrylamide. Design of a sensitive and reproducible behavioral testing protocol to detect subtle and small behavioral variations in living animals was not easy; there were limitations in the two testing methods, i.e., open field test and ladder test, which were selected for this study. It was found from the study that testing protocols and equipment should be standardized in order to warrant data reliability and reproducibility. A minor change of the testing parameters could inadvertently lead to major variations in study results. Test animals were highly sensitive and perceptive to environmental conditions during behavioral experiments. Their behavioral outcomes could be profoundly influenced by how they were housed (individual versus grouped, normal light-dark cycle versus reversed light-dark cycle) and handled (handling training versus minimal handling, tested in colony room versus tested in a designated room) and who were to perform the testing (same operators versus different operators, operators wearing different laboratory garments on different study days) [1]. In addition, the sample size utilized in the behavioral study was somehow limited; assessing subtle behavioral changes potentiated by DEET and/or oxybenzone would need much larger testing animal numbers. Conducting these types of animal studies, with 50 or more animals in each treatment group, would necessitate a larger research facility and significant resources, such as those of a regulatory agency or a pharmaceutical/consumer-care product manufacturer.

5.2. Overall Conclusions

Compiling the data from this entire study on the concurrent use of the insect repellent DEET and the sunscreen oxybenzone has clearly generated some insight into how these consumer-care products could be utilized efficiently and safely. The pharmacokinetic data obtained from this project was important in understanding and elucidating the absorption and biodisposition of the compounds in the rat model. The toxicological data obtained from this project suggested that the risk for increasing adverse effects from concurrent skin application of repellents and sunscreens would be low and marginal in healthy individuals. Nevertheless, uncertainty could still remain regarding long-term skin application of these compounds in susceptible subjects, especially at higher concentration doses.

Concurrent use of DEET and oxybenzone increases the percutaneous permeation of both chemical compounds in a synergistic manner; mixing DEET-based repellent products with sunscreen preparations also reduces the sun-blocking efficacy of the active sunscreen ingredients. Health Canada decided to discontinue combined sunscreen/repellent products in 2003 based on the finding that neither protective efficacy of repellency and UV blockage would be achieved from a combined sunscreen/repellent product. This decision would be strongly supported by the results obtained from this project.

DEET and oxybenzone have been used commercially for more than three decades. DEET is classified as a pesticide by the regulatory agencies. Oxybenzone, on the other hand, is categorized as a cosmetic in Europe, but as a drug in the US by the Food and Drug Administration (FDA). Current user guidelines and recommendations for

DEET are available; suspected incidents of toxicity associated with DEET use including death have also been recorded and reported. The etiology of these serious adverse effects, whether neurological or other symptoms, hence remains somewhat unclear. A recent update on oxybenzone (June 2011) had the FDA implement new marketing guidelines for all sunscreens, which included adding the words "broad spectrum" to formulations that block both UVA and UVB, among other labeling modifications. These added guidelines are especially beneficial to sunscreen use safety that had been lacking for several decades. Even though *in vitro* and *in vivo* data from this project did not stipulate a greater incidence of toxicity from using DEET and oxybenzone simultaneously, it had added further benefit and understanding in formulating clearer conclusions regarding the pharmacokinetic and toxicological properties of these two chemical compounds.

5.3. Future Research Directions

The data from this thesis, together with previous findings identifying health problems associated with DEET use and insufficient data regarding overall safety of oxybenzone, further warrants the implementation of large-scale toxicological studies on both compounds in the future. In spite of an absence of behavioral abnormality in this project, mechanistic and potential neurotoxic aspects of DEET should still be the primary focus of future investigations. In addition, metabolic disposition of the two compounds should be further assessed in order to minimize any risk potentiated from using both compounds. DEET overdosing is a known cause to death, especially in children. It remains unclear whether concurrent use of DEET and oxybenzone would exacerbate this risk in susceptible individuals. Nevertheless, supplementary data on any toxicological

property of DEET and oxybenzone, whether as single component or as combined entity, will be beneficial to the general public in using repellent and sunscreen products efficiently and safely.

Development of novel preparations to reduce overall percutaneous permeation and systemic disposition of active repellent and sunscreen ingredients would be another direction for future research. Long-acting repellent formulations would be beneficial, because they can reduce the need for frequent, repeat skin applications, yet minimize overall percutaneous absorption and maintain prolonged repellency on the skin surface. Regular daily use of sunscreens by an average consumer, in accordance to current dermatological recommendations of frequent, liberal applications, will also increase the likelihood for active sunscreen ingredients to penetrate through the skin and deposit throughout the human body. Newly developed sunscreen preparations are able to minimize percutaneous absorption of the active ingredients, leading to negligible systemic disposition of the substances [2]. Therefore, it would be desirable and beneficial to further characterize the pharmacokinetic and toxicological properties of these novel formulations in animal and/or human subjects.

As the human race approaches seven billion around the world, concerns and pressures from food shortages, environmental pollution, global warming, and disease management have become an emerging problem for governments and healthcare professionals. It is anticipated that our society will take the responsibility to become more environmentally conscious and to actively engage in disease control and prevention. Insect repellents and sunscreens are two types of consumer-care products that have played an important role in minimizing vector-borne diseases and skin cancer. They will

continue to help the general public ensure healthy life styles and improved quality of life in years to come.

5.4. References

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