EVALUATION OF PERCUTANEOUS PENETRATION OF INSECT REPELLENT DEET AND SUNSCREEN OXYBENZONE FROM EMULSION-BASED FORMULATIONS

BY

TAO WANG

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

DOCTOR OF PHILOSOPHY

Faculty of Pharmacy
University of Manitoba
Winnipeg, Manitoba
Canada

(c) March, 2014

ABSTRACT

Insect repellent N,N-diethyl-*m*-toluamide (DEET) and sunscreen oxybenzone are commonly incorporated into commercially available consumer care products that are utilized to prevent vector-borne diseases and skin aging and damage. Semisolid emulsion-based formulation has been extensively selected for active repellent and sunscreen ingredients due to their excellent solubilizing properties for both lipophilic and hydrophilic components and satisfactory customer acceptance. Under the ideal use situation, an appropriate and elegant emulsion-based formulation can minimize transdermal absorption of DEET and oxybenzone and maximize their skin protection effect.

The percutaneous penetration profiles of DEET and oxybenzone from several emulsion-based formulations were evaluated and compared by using the *in vitro* diffusion experiments in this thesis. Various parameters in the emulsion formulations, such as emulsion type, emulsifier, emollient, penetration enhancer, thickening agent, and thixotropic properties of the emulsions, could exert significant influence on percutaneous permeation of the active ingredients from the preparations. An emulsion-based preparation using the emulsifier, Emulium 22, possessed the relatively lower skin permeation of DEET and oxybenzone than other emulsion-based preparations; it was selected for the *in vivo* animal study. This emulsion-based formulation containing DEET and/or oxybenzone was topically applied to rats once daily for a 60-day period to investigate systemic concentration and tissue deposition of DEET, oxybenzone, and their respective metabolites. This formulation produced lower systemic absorption of DEET and oxybenzone than the commercially available products reported in a previous animal study. The concurrent application of DEET and oxybenzone in this study did not show synergistic enhancement between the two compounds in plasma.

The percutaneous penetration profiles of DEET and oxybenzone from different semisolid emulsions in the *in vitro* diffusion experiment provided the important information in selecting the appropriate formulation of DEET and oxybenzone for topical application. The *in vivo* animal study identified pharmacokinetics and biodistribution characteristics of DEET and oxybenzone from the promising lab-developed emulsion. Studies are ongoing to further improve formulation characteristics so that this emulsion-based preparation could be used to minimize overall transdermal permeation of DEET and oxybenzone from topical skin application.

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THIS THESIS IS DEDICATED TO

MY FAMILY

ACKNOWLEDGEMENTS

My Ph.D. studies at the Faculty of Pharmacy, University of Manitoba have been an extremely rewarding experience as well as a wonderful personal journey. I have been helped and supported by many people throughout this incredible period of time. Without them I would not have arrived at this important career milestone, so I would like to take the opportunity to thank all of them for their mentoring and friendship.

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STATEMENT OF ORIGINALITY

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

Tao Wang
March 2014

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LIST OF ABBREVIATIONS

ALAB:	l-alanine benzyl ester
Apifil ®:	PEG-8 beeswax
AUC:	Area under the plasma concentration *vs.* time curve
AUMC:	Area under the first moment plasma concentration *vs.* time curves
BMDBM:	Butyl methoxydibenzoylmethane
Brij98:	PEG 20 oleyl ether
CAS:	Chemical Abstracts Service
CCS:	Canadian Cancer Society
CDC:	Centers for Disease Control and Prevention
CMC:	Sodium carboxymethylcellulose
Compritol 888:	Glyceryl behenate
CYP:	Cytochrome P450
DEET:	N,N-diethyl-m-toluamide
DFF:	N,N-diethyl-3-formylfenzamide
DFP:	Diisopropylfluorophosphate
DHB:	2,4-dihydroxybenzophenone
DHMB:	N,N-diethyl-3-carboxylbenzamide

DLS: Dynamic Light Scattering

DMB: 2,2'-dihydroxy-4-methoxybenzophenone

DPPC: Dipalmitoylphosphatidylcholine

EHMC: Ethylhexyl methoxycinnamate

Emulfree CBG: Butylene glycol cocoate, ethyl cellulose, and isostearic

Emulium 22 ®: Tribehenin PEG-20 esters

EPA: Environmental Protection Agency

ET: N-ethyl-3-carboxylbenzamide

ETM: N-ethyl-3-hydroxymethylbenzamide

F#1: Formulation #1

F#2: Formulation #2

F#3: Formulation #3

F#4: Formulation #4

F#5: Formulation #5

FA1: Formulation A1

FA2: Formulation A2

FB1: Formulation B1

FB2: Formulation B2

FC1: Formulation C1

FC2: Formulation C2

FDA: Food and Drug Administration

Geleol: Glyceryl monostearate 40-55

GSH: Glutathione

HDPE: High density polyethylene

HLB: Hydrophilic-Lipophilic Balance

HPLC: High Performance Liquid Chromatography

IPP: Isopropyl palmitate

IPPSF: Isolated Perfused Porcine Skin Flap

IUPAC: International Union of Pure and Applied Chemistry

Labrafac™ PG: Propylene glycol dicaprylocaprate

Labrafil M1944CS: Polyoxylglycerides

Labrasol: Caprylocaproyl macrogolglycerides

LAS: Linear alkylbenzene sulfonate

LDPE: Low density polyethylene

LogKo/w: Log partition coefficient between octanol and water

$LogK_{SC/Vehicles}$: Log partition coefficient between stratum corneum and vehicles

MB: 3-methylbenzamide

MCZ: Miconazole nitrate

MED: Minimum Erythema Dose

MNA:	Methyl nicotinate
MOD™:	Octyldodecyl myristate
MRT:	Mean Residence Time
MSDS:	Material Safety Data Sheet
NCIC:	National Cancer Institute of Canada
NTP:	National Toxicology Program
OBZ:	Oxybenzone
OSNs:	Olfactory Sensory Neurons
OR83b:	Odorant Receptor 83b
PCP:	Pentachlorophenol
PDMS:	Polydimethylsiloxane
PEG-400:	Poly(ethylene glycol)-400
Plurol diisostearique:	Polyglyceryl diisostearate
Plurol oleique CC 497:	Polyglyceryl oleate
PNP:	4-nitrophenol
PPD:	Persistent Pigment Darkening
Precirol Ato 5:	Glyceryl palmitostearate
PS:	Phytosphingosine
Ritadecene 50:	Hydrogenated polydencene
RPI:	Relative Polarity Index

SKN-HMN TDLO: Skin human toxic dose low

ORL-WMN LDLO: Oral woman lethal dose low

ORL-MAN LDLO: Oral man lethal dose low

ORL-RAT LD50: Oral rat median lethal dose

SKN-RBT LD50: Skin rabbit median lethal dose

SLN: Solid Lipid Nanoparticles

SLS: Sodium lauryl sulfate

SMILES: Simplified Molecular-Input Line-Entry System

SPE: Solid Phase Extraction

SPF: Sun Protection Factor

TEWL: Transepidermal Water Loss

THB: 2,3,4-trihydroxybenzophenone

Transcutol HP: Diethylene glycol monoethyl ether

USP: United State Pharmacopeia

UV: Ultraviolet

UVA/UVB: Ultraviolet A/Ultraviolet B

LIST OF SYMBOLS

A:	Diffusion area (cm^2)
a:	Zero viscosity (Pa.s)
b:	Infinite viscosity (Pa.***s)***
c:	Consistency (s)
$\bar{C}_{ss}$**:**	Steady state concentration
C_m**:**	Drug concentration in membranes (g/ml)
C_{max} **:**	Peak plasma concentration *(g/ml)*
C_v**:**	Saturated drug concentration in vehicle (g/ml)
d(0.1):	Volume median diameter where 10% of the distribution is below (µm)
d(0.5):	Volume median diameter where 50% of the distribution is below (µm)
d(0.9):	Volume median diameter where 90% of the distribution is below (µm)
d:	Rate index
D[4,3]:	Volume weighted mean diameter (µm)
Di:	Diameter of particles (µm)
D_m**:**	Diffusion coefficient (cm^2/h)
h_m**:**	Thickness of the membrane (cm)
J_{ss} **:**	Steady-State Flux (g/cm^2h)
k_e**:**	Elimination rate

K_m:	Partition coefficient between membrane and vehicle
K_p:	Permeability coefficient (cm/h)
Lag :	Lag time (h)
Ni:	Number of particles
Q(t):	Accumulative drug amount permeating through the membrane (g)
Span:	Particle distribution span
T_{max}:	Peak time at which the peak plasma concentration was reached *(t)*

CHAPTER 1

DEET and Oxybenzone

1.1. DEET

Mosquitoes have always been a nuisance to those participating in field jobs and/or taking outdoor recreational activities during summer months. Recently mosquito infestation has become an imminent health hazard to the general public with the arrival of West Nile Virus in North America and its widespread dissemination by mosquitoes (CDC, 2010; Pile, 2001). The application of topical insect repellents has been recommended as an essential preventive approach for all those involved in outdoor activities. *N,N*-diethyl-3-methylbenzamide (DEET) has been utilized as a broad-spectrum insect repellent for over six decades (Fradin, 1998); it is also the most common and effective active ingredient in over 90% commercial insect repellent products (Staub, *et al.*, 2002). Both healthcare professionals and government regulatory agencies have strongly recommended that people apply insect repellent products for summer outdoor activities in order to reduce mosquito biting and to minimize potential for vector-borne diseases.

1.1.1. Physical and Chemical Properties of DEET

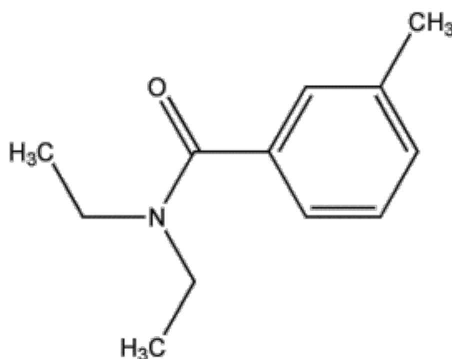
DEET was originally invented as a pesticide for farming purpose. It was subsequently used by the US military in 1946 and by the general public in 1957 as an insect repellent, respectively (EPA, 2009). DEET is considered one of the most effective, long-lasting, and broad-spectrum insect repellents in commercial applications (Qiu, *et al.*, 1998). It can be applied directly to the skin surface or to clothing and other fabric

materials; it is also utilized in farming animals to repel biting insects. It is estimated that approximately 30% US population (Osimitz, *et al.*, 1997) and over 50% Canadians (Wilson, *et al.*, 2005) apply DEET to minimize vector-borne disease transmission during insect-biting seasons. Commercially available insect repellent preparations contain DEET at a concentration range of 5-95%.

Figure 1.1 shows the chemical structure of DEET. It is synthesized by converting *m*-toluic acid (3-methylbenzoic acid) to the corresponding acyl chloride and then reacting with diethylamine (**Figure 1.2**) (Pavia, 2004; Wang, 1974). The reaction products normally contain 85-95% *meta*-isomer (DEET) and two minor by-products, *ortho*-isomer and *para*-isomer. Therefore commercially available DEET is a mixture of three isomers, in which *meta*-isomer is the primary active repellent ingredient (Stinecipher, *et al.*, 1998).

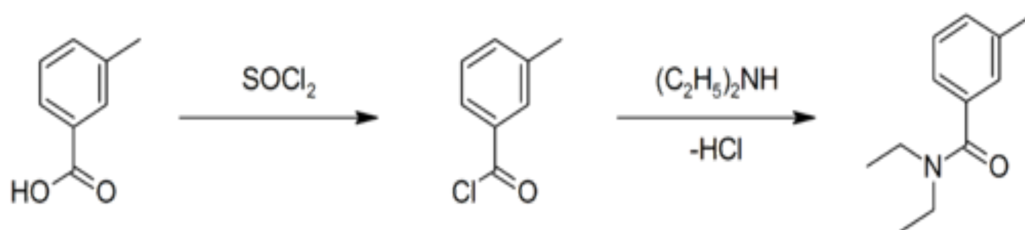
At room temperature, technical grade DEET is a colorless to amber liquid and has a slightly aromatic scent. It is insoluble in water, sparingly soluble in petroleum ether, and soluble in alcohol, ether and benzene. **Table 1.1** lists the various physicochemical characteristics of DEET.

Figure 1.1. The chemical structure of DEET†



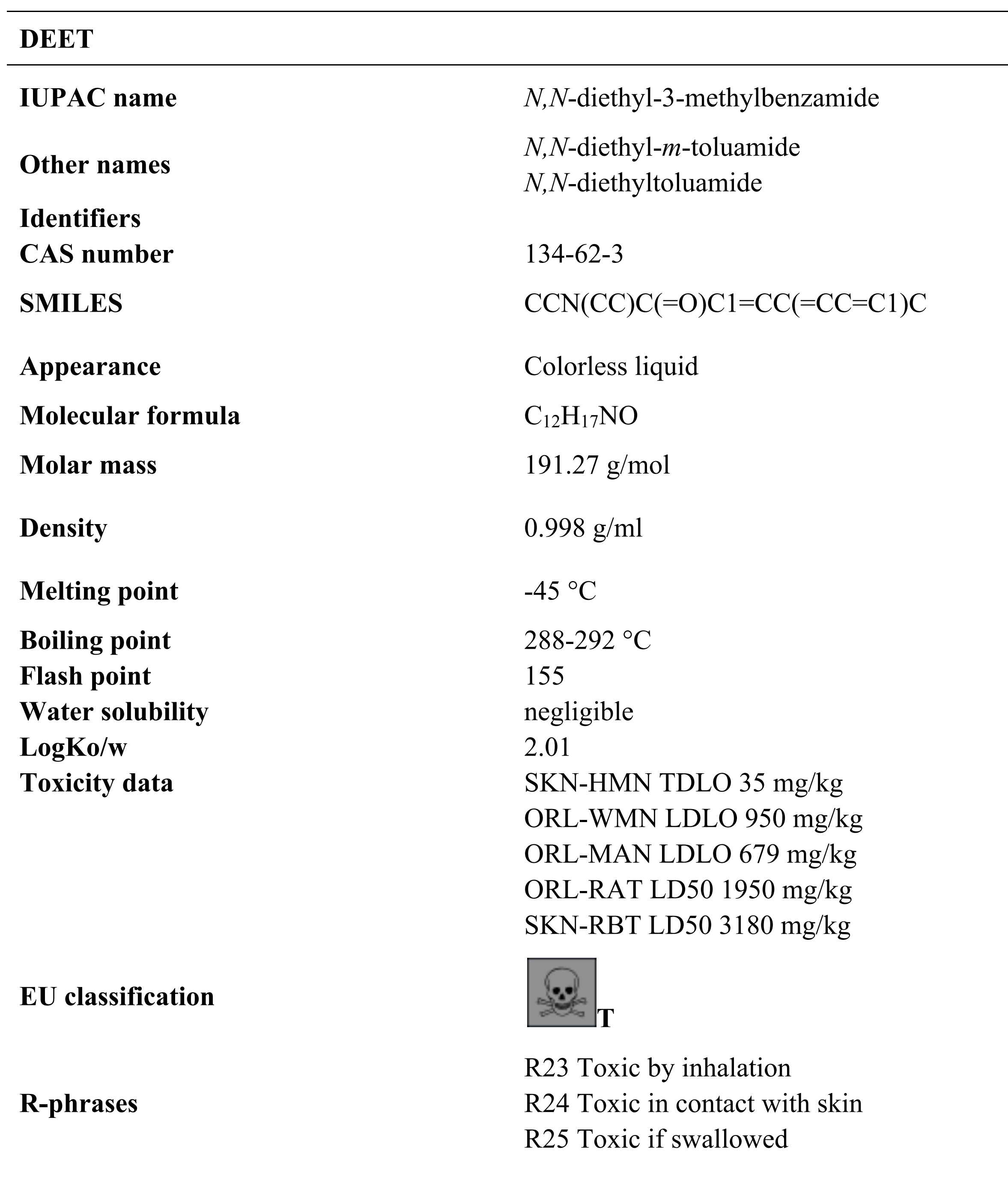
†DEET is *N,N*-diethyl-3-methylbenzamide; it is the most common active ingredient in insect repellents. (Copyright Permitted by Pesticide Research Institute at March 2013)

Figure 1.2. The synthetic route of DEET†



†The synthetic route of DEET involves two steps. The first step is to convert *m*-toluic acid (3-methylbenzoic acid) to the corresponding acyl chloride; the second step is to react with diethylamine. (Copyright Permitted by the free encyclopedia at July 2013)

Table 1.1. Physicochemical properties of DEET†



DEET	
IUPAC name	*N,N*-diethyl-3-methylbenzamide
Other names	*N,N*-diethyl-*m*-toluamide *N,N*-diethyltoluamide
Identifiers	
CAS number	134-62-3
SMILES	CCN(CC)C(=O)C1=CC(=CC=C1)C
Appearance	Colorless liquid
Molecular formula	$C_{12}H_{17}NO$
Molar mass	191.27 g/mol
Density	0.998 g/ml
Melting point	-45 °C
Boiling point	288-292 °C
Flash point	155
Water solubility	negligible
LogKo/w	2.01
Toxicity data	SKN-HMN TDLO 35 mg/kg ORL-WMN LDLO 950 mg/kg ORL-MAN LDLO 679 mg/kg ORL-RAT LD50 1950 mg/kg SKN-RBT LD50 3180 mg/kg
EU classification	**T**
R-phrases	R23 Toxic by inhalation R24 Toxic in contact with skin R25 Toxic if swallowed

†(MSDS, 2005)

1.1.2. Repellent Efficiency of DEET

As one of the most effective, broad-spectrum insect repellents, DEET is primarily used to repel physical contact between humans or domesticated animals and biting insects and thus minimize vector-borne disease transmissions. In military field, application of insect repellents like DEET has shown a practical and crucial means of maintaining troop fighting strength and preventing vector-borne diseases in disease-endemic regions (Hooper, *et al.*, 1983). For general civil usage, DEET-containing insect repellent products have been commonly recommended as an effectively protective approach for Lyme Disease, a multiple-organ-system, immune-mediated inflammatory disorder mainly transmitted by ixodid ticks (Sudakin, *et al.*; 2003; US EPA, 1998), and West Nile fever and encephalitis, an emerging infectious disease transmitted by *Culex* mosquitoes. In addition, DEET (35% DEET solution in isopropanol) is utilized in farming livestock to repel biting flies (Taylor, *et al.*, 1994).

The repellency of DEET has been confirmed by numerous efficacy studies. DEET was found to be highly protective against yellow fever mosquitoes (*Aedes aegypti*), malaria mosquitoes (*Anopheles quadrimaculatus*), and salt marsh mosquitoes (*Aedes taeniorhynchus*) (Schreck, *et al.*, 1989). It was effective against some fly species, such as deer flies (*Chrysops atlanticus*), biting stable flies (*Stomoxys calcitrans*), and biting midges (*Culicoides canithorax*) (Gilbert, *et al.*, 1955; Gilbert, 1966). DEET also exhibited repellency against deer ticks (Schreck, *et al.*, 1986), lone star ticks (Grothaus, *et*

al., 1976), rat fleas (Rutledge, *et al.*, 1982), and squirrel fleas (Mehr, *et al.*, 1984). In addition, DEET was proficient in repelling black flies (Robert, *et al.*, 1992), phlebotomine sand flies (Schreck, *et al.*, 1982), and the mosquitoes *Cx. gelidus, Culex vishnui, Cx. tritaeniorhynchus, Anopheles albimanus and An. dirus* (Frances, *et al.*; 1996; Schreck, 1985).

Even though DEET has been a common and effective insect repellent against vectors for years (Carroll, *et al.*, 2005; Klun, *et al.*, 2006), the exact mechanics of its repellency and molecular targeting have not been fully elucidated (Davis, *et al.*, 1975; Dogan, *et al.*, 1999; Klun, *et al.*, 2006; McIver, 1981; Schreck, *et al.*, 1970; Xue, *et al.*, 2007). As a volatile reagent, DEET does evaporate to repel mosquitoes at a distance of approximately 38 cm around its host (Schreck, *et al.*, 1970) and to repel ticks in the wider vapor phase (Carroll, *et al.*, 2005). DEET was found to block the attraction by mosquitoes to lactic acid, a substance in human sweat (Dogan, *et al.*, 1999), and more specifically, to inhibit the electrophysiological activity to lactic acid on the sensitive olfactory sensory neurons (OSNs) at the antennae of *Aedes aegyptii* (Davis, *et al.*, 1975). Mathias Ditzen and his colleagues conducted a behavioral and electrophysiological study in malaria mosquitoes and fruit flies to investigate a molecular mechanism of DEET in the observed olfactory repellency. They found that DEET blocked electrophysiological responses of olfactory sensory neurons from attractive odor, carbon dioxide and 1-octen-3-ol, in *Anopheles gambiae* and *Drosophila melanogaster*. The attractive odor

was masked through inhibiting the subsets of heteromeric insect odorant receptors that required OR83b as a co-receptor. The identification of this molecular target in DEET repellency might aid in designing a safer and more efficient insect repellent in the future (Ditzen, *et al.*, 2008). Further investigation is still ongoing.

Two essential attributes of a topical insect repellent preparation are the effective dose and protection time. In 1963, Smith *et al.* defined the minimum effective dose of an insect repellent as the minimal amount of the test material per unit of surface required to protect against a given population of insects (Smith, *et al.*, 1963). The protection time was defined as the time elapsing between applying a testing material to the first bite from the test population. The minimum effective dose of DEET against *Ae aegypti* was first reported by Smith *et al.* (1963) at 46-78 μg/cm^2; the application of a 10% DEET solution in ethanol provided a protection time of 5.6-6.2 hours to the forearms of human volunteers (Smith, *et al.*, 1963). In another repellency testing, the average minimum effective dose of DEET against *Ae. aegypti* was found to be 25 μg/cm^2 in 16 human volunteers, and a dose of 0.32 mg/cm^2 provided an average protection time of 6.8 hours (Gabel, *et al.*, 1976). Maibach *et al.* yet reported the minimum effective dose of DEET against *Ae. aegypti* as low as 16 μg/cm^2 (Maibach, *et al.*, 1974a). Variations in these two parameters were largely attributed to different experimental protocols from various laboratories. The protection time was dependent on doses applied and the density of insect populations in the testing, while the minimum effective dose was rarely estimated

with accuracy in very large experiments (Rutledge, 1985).

In reality, the efficacy and protection time of DEET are readily influenced by a variety of environmental factors, such as temperature, air flow and speed, and loss from evaporation or abrasion as well (Gossel, 1984; Maibach, *et al.*, 1974a; Maibach, *et al.*, 1974b). In studies correlating environmental temperature (26-50 ºC) and DEET repellency, protection time of DEET was decreased by one-half as the temperature increased 10 ºC with all other conditions remained the same (Khan, *et al.*, 1973). At an application dose of 0.16 mg/cm^2, DEET exhibited protection efficacy for 200 minutes at 26 ºC with a static air current. When air flow was increased to 192 m/min, the protection time of DEET was decreased to 73 minutes (Khan, *et al.*, 1973). High DEET concentration also helped prolong the repellent efficiency due to less physical loss. For example, 10% DEET protected against *Ae. aegypti* between 3-14 hours in human volunteers, while 95% DEET increased the protection up to 20 hours (Anonymous, 1980).

1.1.3. Biodistribution of DEET

DEET is considered a toxic substance in MSDS (Material Safety Data Sheets); its toxicity is categorized as a 3 on a scale of 4 (e.g., 1 as highly toxic, 2 as moderately toxic, 3 as slightly toxic, and 4 as non-toxic) (US EPA, 1998). As a result, DEET has subjected to little *in vivo* evaluation in humans, even though over 90% commercial insect repellent

products contain DEET as an active ingredient. A majority of assessment has been carried out in various animal models to understand absorption, distribution, metabolism and elimination of DEET *in vivo*. Information regarding DEET usage in humans has been largely derived from data obtained from these animal studies.

Numerous studies have demonstrated that DEET is capable of penetrating across the skin rapidly and extensively, though results were quite variable among these studies. In an *in vitro* study using [^{14}C] DEET dissolved in acetone, total skin permeation of DEET obtained at a 48-hour exposure was 36% for rhino mouse skin, 21% for rat skin, 15% for pig skin, and 11% for hairless guinea pig skin, respectively (Moody, *et al.*, 1993). In an *in vitro* permeation study using abdominal human cadaver skin, DEET permeation was 50.8% one hour after topical application of an ethanolic DEET solution at 25 μg/cm^2 (Spencer, *et al.*, 1979). In another *in vitro* study with human skin specimens, approximately 30% of DEET permeated across the membrane one hour after topical application of [^{14}C] DEET at 27-32 μg/cm^2, which was 41% lower than the finding by Spencer *et al.* (Reifenrath, *et al.*, 1982).

Similarly, *in vivo* permeability of DEET was also variable from various studies. In the earliest *in vivo* penetration study by Schmidt *et al.*, a dermal dosing of 0.99-1.14 mg/cm^2 DEET yielded 19-48% DEET penetration in guinea pigs (Schmidt, *et al.*, 1959). In 1963, Smith *et al.* reported that the penetration of DEET ranged 7-30% in guinea pigs after topical application of approximately 1mg/cm^2 of DEET (Smith, *et al.*, 1963). In the

same year, Smith *et al.* also reported that the average DEET penetration in two male human volunteers was 9%, 11%, 38%, and 54% two hours after an ethanol DEET solution was applied to the forearms at dosing of 1.86, 0.93, 0.16, and 0.077 mg/cm^2, respectively (Smith, *et al.*, 1963).

Systemic distribution of DEET after topical skin application has also been confirmed in animals and humans. In 1982, Snodgrass *et al.* found that absorption of [^{14}C] DEET amounted 48% in 7 days after topical application of 4μg/cm^2 [^{14}C] DEET in male rats (Snodgrass, *et al.*, 1982). In 1996, Schoening *et al.* reported that 82% of dermally applied DEET dose (100 mg/kg [^{14}C] DEET, or 2 mg/cm^2) permeated across the skin in rats within 7 days and that peak radioactivity of [^{14}C] DEET occurred 2 hours after the dermal administration, indicating fast transdermal absorption of DEET *in vivo* (Schoenig, *et al.*, 1996).

Regarding DEET absorption in humans, Feldmann and Maibach (1970) reported that the average transdermal absorption of DEET was 16.7%, as determined by the radioactivity recovered from urine samples in 5 days after dermal application of [^{14}C] DEET at 4 μg/cm^2 on the ventral surface of forearms in 4 human volunteers (Feldmann, *et al.*, 1970). In 1995, Selim *et al.* found that accumulation of DEET following dermal application of technical grade DEET and 15% DEET solution (in ethanol) was 5.6% and 8.4% in 5 days, respectively. (Selim, *et al.*, 1995). The peak radioactivity of [^{14}C] DEET in human plasma occurred at 6 hours for technical grade DEET and at 2 hours for 15%

DEET solution (in ethanol), respectively. It appeared that transdermal absorption of DEET was greater in animals than in humans at the same dosing level. This discrepancy might be attributed to different physiological and anatomical characteristics of skin between animals and humans. For compounds that are lipid-soluble with low molecular weight, transdermal absorption initiates with a transient diffusion predominantly through hair follicles and ducts, followed by a steady-state diffusion through stratum corneum (Snodgrass, *et al.*, 1982). DEET is a lipid component (LogKo/w 2.01) with a small molecular weight (Balmer, *et al.*, 2005). In general, animal skin has more hair follicles and duct networks than human skin, which may offer a greater appendageal permeation rate during initial transient diffusion phase. In addition, stratum corneum of animal models also contains more lipid components, which could further facilitate skin absorption of lipophilic compounds like DEET during steady-state diffusion phase.

Biodistribution of DEET was investigated in several animal species including rats and mice. Blomquist and Torsell observed [^{14}C] radioactivity in liver, kidney, lacrimal gland, nasal mucosa, and skin following dermal application of [^{14}C] DEET at 15 mg/kg in mice (Blomquist, *et al.*, 1977). In 1996, Schoenig *et al.* reported that [^{14}C] radioactivity was present in following tissues: heart, kidneys, liver, lungs, brain, stomach, cecum, small and large intestines, bone, muscle, fat, spleen, sciatic nerve, spinal cord, plasma, blood, and carcass, after study rats were sacrificed at peak blood concentration following dermal or oral administration of [^{14}C] DEET (Schoenig, *et al.*, 1996). In addition, liver

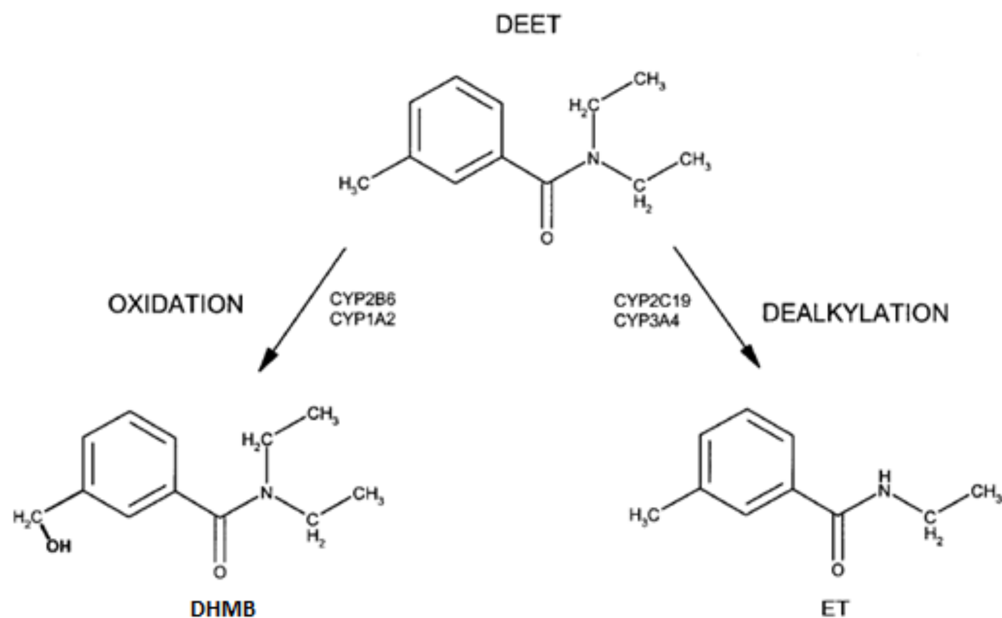
and kidneys showed higher radioactivity levels than plasma level, which was consistent with previous findings by Blomquist *et al.* (1977).

Only two postmortem cases reported tissue distribution of DEET in humans. In 1987, Tenenbein reported that a 33-year-old woman died of ingesting 50ml 95% DEET. DEET concentrations in tissues were 10.4 mg/dl in gastric lavage returns, 16.8 mg/dl in blood, 11.2 mg/dl in postmortem blood, and 17.7 mg/dl in liver, respectively (Tenenbein, 1987). Another 26-year-old man committed suicide by ingesting 50 ml of 95% DEET solution. The tissue concentrations of DEET were 24 mg/dl in blood, 15 mg/dl in vitreous, and 10 mg/dl in urine. The data indicated that DEET was absorbed and distributed into systemic circulation quickly and extensively through the gastrointestinal tracts (Tenenbein, 1987).

DEET primarily undergoes hepatic metabolism before excretion. The metabolism of DEET had been confirmed in numerous animal models. *In vitro* studies using rat liver microsomes had identified two major metabolites, N-ethyl-3-carboxylbenzamide (ET) and N,N-diethyl-3-carboxylbenzamide (DHMB) (Taylar, 1986), and three minor metabolites, N-ethyl-3-hydroxymethylbenzamide (ETM), 3-methylbenzamide (MB), and N,N-diethyl-3-formylfenzamide (DFF) (Yeung, *et al.*, 1988). ET was formed by dealkylation of an N-ethyl group; DHMB was formed by oxidative hydroxylation of the aromatic methyl group in the meta-position. The yield of three minor metabolites resulted from subsequent oxidation, hydroxylation, and glucuronidation (**Figure 1.3**).

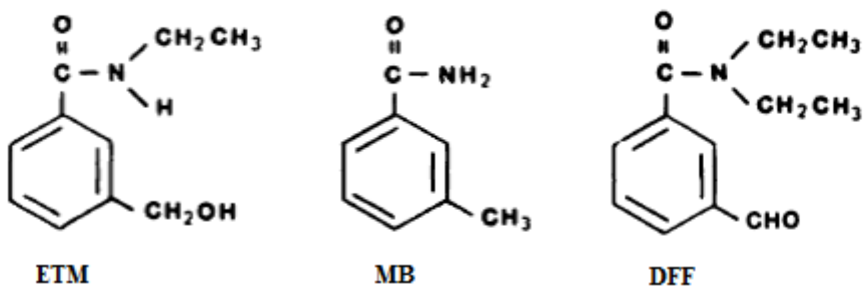
Figure 1.3. The metabolites of DEET

a: Major metabolites of DEET†



†ET and DHMB are two major metabolites of DEET. ET is formed by dealkylation of an N-ethyl group; DHMB is formed by oxidative hydroxylation of the aromatic methyl group in the meta-position. (Copyright Permitted by Informa Healthcare at December 2013)

b: Other metabolites of DEET†



†ETM, MB, and DFF are three minor metabolites. They result from subsequent oxidation, hydroxylation, and glucuronidation of two major metabolites. (Copyright Permitted by American Society for Pharmacology & Experimental Therapeutics at December 2013)

These metabolites had also been confirmed *in vivo* from subsequent animal studies. The two major metabolites were identified in rat urine after ^{14}C-labled DEET was administered dermally or orally (Schoenig, *et al.*, 1996), while the three minor metabolites were detected in rat urine (Taylor, *et al.*, 1990).

Metabolism of DEET in humans was reportedly similar to the findings in the animal models. *In vitro* metabolic study using pooled human liver microsomes identified two major metabolites, and reported significantly higher affinity for the ring methyl hydroxylation reaction (DHMB) than for N-deethylation (ET). The P450 isoforms in liver microsomes that had the highest activity to form DHMB were CYP2B6 and CYP1A2. Isoforms CYP2C19, CYP3A4, 3A5, and 2A6 produced detectable amounts of ET (Usmani, *et al.*, 2002). The study also revealed the inter-individual variability in activity of CYP450 isoforms relevant to DEET metabolism in human liver microsomes. For *in vivo* DEET metabolism, ET and DHMB were detected in the urine of a 30-year-old male who was exposed to 10.4 g DEET in commercial lotion on 75% of the skin surface (Wu, *et al.*, 1979). In addition, at least 6 metabolites were detected in the urine of human volunteers who had topically applied 12-15 mg of DEET on the forearms (Selim, *et al.*, 1995).

DEET appeared to be eliminated rapidly and completely in animals and humans. In animal models, DEET was primarily eliminated in urine within the first 24 hours after administration. Schmidt *et al.* reported that 82% [^{14}C] DEET was accountable in urine

within the first 24 hours after dermal administration of 1000 μg/cm^2 DEET in guinea pigs (Schmidt, *et al.*, 1959). In the study by Lure *et al.*, 68% of [^{14}C] DEET was eliminated from urine in rats within the first 24 hours after topical application of DEET at 330-400 μg/cm^2 (Lure, *et al.*, 1978). In addition, the enterohepatic elimination of DEET was reported to be very small; very little radioactivity of [^{14}C] DEET was detected in feces (Lure, *et al.*, 1978; Schmidt, *et al.*, 1959; Snodgrass, *et al.*, 1982). DEET elimination from repeated dermal exposure was also reported; [^{14}C] DEET radioactivity level kept in a steady state level of 45% of applied dose within the first 24 hours and throughout the entire study duration when pregnant rabbits were treated with repeated topical doses for 29 days (Snodgrass, *et al.*, 1982).

In human subjects, over 99% of the absorbed ^{14}C-labeled DEET was eliminated from the urine within 5 days following dermal application of technical DEET or 15% DEET solution in ethanol. Meanwhile, elimination of DEET in feces accounted for less than 0.1% of the dermal dose, which further verified a minimal enterohepatic elimination of DEET that had been previously observed in animal models (Selim, *et al.*, 1995).

In summary, DEET is capable of penetrating across the skin and rapidly distributing into the circulation *in vivo*. The compound is primarily metabolized in liver and eliminated rapidly from the urine. Fecal excretion of DEET is minimal. Because elimination rate of DEET was high, no evidence had suggested DEET accumulation in vital organs in study animals after single-dose and repeated-dose applications of DEET *in*

vivo. DEET demonstrated similar biodisposition characteristics in humans as well; it experienced very similar metabolic pathways in the body. DEET is rapidly eliminated, but not as completely as in animal models. The distribution and accumulation data of DEET in humans has been very limited due to the nature of the compound; information available has been largely derived from findings found in animal models.

1.1.4. Adverse Effects of DEET

Adverse effects associated with DEET exposure have been extensively investigated in various animal models; these studies particularly focused on neurotoxicity, teratologic and developmental toxicity, and cardiovascular toxicity that might be attributed to DEET applications.

According to collective data available in literature, neurotoxicity from DEET exposure was apparently dependent upon DEET dose applied and/or co-administration of DEET together with other chemicals such as insecticides. In an acute neurotoxicity study by Schoenig *et al.* (1993), rats treated with oral dose of DEET up to 500 mg/kg did not exhibit any adverse effect on clinical signs or survival. Neither was there gross or microscopic alternation occurred in the central or peripheral nervous system (Schoening, *et al.*, 1993). In another acute neurotoxicity study in rats, a single oral dose of DEET was raised to 1-3 g/kg; this higher oral dose did induce a spongiform myelinopathy in study animals, which was primarily localized to cerabellar roof nuclei (Verschoyle, *et al.*, 1992).

When the single oral dose of DEET was approaching the oral LD_{50} (2-3 g/kg) in rats, more severe symptoms were observed, such as a decrease of reactivity and muscle tone and clear electrophysiological signs (spike discharges) of prolonged suppressed seizure activity (Macko, *et al.*, 1980; Morre, 2000). In one chronic toxicity study, no selective target organ toxicity was observed following oral dosing of DEET in dogs (30-400 mg/kg/d), rats (10-400 mg/kg/d), and mice (250-1000 mg/kg/d) (Schoenig, *et al.*, 1999). Nevertheless, DEET did induce higher incidents of neurotoxicity after its co-administration with other chemical substances. Abou-Donia *et al.* (1996) reported that treatment of DEET alone (500 mg/kg/d, 5 d/week, 2 month study duration) produced little neurotoxicity in hens, but co-administration of DEET with anti-nerve-gas agent pyridostigmine bromide (5 mg/kg/d) or insecticide permethrin (500 mg/kg/d) led to greater neurotoxicity under similar experimental conditions (Abou-Donia, *et al.*, 1996). Abou-Donia *et al.* (2003) also reported that combined exposure to pyridostigmine bromide (1.3 mg/kg/d in water, oral), DEET (40 mg/kg/d in ethanol, dermal), and permethrin (0.13 mg/kg/d in ethanol, dermal) caused apoptosis in rat testicular germ-cells, sertoli cells, and leydig cells, as well as in the endothelial lining of blood vessels (Abou-Donia, *et al.*, 2003).

No severe teratologic and developmental toxicity of DEET was observed in animal studies. Schoenig *et al.* (1994) reported that after female rats were treated with 125, 250, and 750 mg/kg/d technical grade DEET on gestational day 6-15, the maternal

toxicity occurred only in treatment group at the highest; the clinical signs observed included depressed body weight and food consumption, and two cases of death. In female rabbits that were treated with technical grade DEET at 30, 100, and 325 mg/kg/d on gestational day 6-18, maternal toxicity also occurred to study animals in the highest dosing group, and depressed body weight and food consumption were observed (Schoenig, *et al.*, 1994). Results from these two animal studies of teratogenic and developmental toxicity were consistent with those by Angerhofer and Weeks (1980) and Wright *et al* (1992), in which no severe teratogenic toxicity was observed in rabbits and rats after dermal or subcutaneous treatment with DEET, respectively (Angerhofer, *et al.*, 1980; Wright, *et al.*, 1992). Regarding teratologic and developmental toxicity in male rats, no direct evidence was found in sperm abnormalities and sperm motility after repeated dermal administration of DEET at dosing range of 100-1000 mg/kg/d for 9 weeks or 6 months (Gleiberman, *et al.*, 1976; Lebowitz, *et al.*, 1983).

Two studies reported DEET-induced hypotension in rats and dogs (Leach, *et al.*, 1988). After anesthetized rats were intraperitoneally injected with DEET (in 25% ethanol solution) at 56, 113, and 224 mg/kg doses, blood pressure and heart rate were decreased in dose-dependent patterns in the animals. Similar hypotensive symptoms were also observed in dogs after intraperitoneal injection of 224 mg/kg DEET. The hypotension was attributed to a reduced cardiac output, for there was no variation in peripheral resistance or stroke volume. However, it had been reported that intravenous

administration of ethanol (40 mg/100 g b.w.) also induced hypotensive effects in rats due mainly to bradycardia (Penna, *et al.*, 1985). Study by Leach *et al.* (1988) did not clarify whether the observed hypotension was induced by DEET, or by the testing vehicle, or by the combination of DEET/testing vehicle.

According to information collected by the Poison Control Centers, exposure to DEET in humans could induce both local and systemic adverse effects. The local adverse effects from using insect repellents containing DEET included acute dermatitis, red raised lesions, a baffling bullous eruption, slight tingling sensation and a feeling of dryness and astringency (Ambrose, *et al.*, 1959; Lamberg, *et al.*, 1969; Maibach, *et al.*, 1975; Reuveni, *et al.*, 1982;). Accidental ocular exposure to DEET products was linked to some ocular symptoms such as corneal abrasion (Bell, *et al.*, 2002). The systemic adverse effects from DEET usage included acute manic psychosis, cardiovascular toxicity, anaphylaxis, and reproductive toxicity.

Clem *et al.* (1993) reported that a healthy 61-year-old Caucasian female developed a hypotension with a systolic blood pressure of 80 mmHg, and experienced vomiting, diarrhea, and nausea after she applied a sunscreen and a DEET-containing repellent spray while working in the garden (Clem, *et al.*, 1993). The patient had her blood pressure stabilized several hours after she was admitted into a hospital. Miller (1982) reported that a healthy 42-year-old woman who had no prior atopic allergic history developed generalized pruritus and processed to a generalized angioedema after

contacting with an insect repellent containing 52% DEET (Miller, 1982). According to report by Schaefer and Peters (1992), a mother applied DEET daily during her whole pregnancy in addition to the prophylactic use of chloroquine. Her son, a 4-year-old boy, suffered from impaired sensorimotor coordination, mental retardation, and craniofacial dysmorphology (Schaefer, *et al.*, 1992).

Topical application of DEET might also lead to more serious incidents of systemic adverse effects, such as encephalopathy and death in humans. Three cases of death from dermal application of DEET in young children had been reported (Heick, *et al.*, 1983; Pronczuk, *et al.*, 1983; Zadikoff, 1979). Zadikoff (1979) reported that a 5-year-old girl died following dermal application of a 10% DEET spray daily for 3 months. The major symptoms prior to the incident included headache, agitation, athetosis, and disorientation; a generalized edema with intensive congestion of the meninges was observed in brain at autopsy. Heick *et al.* (1983) reported that a 6-year-old girl died 8 days after her admission to hospital; she had been applying a 15% DEET spray extensively to her skin surface for 10 occasions (Heick, *et al.*, 1983). Pronczuk *et al.* (1983) reported that a 17-month-old girl died of acute encephalopathy after frequent skin exposure to a DEET lotion for 3 weeks (Pronczuk, *et al.*, 1983). In addition, there were eight reports of toxic encephalopathy involving dermal application of insect repellent products containing DEET (Briassoulis, *et al.*, 2001; Edwards, *et al.*, 1987; Gryboski, *et al.*, 1961; Hampers, *et al.*, 1999; Lindsay, *et al.*, 1978; lipscomb, *et al.*, 1992; Oransky, *et*

al., 1989; Roland, *et al.*, 1987). Six of the patients utilized insect repellents containing 15% DEET or above. The most common symptoms were convulsions and seizures among these patients.

Similarly, severe toxic effects could result from ingestion of DEET in humans. A 33-year-old man drank 8 ounces of insect repellent, and developed cerebral edema and died in hospital 3 months later (Veltri, *et al.*, 1994). Another incident report described 5 cases associated with oral ingestion of DEET. Two of the subjects died and the other three survived without sequelae; all patients experienced seizures, coma, and hypotension within one hour of the ingestion (Veltri, *et al.*, 1994).

1.1.5. Formulations of DEET

Insect repellents are commercially categorized as over-the-counter, consumer-care products; DEET-based repellent products are sold worldwide under various brand names. There are a variety of preparations available to offer application convenience and product preference. Aerosol spray, pump spray, washable hydrogel, lotion, stick, soap, and impregnated towelette are several common preparation types. In addition, DEET is also embedded in fabric materials to make jackets, pants, sleeping bags, camping tents and nettings; as such they may supply additional protection against biting insects for outdoor work and recreational activities.

Used as topical skin preparations, DEET-based insect repellent products should

technically possess many characteristics of a dermatological product; more specifically, formulation design would emphasize the aesthetic qualities of a final finish product, such as appearance of the preparation, the amount of active ingredient incorporated, skin feeling and use convenience of the product. It is always a technical challenge to formulate an efficient and aesthetically balanced preparation for DEET-based insect repellents.

DEET concentration in commercial repellent products varies significantly depending on use indications and protection purposes. Certain insect repellent products may contain 100% DEET to achieve maximal repellency (Sadik, 1990). The US military has utilized insect repellents containing 75% DEET in alcohol; workers of the National Park applied repellents containing 71% DEET. The Singapore Armed Forces had also employed a 75% DEET-based insect repellent preparation for personal protection during field operations (Fai, *et al.*, 1996). Nevertheless, insect repellents of high DEET concentrations do not necessarily result in benefits in additional protection time and efficacy against biting insects. The Occupational Health Group had demonstrated that there was no clear incremental benefit when DEET concentrations were raised above 35% (Occupational Health Group, 2012). Moreover, high DEET concentration may potentiate local or systemic adverse effects in humans. Common DEET concentration in commercially available insect repellents nowadays ranges between 5-30% (Sue, 2013).

Even though DEET is one of the most effective insect repellents, its repellency

duration is comparatively short. Attempts have been made to prolong its protection time by using various formulation strategies. Newer formulation designs of DEET-based products have included hydrogel, bi-gel, emulsion, liposphere, microcapsule, and microparticle.

Domb *et al.* (1995) evaluated a DEET-encapsulated liposphere system to decrease overall transdermal penetration of DEET and to increase protection time of the preparation. In one of the liposphere formulations containing 10% DEET, transdermal bioavailability of DEET was decreased by 29% in rabbits when compared to an ethanol DEET solution of the same strength (Domb, *et al.*, 1995). Rutledge *et al.* (1996) prepared a polymer formulation containing a high-molecular-weight fatty acid and several microcapsule formulations containing lanolin, gelatin, gum arabic, tannic acid, stearic acid, and propylene glycol. These formulations offered higher effective repellency than unformulated DEET of the same concentration in a repellency test by using *Ae. aegypti* and *An. albimanus* mosquitoes and rabbits (Rutledge, *et al.*, 1996). Qiu *et al.* (1997) developed an emulsion-based DEET hydrogel using PEG-polyacrylic acid polymer. The formulation decreased the skin/vehicle partition coefficient of DEET and modified DEET release property from the hydrogel. Consequently, the preparation reduced transdermal bioavailability of DEET by 23% in beagle dogs when compared to a commercial DEET cream of the same strength; it also improved repellency against fasted *Ae. aegypti* mosquitoes in lab conditions for 6 h (Qiu, *et al.*, 1997). Kasting *et al.* (2008) prepared a

walled polysaccharide microcapsules containing 15% [^{14}C] DEET (w/w), and other excipients, such as cetyl esters wax, emulsifying wax, sodium carboxymethylcellulose (CMC) and benzalkonium chloride. This microcapsule formulation reduced the permeation of radiolabeled [^{14}C] DEET by 25-30% in comparison to the control, DEET in ethanol. DEET evaporation from this formulation was greater than or comparable to that from the control (Kasting, *et al.*, 2008). Fei *et al.* (2007) designed a DEET microcapsule containing copolymer butyl acrylate and chitosan in an aqueous solution. When applied to cotton textile, the repellency of microcapsules remained 100% against *Aedes albopictus* after 8 h, while the repellency of DEET in ethanol kept only for 4 hours at 100% level (Fei, *et al.*, 2007).

Another recent development in material science was to treat fabrics with DEET to provide repellency versatility while avoiding direct skin contact of the compound in humans. Romi *et al.* (2005) prepared a DEET/β-cyclodextrin grafting to cellulosic textiles to increase the repellence of the cotton fabric, which could be further made into special clothing to protect against mosquitoes or other insects and to minimize direct contact of DEET with human skin (Romi, *et al.*, 2005). N'Guessan *et al.* (2007) prepared a melamine microcapsule that enclosed 30% DEET and then applied the DEET microcapsules to polyester camp netting at 8 g DEET/m^2; the material inhibited the blood-feeding of female *Anopheline* mosquitoes (malaria vector) with a stable protection level for 6 months. In comparison, polyester netting treated with unencapsulated DEET

formulation showed inhibition decrease to 70% by 3 months and completely lost inhibition between 3-6 months (N'guessan, *et al.*, 2008).

Specialty DEET formulations had been developed for military purposes in order to protect military personnel in combat fields and to maintain effective combating strength. Gupta and Rutledge (1991) reported a repellency test by using a polymer cream formulation (33% DEET) developed by Consumer Specialties Division of 3M Company and a microparticulate formulation (41.8% DEET) developed by Biotek Corporation. The results demonstrated that the polymer cream formulation afforded better protection against mosquitoes, even though it had lower DEET concentration than the microparticulate formulation and the standard repellent control (75% DEET) (Gupta, *et al.*, 1991). Kroeger *et al.* (1997) reported a soap preparation that contained 20% DEET together with 0.5% permethrin. This combination provided additional protection against malarial dissemination; study subjects who utilized the soap received 94% fewer bites for 2 hours than those who did not use the soap (Kroeger, *et al.*, 1997).

Adding fixatives to perfume preparations prolongs and preserves the fragrance. This approach has also been utilized to create long-lasting DEET-based insect repellents. Khan *et al.* (1975a) showed that concurrent dermal application of the synthetic fixative Tibetene® (2,6-dinitro-3,4,5-triethyl-t-butyl-benzene) and DEET (0.16 mg/cm^2) at a ratio of 1:1 increased the protection time against *Ae. Aegypti* by 29%. The protection time was further prolonged by 47% and 88% when the dose ratio was changed to 2:1 and 3:1,

respectively. Similar patterns were also observed when DEET dose was doubled. Other synthetic fixatives such as musk ambrette (2,4-dinitro-3-methyl-6-t-butyl anisole), musk xylol (5-t-butyl-2,4,6-rinitro-m-xylene), and givambrol (2,4-di-butyl-4-methoxybenzaldehyde) also enhanced protection time of DEET (Khan, *et al.*, 1975a). Khan *et al.* (1975b) reported that concurrent application of vanillin and DEET (0.16 mg/cm^2) at the ratio of 1:1, 2:1, 3:1 increased the protection time of DEET against *Ae. Aegypti* by 95%, 142%, and 179%, respectively (Khan, *et al.*, 1975b).

In order to increase protection time of DEET and to reduce transdermal absorption of DEET at the same time, two formulation approaches could be utilized, i.e., to control DEET release/permeation into the systemic circulation through skin membrane, and to control DEET evaporation from a formulation.

1.2. Oxybenzone (OBZ)

Skin cancer has become the most common cancer in Canada; approximately 30% of newly diagnosed cancer cases belong to skin cancer and its incidence rate is continuously increasing (CCS/NCIC, 2008). Basal cell carcinoma and squamous cell carcinoma are two commonly encountered forms of skin cancer, but malignant melanoma is the most life-threatening skin cancer. Over 90% of skin cancer is caused by overexposure to ultraviolet (UV) radiation from the sunlight. Chronic exposure to sunlight for an extended period of time is attributed to 95% basal cell carcinomas (U. S.

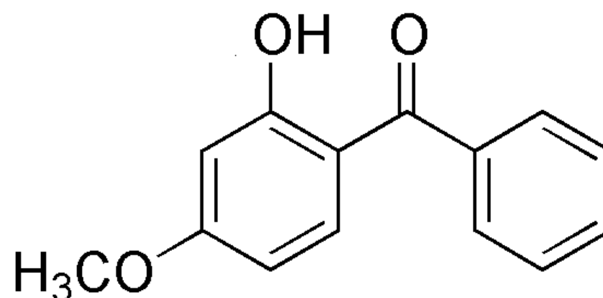
Preventive Services Task Force, 1996). Numerous studies have proven that skin damages from sunlight radiation accumulate over the time, regardless of whether or not sunburn actually occurs. Healthcare professionals have always recommended that sunscreens be used as an essential preventive weapon against sunburns and skin cancers.

1.2.1. Physical and Chemical Properties of OBZ

OBZ (2-hydroxy-4-methoxybenzophenone) is a UVA/UVB sun-blocking ingredient found in common commercially available sunscreen preparations. OBZ is also found in various cosmetic products, such as lipsticks, shampoos, conditioners, skin moisturizers, facial cleansers, as a supplemental sun-blocking agent.

OBZ is a monomethoxylated congener of 2-hydroxyhenzophenone (**Figure 1.4**) that naturally occurs in plant pigments. It is synthesized by the reaction of 2-hydroxyanisole and benzoyl chloride (Cosmetic Ingredient Review Panel, 1983; Okereke, *et al.*, 1995; Stecher, 1958). The physical appearance of OBZ in its standard state (25° C, 100 kpa) is white to yellowish crystal. OBZ is insoluble in water, and readily soluble in many organic solvents, such as acetone, ethanol, ethyl acetate, methanol, with a melting point of 63° C and a boiling point of 220° C. **Table 1.2** lists various physicochemical properties of OBZ.

Figure 1.4. The chemical structure of OBZ†



†OBZ is 2-hydroxy-4-methoxybenzophenone; it is a UVA/UVB sun-blocking ingredient in commercially available sunscreen preparations. (Copyright Permitted by Wikimedia Commons, the free media repository at August 2013)

1.2.2. Efficiency of OBZ

OBZ is approved for use as an active sunscreen agent in over-the-counter pharmaceutical and cosmetic products by the US Food and Drug Administration (FDA) (FDA Title 21 Part 352, 2009). OBZ is also approved by the FDA for use as a photostabilizer in food industry and agriculture (FDA Title 21 Part 177, 2009; Okereke, *et al.*, 1995). The annual demand for OBZ as a cosmetic and sunscreen additive was estimated to be approximately 3600 kg in 1977 (National Cancer Institute/Scientific Research Inc., 1978). However, use of OBZ has significantly increased over the years due to increasingly awareness of UV radiation and skin cancer (Okereke, *et al.*, 1994). It has become one of the most common active sunscreens in current market (El Dareer, *et al.*, 1986).

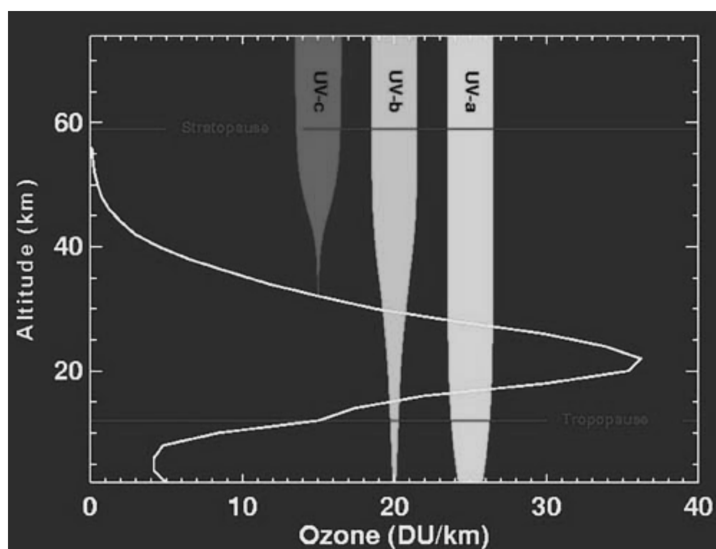
Table 1.2. Physiochemical properties of OBZ†

OBZ	
IUPAC	(2-Hydroxy-4-methoxyphenyl)-phenylmethanone
Synonyms	Oxybenzone; Benzophenone-3
	2-hydroxy-4-methoxybenzophenone
	4-Methoxy-2-hydroxy-benzophenone
	(2-hydrozy-4-methoxyphenyl)phenyl-methanone
Identifiers	
CAS number	131-57-7
SMILES	O=C(C2=CC=CC=C2)C1=C(O)C=C(OC)C=C1
Properties (25 °C 100 kpa)	
Appearance	White to yellowish crystals
Molecular formula	$C_{14}H_{12}O_3$
Molar mass	228.24 g/mol
Melting point	62-65 °C
Boiling point	224-227 °C
LogKo/w	3.79
Toxicity DATA	ORL-RAT LD50 7400 mg/kg

†(Sciencelab, 2005)

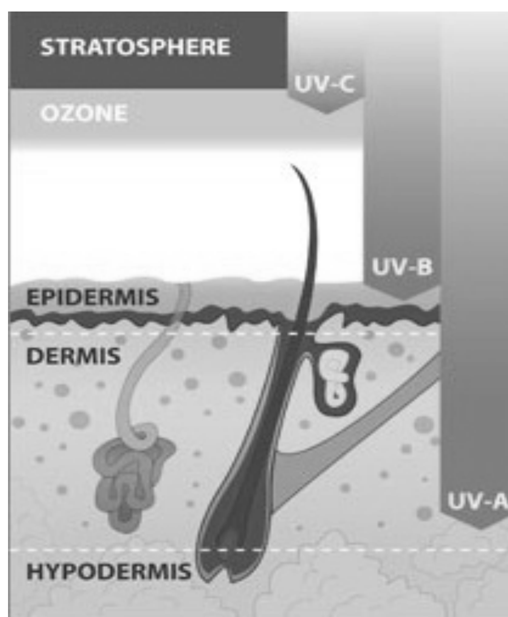
Sunscreens mainly provide protection to human skin against sunlight by absorbing and blocking UVA and UVB radiations. The FDA typically defines sunscreen substances as active ingredients that absorb, reflect, and/or scatter UV radiation in the range of 290-400 nm. As an active chemical sunscreen approved by the FDA, OBZ absorbs ultraviolet radiation A and B (UVA/UVB) and protects the skin from damages induced by sunlight exposure, such as erythema (reddish) and melanoma. The sun emits ultraviolet radiation in the wavelengths of 200-400 nm, i.e., UVA (315-400 nm), UVB (290-315 nm), and UVC (200-290 nm). Approximately 99% of the ultraviolet radiation that is capable of reaching the earth is UVA, due mainly to little absorption within the ozone layer of the atmosphere (**Figure 1.5**). The amount of UVA radiant is constant throughout the day (Kim, 2001); it is a less harmful radiation among three UV bands. UVA is able to penetrate deeply into the dermis of the skin (**Figure 1.6**) and to induce skin photoaging, erythema, and carcinomas while acting together with UVB. The majority of UVB is screened out by the ozone layer, but a small amount of UVB still reaches the earth surface. The intensity of UVB radiation is variable during the day, with the highest levels between 10:00 AM and 5:00 PM. Even though UVB only penetrates into the epidermis, its efficiency to cause skin erythema and melanogenesis is 1000 times more than UVA (Kim, 2001; Pathak, 1987). UVC is completely absorbed by ozone layer, therefore it does not contribute to skin aging and skin damages in humans (Kim, 2001).

Figure 1.5. UV blocking of ozone at various altitudes†



†UVA is hardly absorbed by ozone layer; it is capable of reaching the earth. Most of UVB is absorbed by ozone layer; a small amount of UVB reaches the earth surface. UVC is completely absorbed by ozone layer (Copyright Permitted by Wikimedia Commons, the free media repository at August 2013).

Figure 1.6. UV penetration in the skin†



†UVA is able to penetrate deeply into the dermis of the skin. UVB only penetrates into the epidermis. UVC is not able to reach the skin surface in humans (Natural Organic Sunscreen, 2008).

In addition to human usage, OBZ is also utilized as a photostabilizer in industrial inks and paints, and agricultural films. Specifically, OBZ is allowed for use in formulations of rigid acrylics and modified acrylic plastics, which are components of articles in direct contact with food, since the FDA has approved OBZ for use as a photostabilizer under the section of indirect food additives. The use of plastic packaging containing OBZ as a UV blocker allows manufacturers to pack their products in clear glass or plastics for better presentation; otherwise opaque or dark packaging materials would have been required.

The sun-blocking efficacy of sunscreen compounds is measured in terms of Sun Protection Factor (SPF), which is defined as the ratio of the minimum erythema dose (MED) required to redden protected skin with a sunscreen to the MED required to redden unprotected skin (Equation 1.1). The higher an SPF is, the more protection a sunscreen product offers against UVB radiation, as the definition of SPF primarily pertains to UVB protection, not UVA protection (Kim, 2001).

$$\text{SPF(UVB)} = \frac{\text{MED in Sunscreen−Protected Skin}}{\text{MED in Non−Sunscreen−Protected Skin}} \qquad \text{Equation 1.1}$$

For UVA protection, the effectiveness of an active sunscreen ingredient is measured by term Persistent Pigment Darkening (PPD). Instead of measuring skin erythema or reddening, PPD measures the persistent darkening or tanning of the skin

caused by UVA radiation (Equation 1.2). Theoretically, a sunscreen with a PPD rating of 10 should filter out 10 times of UVA in comparison to that without protection.

$$\text{SPF (UVA)} = \frac{\text{PPD in Sunscreen}-\text{Protected Skin}}{\text{PPD in Non}-\text{Sunscreen}-\text{Protected Skin}} \qquad \text{Equation 1.2}$$

OBZ, as a chemical sunscreen, completely screens out UVB and partially filters out UVA to protect the skin from sunlight damages. OBZ and other chemical sunscreens contain numerous double bonds in their chemical structures. This molecular arrangement creates many electrons in the lower-energy orbital that can absorb the energy of ultraviolet radiation and be excited into the higher-energy orbital (Stryer, 1981). The wavelength at which the sunscreen molecules absorb the sunlight is proportional to the number of double bonds; for example, compounds that absorb UVA (315-400 nm) radiant would have more double bonds in their structures. OBZ is a UVA/UVB absorber, but its absorption is significantly reduced above the wavelength of 330 nm. Consequently, in commercially available sunscreen preparations OBZ is commonly combined not only with other chemical sunscreen ingredients such as avobenzone and ecamsule, but also with physical sunscreen ingredients such as zinc oxide and titanium dioxide, in order to provide a broad-spectrum UV protection. Physical sunscreens protect the skin from damaging sunlight by scattering, reflecting, and/or physically blocking UV radiation. They provide satisfactory protection against UVA, as well as a portion of visible light

(Kim, 2001). It is a necessary and practical approach to combine physical and chemical sunscreens together to create a broad-spectrum sunscreen product.

1.2.3. Biodistribution of OBZ

OBZ, an active sunscreen agent, had been detected in the plasma and urine following topical skin application in rodents (Dareer, *et al.*, 1986; Kadry, *et al.*, 1995; Okereke, *et al.*, 1993; Okereke, *et al.*, 1995a) and humans (Gustaysson, *et al.*, 2002; Hayden, *et al.*, 1997; Janjua, *et al.*, 2004). A number of studies had been carried out to investigate the absorption, distribution, metabolism, and excretion of OBZ in animals and in humans.

Skin penetration of OBZ had been demonstrated *in vitro* using human epidermis model. Jiang *et al.* (1999) assessed the percutaneous penetration of five active ingredients in commercial sunscreens with a finite dose of 2-2.5 mg/cm^2 across excised human epidermis. Up to 10% of the applied doses permeated into the receptor fluid within an 8-hour testing period. Moreover, systemic absorption of OBZ from oral digestion and dermal application was also observed in animal models (Jiang, *et al.*, 1999). Kadry *et al.* (1994) reported that OBZ was promptly absorbed through the gastrointestinal tract in rats, as OBZ was detected in plasma 5 minutes after oral digestion. The maximal plasma concentration (C_{max}) of OBZ was 25.6±4.6 μg/ml at the average peak time (T_{max}) of 3.0±0.4 hours; the elimination half-life of OBZ after oral administration was 0.71 hours

(Kadry, *et al.*, 1995). Jeon *et al.* (2008) found very similar results, i.e., C_{max} of 21.21±11.61 μg/ml and T_{max} of 3 hours after a single oral dose of OBZ in rats (Jeon, *et al.*, 2008). Dermal absorption of OBZ in rats was reported by Okereke *et al.* (1994). OBZ was detected in plasma 5 minutes after dermal application of 100 mg/kg in Sprague-Dawley rats; C_{max} of 35±4.5 μg/ml was observed at 2.5 hours post-administration (Okereke, *et al.*, 1994). In human studies, it was found that OBZ was capable of rapidly entering into systemic circulation following dermal application. Janjua *et al.* (2004) reported C_{max} and T_{max} of OBZ at 200 ng/ml and 3-4 hours in women and 300 ng/ml and 3 hours in men, respectively, after 2 mg/cm^2 basic cream formulation had been topically applied to the whole-body skin surface daily for one week in healthy human volunteers (Janjua, *et al.*, 2004). These results were similar to findings by Okereke *et al.* (1994). T_{max} of OBZ in humans after dermal administration was comparable to that in rats, although C_{max} of OBZ in humans after dermal administration was slightly lower than that in rats. Based on these reports, OBZ was able to permeate across skin barrier after dermal application and to distribute into the systemic circulation afterward.

Little has been known about OBZ tissue distribution in humans, since it is impossible to conduct such a conventional acute toxicity study in human subjects (Anonymity, 1983). Information regarding *in vivo* tissue distribution of OBZ was largely obtained from rodent animals following oral or dermal administration. After

topical application of an ethanol solution of [^{14}C] OBZ at doses of 50, 80, 200 μg per rat and a lotion of [^{14}C] OBZ at 50 μg per rat, the radioactivity of [^{14}C] OBZ was detected 72 hours post-administration in gut contents, gut tissue, liver, lungs, kidneys, muscle, fat, skin, plasma, and whole blood (El Dareer, *et al.*, 1986). Following oral administration of [^{14}C] OBZ at dose of 3.01-2570 mg/kg in rats, the distribution of radioactivity 72 hours post-administration spread even more extensively than that from topical administration, with detection in stomach contents, small intestine contents, large intestine contents, stomach, small intestine, large intestine, liver, lungs, kidneys, brain, muscle, spleen, fat, skin, plasma, and whole blood (El Dareer, *et al.*, 1986). The level of radioactivity found in gut contents, gut tissue, and kidneys was higher than that in other tissues for both oral and topical administration. In another study male Sprague-Dawley rats were orally administrated OBZ at 100 mg/kg, and tissue samples collected 6 hours after doing. Free OBZ and total OBZ (free and conjugated) were respectively detected as 8.9 ± 23.8 μg and 2087 ± 60.1 μg in liver, and 5.65±0.16 μg and 312.6±181.80 μg in kidneys. OBZ was also detected in testes, spleen, heart, and intestine (Kadry, *et al.*, 1995). This study reported higher OBZ amount in liver and kidneys compared to study by El Dareer *et al.* (1986), because the former collected samples 6 hours after dosing while the latter collected samples 72 hours after dosing. This demonstrated that OBZ reached vital metabolic organs at a relatively fast rate, and that it was also remained in body for several days.

OBZ was metabolized to at least three compounds, namely, 2,4-dihydroxybenzophenone (DHB), 2,3,4-trihydroxybenzophenone (THB), and 2,2'-dihydroxy-4-methoxybenzophenone (DMB) after an oral dosing of 100 mg/kg in rats (Okereke, *et al.*, 1993). Jeon *et al.* (2008) reported similar metabolites in rats (Jeon, *et al.*, 2008). DHB was the primary metabolite of OBZ, which was formed by O-dealkylation of the methoxy side chain on ring A of OBZ. DMB and THB were detected at trace concentrations in a majority of study samples. DMB was formed by aromatic hydroxylation of ring B of OBZ at the ortho position; THB was probably formed by aromatic hydroxylation of ring A of DHB at the meta-position. Metabolic reactions of OBZ were apparently catalyzed by cytochrome P-450 enzyme system that metabolizes many endogenous and exogenous xenobiotics (Ingelman-Sundberg, *et al.*, 1982; Porter, *et al.*, 1991). Both OBZ and its metabolites also conjugated with plasma macromolecules, which typically contained ketonic compounds (Okereke, *et al.*, 1993; Harding, *et al.*, 1971). Metabolism of OBZ in humans was only available for topical application of sunscreens containing OBZ. Hayden *et al.* reported that OBZ and its metabolites (THB and DHB) were detected in urine samples after topical application of sunscreens in healthy human volunteers; OBZ and its metabolites also underwent conjugation with plasma proteins *in vivo* (Hayden, *et al.*, 1997).

OBZ and its metabolites were detected in urine and feces after intravenous, oral, and dermal administration of OBZ in rats (El Dareer, *et al.*, 1986; Kadry, *et al.*, 1995;

Okereke, *et al.*, 1993; Okereke, *et al.*, 1994;). El Dareer *et al.* reported that radioactivity accounted for 67.4% of the applied dose in urine and 21.2% in feces after intravenous administration of [^{14}C] OBZ at 4.63 mg/kg. After oral administration at 3.01-2470 mg/kg, urinary and fecal excretion of [^{14}C] OBZ radioactivity respectively ranged 63.9-72.9% and 19.3-41.7% of the applied doses within 72 hours (El Dareer, *et al.*, 1986). In study by Kadry *et al.*, about 71% of the applied OBZ was excreted in urine within 72 hours while about 25% of the applied OBZ appeared in fecal excretion within 72 hours, after an oral administration of OBZ (100 mg/kg) in rats. Okereke *et al.* studied elimination of OBZ after dermal administration of the compound at 100 mg/kg twice daily for four weeks in rats, and recorded an average 51% excretion in urine and an average 42% excretion in feces. Therefore, urine was the primary elimination route for OBZ, followed by the feces. (Kadry, *et al.*, 1995; Okereke, *et al.*, 1993). In humans elimination of OBZ had only been detected in urine following topical skin application. After daily dermal application of 2 mg/cm^2 sunscreen cream formulation to whole body for two weeks in healthy volunteers, approximately 60 ng/ml and 140 ng/ml of OBZ were recovered in the urine samples of female and male volunteers respectively (Janjua, *et al.*, 2004). Hayden *et al.* (1997) reported that OBZ was detected in the urine following topical application of a commercial sunscreen containing 6% OBZ at dosing of 12.4 mg/cm^2 on the forearms of healthy volunteers for 10 hours; 1-2% of the applied OBZ was excreted in the urine. In addition, OBZ might be excreted through human breast milk after topical application

(Hany, *et al.*, 1995).

In summary, OBZ experienced significant skin penetration, tissue disposition, metabolism, and elimination from urine and feces (Benson, 2000). Topical skin application of OBZ resulted in systemic absorption in both animals and humans. Nevertheless, little is known regarding tissue biodistribution of OBZ in humans, and a majority of *in vivo* data has been derived from those of animal studies. Liver was possibly the primary metabolizing organ for OBZ, where OBZ was metabolized to at three compounds, DHB, THB, and DMB.

1.2.4. Adverse Effects of OBZ

OBZ may induce visible, external adverse effects to skin including contact irritation, allergic contact dermatitis, phototoxicity, photoallergy, and skin discoloration; these symptoms were observed in healthy human volunteers but they do not necessarily potentiate harmful implications on human body. In a study of photoallergic contact dermatitis of sunscreen chemicals using patients at 17 medical centers in the UK, Ireland, and the Netherland, Bryden *et al.* (2006) reported that OBZ was the most common photoallergen, because it induced 21% photoallergy in total allergic reactions (51/130) and 7% contact allergy in total allergic reactions (9/130) (Bryden, *et al.*, 2006). Another study of photoallergic contact dermatitis conducted at Columbia also indicated that OBZ was the most common photoallergen among ultraviolent filters with 26.8% photoallergy

in total allergic reactions (22/82), which was comparable to the UK study (Rodriguez, *et al.*, 2006). Veysey *et al.* (2006) reported that OBZ contained in a cosmetic lip product caused a case of photoallergic lip inflammation in a female patient (Veysey, *et al.*, 2006).

Little information is available regarding chronic adverse effects of OBZ in humans. The documented chronic toxicity of OBZ included repeated dose toxicity (Repeat-dose toxicity studies, 2010), developmental and reproductive toxicity, estrogen-like effects, antiandrogen effects, potential mutagenic and carcinogenic toxicity, and cell growth inhibiting effects. In a 90-day toxicity study of OBZ with repeated, daily oral dose of 0.02-1.0% OBZ in Wistar rats, Lewerenz *et al.* (1972) reported depressed growth, leukocytosis, anemia, reduced organ weights, and tubular dilatation of the renal organ (Lewerenz, *et al.*, 1972). The developmental and reproductive toxicity of OBZ was tested by NTP (National Toxicology Program) in 1990 with male Fisher F344/N rats and B6C3Fl mice by oral dose of 3125-50000 ppm. The study indicated that male reproductive toxicity ranged from decreased sperm count and increase in abnormal sperms at higher doses to decreased sperm density at lower doses. Renal toxicity was also observed in rats (French, 1992). The estrogen-like effects of OBZ were reported *in vitro* by Schlumpf *et al.* (2001); OBZ increased the proliferation of MCF-7 breast cancer cells (Schlumpf, *et al.*, 2001). Ma *et al.* (2003) also found antiandrogen effects of OBZ *in vitro*, as OBZ acted as estrogen agonists on MCF cells (Ma, *et al.*, 2003). Suzuki *et al.* (2005) reported both estrogenic and antiandrogenic effects of OBZ in human breast cancer cell

line MCF-7 (Suzuki, *et al.*, 2005). Moreover, Knowland *et al.* demonstrated that octyldimethyl-p-aminobenzoate had photomutagenic effects under UV illumination due to generation of free radicals. Since OBZ had similar chemical structure as octyldimethyl-p-aminobenzoate, it may possess similar photomutagenic effects under UV illumination (Knowland, *et al.*, 1993). In addition, topical treatment of OBZ at a dose of 100 mg/kg for 4 weeks was observed to exert an influence on blood glutathione (GSH) level in rats, but the reading reversed to normal level after 60 minutes (Okereke, *et al.*, 1995). OBZ was also found to inhibit cell growth and DNA synthesis at doses ranging between 25-100 mg/ml in human cell cultures (Xu, *et al.*, 1999).

In summary, OBZ has shown variable skin irritations in humans after topical applications. It has also demonstrated chronic toxicity effects in animal models. Further studies are still required to systemically investigate its acute and chronic adverse effects from well-designed risk assessment experimentation.

1.2.5. Formulations of OBZ

Various sunscreen preparations are commercially available; they include cream, lotion, gel, oil, stick, and spray. Newer materials and formulation techniques have also been utilized to modify overall percutaneous penetration of active sunscreen ingredients and to improve sun-blocking efficacy of the finished products. Among them there are applications of cyclodextrins, microparticles, solid lipid microspheres, solid lipid

nanoparticles, and microcapsules.

Percutaneous permeation of chemical substances is partially dependent upon preparation type and manufacture method. Solubility or miscibility of an active ingredient in a semisolid emulsion or ointment can dictate material release and interaction between preparation and stratum corneum. In addition, barrier functions of stratum corneum may be altered by many lipophilic additives, as such percutaneous permeation of an active ingredient is to be enhanced. Influences of sunscreen products on skin permeation of sun-blocking ingredients have been investigated, both *in vitro* and *in vivo*. Results from these studies have also helped design and develop newer sunscreen preparations that showed improved characteristics in sun protection and overall product quality.

Studies showed that *in vitro* epidermal retention of OBZ from an oil-in-water emulsion-gel and a petroleum-based jelly within a 6-hour period was 3.78% and 1.83% respectively, while skin penetration of OBZ from the same preparation and same timeframe was 1.05% and 4.87% respectively (Treffel, *et al.*, 1996). Similar patterns were also observed in human volunteers, with OBZ recovery from the oil-in-water emulsion-gel preparation in human stratum corneum significantly higher than that from petroleum jelly counterpart. Furthermore, SPF value of the emulsion-gel was measured to be 14, while SPF of petroleum jelly was 5. The higher SPF value of the emulsion-gel was mainly attributed to the higher retention of sunscreen ingredients in stratum cornuem from emulsion-gel preparation (Treffel, *et al.*, 1996). Emulsion-gel formulation might

have increased the solubility of OBZ within lipids of stratum corneum, enhanced partitioning of OBZ in the skin, and promoted OBZ release rate from formulation to the skin surface (Treffel, *et al.*, 1996). OBZ penetration into and through micro-Yucatan pig skin was also reportedly dependent upon formulation types (Gupta, *et al.*, 1999). Permeability coefficient and flux of OBZ were associated with its solubility characteristics in preparation vehicles. For example, hydroalcoholic solution readily evaporated on the skin surface after application, which would result in decrease of OBZ in the vehicles. Consequently thermodynamic activity of OBZ would increase, partitioning of OBZ from vehicles to skin membrane would enhance, and diffusivity of OBZ would also increase in comparison to oil-based vehicles such as diisopropyl adipate (Benson, *et al.*, 1999; Jiang, *et al.*, 1998). Similar results were observed in sunscreen agent octyl salicylate and hydroalcoholic vehicle (Walters, *et al.*, 1997). Even though accumulated skin penetration of octyl salicylate from hydroalcoholic solution was similar to that from an O/W emulsion, epidermis retention of the agent after 48 hours was higher from hydroalcoholic solution than from the emulsion. It was therefore apparent that different preparation vehicles could alter skin retention and penetration for OBZ and other sunscreen ingredients.

Cyclodextrins are cyclic oligosaccharides with hydrophobic internal cavities and hydrophilic bucket-like exterior. This unique structural configuration is capable of entrapping appropriate lipophilic molecules into hydrophobic cavities to form inclusion

complexes. Cyclodextrins have been used in pharmaceutical preparations to increase apparent aqueous solubility of medications (Loftsson, *et al.*, 1999; Loftsson, *et al.*, 2001; Nagase, *et al.*, 2001) and to improve stability of labile molecules in air and light (Antoniadou-Vyza, *et al.*, 1997; Scalia, *et al.*, 1998; Williams, *et al.*, 1999;). Researchers have also studied the relationship between cyclodextrins and transdermal penetration (Legendre, *et al.*, 1995; Loftsson, *et al.*, 1995; Loftsson, *et al.*, 2002; Williams, *et al.*, 1998). Felton *et al.* (2002) reported the effects of hydroxypropyl-β -cyclodextrin (HPCD) on percutaneous penetration and skin accumulation of OBZ. HPCD concentration tested was 0-20% (w/w) and OBZ concentration was constant at 2.67 mg/ml. Percutaneous permeation and skin accumulation increased with HPCD from 0% to 10%; the maximum flux occurred at 10% HPCD. Both transdermal permeation and skin accumulation of OBZ decreased at 20% HPCD. Felton *et al.* (2004) also investigated the effect of HPCD inclusion on photoprotection of OBZ. HPCD-OBZ complexes exerted substantial photoprotective effect at 5% and 20% HPCD concentrations. It was concluded that HPCD inclusion in sunscreen preparation could enhance photoprotection of UV absorbers *in vivo*.

Microspheres are homogeneous matrix particles in which pharmaceutical or cosmetic ingredients are dissolved or dispersed (Anselmi, *et al.*, 2002). Microsphere sunscreen delivery systems have been studied to achieve adequate SPF efficacy and to reduce exposure concentration of organic sunscreens to users. Commercial sunscreen

ingredients were encapsulated into two different polymer matrix systems, semi-synthetic hyaluronic acid benzyl ester and a synthetic polymer (patent pending). The two preparations were compared in terms of morphology, particle size, substantivity, and *in vitro* SPF. Both microspheres appeared as spherical shape with smooth surface, and displayed uniform particle size distribution. Microspheres formulated by synthetic polymer provided a better substantivity and higher SPF *in vitro*, which demonstrated an enhancement of skin protection activity. In 2006, Patel *et al.* prepared gelatin microspheres containing OBZ; microspheres of OBZ:gelatin in a ratio of 1:6 displayed the slowest release of OBZ, while those of OBZ:gelatin in a ratio of 1:2 showed the fastest release of OBZ. The formulation with OBZ gelatin microspheres in aloe vera gel possessed the best photoprotective efficacy.

Solid lipid nanoparticles (SLN) are an aqueous colloidal dispersion with particles in the range of 50-1000 nm. These particles are composed of biodegradable lipids and drugs. SLNs have been introduced as a carrier system for pharmaceutical compounds and cosmetic ingredients (Wissing, *et al.*, 2001). Incorporation of sunscreens into SLNs led to synergistic photoprotective characteristics, i.e., photoprotective effect of sunscreens encapsulated in SLNs was higher than the additive effect of UV scattering by the SLNs and UV absorption by the sunscreens. Furthermore, photoprotective effect of OBZ in SLN dispersion was found to be three fold higher than that from a conventional sunscreen emulsion (Wissing, *et al.*, 2001). SLNs also inhibited the release rate of active sunscreens.

In studies comparing SLNs and traditional O/W emulation containing OBZ, OBZ release rate from SLN formulations decreased by up to 50% *in vitro* and by 30-60% *in vivo*, respectively. On contrary, OBZ in conventional emulsions was released and penetrated across human skin at a higher rate and to a greater extent in all testing models. (Wissing, *et al.*, 2002).

Different types of preparation may exert variable influences on percutaneous penetration of OBZ and subsequently its sun-blocking efficacy. Newer formulation techniques and materials have demonstrated desirable characteristics in minimizing skin penetration of OBZ and enhancing overall effective sun protection of the final products for human usage. These properties are still being explored by researchers, and hopefully there will be more sunscreen preparations with improved safety and efficacy in the future.

1.3. Dermal Exposure of Chemical Mixtures

Risk assessment of chemical substances is an important aspect of pharmaceutical and consumer-care industry where thousands of chemical compounds are used for many different applications. Regulatory agencies such as the Environmental Protection Agency, Occupational Safety and Health Administration, and Agency for Toxic Substance and Disease Registry regularly conduct experiments and collect data regarding safety and efficacy of chemical substances (EPA, 1995; EPA, 1999; Poet, *et al.*, 2002). Under most circumstances, profiles of these chemical substances are based on individual compounds.

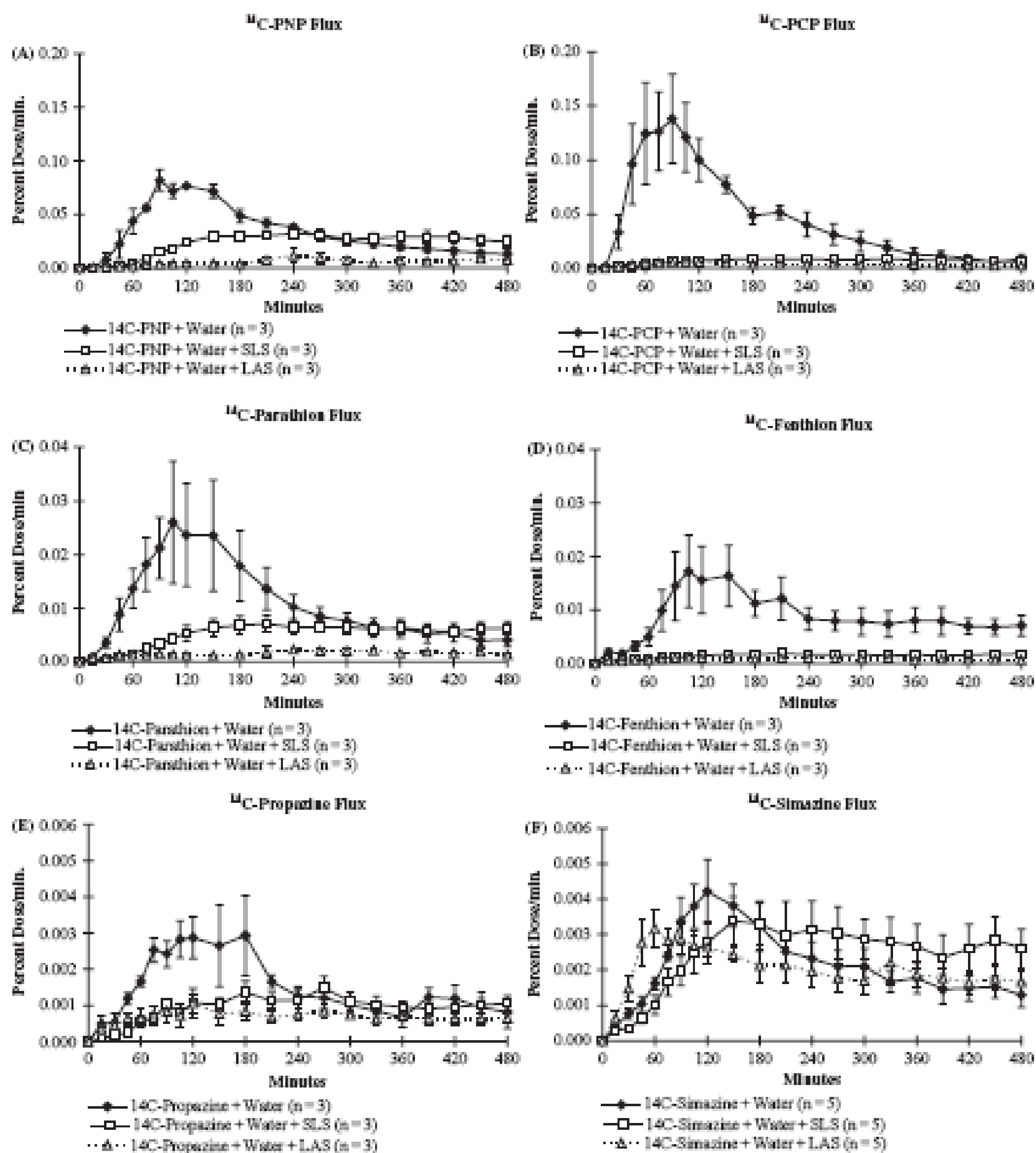
Nevertheless, it is not uncommon in reality for humans to utilize complex chemical mixtures in pharmaceutical and cosmetic products; as a matter of fact, a majority of medications and consumer-care products do contain more than one chemical ingredient in their final forms. For skin application of chemical mixtures, presence of multiple chemicals may often alter the rate and extent of dermal absorption of each individual chemical in the mixtures. Concerns do exist for a lack of risk assessment data on many chemical mixtures (Borgert, *et al.*, 2001; EPA, 1986; EPA, 1988; Pohl, *et al.*, 1997). Regulatory agencies have subsequently recommended moving beyond individual chemical risk assessments and measuring collective toxicity of the chemical combinations (CRARM, 1997).

1.3.1. Mechanisms of Chemical Interactions

Chemical and physical interactions may occur when multiple compounds are combined to form a mixture; consequently this may also lead to modifications in dermal and transdermal absorption of individual substances after topical skin application. Human skin can be visualized as divided into several physiological layers, i.e., exterior skin surface, stratum corneum, epidermis, dermis, and subcutaneous fat tissues; interactions with a chemical mixture can even exert different mechanisms when it is passing through these anatomical layers. For example, mechanisms of chemical interactions on the skin exterior may include chemical-chemical binding, altered physicochemical properties (in

solubility, volatility, and critical micelle concentration, etc.), altered rate of surface evaporation, occlusive behavior, and binding with adnexial structures or their secretions. Surfactants are usually one of the common additives in many dermal mixtures. They are capable of modulating dermal absorption of other components in the mixture by altering the solubility and surface tension of these compounds in aqueous vehicles. Sodium lauryl sulfate (SLS) and linear alkylbenzene sulfonate (LAS), two widely used anionic surfactants, were observed to influence dermal absorption of six chemical substances, i.e., pentachlorophenol (PCP), 4-nitrophenol (PNP), parathion, fenthion, simazine, and propazine *in vitro*. **Figure 1.7** shows the study results from isolated perfused porcine skin flap (IPPSF); both SLS and LAS decreased dermal absorption of these six compounds from water solution, and absorption from SLS solutions was greater than that of LAS (Riviere, *et al.*, 2010). The six compounds were all lipophilic with relatively low aqueous solubility, and their LogKo/w values were in the range of 1.91-5.12. Lipophilic compounds possessed a tendency to partition into stratum corneum from aqueous solution (Rhee, *et al.*, 2007). The presence of surfactants increased the solubility of these compounds in aqueous solution and subsequently decreased their partition into the stratum corneum. As a result, absorption of the test substances from aqueous solution was generally higher than that from aqueous solution with surfactants. Both SLS and LAS were anionic surfactants with a 12-carbon chain. The diameter of SLS micelles was

Figure 1.7. Flux profiles of six different chemicals†



†Mean (SEM) flux profiles (percent dose/min) following topical doses of (A) *p*-nitrophenol, (B) pentachlorophenol, (C) parathion, (D) fenthion, (E) propazine, and (F) simazine in water, 10% SLS, and 10% LAS. Both SLS and LAS decreased dermal absorption of these six compounds from water solution, and absorption from SLS solutions was greater than that of LAS (Copyright Permitted by Toylor & Francis at December 2013).

uniformly half the diameter of LAS micelles. This size difference likely attributed to a greater absorption by SLS than by LAS.

Compounds such as glyceryl monocaprylate, n-methyl pyrrolidone, and diethylene glycol monoethyl ether (Transcutol HP) also demonstrated modifications to dermal absorption of other components in mixtures. 10% glyceryl monocaprylate in isopropyl myristate solution enhanced the permeation of pentazocine by 4-folds when compared to that of isopropyl myristate alone (Furuishi, 2007). The addition of glyceryl monocaprylate increased skin-moisturizing capacity, thereby enhancing skin permeation of pentazoncine (Okumra, *et al.*, 1991). In another report, 75% n-methyl pyrrolidone in isopropyl myristate solution significantly improved lidocaine flux across human skin by 25-folds in comparison to that of isopropyl myristate alone (Lee, 2006). As a co-transporter, n-methyl pyrrolidone had the high permeability through human stratum corneum in n-methyl pyrrolidone/isopropyl myristate system and was able to form hydrogen bonding between n-methyl pyrrolidone and lidocaine (Lee, *et al.*, 2005). This bond could serve as a driving force for lidocaine flux. In study by Rhee *et al.*, 40% Transcutol HP in an isopropyl myristate solution increased skin permeability of clebopride by 80-fold when compared to that of isopropyl myristate alone (Rhee, *et al.*, 2007). Transcutol HP increased solubility of clebopride in isopropyl myristate and enhanced drug load in the binary vehicle, thereby facilitating skin permeation rate of clebopride.

Potential interactions may also involve constituents of stratum corneum. Oleic acid, oleyl alcohol, decanoic acid, and butene diol may act as lipid disrupting agents; they increased the fluidity of lipids in stratum corneum, and subsequently altered the permeability of other compounds within intercellular lipids of stratum corneum (Francoeur, *et al.*, 1990; Kim, *et al.*, 1996). Ethanol is a common pharmaceutical solvent. It is capable of extracting large amount of lipids from stratum corneum and increasing the mobility of alkyl chain in the lipid layer of stratum corneum. When ethanol was applied with other chemicals, it enhanced transdermal absorption of these substances (Krill, *et al.*, 1992; Liu, *et al.*, 1991; Sugibayashi, *et al.*, 1992). *N*-methyl pyrrolidone, a dipolar aprotic solvent, also enhanced the transport of chemical substances across the skin by altering lipid fluidity in stratum corneum (Yoneto, *et al.*, 1995). Skin depot formed within stratum corneum is another example. Ethyl parathion is a potent insecticide with high lipophilicity (LogKo/w 3.83); the compound exhibited high affinity to cutaneous lipid and formed skin depot after topical application. Subsequently this skin depot could alter solubility of other chemicals in the skin or modify lipid structure of the skin, leading to changes of percutaneous penetration by other chemical mixtures (Moody, *et al.*, 2007).

A eutectic mixture of two or more active ingredients may also alter dermal absorption through interaction with the constituents of stratum corneum. Eutectics create mixtures with lower melting point than the original individual components. This lowered melting point reflected the relatively higher hydrophobic property and was associated

with low level of crystalline interactions in the substances (Roy, *et al.*, 1988). Calpena *et al.* reported that permeability of chemicals varied inversely with their melting points; the lower the melting point of a chemical is, the higher the permeability value is (Calpena, 1994). A eutectic system may create two types of mechanism in enhancing dermal absorption, i.e., eutectics produces a mixture of lower melting point to act on skin and to alter permeation profiles of the components in this mixture; eutectic mixture of lower melting point may also directly disrupt the skin structure and consequently to alter the permeation of other chemical ingredients. Eutectic systems of ibuprofen-terpenes and propranolol-fatty acids had been successfully utilized to improve dermal permeation of the active components. (Stott, *et al.*, 1998; Stott, *et al.*, 2001). Terpenes interacted with ibuprofen to disrupt hydrogen-bonded dimmers in ibuprofen; eutectic mixture of ibuprofen-terpenes would form a saturated ibuprofen solution and thus produce maximum thermodynamic activity and driving force for its percutaneous penetration. For propranolol and fatty acids, chemical bonds were formed between amino group of propranolol and carbonyl group of fatty acid. The oppositely charged ions would permeate stratum corneum by an ion-pair mechanism, therefore promoting molecular permeation. Kaplun-Frischoff and Touitou also reported that permeation of testosterone through human cadaver skin was enhanced when testosterone was combined with menthol to form a eutectic system (Kaplunfrischoff, *et al.*, 1997). Menthol was able to alter skin lipids by decreasing melting point of cholesteryl oleate and ceramides and

modifying thermogram profile of stratum corneum; this in turn enhanced skin permeability. This mechanism was also known as “push-pull mechanism” (Kadir, *et al.*, 1987).

The third level of interactions is biotransformation of chemical substances within viable epidermis; possible mechanisms included competitive and noncompetitive inhibition for occupancy at an enzyme’s active site, and induction or inhibition of drug-metabolizing enzymes (Haberland, *et al.*, 2006; Stoppie, *et al.*, 2000). A chemical molecule may induce keratinocyte to release cytokines or other inflammatory mediators (Allen, *et al.*, 2000; Luger, *et al.*, 1990; Monteiro-Riviere, *et al.*, 2003), which will subsequently lead to changes in barrier function of stratum corneum or vascular function of dermis. Alternatively, cytokines may also modulate activities of biotransformation enzymes (Morgan, 2001). The final level of potential interactions would occur in dermis where a compound may directly or indirectly modulate vascular uptake of other chemical substances (Riviere, *et al.*, 1992; Williams, *et al.*, 1993). This kind of vascular modulation may affect the depth and extent of chemical penetration into underlying tissues. Potential interactions from chemical mixtures after dermal exposure are often quantitated by studying chemical absorption properties and transdermal permeability; this may sometimes simplify investigational complexity. One of the quantitative values in differentiating permeability of components in a chemical mixture is partition coefficient; this specific parameter provided useful approach to assessing effects of a mixture

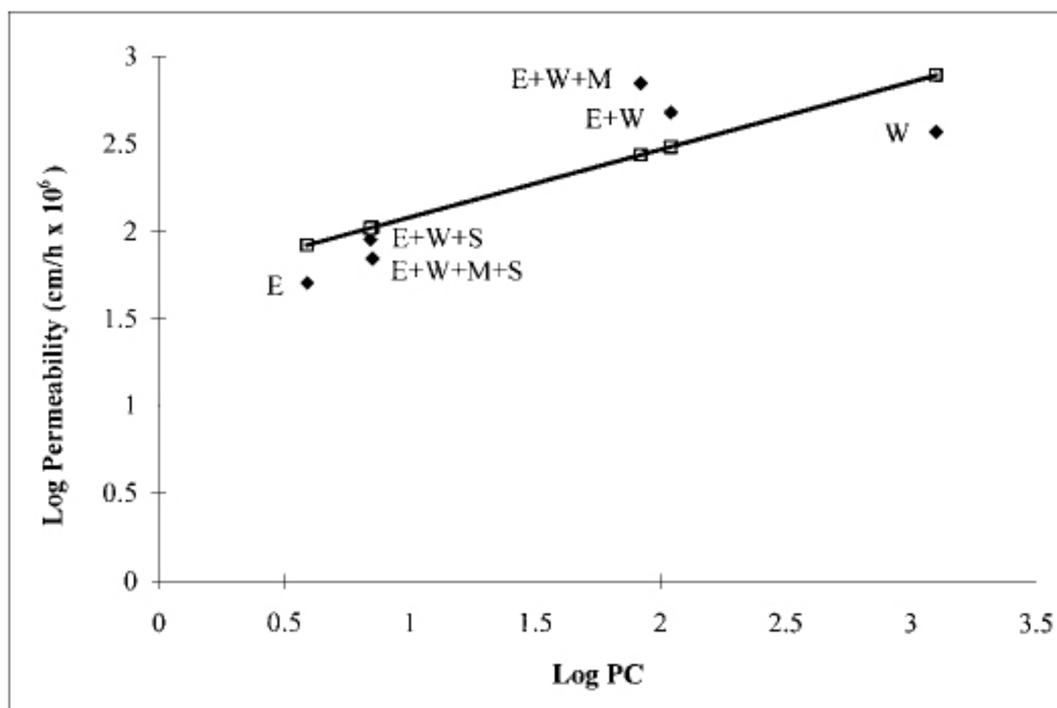
interaction on subsequent drug absorption. **Table 1.3** lists partition coefficient of pentachlorophenol (PCP) between porcine stratum corneum and six vehicles (Baynes, *et al.*, 2002). The six vehicles respectively are water, water + ethanol + methyl nicotinate, water + ethanol, water + SLS, ethanol + methyl nicotinate, and ethanol. **Figure 1.8** illustrates the relationship between partition coefficient of PCP in porcine stratum corneum and six vehicles and skin penetration of PCP from these six vehicles. Skin absorption of PCP from different chemical mixtures was correlated with partition coefficient of PCP. PCP was a lipophilic compound and hence barely soluble in water. It was more likely to partition from an aqueous solvent to stratum corneum than with 100% ethanol. A mixed system (e.g., 40% ethanol + 60% water) significantly increased PCP solubility, thus decreased solute activity and partition coefficient of the compound from vehicle to stratum corneum (Ashton, *et al.*, 1986; Li, *et al.*, 1994; Sloan, *et al.*, 1986). Partition coefficient is an important parameter in skin penetration, and can be utilized to predict dermal absorption and permeability in a linear regression analysis. Few studies hve been performed to measure partition coefficient and permeation coefficient in a chemical mixture regarding synergistic transdermal permeation of two or more chemical ingredients. More efforts are needed to expand experiments in the field in order to understand and quantitate skin absorption of chemical mixtures.

Table 1.3. Physicochemical properties of PCP mixtures†

Mixture	**PCP solubility (%)**	**$LogK_{SC/vehicle}$**	**Viscosity (Pa S)**
100% water	0.008	3.10	0.0011
100% EtOH	76.74	0.59	0.0015
60% water + 40% EtOH	0.048	2.04	0.0015
60% water + 40% EtOH +MNA	0.15	1.92	0.0025
60% water + 40% EtOH +SLS	1.36	0.84	0.0037
60% water + 40% EtOH + SLS + MNA	1.28	0.85	0.0038

†$LogK_{SC/vehicle}$: Log Partition coefficient between porcine stratum corneum and six vehicles; EtOH: Ethanol; MNA: Methyl nicotinate (Baynes, *et al.*, 2002).

Figure 1.8. Log permeability-log partition coefficient line fit of PCP†



†Skin absorption of PCP from six vehicles, including E (ethanol), W (water), E+W, E+W+M (methyl nicotinate), E+W+S (SLS), E+W+M+S, was correlated with partition coefficient of PCP (Copyright Permitted by Oxford University Press at December 2013).

1.3.2. Concurrently Dermal Exposure of DEET and OBZ

Windheuser *et al.* reported that DEET enhanced *in vitro* permeation of a wide variety of drugs through hairless mouse skin, including hydrocortisone, hydrocortisone acetate, hydrocortisone 17-butyrate, hydrocortisone 17-valerate, dibucaine, benzocaine, indomethancin, ibuprofen, erythromycin, tetracycline hydrochloride, griseofulvin, mycophenolic acid, metyl salicylate, and triethanolamine salicylate. The largest

penetration enhancement appeared in tetracycline hydrochloride from not detectable (without DEET) to 100% (with DEET) (Windheuser, *et al.*, 1982). As one of the most efficient insect repellents for the past six decades, DEET was commonly applied with other compounds, such as permethrin. Combined exposure to DEET and permethrin induced greater biochemical, behavioral, and metabolic alterations in animals compared to each individual compound (Abu-Qare, *et al.*, 2003). Literature has also shown that combination of DEET and permethrin led to greater impairment in inclined performance of sensorimotor behavioral test than permethrin alone (Abdel-Rahman, *et al.*, 2004). Nevertheless, mechanism of permeation enhancement by DEET has not been known or discussed. The only fact known to date was that DEET was a good solvent, and many medicinal ingredients were mixable in DEET. Alternation of solubility by another compound may modulate partition coefficient of the molecules from a vehicle to stratum corneum, hence enhancing their permeation profiles. Furthermore, DEET as a lipophilic solvent may fluidize lipid layer of stratum corneum and enhance permeation of other compounds.

OBZ, as one of the active organic sunscreens approved by FDA, was detected to enhance skin penetration of herbicide 2,4-dichlorophenoxyacetic acid, a moderately lipophilic ingredient with a LogKo/w 2.01 (Pont, *et al.*, 2004). Accumulative release of 2,4-dichlorophenoxyacetic acid through hairless mouse skin was increased from 54.9% without OBZ pretreatment (0.6% ethanol solution) on the skin to 74.9% with OBZ

pretreatment. While the exact enhancement mechanism was unknown, it appeared that interaction between OBZ and 2,4-dichlorophenoxyacetic acid might increase partition of 2,4-dichlorophenoxyacetic acid to stratum corneum from ethanol and subsequently enhance its percutaneous penetration.

Because both DEET and OBZ were capable of enhancing skin penetration, studies were carried out to evaluate synergistic penetration of their own when simultaneous presence. Gu *et al.* (2004) reported that concurrent application of DEET and OBZ in three different medium, propylene glycol, ethanol, and poly(ethylene glycol)-400 (PEG-400), increased permeation percentage through PDMS and pig skin (Gu, *et al.*, 2004). Gu *et al.* (2005) also reported that synergistic penetration of DEET and OBZ from commercial insect repellent products and sunscreen products through pigskin depended on formulation type, application amount, and application sequence (Gu, *et al.*, 2005). Kasichayanula *et al.* (2007) reported that combined application of DEET and OBZ resulted in synergistic percutaneous penetration of DEET and OBZ after topical application of three commercial repellent and sunscreen products in piglets (Kasichayanula, *et al.*, 2007). These studies all indicated that potential chemical and physical interactions might occur from concurrent application of DEET and OBZ, which would further lead to changes in skin penetration characteristics of the two active compounds. Synergistic transdermal characteristics of DEET and OBZ in pharmaceutical vehicles, commercially available preparations, and emulsion-based formulations have

been investigated for many years in our laboratory. Further investigations are being carried out to elucidate the exact mechanisms of synergistic percutaneous penetration of DEET and OBZ**.** After concurrent application of DEET and OBZ, they may form a special chemical bond, which could alter individual solubility of the compounds in various vehicles. One or both chemicals may also fluidize lipid layer of stratum cornuem. Thereby, interactions between these two compounds or between chemicals and the skin may influence partition coefficient from a vehicle to stratum corneum and thus modify the skin penetration of DEET and OBZ in the mixture. More studies are certainly required in the future.

1.4. Conclusion

DEET is utilized as a broad-spectrum insect repellent in over 90% commercial insect repellent products. When topically applied on skin surface, DEET is capable of penetrating through the skin and rapidly distributing into the blood circulation. The compound is primarily metabolized in the liver and eliminated rapidly through the urine. Systemic disposition of DEET may lead to adverse effects in susceptible individuals. OBZ is a common UVA/UVB sun-blocking ingredient found in many commercially available sunscreen preparations. Topical application of OBZ may also experience significant skin penetration, tissue disposition, metabolism, and elimination in urine and feces. Skin irritations are frequently encountered in humans after topical administration

of OBZ.

A variety of new preparations have been developed and tested in order to minimize systemic absorption of DEET and OBZ from topical skin applications. Many new formulation techniques have also attempted to enhance overall protection efficacy of the final products in repellency and sun protection. Concurrent topical exposure to DEET and OBZ produced a synergistic percutaneous penetration of both chemicals, which is not desirable for topical skin products like repellents and sunscreens. There are numerous physical and chemical properties that may alter skin penetration of substances present in semisolid skin preparations. Identification of possible mechanisms in physical and chemical interactions on the skin surface, followed by appropriate formulation of emulsion-based topical delivery systems, can benefit designing safer and more efficient topical preparations for compounds like DEET and OBZ.

CHAPTER 2

Skin Penetration and Topical Drug Delivery

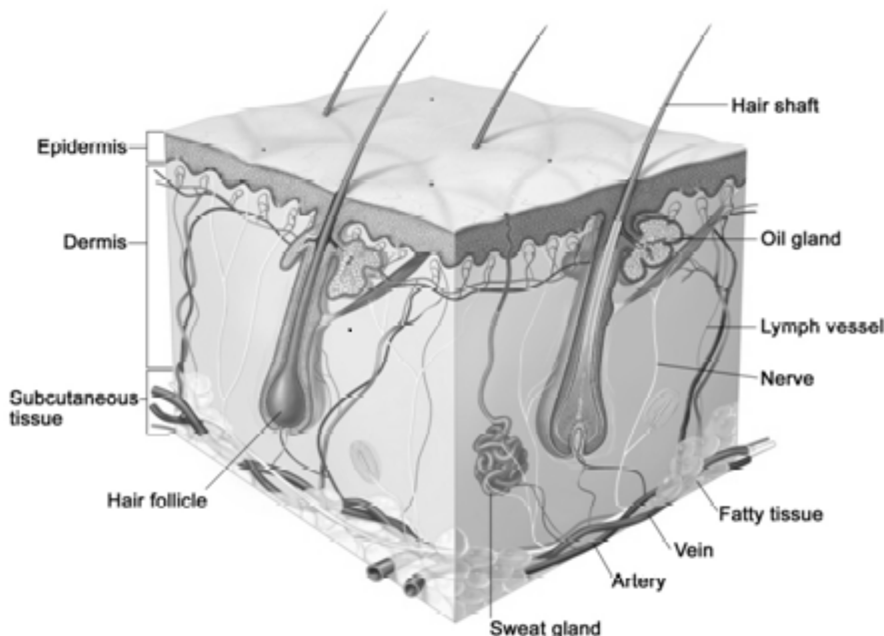
2.1. Skin

The skin is the largest organ of the human body, which consists of stratum corneum (nonviable epidermis), viable epidermis, dermis, subcutaneous tissue, such as artery, vein, nerve, lymph vessel, and fatty tissue, and skin appendages, such as hair follicles and sebaceous glands (**Figure 2.1**). There are blood and lymph vessels, and nerve endings embedded in subcutaneous tissue. One of the most important functions of human skin is to protect the internal organs and tissues against invasion of external pathogens, such as fungi, yeasts, and bacteria, and physicochemical assaults of solvents, detergents, cosmetics, and drugs. Skin also prevents unregulated loss of water and essential electrolytes from the human body, and senses various feelings of the external environment (Bronaugh, *et al.*, 1999).

2.1.1. Stratum Corneum (Nonviable Epidermis)

Stratum corneum, the outmost layer of the epidermis, is a principal rate-limiting barrier against percutaneous penetration of any chemical substance. It is important to understand the physicochemical properties of stratum corneum in order to develop and evaluate topically applied dosage forms. Stratum corneum is formed from continuous protein-enriched cells (corneocytes) connected by corneodesmosomes and embedded in an intercellular lamellar non-polar lipid matrix (Proksch, *et al.*, 2008).

Figure 2.1.The structure of human skin†

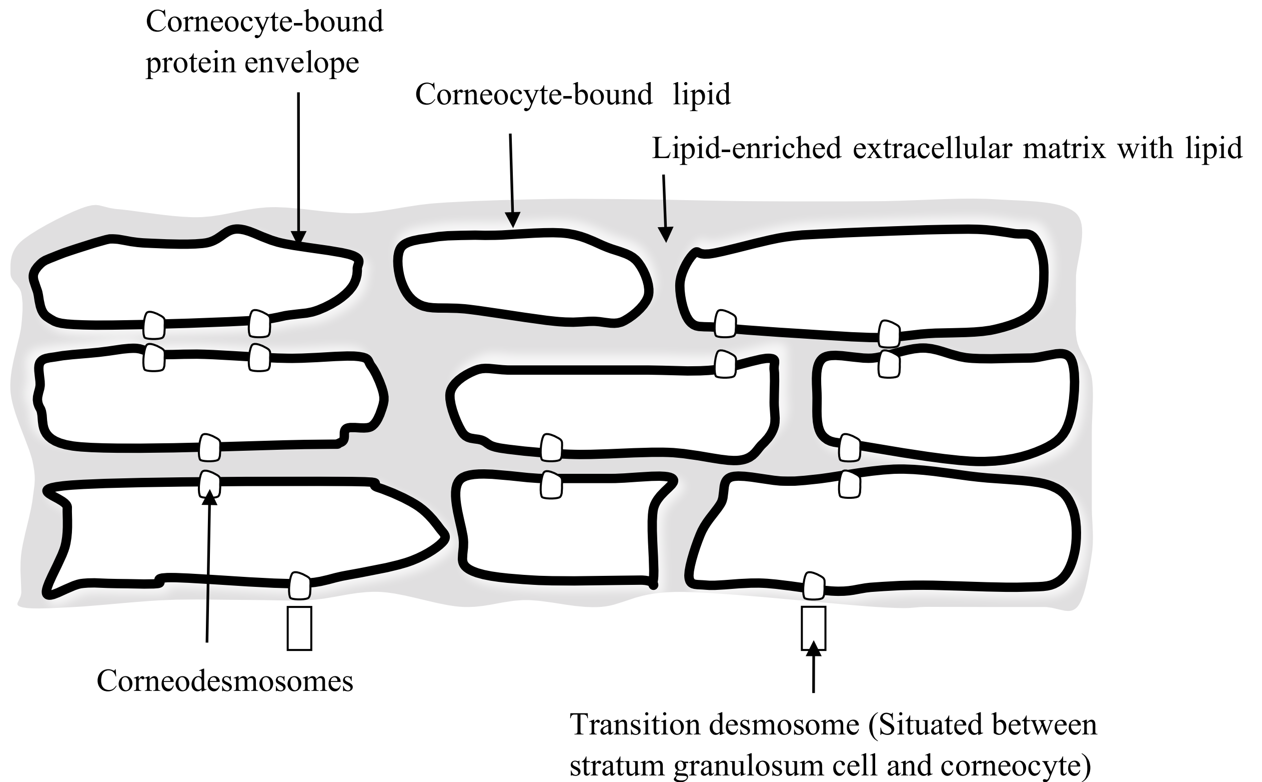


†The human skin consists of stratum corneum, viable epidermis, dermis, subcutaneous tissue, and skin appendages (Copyright Permitted by Terese Winslow, U.S. Govt. at August 2013).

The final steps of keratinocyte differentiation result in a profound structural transformation of keratinocytes through the formation of flat and anucleated corneocytes in stratum corneum. Coneocytes are composed of keratin filaments and surrounded by cross-linked protein envelope and a covalent-bound lipid envelope (**Figure 2.2**).

Corneocytes are formed during the final stage of normal differentiation of skin cells. These flattened, organized cells are composed of condensed keratins incorporating with filaggrin. Both keratin and filaggrin constitute 80-90% of proteins in the mammalian nonviable epidermis and viable epidermis (Nemes, *et al.*, 1999; Roop, 1995).

Figure 2.2. The structure of stratum corneum†



†In the human stratum corneum, corneocytes are surrounded by the protein/lipid envelope, embedded in the lipid-enriched extracellular matrix with lipid bilayer, and interacted through corneodesmosomes (Rawlings, 2010).

Filaggrin is a filament-associated protein that binds to keratin fibers and aggregates keratin filaments into tight bundles. Filaggrin also undergoes further processing in the upper stratum corneum to release free amino acid that assists in water retention by corneocytes (Ovaere, *et al.*, 2009). Loss of normal profilaggrin was found in flaky tail mice, which characteristically display dry and flaky skin. With dry skin, transepidermal water loss (TEWL) increases; the water content in stratum corneum falls

below 10% of overall skin weight. Because diffusion is the primary mechanism of transdermal penetration, the degree of skin hydration may ultimately dictate the role of stratum corneum as a skin barrier (Rougier, *et al.*, 1999). As a matter of fact, an increase of water content in skin corresponds to an increase in permeability by topically applied compounds. Hence, skin hydration, especially corneocytes hydration, is important in determining the rate of percutaneous penetration of a given solute, which may penetrate through the inner lumens of corneocytes. Filaggrin in healthy skin is capable of assisting in water retention by corneocytes; this could further facilitate percutaneous penetration of chemical substances across the skin membrane.

Residing on the exterior of corneocytes, cornified cell envelope is a tough protein or lipid polymer structure formed below the cytoplasmic membrane. It consists of two parts, a corneocyte-bound protein envelope and a corneocyte-bound lipid envelope (Proksch, *et al.*, 2008). The corneocyte-bound protein envelope contributes to the biomechanical properties of the cornified cell envelope as a result of cross-linking of structural proteins, including involucrin, loricrin, trichohyalin. The corneocyte-bound lipid envelope has a plasma membrane-like structure, which replaces the plasma membrane on the external side of mammalian corneocytes (Swartzendruber, *et al.*, 1987). These lipids not only provide a coating to the cells but also interdigitate with the intercellular lipid lamellae (Nemes, *et al.*, 1999).

Corneodesmosomes are a desmosome on corneocytes, whereas transition

desmosomes are desmosomes that connect corneocytes in stratum corneum to keratinocytes of the granular layer in epidermis (Figure 2.2). Adjacently interconnected corneocytes are important to cohesion of stratum corneum; they are shed during desquamation process in stratum corneum (Proksch, *et al.*, 2008).

Major lipids surrounding corneocytes in the intercellular lamellar of stratum corneum include ceramides, free fatty acid, and cholesterol. These ceramides are amide-linked fatty acids containing sphingoid base, long-chain amino alcohol, which accounts for 30-40% of stratum corneum lipid. There are at least nine different free ceramides existing in stratum corneum (Uchida, *et al.*, 2006), two of which are ceramide A and ceramide B, covalently binding to cornified envelope proteins, especially involucrin (Bouwstra, *et al.*, 2006). Stratum corneum also contains free fatty acids as well as fatty acids bound in triglycerides, phospholipids, glycosylceramides, and ceramides. The chain length of free fatty acid ranges from C_{12} to C_{24}. The closely packed fatty acid molecules are able to form either solid waxy or liquid crystalline areas within the intercellular lipid matrix (Downing, 1987; Friberg, *et al.*, 1985). When the lipid matrix is solid and rigid, stratum corneum will be more crystal-like and less permeable. On the other hand, it will become more fluidic and permeable. Hence, how fatty acids are packed could regulate skin barrier functions and influence percutaneous skin penetration. Cholesterol is another major lipid found in stratum corneum. Despite the fact that basal cells absorb cholesterol from circulation, a majority of cholesterol is synthesized in situ

from acetate in epidermis and is brought to stratum corneum during cell differentiation. Structurally, cholesterol contains four rigid carbon rings tightly bound to each other; this makes the membrane less flexible and less permeable (Feingold, *et al.*, 1986; Feingold, *et al.*, 1990; Menon, *et al.*, 1985). Consequently, intercellular lipid matrix with higher level of cholesterol increases the rigidity of stratum corneum. Overall, ceramides, free fatty acids, and cholesterol collectively play important roles in skin barrier of stratum corneum. They may alter permeability and physical properties of stratum corneum at different concentration levels and by different interactions with one another (Bouwstra, *et al.*, 1997).

2.1.2. Viable Epidermis

The viable epidermis is composed of four distinctive layers with cells in various stages of differentiation; they are basal layer, spinous layer, granular layer, and lucid layer. The basal layer contains an extensive keratin network, primarily made of keratin K5 and K14. This structure contributes to minimizing TEWL and preventing foreign substance absorption (Moll, *et al.*, 1982). Cells begin migrating upward from basal layer. Once skin cells migrate from basal layer into spinous layer, they lose the ability to proliferate and divide. Subsequently, the size of cells increases, the shape of cells flattens, and the water content in the cells diminishes. Synthesis of proliferation-specific keratin K5 and K14 is interrupted in spinous layer, as such keratin K1 and K10 aggregate to form

filaments (Warhol, *et al.*, 1985). The precursor of cornified envelope protein, involucrin (Warhol, *et al.*, 1985) appears in spinous layer, and it is transferred into insoluble cornified envelope (Thacher, *et al.*, 1985). After spinous layer, skin cells migrate into granular layer. The filaments are further packed up tightly with filaggrin, leading to formation of macrofibrils. Certain granule-like structures, such as lamellar bodies and stable protein envelopes, are also formed in granular layer. Ultimately, differentiation of keratinocyte produces corneocytes, which consist of cornified protein envelopes filled with keratin bundles and are embedded in a lipid-enriched intercellular matrix. In the final state, passing from granular layer to stratum corneum, flat horny cells are formed and lipid lamellae are released into the intracellular space. With these changes, transition from viable basal cells to keratinized horny cells is completed. Overall, structural changes of the skin evolve from a closely packed aggregate of cubital cells with conventional phospholipid membranes to a bricks-and-mortar configuration in which major lipids form the mortar between the bricks, enveloped corneocytes. In this way, these lipids will block diffusion of hydrophilic compounds across epidermis, and water-bound substance cannot penetrate beyond lipid-rich granular layer. Uncharged molecules with a low molecular weight and an appropriate Ko/w are capable of permeating across epidermis. The primary penetration pathway is through intercellular lipid, even though possibility of these molecules entering inner lumens of corneocytes does exist, especially after an extended exposure (Brandau, *et al.*, 1982; Schaffer, *et al.*, 1982; Zatz, 1993).

2.1.3. Dermis

The stratum basal layer separates dermis from epidermis. Under the basal layer is dermis, which consists of a meshwork of structural fibers, reticulum, collagen, and elastin, filled with a ground substance of mucopolysaccharidic gel (Wilkes, *et al.*, 1973). The thickness of dermis is in the range of 1-5 mm (Woodburne, 1965). The upper one-fifth of the tissue is known as papillary dermis, which is finely structured and physically supports a delicate capillary plexus that nurtures the epidermis. Loop of capillaries goes from capillary plexus into individual dermal papilla. This transfers nutrients to the epidermis through an ascending arterial component and removes waste from epidermis by a descending venous limb. The entire skin microcirculation system is located in dermis. At room temperature blood flows into the skin at approximately 0.05 ml per minute per gram of the tissue (Scheuplein, *et al.*, 1971); blood supply should increase when the skin is warmer (Rothman, 1954). By entering blood vessels in dermis, substances applied to the skin surface may further travel into systemic circulation or permeate deeper into tissue layers. Blood flow rate in the skin not only determines dermis capillary clearance, but also influences concentration gradient between dermis and skin surface (Kretsos, *et al.*, 2007).

Underneath papillary dermis, the skin merges with coarser fibrous reticular dermis, a major structural element of dermis. The dermis is also penetrated with a sensory nerve network and a lymphatic network. Numerous cells, such as fibroblast, mast cells,

blood cells, endothelial cells, nerve cells and endings, are found in dermis. These cells exhibit a variety of important functions in the skin. For example, fibroblasts synthesize structural fibers, and mast cells play a role in synthesizing ground substance. They are also a source of histamine, to be released when skin is immunologically provoked by allergens.

2.1.4. Appendages

Hair follicles, sebaceous glands, eccrine glands, apocrine glands, and nail plates are collectively known as skin appendages. Hair follicles lie within the skin; they are formed from epidermal cells in fetal life. Hair shafts grow up from hair follicles. They are a compact of keratinized and fused cells and continuously formed by division, differentiation, and compaction of cells in the bulb of hair follicles. Hair follicles occupy approximately 1/1000 of the skin surface. Each hair follicle has one or more sebaceous glands, which have ducts leading to opening space of hair shaft. Sebum is generated by dead sebaceous gland cells and other follicular debris within follicular orifice, and is forced upward to skin surface through these outlets.

Eccrine glands (salty sweat glands) are spread all over the body, except for the genitalia. There are 150-600 sweat glands per cubic centimeter on the body surface (Katz, *et al.*, 1971). The glandular openings represent about 1/10,000 of the skin surface. Eccrine sweat is a slightly acidic hypotonic aqueous solution (Scheuplein, 1965). Its

secretion is stimulated when the body becomes overheated in warm temperature or through exercise. In addition, eccrine sweating is also stimulated by emotions, as these glands are innervated by autonomic nervous systems.

Apocrine glands are located in axillae, in anogenital regions and around nipples. They are coiled into tubular structures and ten times larger than eccrine glands. Apocrine glands may extend throughout the dermis and into subcutaneous layers (Wilkes, *et al.*, 1973; Woodburne, 1965). Each apocrine gland is paired with a neighboring hair follicle. Its secretion is vented into sebaceous duct of the hair follicle beneath skin surface and combined with sebum before release. Human body odor is attributed to bacterial decomposition of this secretion.

Skin appendages, i.e., sweat glands and hair follicles, had not been regarded as a significant skin penetration route in the past, since they accounted for approximately 0.1% of the skin surface area (Schaefer, *et al.*, 1996). This calculation, however, did not take into consideration that hair follicles might represent invaginations, which extended deep into dermis with a significant increase in actual surface area available for drug penetration. Recent studies have suggested that hair follicles represented a highly relevant and efficient penetration pathway and reservoir for topically applied substances. With a rich perifollicular vascularisation and special differentiation pattern (smaller and crumbly corneocytes) in lower follicular duct, hair follicles possessed distinct characteristics favorable for drug penetration. Multiple references have indicated that follicular

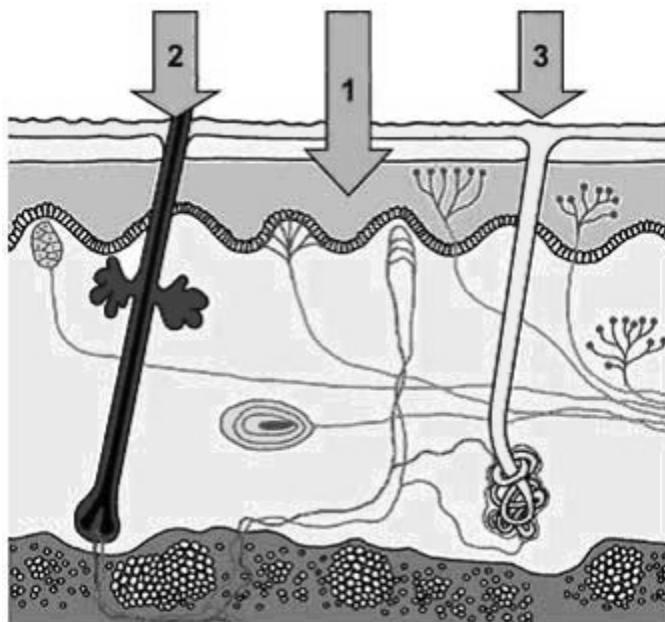
penetration route might be especially relevant for hydrophilic and high molecular weight molecules, as well as by particle-based drug delivery systems (Dokka, *et al.*, 2005; Essa, *et al.*, 2002; Lademann, *et al.*, 2001; Lademann, *et al.*, 2003; Mitragotri, 2003; Ogiso, *et al.*, 2002; Ossadinik, *et al.*, 2006; Schaefer, *et al.*, 2001; Toll, *et al.*, 2004).

2.1.5. Percutaneous Penetration Routes

When a topical delivery system is applied to the skin surface, an active ingredient passively diffuses from the vehicle to the skin surface first. Subsequently the compound permeates from skin surface into internal layer of skin along a concentration gradient. There are two routes of percutaneous permeation, i.e., transepidermal route and transfollicular route. The former involves direct diffusion across stratum corneum, whereas the latter involves diffusion through follicular pores and ducts of pilosebaceous glands (**Figure 2.3**).

In accordance to cellular structure of stratum corneum, transepidermal route is categorized into two permeation subroutes, transcellular route and intercellular route. A penetration across lipid lamellae and corneocytes of stratum corneum is known as transcellular route of penetration; a penetration through the tortuous lipid lamellae pathway is termed intercellular route (**Figure 2.4**). In general a chemical molecule may permeate stratum corneum by both transport mechanisms at the same time.

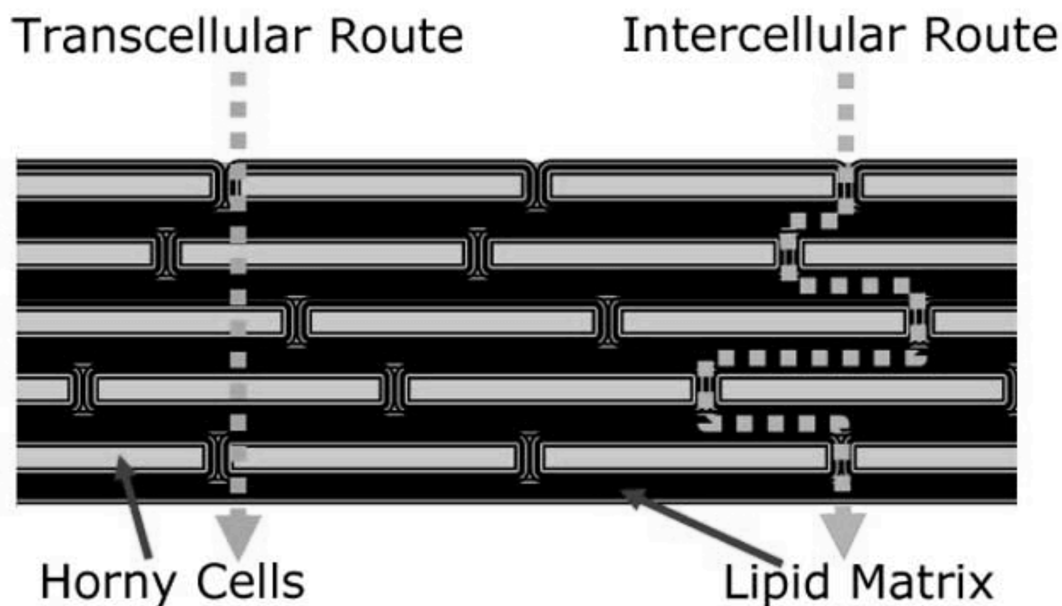
Figure 2.3. Possible pathways of a penetrant cross the skin barrier†



†(1) transepidermal route across the intact horny layer, (2) transfollicular route through the hair follicles with the associated sebaceaous glands, or (3) transfollicular route via the sweat glands (Daniels, 2010)**.**

For appendageal route or shunt route, drug penetration takes place via either eccrine sweat gland ducts or follicular ducts. The content within eccrine sweat glands is hydrophilic in nature, which may be favorable to hydrophilic molecules. On contrary, the content of follicular ducts is generally lipophilic because sebum is excreted into the opening of follicular ducts. Therefore it attracts lipophilic compounds. Even though skin appendages cover approximately 0.1% of entire skin surface, this shunt route may exert an important influence on initial phase of drug diffusion. This route of penetration should not be underestimated (Illel, *et al.*, 1988).

Figure 2.4. Schematic diagram of the two microroutes of penetration†



†The transcellular route is a penetration route across lipid lamellae and corneocytes of stratum corneum; the intercellular route is a penetration route through the tortuous lipid lamellae pathway (Daniels, 2010).

Once the diffusive movement extends from stratum corneum and follicular pores to viable epidermis and dermis, a concentration gradient is established throughout the skin layers, allowing for continuous diffusion of drug molecules from stratum corneum into deeper skin layers. The capillary network will then carry drug molecules away and distribute throughout the body. For drugs that are meant for systemic effects, maximal percutaneous penetration is required to achieve optimal pharmacological outcomes. For cosmetic ingredients or topical skin preparations, systemic absorption through percutaneous permeation might not be desirable or productive, as these products are

intended for topical skin effects.

2.2. In Vitro Diffusion Protocols

To characterize percutaneous drug penetration and absorption, the most practical and cost-saving approach is to utilize *in vitro* diffusion experimentation. Fundamental theories and experimental settings have been extensively explored and well established over the years to describe drug transport across skin membrane; they include Fick's Diffusion Laws, diffusion cells, diffusion membrane models, and receptor fluids. Different types of preparation can also affect the rate and extent of drug permeation and absorption across skin membrane.

2.2.1. Mathematical Models of Diffusion

Human skin is a complex and viable organ that is composed of stratum corneum, epidermis, dermis, subcutaneous tissues, and skin appendages. Nevertheless it is usually regarded as a simple and homogeneous membrane *in vitro* as far as transdermal mathematical models are concerned. A simplified *in vitro* diffusion experiment will study drug diffusion from an infinite volume, well-stirred vehicle in a donor cell, across a select membrane model, and into a receptor fluid under sink conditions.

Fick's Diffusion Laws have been extensively used to quantify percutaneous permeation in *in vitro* diffusion experiments. An *in vitro* permeability study with constant

donor concentration and receptor conditions can be expressed using Fick's Second Diffusion Law as the following (Roberts, *et al.*, 1999),

$$\frac{\partial C}{\partial t} = D_m \frac{\partial^2 Cm}{\partial x^2} \qquad \text{Equation 2.1}$$

Above equation presumes that a) concentration of solute in the donor cell remains constant; b) "sink conditions" exist in the receptor cell for the duration of the experiment; and c) the initial concentrations in model membrane are C_m (x,0)=0, $C_m(0,t)=K_mC_v$, and $C_m(h_m, t)=0$, where C_m: Drug concentration in membranes (g/ml), C_v: saturated drug concentration in vehicle (g/ml).

Under most circumstances, Fick's Second Diffusion Law is solved in terms of the amount of solute Q(t) exiting from the membrane in time t, and is expressed as the following (Roberts, *et al.*, 1999),

$$\frac{Q(t)}{A} = K_m h_m C_v \left[\frac{D_m t}{{h_m}^2} - \frac{1}{6} - \frac{2}{\pi}\sum_{n=1}^{\infty} \frac{(-1)^n}{n^2} \exp\left(\frac{D_m \pi^2 n^2 t}{{h_m}^2}\right)\right] \qquad \text{Equation 2.2}$$

where Q(t): accumulative drug amount permeating through the membrane (g)

K_m: partition coefficient between membrane and vehicle

C_v: saturated drug concentration in vehicle (g/ml)

h_m: thickness of the membrane (cm)

A: diffusion area (cm^2)

D_m: diffusion coefficient (cm^2/h).

When t→∞, Equation 2.2 is simplified to the following (Roberts, *et al.*, 1999)

$$\frac{Q(t)}{A} = K_m h_m C_v \left(\frac{D_m t}{{h_m}^2} - \frac{1}{6}\right)$$

$$= \frac{K_m C_v D_m}{h_m}\left(t - \frac{{h_m}^2}{6D_m}\right) \quad \text{Equation 2.3}$$

$$= K_p C_v (t\text{-lag}) \quad \text{Equation 2.4.}$$

where permeability coefficient K_p of a solute is expressed in,

$$K_p = \frac{K_m D_m}{h_m} \quad \text{Equation 2.5.}$$

Lag time is given by,

$$\text{Lag} = \frac{{h_m}^2}{6D_m} \quad \text{Equation 2.6.}$$

Once a steady-state status is reached in percutaneous penetration, permeation parameters can also be calculated by Equation 2.3 using linear regression of the experimental data. In this linear equation,

$$\text{Slope} = \frac{K_m C_v D_m}{h_m} \qquad \text{Equation 2.7}$$

$$\text{Intercept} = \frac{K_m C_m h_m}{6} \qquad \text{Equation 2.8.}$$

Subsequently, permeation parameters can be calculated with the following,

$$K_m = \frac{6 * \text{Intercept}}{C_v} \qquad \text{Equation 2.9}$$

$$D_m = \frac{\text{Slope} * h_m}{K_m C_v} \qquad \text{Equation 2.10}$$

$$K_p = \frac{K_m D_m}{h_m} \qquad \text{Equation 2.11}$$

$$\text{Lag} = \frac{{h_m}^2}{6 D_m} \qquad \text{Equation 2.12.}$$

A steady-state flux is defined as the following (Roberts, *et al.*, 1999),

$$J_{ss} = \frac{Q(t)}{A(t - \text{lag})} = K_p C_v \qquad \text{Equation 2.13.}$$

Therefore, the permeation coefficient can be simplified into the following equation,

$$K_p = \frac{J_{ss}}{C_v} \qquad \text{Equation 2.14.}$$

Data collected from an *in vitro* diffusion study is often used to quantify

percutaneous permeation of diffusants using Fick's Diffusion Laws. For example, permeation coefficient (K_p) is associated with capability of percutaneous diffusion and permeation of a diffusant; the parameter is utilized to compare permeation profiles of a diffusant under different experimental conditions. In addition, permeation coefficient is dependent upon other parameters such as partition coefficient, diffusion coefficient, and membrane thickness; adjusting these parameters would consequently alter permeation coefficient. Modifying formulation components may also change solubility of chemical components in preparation and in skin, subsequently leading to changes in partition coefficient, diffusion coefficient, and eventually permeation coefficient or permeability. Lag time is defined as the time period from the first contact of a diffusant with skin surface to that a steady state flux has been established. According to Equation 2.12, lag time is also dependent upon diffusion coefficient, as such release of drugs from a topical dosage form may also influence their diffusion capability through the skin.

2.2.2. Factors Influencing in Vitro Diffusion Models

Design of a practical and reproducible *in vitro* percutaneous absorption study involves selection of appropriate diffusion cell, skin membrane, donor and receptor configuration, sampling interval, and assay detail, in order to obtain reliable experimental data. One of the primary objectives of conducting an *in vitro* diffusion experiment is to obtain relevant percutaneous absorption characteristics important to formulation

development and product assessment, as *in vivo* studies in living animals are often extremely expensive and ethically regulatory. Consequently a number of protocols have been proposed and utilized for *in vitro* diffusion experimentation in the hope of acquiring alternative results prior to any *in vivo* study.

There are various types of diffusion cells available for *in vitro* diffusion studies; appropriate selection of a specific diffusion configuration may achieve different experimental objectives. For example, Franz-style diffusion cells, or vertical diffusion cells, are of great benefit to controlling testing dose size and application occlusivity, which is the most common diffusion configuration for experimentation. The applied dose remains static in the donor compartment of a vertical diffusion cell, which is more realistic to simulating actual topical applications *in vivo*. Horizontal diffusion cell is another study setting that is capable of adjusting temperature and agitation of the testing content in a donor compartment. However, it is inflexible to modify dose application and dosing amount for this type of diffusion cells because both donor and receptor compartments are completely sealed systems for the entire study period. As such horizontal diffusion cells may not realistically simulate topical application *in vivo*. In addition, diffusion cells can also be categorized into static and flow-through according to receptor fluid mobility. A static diffusion cell maintains its receptor fluid for the entire duration of the experiment. A flow-through diffusion cell on the other hand keeps its receptor fluid recycled or replaced during the experiment, which might simulate actual

physiological condition of skin *in vitro*.

Whenever permitted, excised human skin is the recommended membrane model for all diffusion studies because of its relevance to real skin applications. Nevertheless human skin is not always available in ample amount under most circumstances; a variety of animal skin membranes have to be used as substitutes in diffusion experiments (Bronaugh, *et al.*, 2005). In general, animal skin membranes are more permeable to chemical substances than human skin. This may sometimes lead to discrepancies in predicting permeation characteristics for drug efficacy and safety. To prepare skin membrane for proper diffusion experiments, the excised skin specimens are often dermatomized to 200-380 μm to include only stratum corneum and epidermis, the primary skin barrier layers to foreign substances; full skin is not commonly used for diffusion experiments because it can sometimes artificially retain testing chemicals as a reservoir (Bronaugh, *et al.*, 1984; Bronaugh, *et al.*, 1986). The most common species for skin samples include hairless mouse (Son, *et al.*, 2012), hairless rat (Siddoju, *et al.*, 2012), guinea pig (Aggarwal, *et al.*, 2013), piglet (Kasichayanula, *et al.*, 2005), and rabbit (Mannem, *et al.*, 2013)

Whereas animal skin specimens are unavailable, artificial membranes, such as nitrocellulose (Mitri, *et al.*, 2011), high density polyethylene (HDPE) (Jiang, *et al.*, 1997), low density polyethylene (LDPE) (Jiang, *et al.*, 1997), and silicone (Oliveira, *et al.*, 2012), etc., are also useful as substitutes of biological membranes. Artificial membranes may

exert certain advantages over biological skin samples, simple pretreatment process, easy storage condition, low cost, and capability in imitating chemical permeation, to just name a few. Nevertheless, artificial membrane models are appropriate only for assessing substance release from a topical vehicle or delivery system; they are not suitable for predicting drug penetration characteristics across human skin.

To maintain *in vitro* skin viability, a physiological buffer such as balanced salt solution and tissue culture medium is used to treat the skin specimens for at least 24 h (Collier, *et al.*, 1989). Skin treatment with a physiological buffer prior to *in vitro* diffusion experiment is preferred, in order to produce reliable and reproducible simulation to *in vivo* conditions. For diffusion (receptor) medium, bovine serum albumin is added sometimes to increase solubility of lipophilic compounds. Solubilizing agents (e.g., surfactants and organic solvents) may also be included in the receptor fluid, so that it is easier to quantify skin permeation using routine analytical methods. For example, U.S. Environmental Protection Agency (EPA) Protocols utilized Brij98 (PEG 20 oleyl ether) in receptor fluid to facilitate screening of various chemical compounds (EPA, 2009). Care should be taken in using other solubilizing agents, as they might damage skin barrier and lead to unreliable results.

2.3. Topical Drug Delivery Systems

Percutaneous penetration of chemical substances following topical skin

application depends not only on physicochemical properties of individual compounds, but also on composition of the vehicles in which they are incorporated. Skin formulations are capable of influencing the release of an active ingredient from its delivery system. An advanced skin delivery system may serve multiple purposes in modifying chemical penetration, improving therapeutic efficacy, controlling drug release, separating incompatible ingredients, stabilizing active ingredients, prolonging preparation shelf-life, minimizing skin irritation/sensitization, and creating desirable product aesthetics.

There are a variety of topical skin preparations available in commercial applications. Traditional topical formulations in cosmetics and pharmaceuticals range from simple liquid form, such as suspensions, to semisolid form, such as emulsions and hydrogels. Emulsion-based preparations have been found in a majority of healthcare skin products due to their excellent amphiphilic solubilizing properties and good consumer acceptance. Recently, some novel, advanced topical skin delivery technologies have also been developed and tested for cosmetic and pharmaceutical formulations. They include lipid systems, nanoparticles, microcapsules, polymers, and films, which may be further prepared into conventional dosages such as creams, liquids, hydrogels, and patches. In particular, these newer topical delivery technologies are promising for effective delivery of macromolecular drugs such as protein (van der Maaden, *et al.*, 2012), peptides (Abu Samah, *et al.*, 2011), and DNA (Choi, *et al.*, 2006).

2.3.1. Emulsions

Emulsions are heterogeneous disperse systems containing at least two immiscible phases, in which one phase is dispersed as globules (dispersed phase) in the other phase (continuous phase). The size of globules in emulsions ranges from 0.5-10 μm. When an oil phase is dispersed in an aqueous phase, the preparation is termed oil-in-water emulsion. Conversely, if an aqueous phase is dispersed in an oil phase, the preparation is known as water-in-oil emulsion.

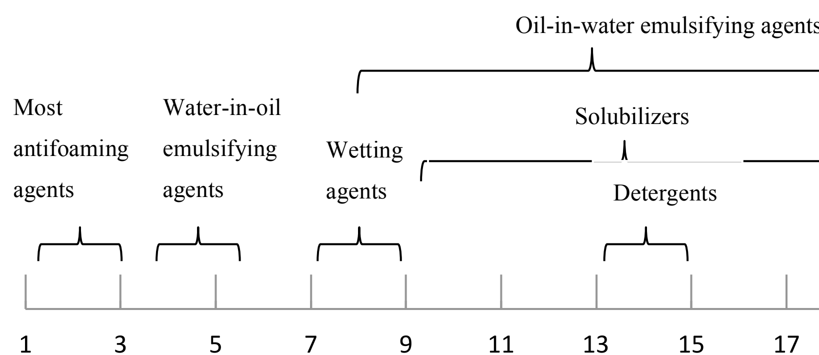
Emulsions are stabilized by the presence of a film formed at the interface between the oil phase and the water phase. The materials used to create such a film in emulsions are also known as emulsifying agents or emulsifiers. Emulsifying agents are the most important component in emulsions to achieve physical stability; they prevent coalescence of dispersed droplets in the preparations. Based on the stabilizing film formed at the interface of the two phases, emulsifying agents are classified into monomolecular, multilayer, and solid particle types (Martin, 1993). Emulsifying agents that form monomolecular films are usually highly surface-active, synthetic surfactants. They lower surface tension in proportion to their tendency to be adsorbed at the oil and water interfaces. Coalescence of the dispersed droplets is prevented by a coherent and flexible film and reduction of excess surface free energy. Multilayer emulsifying agents are mostly hydrophilic natural colloids, such as gelatin and acacia; they generate strong and rigid multilayer films at the oil and water interfaces. These ingredients stabilize an

emulsion through mechanical strength of films rather than negligible ability to lower interfacial tension. Multilayer emulsifying agents were extensively used in the past, but they have been gradually replaced by synthetic surfactants. Solid particle emulsifying agents create the film at the interfaces in the form of fine particles; they stabilize emulsions by producing steric hindrance at the interfaces. These ingredients have also been replaced recently by monomolecular emulsifying agents in emulsion preparations.

An emulsifying agent must exhibit strong adsorption at the interface of an oil phase and a water phase. This property requires a proper balance between hydrophilic and lipophilic tendency of a surfactant. When an emulsifying agent is predominantly hydrophilic, it tends to produce an oil-in-water emulsion. When a surfactant is predominantly lipophilic, it favors the formation of a water-in-oil emulsion (Martin, 1993). For example, sodium oleate is a good oil-in-water emulsifying agent, since it possesses a hydrophilic carboxyl group (-COO-) that predominates over the lipophilic hydrocarbon group ($C_{17}H_{33}$-). On the contrary, calcium oleate and other polyvalent soaps are predominately lipophilic in nature and hence selected to formulate water-in-oil emulsions. The hydrophilic-lipophilic balance of surface active agents was described by Griffin (Griffin, 1949; Griffin, 1954) in terms of the value of hydrophilic-lipophilic balance (HLB). The HLB values are scaled between 1-50. A surfactant with a higher HLB value is hydrophilic, whereas a compound with a low HLB value is lipophilic. Emulsifiers with high HLB values (8-18) tend to facilitate the formation of oil-in-water

emulsions, while emulsifiers with low HLB values (3-8) tend to form water-in-oil emulsions. **Figure 2.5** shows the most favorable range for various classes of surfactants in HLB scale (Atlas Chemical, 1963).

Figure 2.5. The atlas HLB system†



†The emulsifiers with HLB values (1-3) tend to be antifoam agents. The emulsifiers with low HLB values (3-8) tend to form water-in-oil emulsion. The emulsifiers with high HLB values (8-18) tend to form oil-in-water emulsions (Atlas Chemical, 1963).

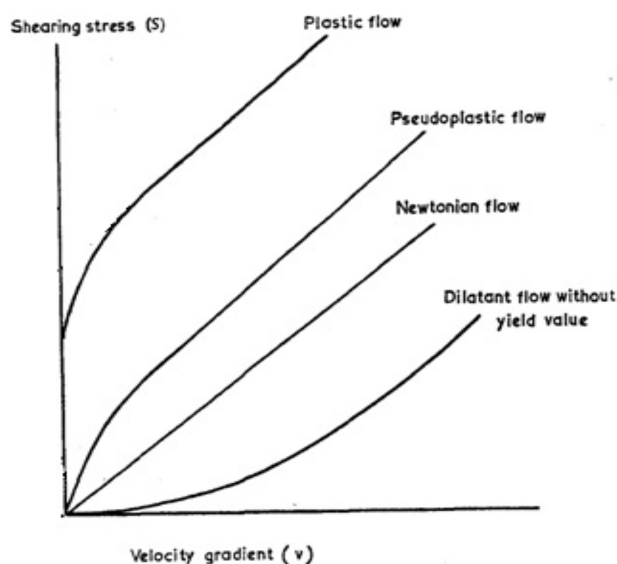
HLB value was closely associated with distribution of emulsifiers between an oil phase and a water phase in an emulsion (Lalor, *et al.*, 1995). Selection of an emulsifier (surfactant) and its distribution between oil and water phases play an important role in thermodynamic activity of a diffusant in the vehicle. Tween 60, an emulsifier with high HLB value, is usually used to form o/w emulsions, and it is strongly absorbed at the interface of the oil and water phase. Lalor *et al.* found that additional secondary

emulsifiers were mainly distributed into aqueous phase of an emulsion, in which it aggregated into micelles and solubilized the diffusants, methyl, ethyl and butyl p-aminobenzoate in aqueous phase, thereby reducing thermodynamic activity of the permeants. However solubility of a diffusant in oil phase of an oil-in-water emulsion was not apparently influenced by an emulsifier. Studies have revealed that thermodynamic activity of the external phase was the driving force in percutaneous permeation. Chemical permeation could be lowered by an emulsifier present in the water phase of an oil-in-water emulsion; similar results were observed in water-in-oil emulsions formulated with emulsifier Arlacel 83.

Rheology is the science of studying deformation and flow of matter. There are many important applications of rheology in pharmaceutical preparations. For example, pushing an injection through a hypodermic needle, pouring a suspension from a bottle, spreading a lotion on the skin, and squeezing a facial cleanser from a tube all involve rheological principles. Under rheology all liquid and semisolid materials can be classified into Newtonian systems or Non-Newtonian systems. Flow properties of a Newtonian system fit the Newton's Law, i.e., viscosity of the matter is dependent only upon composition and temperature, but is independent upon shear stress, shear rate, and duration of testing. The flow curve of a Newtonian system can be plotted using shear rate versus shear stress, which will yield a straight line going through the origin as shown in Figure 2.6. Non-Newtonian systems include plastic, pseudoplastic, and dilatant flow

bodies, also displayed in **Figure 2.6**. A plastic flow curve does not pass through the origin; the line intersects axis of shear stress at a certain point. A pseudoplastic flow curve has lower viscosity following increase in shear stress. On contrary, a dilatant flow curve has higher viscosity following increase in shear stress.

Figure 2.6. Types of flow behavior†



†The newtonian flow is a straight line going through the origin. Non-Newtonian systems include plastic, pseudoplastic, and dilatant flows displayed above (Sherman, 1968).

Emulsions in general exhibit non-Newtonian flow characteristics, except with emulsions in which the dispersed phase has a low concentration proportion (Buhidma, *et al.*, 1997). Emulsions may possess plastic or psuedoplastic flow properties accompanied by thixotropic properties (shear-thinning properties). For optimal topical application, emulsions should possess thixotropic characteristics; as such a lotion product could be

readily picked up from a container, spread smoothly on the skin, and form a stable and uniform film on skin surface. There are many formulation factors that would dictate rheological properties of an emulsion preparation. **Table 2.1** summarizes some common factors involved (Sherman, 1968). These factors may further influence percutaneous penetration of an active ingredient from emulation-based formulations (Li, *et al.*, 2011). Subsequently emulsion rheology is very important in clarifying correlation between formulation and percutaneous penetration and designing appropriate emulsion-based topical formulations.

2.3.2. Other Emulsion Forms

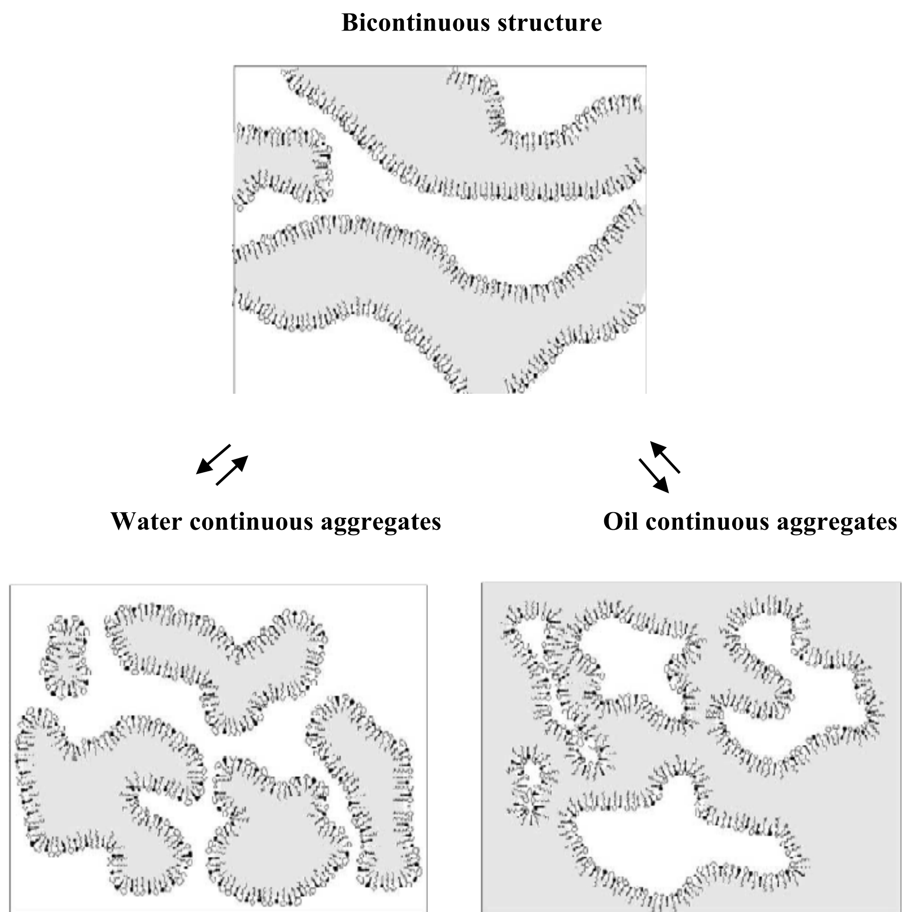
Compared to regular emulsions with droplet diameter of 1 μm and above, microemulsions are thermodynamically stable dispersions with droplet diameter around 100 nm. Microemulsions are single-phase systems composed of fused oil droplets and fused water droplets without a dispersed phase; this plausible transition occurs by increasing oil fraction or water fraction (**Figure 2.7**). They are thermodynamically stable with extremely low interfacial tension, and typically transparent due to tiny droplet size. Numerous studies have reported percutaneous penetration of drugs using topical microemulsion formulations; these microemulsion vehicles composed of different constituents (e.g., surfactants, oil phases, and water phases) all appeared to increase percutaneous penetration of active ingredients across the study membranes *in vitro*.

Table 2.1. Factors associated with emulsion ingredients that influence rheological behaviors†

Emulsion ingredients	Influencing Factors
Internal phase	(a) Volume concentration (ϕ); hydrodynamic interaction between globules; flocculation (b) Viscosity (η_i); deformation of globules in shear (c) Globule size and size distribution; interfacial tension between the two liquid phases; globule behavior in shear; interaction with continuous phase; globule interaction (d) Chemical constitution
Continuous phase	(a) Viscosity (η_o) (b) Chemical constitution; polarity; pH; potential energy of interaction between globules (c) Electrolyte concentration if polar medium
Emulsifying agent	(a) Chemical constitution; potential energy of interaction between globules (b) Concentration and solubility in internal and continuous phases; emulsion type; emulsion inversion; solubilization of liquid phase in micelles (c) Thickness of film adsorbed around globules; deformation of globules in shear; fluid circulation within globules (d) Electroviscous effect
Additional stabilizing agents	(a) Hydrocolloids; hydrous oxides; interfacial boundary region

†(Sherman, 1968)

Figure 2.7. Basic microemulsions†



†Microemulsions are formed by oil phase (grey), aqueous phase (white) and surfactant/co-surfactant interfacial film; the plausible transition between the structures occurs by increase of oil fraction or water fraction, respectively (arrows indicated) (Copyright Permitted by Elsevier at December 2013).

Dreher *et al.* developed a topical microemulsion using lecithin and compared it with isopropyl palmitate (IPP) vehicle for percutaneous penetration of two nonsteroidal anti-inflammatory drugs, diclofenac and indomethacin. Steady-state flux of both drugs from the microemulsion was significantly increased by 3-6 folds compared to the neat IPP vehicle, which was likely attributed to higher drug solubility in lecithin-based microemulsion. Drug loading in microemulsion was also much higher than IPP, 35 mg/ml for both indomethacin and diclofenac in the former, and 1.5 and 0.1 mg/ml for indomethacin and diclofenac in the latter (Dreher, *et al.*, 1997). Delgado-Charro *et al.* formulated a sucrose microemulsion using Labrasol, a novel low irritant surfactant, and Plurol Isostearique, a non-alcohol co-surfactant. Permeation rate of sucrose through mouse skin from the microemulsion was found to be significantly higher than an equivalent and saturated sucrose aqueous solution. It was concluded that microemulsion might possess good potential to deliver hydrophilic drug across the skin (Delgado-Charro, *et al.*, 1997).

Nanoemulsions are true emulsions that include a dispersed phase and a continuous phase; droplets in nanoemulsions are in the submicron range, after which the term nanoemulsion is named. The dispersed droplets in a nanoemulsion are so small that the gravity exerts little effect on them; therefore nanoemulsions are very stable as a dispersion system. Nanoemusions are considered effective delivery systems for the skin, because small droplets possess high surface areas and uniformly spread over rough skin

surface. The ability to hold hydrophobic compounds in oil phase also enhances solubilization of poorly water-soluble compounds and increases percutaneous penetration of the substances. Shakeel *et al.* prepared an oil-in-water nanoemulsion to investigate skin permeation and anti-inflammatory effect of aceclofenac in comparison to conventional gel (shakeel, *et al.*, 2007). Permeability parameters of aceclofenac in optimized nanoemulsion formulation significantly increased as compared with conventional aceclofenac gel. Anti-inflammatory effects of aceclofenac from nanoemulsion also increased for a 24-hour application period in rats. Components of the nanoemulsion apparently enhanced the skin penetration by extracting lipids from stratum corneum and denaturing keratin in stratum corneum (Shakeel, *et al.*, 2008). In another study Dixit *et al.* investigated skin penetration of carvedilol from nanoemulsion. Drug solubility was increased by 4500 folds when it was incorporated in nanoemulsion; steady state flux and permeation coefficient of carvedilol in nanoemulsion was significantly enhanced as compared to the control. Higher drug solubility in nanoemulsion, as well as similar characteristics of lipid extraction and keratin denaturation in stratum corneum, was attributed to percutaneous enhancement of carvedilol across the skin (Dixit, *et al.*, 2008).

Cationic emulsion systems can strongly interact with epithelial cells of the skin, which carry negative charges on the surface due to negatively charged residues of proteins in the cell membrane (Peira, *et al.*, 2008). As a result such a delivery system

improves percutaneous penetration, in particular for large molecules that are commonly less soluble in water. With cationic emulsions, an active ingredient is dissolved in the oil droplets surrounded by lipids of positive charges. The droplets are smaller than 200 nm in diameter, and form a colloidal suspension in water. The positively-charged droplets can easily penetrate negatively-charged cell membrane by electrostatic attraction, leading to an improved permeation of the drug embedded in the emulsion. In an *in vitro* transdermal delivery study, the roles of neutral and positively-charged surfactants in emulsions were investigated for skin permeation of fludrocortisone acetate and flumethasone pivalate (Hoeller, *et al.*, 2009). The tested neutral surfactants included sucrose laureate and polysorbate-80, and the positively-charged surfactant was phytosphingosine (PS). Emulsion made of positively-charged surfactant provided an enhancement factor in the range of 1.1-1.5 for fludrocortisone acetate and flumethasone pivalate as compared to the control. The penetration enhancement was attributed to the electrostatic attraction between positive droplets and negatively skin cell membrane. In another study using miconazole nitrate (MCZ) and positively-charged microemulsion, l-alanine benzyl ester (ALAB), an ester of a natural amino acid, was applied as a positively charge-inducing agent (Peira, *et al.*, 2008). Skin accumulation of MCZ from this microemulsion was nearly twice that from its negatively-charged counterpart. It was apparent that cationic microemulsions could be used to optimize skin penetration of drug substances.

2.3.3. Microcapsules, Microspheres and Microparticles

Techniques to encapsulate various active ingredients have been considerably investigated and developed for years. Microencapsulation is to prevent a core active ingredient from contacting external environment by using protective coating. Microcapsules, microspheres, or microparticles are hence defined as substantial spherical colloidal systems with particle diameters in the range of 10 nm to 2 mm, with liquid or solid active ingredients being encapsulated inside polymeric or waxy material. Depending on microencapsulation methods, microparticles are categorized in two types, i.e., shell system and matrix system. The former has active ingredients surrounded by one or more layers of coating, while the latter has active ingredients dissolved or suspended within a matrix material. Various preparation methods have been successfully utilized. The coacervation method has been used commonly and frequently (Bakan, *et al.*, 1978). The liquid phase wraps as a uniform layer around suspended core particles. Solidification of coating material is achieved by means of heating, cross-linking, or solvent removal techniques. Microcapsules are collected by centrifugation or filtration, and then dried by techniques like fluid-bed drying or spray drying, to yield free flowing particles.

Microencapsulating active components for topical preparations is a very common approach to improve product safety and minimize skin adverse effects through reducing drug release amount at the application site. For example, sunscreens should act on the skin surface and generate minimal percutaneous penetration to prevent possible adverse

risks. Lipid microparticles are particularly an appropriate vehicle for sunscreens. They exhibited high loading capacity for lipophilic ingredients like sunscreens, and proper particle size for reduced skin penetration (Toll, *et al.*, 2004; Yener, *et al.*, 2003). Scalia *et al.* evaluated a microparticle loaded with the UVB filter ethylhexyl methoxycinnamate (EHMC) and the UVA filter butyl methoxydibenzoylmethane (BMDBM) for the effect on percutaneous penetration. Microparticles were composed of stearic acid or glyceryl behenate as lipid material, hydrogenate phosphatidylcholine as surfactant and distilled water containing 1% (w/w) hydrogenated soybean phosphatidylcholine. The encapsulated UV filters showed a significantly lower skin penetration *in vivo* than the nonencapsulated UV filters. The reduced percutaneous penetration of BMDBM and EHMC achieved by these lipid microparticles preserved the UV filter efficacy and limited potential toxicological risks (Scalia, *et al.*, 2011). Microencapsulation was also applied to reduce skin penetration of the insect repellent citronella oil. Solomon *et al.* prepared a microcapsule by using gelatin coacervation method. The microcapsule consisted of citronella oil, gelatin as surfactant, and distilled water with sodium sulfate solution (20% w/v). Citronella oil in microcapsules decreased its membrane permeation by at least 50% compared to the PEG base (Solomon, *et al.*, 2012). Therefore, microencapsulation was especially beneficial for topical preparation development because it was able to provide a protective barrier for the active components and to improve and overcome issues regarding use safety, efficacy, and stability.

2.3.4. Nanotechnology

Nanotechnology is considered the next "Industrial Revolution" according to government research and development planners. Nanoparticles are defined by the National Nanotechnology Initiative as particles with dimensions between 1 nm and 100 nm (Lovestam, *et al.*, 2010; National Nanotechology Initiative, 2010). Nanoparticles are subdivided into lipid nanoparticles and non-lipid nanoparticles based on the encapsulating membrane structure. Lipid nanoparticles are usually called liposomes. Their encapsulating membranes are made up of lipid. Liposomes were introduced in the early 1980s as a novel drug delivery system. More attention has been paid to liposomes since 1980's to facilitate skin transportation of active ingredients. Liposomes enhanced skin deposition via transdermal route and/or skin appendages, reduced systemic adsorption of the chemicals, and minimized possible side effects. The first developed liposome system was a dipalmitoylphosphatidylcholine (DPPC) and cholesterol vesicle. The system was able to increase drug concentration in epidermis and dermis by 4 times as compared to standard treatment (Pegoraro, *et al.*, 2012). Recently, ultradeformable liposomes were developed and studied as a transdermal drug delivery system. This novel liposome system maintained its aggregate stability and was flexible, which facilitated drug penetration through the skin and into deeper tissue layers. In general ultradeformable liposomes were composed of a surfactant component of 10–25% and an ethanol content of 3–10%. Compared to conventional liposomes, they had higher alcohol

content, such as that found in ethosomes (Barupal, *et al.*, 2010). The high alcohol content was capable of fluidizing lipids of the stratum corneoum and increaseing penetration of the liposome vesicles. When the liposomes were applied to the skin surface, they would deform and squeeze across very fine pores of skin, subsequently reaching epidermis and dermis for systemic delivery (Barry, 2001; Cevc, *et al.*, 2003; Massignani, *et al.*, 2010).

Non-lipid nanoparticles are commonly referred to as polymeric nanoparticles, also known as polymersomes. The encapsulating membranes are composed of high molecular weight amphiphilic block copolymers. Polymersomes have been utilized to encapsulate or adsorb various drug substances, such as proteins, vaccines, genes, etc. Moreover, they are also deformable more or less, thereby reaching deeper skin layers and delivering drugs to desired sites at appropriate skin permeation rate (Cevc, *et al.*, 1998; Christian, *et al.*, 2009). By using Dynamic Light Scattering (DLS) Battaglia *et al.* showed that polymersomes deformed sufficiently to penetrate pores that were up to 8 times smaller than the size of polymersomes without fragmenting (Battaglia, *et al.*, 2006). In comparison, conventional liposomes only crossed pores that were half of the liposome size (Cevc, *et al.*, 1998). When polymersome formulation was applied to *ex vivo* human skin, permeation of fluorescently labeled dextran was increased by at least 10 times compared to conventionally passive diffusion (Pegoraro, *et al.*, 2012).

Solid lipid nanoparticles (SLNs) were developed in the early 1990's as a novel nanoparticle delivery system to alternate emulsions, liposomes, and polymeric

nanoparticles. SLNs are an aqueous colloidal dispersion with particles in the range of 50-1000 nm. The particles are composed of biodegradable lipids and drug compounds. A drug is located in the biodegradable lipid matrix with a dissolved state in solid solution. SLNs are produced by high-pressure homogenization and emulsification of melted lipids/glycerides, such as glyceryl behenate, soya lecithin, medium-chain triglycerides, and block copolymer. During cooling stage, the emulsified molten lipids will crystallize and produce particles with a solid core of glycerides surrounded by soft layers of phospholipids and steric stabilizers. Crystalline active ingredients are stored in the lamellar structures.

SLNs are physically stable not only in aqueous dispersions but also with incorporation into dermal creams. When a dermal cream of SLNs is applied to the skin surface, the ultrafine SLN particles can form an adhesive film and subsequently produce an occlusive effect on the skin. Vitamin A palmitate had been formulated into SLNs by Pople and Singh (Pople, *et al.*, 2006). Using *in vitro* diffusion study, drug release from SLNs and gel control was 67.5% and 54.4% respectively. After evaluating the effects of SLN treatment on thickness of stratum corneum in rats, it was found that SLN treatment resulted in an increase of thickness in stratum corneum by almost 3 times as compared to conventional gel control. The increase in thickness was likely to be attributed to hydration effect from SLNs, which would induce higher skin permeation of drugs from SLNs.

SLNs had also been used to topically deliver anti-inflammatory and antifungal drugs. Kuntsche *et al.* investigated SLNs to deliver corticosterone to the skin using excised human skin and rat epidermal keratinocyte organotypic culture model in vertical Franz diffusion cells (Kuntsche, *et al.*, 2008). Cumulative skin permeation of SLNs and PBS (Phosphate Buffered Saline) control was 0.1% and 1.5% in human epidermis, and 0.2% and 0.7% in rat epithelial keratinocyte organotypic culture. In another study, Sanna *et al.* loaded antifungal econazole nitrate into SLNs (Sanna, *et al.*, 2007). Cumulative skin permeation of econazole nitrate from SLN-containing gel into porcine skin was 48.5±0.8 $\mu g/cm^2$ for a 24-hour period, while that of non-SLN-containing gel was 124.7±21.6 $\mu g/cm^2$. These results demonstrated a lower skin permeation by SLNs, possibly attributed to an effect by SLNs to release drug from outer particle surface but to retain drug content within the SLN cores (Muller, *et al.*, 2001; Muller, *et al.*, 2007; Souto, *et al.*, 2004).

2.4. Conclusion

Skin is the largest organ of the human body; its major function is to provide an important protective barrier to separate internal organs from external environment. Stratum corneum, as the outmost layer of the skin, is the principal rate-limiting barrier against percutaneous penetration of any chemical substance. No molecule can pass across stratum corneum readily, but skin permeation is possible, especially for small molecules

with molecular weight under 500 Dalton and appropriate partition coefficient. Passively diffusion from a formulation to skin surface and then into skin layers constitutes percutaneous absorption, which can be further categorized into two routes, i.e., transepidermal route and transfollicular route.

In vitro diffusion experimentation is one of the most practical and cost-effective approaches to characterize percutaneous penetration. Fundamental theories and experimental settings have been extensively explored and well established. Various parameters can influence accuracy and reproducibility of diffusion experiments. For topical skin formulations, one of the critical evaluation criteria is to evaluate their percutaneous permeation across the skin membrane. Conventional preparations such as emulsions are excellent vehicles for topical skin products. Some novel, advanced topical skin delivery technologies have also been developed recently, including microcapsules, microspheres, liposomes, polymersomes, and SLNs. These particles may be further prepared into conventional dosages such as creams, liquids, and hydrogels. It is believed that more new topical skin preparations will be available in the future to improve patient compliance and provide product options.

CHAPTER 3

Hypotheses and Objectives

Mosquito infestation during summer months has always been a widespread problem in the province of Manitoba; people participating in outdoor farming or recreational activities normally have to apply insect repellents in order to minimize assaults from mosquitoes and other biting insects. With the arrival of the West Nile virus in North America since 1999 (CDC, 2010) and its widespread dissemination by mosquitoes, mosquito infestation has now become an imminent health hazard for the general public (Pile, 2001). Skin cancer is another common disease closely associated with summer outdoor activities. Over 60,000 Canadians develop skin cancer each year, and this disease is increasing at an epidemic rate of approximately 5% each year in the world (Canadian Dermatology Association, 2014). UV rays from sunlight have been identified as causing more than 90 % of all skin cancers, and chronic exposure to sunlight for an extended period of time is the source of 95% of all basal cell carcinomas (Guide to clinical preventive services). Numerous studies have proven that skin damage from sunlight exposure builds up over the time, regardless of whether sunburn actually occurs. Therefore, application of repellent and sunscreen products has been widely accepted by the general public as an effective and practical approach to minimize vector-borne diseases and skin damage.

DEET has been the most popular broad-spectrum insect repellent for decades (Qiu, *et al.*, 1998); it is one of effective active ingredients in over 90% commercial insect repellent (Staub, *et al.*, 2002). OBZ is a principal UVA/UVB sun-blocking ingredient in

most commercial sunscreens; OBZ is also available in various cosmetic products including lipsticks, moisturizers, facial cleansers, shampoos and conditioners to supply additional protecting and stabilizing effects. Nevertheless, few studies have investigated percutaneous aspects of concurrent application of DEET and OBZ in the past. Gu *et al.* reported the first study of transdermal absorption from combined use of mosquito repellent DEET and sunscreen OBZ; it was found that enhanced permeation of DEET and OBZ across piglet skin and PDMS, when both compounds were simultaneously incorporated in propylene glycol, ethanol and poly(ethylene glycol) (Gu, *et al.*, 2004). Experiments have also revealed a synergistic absorption between the two components in different application sequences, different application proportions, and different formulations through pigskin (Gu, *et al.*, 2005) and artificial membranes (Wang, *et al.*, 2006), suggesting potential safety concerns from concurrent application of sunscreen and DEET products in susceptible subjects. Therefore, this thesis focused on developing and optimizing emulsion-based lotion preparations, testing them *in vitro* and *in vivo*, in order to minimize synergistic skin permeation of DEET and OBZ.

It was hypothesized with this thesis that,

(1). Appropriate formulation processing and selection of proper excipients would minimize overall transdermal absorption of DEET and OBZ from concurrent application of repellent and sunscreen products;

(2). Repeated topical applications of repellent DEET and sunscreen OBZ would

result in accumulation of the chemicals *in vivo*, but appropriate formulation would reduce percutaneous penetration of DEET and OBZ.

The objectives of the research were,

(1). To develop new emulsion-based lotions that could reduce overall transdermal permeation of the active repellent and sunscreen ingredients;

(2). To evaluate the developed preparations using diffusion experimentation and in an animal model;

(3). To understand mechanisms of percutaneous enhancement between DEET and OBZ, and possible influences from lotion ingredients.

CHAPTER 4

Formulation Preparations

4.1. Introduction

Insect repellents and sunscreens are common consumer care products that are applied to the skin surface to prevent humans from vector-borne diseases and skin cancers (Cokkinides, *et al.*, 2004; Dennis, *et al.*, 2003; Fradin, *et al.*, 2002; Staub, *et al.*, 2002). There are numerous repellents and sunscreens commercially available to the general public, in a variety of formulation types such as sprays, creams, lotions, sticks, foams, and towelettes (EPA, 1998; FDA, 2012). Use concentrations of active ingredients in repellent and sunscreen preparations are also variable and different. So far research and development of many consumer care products has been mainly focused on application convenience and effectiveness, very few studies have investigated potential side effects from skin application of these products and subsequent skin absorption of the active ingredients.

Under ideal use situation, active ingredients like DEET and OBZ should exert minimal transdermal absorption and systemic disposition from topical preparations. However, both DEET and OBZ are capable of permeating across the skin and reaching general circulation after skin applications. Permeation rate and degree of DEET and OBZ across the skin were also dependent upon the dissolving vehicles (Gu, *et al.*, 2004) or the preparation types of commercial products (Gu, *et al.*, 2005). This led to the study hypothesis that different formulation strategies might produce DEET and OBZ preparations that would reduce skin permeation and retention of the active ingredients

and consequently minimize their systemic disposition from topical skin application.

Appropriate formulation strategies are capable of modifying percutaneous permeation characteristics of the active ingredients from a particular preparation. For example, cyclodextrins was proven effective in reducing DEET release from the encapsulation, hence minimizing overall percutaneous permeation (Kasting, *et al.*, 2008; Proniuk, *et al.*, 2002;). On the other hand, transdermal permeation of OBZ increased as concentration of hydroxypropyl-β-cyclodextrin was increased from 0 to 10% (Qiu, *et al.*, 1997). Insect repellent DEET and sunscreen OBZ are commonly formulated as semisolid emulsion-based products because emulsions possess excellent solubilizing properties for both lipophilic and hydrophilic compounds (Förster, *et al.*, 1998). In this study, several emulsion-based preparations were prepared using two different preparation methods, i.e., incorporation method and fusion method. DEET and OBZ were incorporated into these formulas either as individual ingredient or as combined components. Various physical characteristics of the prepared formulations, such as firmness, consistency, cohesiveness, index of viscosity, and thixotropic property, were measured and compared using texture analyzer and rheometer. The purpose of the preparation was to optimize emulsion-based formulations containing repellent DEET and sunscreen OBZ in order to minimize skin penetration and systemic disposition of the two active ingredients. The two preparation methods were also evaluated to prepare a quality final formula for further testing in an animal model.

4.2. Materials

Pure DEET (N,N-diethyl-*m*-toluamide) was purchased from Fluka Chemika GmbH (Buchs, Switzerland), and OBZ (2-hydroxy-4-methoxybenzophenone) was purchased from Riedel-de Haën Laborchemikalien GmbH **(**Seelze**,** Germany)**.** Some of the ingredients used in the formulations, including Emulium 22® (tribehenin PEG-20 esters), MOD™ (octyldodecyl myristate), Emulfree CBG (butylene glycol cocoate, ethyl cellulose, and isostearic alcohol), Labrasol (caprylocaproyl macrogolglycerides), Precirol Ato 5 (glyceryl palmitostearate), Geleol (glyceryl monostearate 40-55), Apifil® (PEG-8 beeswax), Transcutol HP (diethylene glycol monoethyl ether), Labrafil M1944CS (polyoxylglycerides), Isostearate isostearyle, Plurol oleique CC 497 (polyglyceryl oleate), Compritol 888 (glyceryl behenate), Plurol diisostearique (polyglyceryl diisostearate), and Labrafac™ PG (propylene glycol dicaprylocaprate) were supplied by Gattefossé Canada, Inc (Toronto, Ontario, Canada). Ritadecene 50 (hydrogenated polydencene) was provided by RITA (Woodstock, IL, USA). Both cetyl alcohol and xanthan gum were purchased from Medisca Pharmaceutique Inc. (Montreal, Canada). Glycerin and mineral oil was obtained from Mallinckrodt Specialty Chemical Company (Paris, Kentucky, USA). Sodium hydroxide and castor oil were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Arlacel P135 was supplied by UNIQEMA (New Castle, DE, USA). Cabomer 934 was obtained from BF Good-rich-company, Chemical Group (Cleveland

Ohio, USA). Deionized water was purified by a Milli-Q® Pure Water System (Millipore, Nepean, ON, Canada) in the laboratory.

4.3. Methods

4.3.1. Formulations with Different Preparation Methods

4.3.1.1. Preparation Methods

Three different emulsions were prepared by using the incorporation method outlined in **Tables 4.1-4.3**. In the preparation of Formulation A1 (FA1), Cabomer 934 was left at room temperature for natural swelling in water for 24 hours. Both Phase I (oil phase) and Phase II (water phase) were initially heated to 75°C at 500rpm. Phase I was then poured into Phase II at 1500rpm. After agitation for 15 minutes, 0.5 ml 10% sodium hydroxide was added into the mixture for pH adjustment. The mixture was further agitated until congealed. Once emulsion had been formed, 7.5% DEET, 5% OBZ or 7.5% DEET/5% OBZ were respectively mixed into this emulsion base at 1500rpm, and the final formulations were cooled down to room temperature. Preparation procedures for Formulation B1 (FB1) were similar to those of FA1, except that xanthan gum was also added into the formulation as a thickening agent. Formulation C1 (FC1) was a water-in-oil emulsion. In preparing FC1, Phase I (oil phase) and Phase II (water phase) were initially heated to 75°C under agitation. Phase II was then added to Phase I. Agitation was kept until the mixture was cooled down to room temperature; 7.5% DEET,

5% OBZ or 7.5% DEET/5% OBZ were then mixed into the emulsion respectively.

Three other emulsions were prepared by using the fusion method (**Tables 4.4-4.6**), i.e., OBZ was added into the oil phase and heated to 75°C together with other oil components, while DEET was added during congealing of the emulsion base. Agitation speed and duration, DEET and OBZ concentrations were maintained the same as the incorporation preparation method.

All finished formulations were stored in sealed containers and placed at room temperature for one month. Their physical characters were observed and recorded on the last day of the storage.

4.3.1.2. Droplet Size Distribution

The droplet surface morphology of the emulsions prepared by using the fusion method was observed and recorded with an optical microscope fitted with a digital camera (James Swift MP3502, Prior Scientific Instruments LTD). A drop of the diluted emulsion was placed on a microscope slide, and a piece of glass placed over the drop. Observations were made afterwards.

The droplet size of the emulsions prepared by using the fusion method was also measured under a Malvern Mastersizer 2000s (Malvern Instruments Ltd., Malvern, Worcestershire, United Kingdom). In case of laser diffraction measurements, one drop of the oil-in-water emulsion was suspended in the dispersion accessory with distilled water,

Table 4.1. Formulation A1 with incorporation preparation method

Ingredients	Formulation A1 Oil in Water (23/77)		
	A	**b**	**c**
I (Oil phase)			
Emulfree CBG	4%	4%	4%
Geleol	2%	2%	2%
Labrasol	8%	8%	8%
Cetyl Alcohol	4%	4%	4%
II (Water phase)			
Deionized Wate/	QS	QS	QS
Glycerin	5%	5%	5%
Carbomer 934	0.25%	0.25%	0.25%
III			
Sodium Hydroxide Sol (10%)	0.5%	0.5%	0.5%
Blank Gel basis	92.5%	95%	87.5%
OBZ	---	5%	5%
DEET	7.5%	--	7.5%

a: Formulation containing DEET

b: Formulation containing OBZ

c: Formulation containing DEET and OBZ

Table 4.2. Formulation B1 with incorporation preparation method

	Formulation B1 Oil in Water with thickening agent (23/77)		
Ingredients	**a**	**b**	**c**
I (Oil phase)			
Emulfree CBG	4%	4%	4%
Geleol	2%	2%	2%
Labrasol	8%	8%	8%
Cetyl Alcohol	4%	4%	4%
II (Water phase)			
Deionized Wate	QS	QS	QS
Glycerin	5%	5%	5%
Carbomer 934	0.25%	0.25%	0.25%
Xanthan gum	0.4%	0.4%	0.4%
III			
Sodium Hydroxide Sol (10%)	0.5%	0.5%	0.5%
Blank Gel basis	92.5%	95%	87.5%
OBZ	---	5%	5%
DEET	7.5%	--	7.5%

a: Formulation containing DEET

b: Formulation containing OBZ

c: Formulation containing DEET and OBZ

Table 4.3. Formulation C1 with incorporation preparation method

	Formulation C1 Water in Oil (40/60)		
Ingredients	**A**	**b**	**c**
I (Oil phase)			
Arlacel P135	3%	3%	3%
Geleol	3%	3%	3%
Labrasol	7%	7%	7%
Cetyl Alcohol	10%	10%	10%
Labrafac PG	8%	8%	8%
Isostearate isostearyle	5%	5%	5%
Plurol oleique CC 497	5%	5%	5%
Mineral oil	9%	9%	9%
Castor oil	5%	5%	5%
II (Water phase)			
Deionized water	QS	QS	QS
Glycerin	5%	5%	5%
Blank emulsion basis	92.5%	95%	87.5%
OBZ	---	5%	5%
DEET	7.5%	--	7.5%

a: Formulation containing DEET

b: Formulation containing OBZ

c: Formulation containing DEET and OBZ

Table 4.4. Formulation A2 with fusion preparation method

	Formulation A2 Oil in Water (23/77)		
Ingredients	**a**	**b**	**c**
I (Oil phase)			
Emulfree CBG	4%	4%	4%
Geleol	2%	2%	1%
Labrasol	5.5%	8%	3.5%
Cetyl Alcohol	4%	4%	2%
OBZ	--	5%	5%
II (Water phase)			
Deionized Water	QS	QS	QS
Glycerin	5%	5%	5%
Carbomer 934	0.25%	0.25%	0.25%
III			
Sodium Hydroxide Sol (10%)	0.5%	0.5%	0.5%
IV			
DEET	7.5%	--	7.5%

a: Formulation containing DEET

b: Formulation containing OBZ

c: Formulation containing DEET and OBZ

Table 4.5. Formulation B2 with fusion preparation method

	Formulation B2 Oil in Water with thickening agent (23/77)		
Ingredients	**A**	**b**	**c**
I (Oil phase)			
Emulfree CBG	4%	4%	4%
Geleol	2%	2%	1%
Labrasol	5.5%	8%	3.5%
Cetyl Alcohol	4%	4%	2%
OBZ	--	5%	5%
II (Water phase)			
Deionized Water	QS	QS	QS
Glycerin	5%	5%	5%
Carbomer 934	0.25%	0.25%	0.25%
Xanthan gum	0.4%	0.4%	0.4%
III			
Sodium Hydroxide Sol (10%)	0.5%	0.5%	0.5%
IV			
DEET	7.5%	**--**	7.5%

a: Formulation containing DEET

b: Formulation containing OBZ

c: Formulation containing DEET and OBZ

Table 4.6. Formulation C2 with fusion preparation method

	Formulation C2 Water in Oil (40/60)		
Ingredients	**a**	**b**	**c**
I (Oil phase)			
Arlacel P135	3%	3%	3%
Geleol	3%	3%	3%
Labrasol	7%	7%	5.5%
Cetyl Alcohol	10%	10%	10%
Isostearate isostearyle	5%	5%	5%
Labrafac PG	8%	8%	6%
Plurol oleique CC 497	5%	5%	4%
Mineral oil	7%	9%	7%
Castor oil	4.5%	5%	4%
OBZ	--	5%	5%
II (Water phase)			
Deionized water	QS	QS	QS
Glycerin	5%	5%	5%
III			
DEET	7.5%	--	7.5%

a: Formulation containing DEET

b: Formulation containing OBZ

c: Formulation containing DEET and OBZ

which was continuously homogenized at 12,000 rpm and ultrasonicated for 90 seconds prior to measurement of the particle size. For water-in-oil emulsions, acetone as a dispersant was added to the dispersion accessory under identical conditions of homogenization and ultrasonication. Correction measurements were also taken to compensate for background electrical noise and laser scattering interferences from optics or from samples. Three replicates were run for each emulsion sample. A typical analysis interval was 3 minutes per sample after optical alignment and background measurement. Raw data was analyzed using Malvern software; the mean droplet diameter was mathematically expressed as the following equation (Horiba Scietific, 2002),

$$D[4,3] = \frac{\sum Di^4 Ni}{\sum Di^3 Ni} \qquad \text{Equation 4.1}$$

where D[4,3]: the volume weighted mean

Di: the diameter of particles

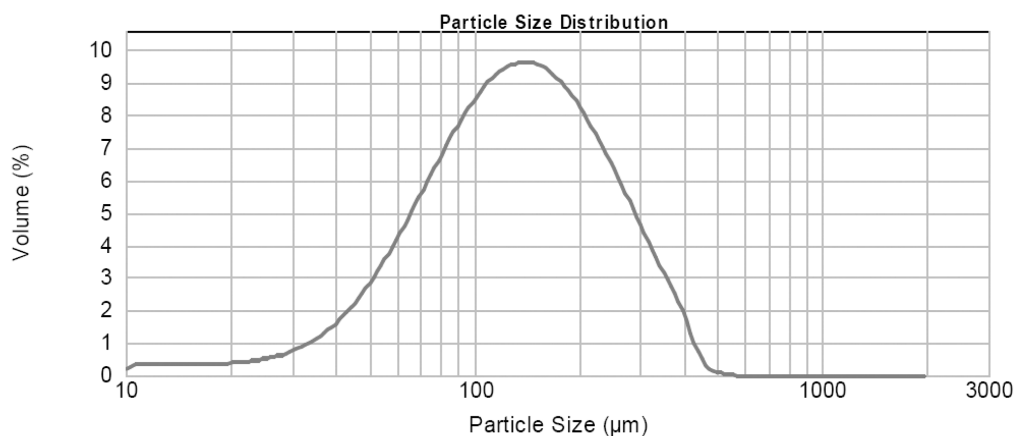
Ni: the number of particles

The particle size distribution was indicated by frequency curve, shown in **Figure 4.1**. The values of d(0.1), d(0.5), and d(0.9) were collected to describe the particle distribution, where d(0.5) was the volume median diameter of droplets, meaning 50% of the particle distribution below this value and 50% above; d(0.1) indicated 10% of particle

distribution below this value; d(0.9) meant 90% of particle distribution below this value. The particle distribution span was calculated using the following equation (Horiba Scietific, 2002),

$$\text{Span} = \frac{[d(0.9) - d(0.1)]}{d(0.5)} \qquad \text{Equation 4.2}$$

Figure 4.1. Particle size distribution†



†The size distribution of oil droplets or water droplets in emulsions (oil-in-water or water-in oil) was indicated by the frequency curve with the bell shape. The values of d(0.1), d(0.5), and d(0.9) were calculated from the curve to describe the particle size distribution span.

4.3.2. Formulations with Five Different Emulsifiers

4.3.2.1. Preparation Method

Five emulsion-based formulations were prepared using five different emulsifiers, and their formulation designs are listed in **Tables 4.7-4.11**. Formulation #1 (F#1) was an oil-in-water bi-gel system. To prepare F#1, Cabomer 934 and xanthan gum were allowed to swell in water for 24 hours at room temperature. Phase I (oil phase) containing OBZ and Phase II (water phase) were heated to 75°C at 300 rpm. Phase I was then added to Phase II at 75°C and 1200 rpm using a kitchen-style blender for 15 minutes. 0.5 ml 10% sodium hydroxide was added to the mixture, and the components were further agitated at 1200 rpm for another 15 minutes. The agitation speed was lowered down to 1000 rpm while the mixture was cooled down. At the end, 7.5% DEET was added to the mixture at 1000 rpm. The agitation was continued until the mixture was cooled to room temperature. Texture properties of the formulations were immediately measured.

Formulation #2 (F#2) and Formulation #3 (F#3) were oil-in-water emulsion-based formulations containing emulsifier Emulium 22 or Apifil, respectively. To prepare F#2, both Phase I and Phase II were heated to 75°C at 300 rpm agitation. Afterwards Phase I was added to Phase II at 75°C and 1200 rpm for 15 minutes. The agitation speed was then reduced to 1000 rpm until the mixture was cooled down to room temperature. According to the requirement of formulation design, 7.5% DEET was added before the mixture congealed. Texture properties of the formulation were measured immediately. To

prepare F#3, Phase I and Phase II were also heated to 75°C at 300rpm. Phase I was then added to Phase II at 75°C and 800 rpm for 15 minutes. Agitation speed was reduced to 500 rpm for another 15 minutes. According to the requirement of formulation design, DEET was added at 500 rpm and room temperature.

Formulation #4 (F#4) and Formulation #5 (F#5) were water-in-oil emulsion-based formulations with emulsifier Arlacel P-135 or Plurol diisostearique, respectively. Preparation procedures of these two formulations were identical. Both Phase I and Phase II were heated to 75°C at 300 rpm. Phase II (water phase) was added to Phase I (oil phase) at 75°C and 800 rpm for 15 minutes. The mixture was cooled down at 500 rpm for 15 minutes. 7.5% DEET was added to the mixture at 500rpm according to requirement of formulation design. The agitation continued at 500rpm until room temperature.

4.3.2.2. Texture Analysis

A TA.XTPlus Texture Analyzer was used to measure physical properties of these five emulsion-based formulations. The preparation was placed in a container (40mm diameter × 4cm height) for each measurement. A P/25P-25 mm diameter perspex cylinder probe was selected for the testing. The probe moved down at a speed of 1.0 mm/s at the beginning of testing. The trigger force and penetration distance of probe were 15 g and 5 mm, respectively. The probe moved up at the speed of 10mm/s after testing.

Table 4.7. Formulation #1 with emulsifying agent Emulfree CBG

	Formulation #1 Oil in Wanter (23/77)		
Ingredients	**a**	**b**	**c**
I (Oil phase)			
Emulfree CBG	4%	4%	4%
Geleol	2%	2%	2%
Labrasol	9.5%	12%	4.5%
OBZ	--	5%	5%
II (Water phase)			
Deionized Water	QS	QS	QS
Glycerin	5%	5%	5%
Carbomer	0.25%	0.25%	0.25%
Xanthan Gum	0.15%	0.15%	0.15%
III			
Sodium Hydroxide 10%	0.5%	0.5%	0.5%
IV			
DEET	7.5%	--	7.5%

a: Formulation containing DEET

b: Formulation containing OBZ

c: Formulation containing DEET and OBZ

Table 4.8. Formulation #2 with emulsifying agent Emulium 22

	Formulation #2 Oil in Water (30/70)		
Ingredients	**a**	**b**	**c**
I (Oil phase)			
Emulium 22	6%	6%	6%
Geleol	2%	2%	2%
MOD	5%	6%	4%
Labrafil 1944	7.5%	9%	3.5%
Precirol Ato 5	2%	2%	2%
OBZ	--	5%	5%
II (Water phase)			
Deionized Water	QS	QS	QS
Glycerin	12%	12%	12%
III			
DEET	7.5%	--	7.5%

a: Formulation containing DEET

b: Formulation containing OBZ

c: Formulation containing DEET and OBZ

Table 4.9. Formulation #3 with emulsifying agent Apifil

	Formulation #3 Oil in Water (30/70)		
Ingredients	**a**	**b**	**c**
I (Oil phase)			
Apifil	4%	4%	4%
Geleol	2%	2%	2%
Labrafac PG	4%	4%	4%
Hydrogenated Polydencene	6%	7%	3.5%
Transcutol HP	6.5%	8%	4%
OBZ	--	5%	5%
II (Water phase)			
Deionized Water	QS	QS	QS
Xanthan Gum	0.15%	0.15%	0.15%
Glycerin	5%	5%	5%
III			
DEET	7.5%	--	7.5%

a: Formulation containing DEET

b: Formulation containing OBZ

c: Formulation containing DEET and OBZ

Table 4.10. Formulation #4 with emulsifying agent Arlacel P-135

	Formulation #4 Water in Oil (40/60)		
Ingredients	**a**	**b**	**c**
I (Oil phase)			
Arlacel P-135	5%	5%	5%
Geleol	2%	2%	2%
LabrafilM1944CS	14.5%	17%	12.5%
Plurol Oleique CC 497	11%	11%	8%
Isostearate Isostearyle	10%	10%	10%
Compritol 888	5%	5%	5%
Transcutol HP	5%	5%	5%
OBZ	--	5%	5%
II (Water phase)			
Deionized Water	QS	QS	QS
Glycerin	5%	5%	5%
III			
DEET	7.5%	--	7.5%

a: Formulation containing DEET

b: Formulation containing OBZ

c: Formulation containing DEET and OBZ

Table 4.11. Formulation #5 with emulsifying agent Plurol Diisostearique

	Formulation #5 Water in Oil (40/60)		
Ingredients	**a**	**b**	**c**
I (Oil phase)			
Plurol Diisostearique	5%	5%	5%
Geleol	2%	2%	2%
Labrafil M 1944 CS	11.5%	11.5%	8.5%
Plurol Oleique CC 497	14%	16.5%	12%
Isostearate Isostearyle	10%	10%	10%
Compritol 888	5%	5%	5%
Transcutol HP	5%	5%	5%
OBZ	--	5%	5%
II (Water phase)			
Deionized Water	QS	QS	QS
Glycerin	5%	5%	5%
III			
DEET	7.5%	--	7.5%

a: Formulation containing DEET

b: Formulation containing OBZ

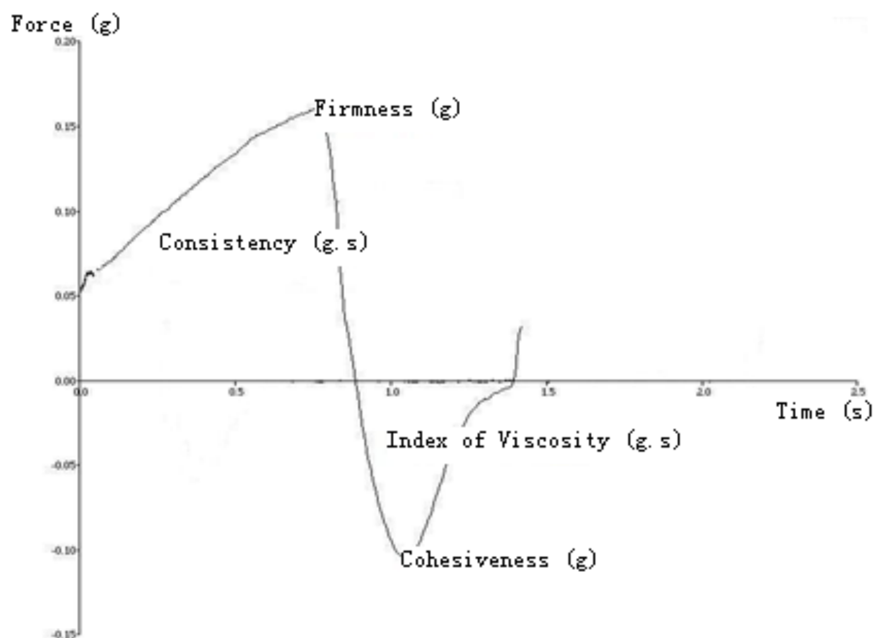
c: Formulation containing DEET and OBZ

Each sample was tested a minimum of three times at room temperature. The TA instrument recorded the resistant force of probe generated from formulations and the distant travelled by probe during testing. The physical properties of formulations, including firmness, consistency, cohesiveness, and index of viscosity were calculated from a typical texture analyzer graph (**Figures 4.2**). As shown in **Figure 4.2**, firmness (positive peak) presented the maximum resistance force to external stress/pressure in a formulation, while consistency (the area under the positive curve) was the work performed by the resistance force during the sustained action of external stress/pressure. Cohesiveness (negative peak) was defined as the minimum resistance force to external strain, while index of viscosity (area under the negative curve) was the work performed by the resistance force changes during the sustained action of external strain. These physical parameters simply and accurately provided the same trend in the deformation ability of semisolid pharmaceutical products under the external forces.

4.3.2.3. Rheological Behavior

Rheological behavior of the five emulsion-based formulations was measured by an AR 1000 Rheometer under the following experiment conditions: temperature 25°C, tested sample weight 0.5 g, and steel cone spindle with 1°59’18” angel and 20mm diameter. The rotation speed was gradually increased from 2 s^{-1} to 20 s^{-1} to obtain an ascendant curve. The descendent curve was obtained by gradually decreasing rotation

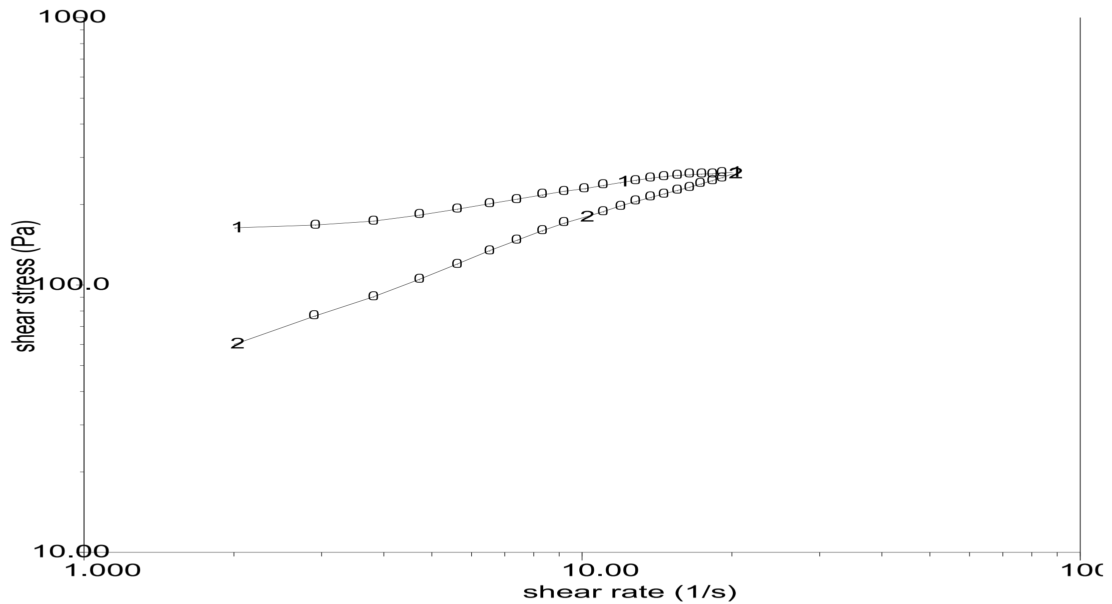
Figure 4.2. The curve of Force *vs.* Time†



†This typical texture analyzer curve described the relationship between the resistant force of probe generated from formulations (y axial) and the testing time (x axial). The physical properties of formulations, including firmness, consistency, cohesiveness, and index of viscosity were calculated from this Force *vs.* Time curve. The definition of four physical properties was described on Page 118.

speed from 20 s^{-1} to 2 s^{-1}. **Figure 4.3** shows a typical rheological graph, in which line 1 was the ascendant curve and line 2 the descendent curve. A rheological graph represented the relationship between shear rate and shear stress. The area of the hysteresis loop between the ascending and the descending curves provided the value of thixotropy.

Figure 4.3. Consistency curve for thixotropic systems†



†This typical rheological curve represented the relationship between shear rate (x axial) and shear stress (y axial). The area of the hysteresis loop between the ascending (line 1) and the descending (line 2) curves provided the value of thixotropy.

Rheological graphs of all formulations were analyzed using the Cross Law as the following equation:

$$\frac{(\text{Viscosity}-\text{b})}{(\text{a}-\text{b})} = \frac{1}{(1+(\text{c}*\text{Rate})^{\text{d}})} \qquad \text{Equation 4.3}$$

where a: zero viscosity (Pa.s)

b: infinite viscosity (Pa.s)

c: consistency (s)

d: rate index

The Cross Law is one of the most commonly-used rheological models. It is used to calculate some meaningful rheological parameters from the plot of shear stress *vs.* shear rate. For example, values of zero-rate viscosity, infinite-rate viscosity, consistency, rate index, and thixotropy were obtained by using this model. Zero-rate viscosity was defined as the viscosity at the zero shear rate, which was a critical material property to assess emulsion stability at static state. Infinity-rate viscosity was defined as the viscosity at the infinite shear rate. Rate index showed the degree of dependence of viscosity on shear rate in the shear-thinning field. The value of zero indicated Newtonian behavior, while the value increased with increasingly shear-thinning behavior. Consistency was also known as Cross Time Constant with the dimension of time. In this study, zero-rate viscosity, rate index and thixotropy were selected to demonstrate the rheological properties of the emulsion-based formulations. These rheological parameters provided us with a simple way to understand rheological profile of a shear thinning flow system.

4.3.3. Data Analysis

Statistical analysis was performed using two-way ANOVA and Tukey's test (PC-SAS 8.02, SAS Institute Inc., Cary, North Carolina, USA). The following statistical analyses of the data were conducted, a) the volume mean and median diameter of particles. b) texture parameters (firmness, consistency, cohesiveness, index of viscosity) and rheological parameters (thixotropy, viscosity, and rate index). Differences were considered statistically significant at $P \leq 0.05$.

4.4. Results and Discussion

4.4.1. Formulations with Different Preparation Methods

4.4.1.1. Appearance and Texture

The prepared formulations showed different appearance and texture properties after preparation and storage. FA1, FB1 and FC1 were prepared using the incorporation method. FA1 containing OBZ or combined DEET/OBZ had a slightly yellow, gritty, and creamy appearance and texture, while FA1 containing DEET was a white, fine, milky gel. OBZ, as an active ingredient in the prepared formulation, is a white or light yellow powder with low water solubility. When oil-in-water emulsion bases were incorporated with OBZ, the products felt gritty. Fine and solid particles of OBZ powder with low water solubility may not be completely incorporated into oil-in-water emulsion bases at room temperature; and a few of OBZ particles was dissolved in the oil phase of emulsion.

The gritty feeling might cause undesirable skin sensory characteristics in users and exert negative influences on the appearance of emulsions. The other active ingredient, DEET, is a liquid component. When DEET was incorporated into the emulsion bases, the products looked and felt fine and milky without gritty-feeling. FB1 containing OBZ or combined DEET/OBZ was a slightly yellow, gritty, and rich creamy gel, while FB1 with DEET was a white, fine, and richly milky gel. In FB1, the thickening agent was added to the external phase. Appearance and texture of FB1 was quite similar to that of FA1, but FB1 showed a better skin-feel after application. FC1 containing DEET or combined DEET/OBZ had a white, fine, and greasy appearance and texture, while FC1 containing OBZ was a slightly yellow, fine, and greasy cream. No gritty feeling was sensed in FC1, which might be attributed to improved solubility capacity of lipophilic chemical, OBZ, in the external oil phase of water-in-oil emulsion. But this water-in-oil emulsion felt greasy and sticky due to the presence of external oil phase, which might somehow decrease user satisfaction and costume elegance.

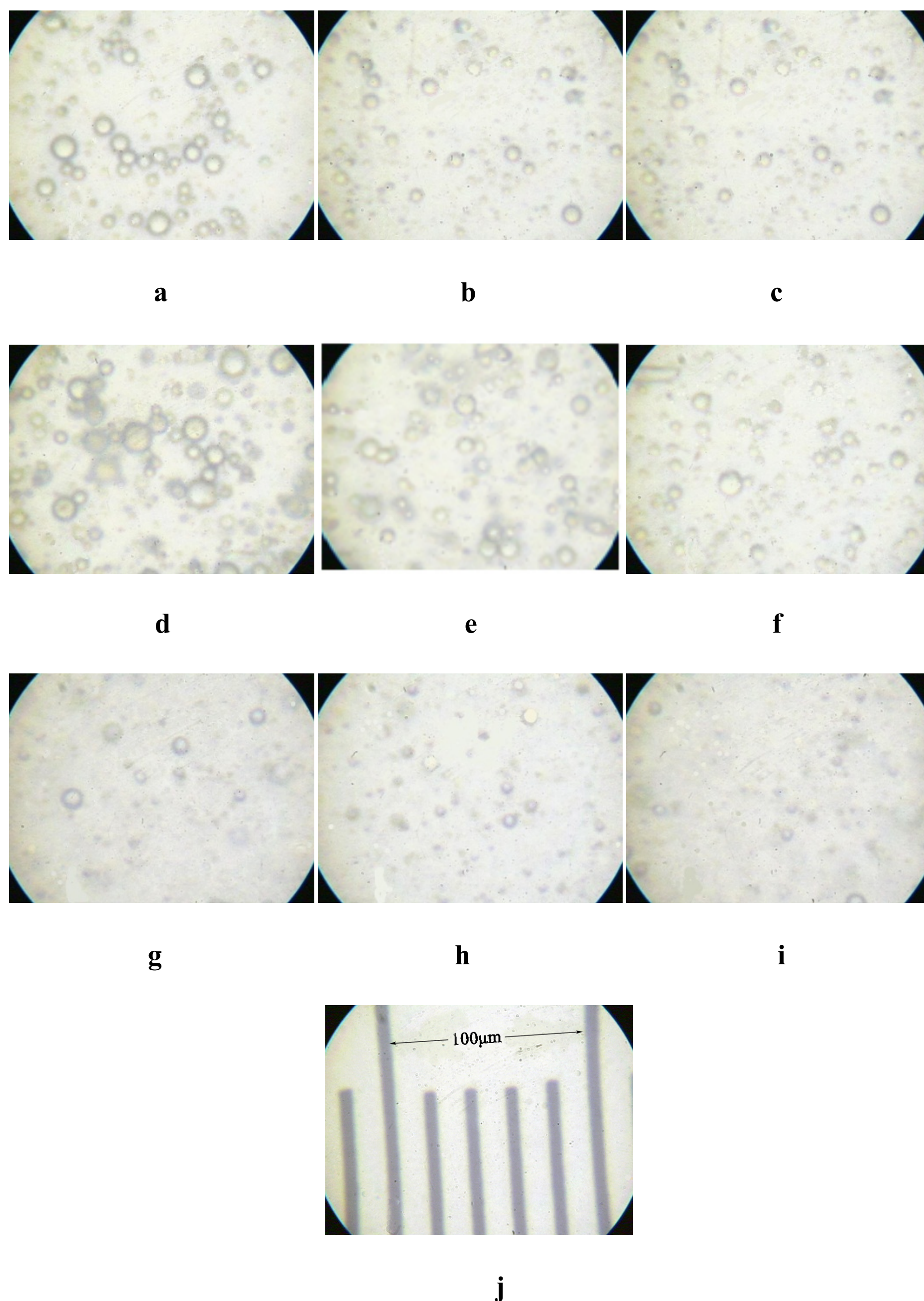
FA2, FB2 and FC2 were prepared by using the fusion method. FA2 containing DEET, OBZ, or DEET/OBZ was a white or slightly yellow fine creamy gel without gritty feeling, when compared to FA1. By using this preparation method, OBZ was combined in oil phase and heated to 75˚C together with all other oil components, so that solid particles of OBZ powder were completely melted and dissolved in the oil phase. The oil-in-water emulsion containing OBZ became a fine and homogeneous product without

gritty feeling. DEET was added during congealing of the emulsion base, which might lead to improved integration of DEET with the oil phase and subsequently stabilized the oil-in-water emulsion containing DEET. FB2 containing DEET, OBZ, or DEET/OBZ had a very similar appearance to FA2, but felt more richness; it was also stable during storage at room temperature. Addition of thickening agent in FB2 gave the product a sensation of richness comparing to FA1, which might be associated with the flexible rotating long-chain molecular structure of the thickening agent. In addition, the rich sensation might improve the value of products in terms of product elegance. FC2 containing DEET, OBZ, or DEET/OBZ was a white or slightly yellow, fine, greasy cream. They all appeared stable during storage at room temperature. By using the fusion method, DEET and OBZ were apparently fully combined into oil components of the emulsions, resulting in satisfactory consistency, stability and appearance. Therefore the fusion method was one of the appropriate methods for preparing emulsions containing DEET and/or OBZ.

4.4.1.2. Droplet Surface Morphology

The droplet surface morphology of the preparations made by the fusion method was observed using an optical microscopy; pictures were taken using a digital camera. As shown in photomicrographs (**Figure 4.4**), three emulsion-based formulations were simple emulsions. Oil globules dispersed throughout continuous aqueous phase in FA2 and FB2,

Figure 4.4. The appearance of droplets in FA2, FB2, FC2†



†The photomicrographs of three emulsion-based formulations with DEET and/or OBZ (**a:** FA2 with DEET and OBZ, **b:** FA2 with OBZ, **c:** FA2 with DEET, **d:** FB2 with DEET and OBZ, **e:** FB2 with OBZ, **f:** FB2 with DEET, **g:** FC2 with DEET and OBZ, **h:** FC2 with OBZ, **i:** FC2 with DEET, **j:** Scale) were obtained using a optical microscopy and a digital camera. All the preparations are simple emulsions (oil-in-water or water-in-oil).

while aqueous globules dispersed throughout continuous oil phase in FC2. The oil globules or aqueous globules were found to be spherical; the surface of oil or aqueous globules was smooth too. In FA2 containing DEET or OBZ, the oil globules appeared to be uniformly distributed in the continuous phase; aggregation of oil globules was observed in FA2 containing combined DEET/OBZ, which may result in larger droplet size. FB2 containing DEET or OBZ also showed uniform distribution of oil globules within continuous phase, while FB2 containing combined DEET/OBZ exhibited aggregation of the oil droplets. In FC2, all emulsions produced uniform distribution of aqueous globules in continuous oil phase without evidence of globule aggregation.

4.4.1.3. Droplet Size Distribution

The droplet size and size distribution of formulations prepared by fusion method were accurately measured using a laser diffraction machine. The volume mean diameters of droplets and droplet span were displayed in **Table 4.12**. The droplet size of FB2 containing DEET or OBZ was significantly different from FA2 counterparts, respectively. The volume mean diameter of droplets in FB2 containing DEET was significantly smaller than that in FA2 by 16%, while the volume mean diameter of FB2 containing OBZ was significantly higher than FA2 counterpart by 43%. Addition of a thickening agent, xanthan gum was able to increase the viscosity of aqueous phase when its concentration was raised above 0.2%, which resulted in decrease of droplet diameter (Ye,

et al., 2004). In this study, addition of xanthan gum only reduced the droplet size in the oil-in-water emulsion containing DEET, but not in the oil-in-water emulsion containing single OBZ or combined OBZ. Oil globules containing OBZ demonstrated different physicochemical properties when compared to DEET counterpart, which might induce different coalescent ability of droplets in the oil-in-water emulsions containing thickening agent.

Furthermore, droplet size of FA2 and FB2 containing combined DEET/OBZ was significantly larger than those containing DEET or OBZ. FA2 containing combined DEET/OBZ had significantly larger droplet size by 33% or by 51% than FA2 containing DEET or OBZ, respectively. FB2 containing combined DEET/OBZ produced significantly higher droplet size by 12% or by 67% compared to FB2 containing DEET or OBZ, respectively. The higher droplet size in the combined preparations of oil-in-water emulsion may be attributed to possible interactions of DEET and OBZ in oil droplets, which would subsequently compromise stability of oil droplets containing combined DEET/OBZ. Previous observation in the photomicrographs (**Figure 4.4**) showed that the oil droplets containing combined DEET/OBZ tended to aggregate in the oil-in-water emulsion. This result was proved by observation of droplets size in the laser diffraction machine.

In addition, FC2 containing DEET, OBZ, or both possessed significantly lower volume mean diameter of droplets than FA2 counterparts, by 40%, 50%, and 45%

respectively. The droplet size in FC2 containing combined DEET/OBZ did not display significant difference from that containing DEET or OBZ. In water-in-oil emulsion, the emulsifier agent was a nonionic synthetic emulsifier, which formed a coherent interfacial film surrounding the dispersed droplets to lower the interfacial tension of droplets and to prevent against coalescence of the droplets. In oil-in-water emulsion, the emulsifier agent was commercially known as Emulfree CBG, including butylene glycol cocoate, ethyl cellulose, and isostearic alcohol. Ethyl cellulose formed multilayers on the interface of water and oil phases to stabilize the oil droplets by steric repulsion, rather than their negligible ability to lower interfacial tension (Brochure Emulfree, 2010). The steric repulsion formed by this multilayer emulsifier agent might not be strong enough to retain oil droplets in smaller size. The nonionic synthetic emulsifier would be expected to confer on the system a high degree of resistance to coalescence. The aqueous droplets in water-in-oil emulsion may coalesce slowly and retain a smaller droplet diameter comparing to oil droplets in the oil-in-water emulsion.

FA2, FB2 and FC2 all showed normal size distribution of the droplets. The distribution span was calculated and listed in **Table 4.12**. The distribution span in FA2 and FC2 with DEET, OBZ, or both ranged between 2.02±0.01 and 2.41±0.01; no difference was observed between each other. Oil-in-water emulsion with thickening agent, FB2, containing OBZ or combined DEET/OBZ showed significantly wider distribution span by 33% or 86% than FA2 counterparts, but FB2 containing DEET had no significant

difference from FA2 containing DEET in their distribution span. A wider distribution span implied that a part of the small droplets had coalesced to form larger droplets. In FB2, d(0.1) and d(0.5) did not significantly increase, but d(0.9) increased distinctively. This indicated that the presence of OBZ might not increase the initial droplet size of the oil droplets, but produced coalescence of part of droplets and increased the distribution span of droplets and the average droplet size in the oil-in-water emulsion with thickening agent.

Table 4.12. The volume mean diameters of droplet and droplet span

Emulsion	Active ingredients	D[4,3] (μm)	d(0.1) (μm)	d(0.5) (μm)	d(0.9) (μm)	Span
FA2	DEET	58.43±1.62	15.93±0.12	45.29±0.63	116.78±3.43	2.23±0.04
	OBZ	51.26±0.41	14.15±0.06	32.85±0.25	93.48±0.62	2.41±0.01
	DEET/OBZ	77.51±1.23[1,2]	19.42±0.24	60.19±1.09	151.14±3.10	2.19±0.01
FB2	DEET	49.16±0.71[1]	14.80±0.05	39.71±0.22	97.01±0.75	2.07±0.00
	OBZ	73.18±1.12[1]	15.35±0.04	43.08±0.26	151.34±2.92	3.16±0.06[1]
	DEET/OBZ	82.18±0.79[1,2]	15.08±0.03	47.67±0.43	200.33±1.10	3.89±0.01[1,2]
FC2	DEET	35.15±0.39[1]	11.89±0.25	31.25±0.44	75.81±0.49	2.02±0.01
	OBZ	27.06±0.30[1]	11.98±0.79	22.02±0.72	62.01±0.87	2.27±0.08
	DEET/OBZ	32.62±1.12[1]	13.64±0.94	32.85±0.25	84.15±2.14	2.16±0.13

Mean±SD (n=3), $P \leq 0.05$

[1]: Significant difference between FA2 and FB2 or between FA2 and FC2

[2]: Significant difference between single preparations and combined preparations

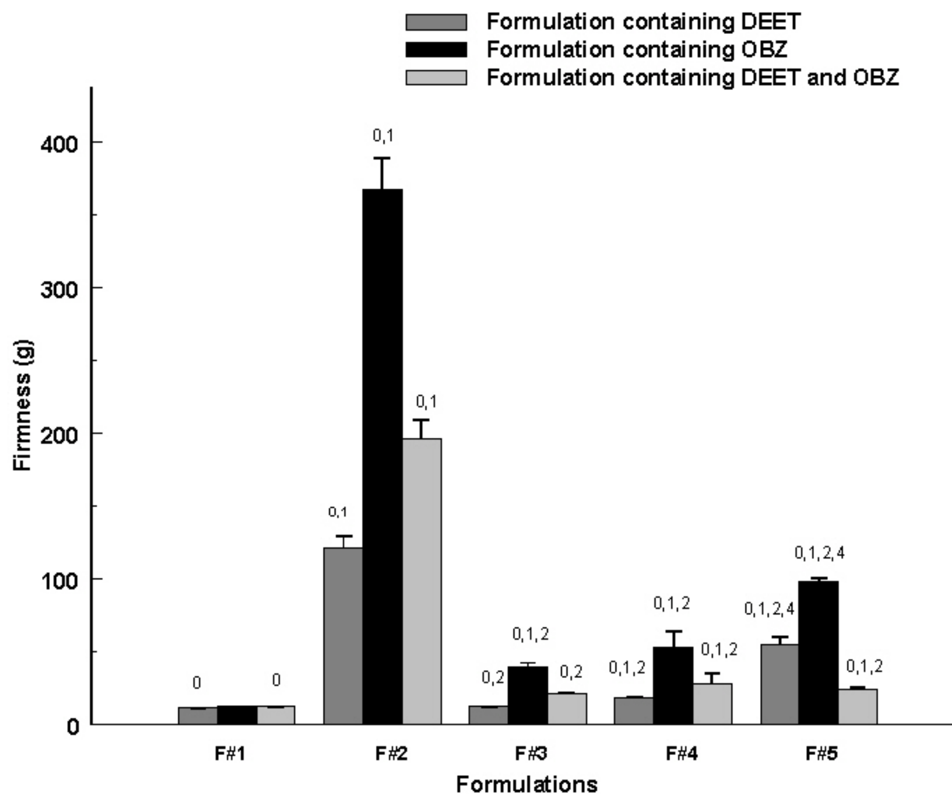
4.4.2. Formulations with Five Different Emulsifiers

4.4.2.1. Texture Analysis

Texture analysis technology has been used in pharmaceutical industry for almost 20 years. A Texture Analyzer instrument provides accurate and reproducible testing results to direct product research and development and to standardize quality control or quality assurance. Texture Analyzer is utilized to test many pharmaceutical products, including various semisolid formulations, such as cream, lotion, ointment, and gel. In this study, five emulsion-based formulations with different emulsifiers were prepared by using the fusion method. The texture parameters, including firmness, consistency, cohesiveness, and index of viscosity were immediately measured by a Texture Analyzer. Since the four measurements were all related to each other, only one of the parameters, firmness, was selected to demonstrate formulation properties and showed in **Figure 4.5**. In oil-in-water emulsions, the firmness of F#2 was significantly higher by 960% and 885% for DEET, and by 2840% and 834% for OBZ in comparison to F#1 and F#3. In water-in-oil emulsions, the firmness of F#5 was significantly higher by 190% for DEET and 84% for OBZ than that of F#4.

The state of firmness presented the resistance to external stress/pressure in a formulation, which simply and accurately indicated the deformation ability of an emulsion preparation under external forces. Because firmness represented the deformation ability of an emulsion, the property collected in a Texture Analyzer could be

Figure 4.5. Texture parameter-Firmness†



Mean±SD (n=3), P≤0.05

[0]: Significant difference between single preparations and combined preparations

[1]: Significant difference with Formulation #1

[2]: Significant difference with Formulation #2

[4]: Significant difference with Formulation #4

†One of the texture parameters, firmness, was selected to demonstrate the formulation properties. In oil-in-water emulsions, the firmness of F#2 was significantly higher than the counterparts of F#1 and F#3 (960% and 885% for DEET and 2840% and 834% for OBZ). In water-in-oil emulsions, the firmness of F#5 was significantly higher than the counterparts of F#4 (190% for DEET and 84% for OBZ).

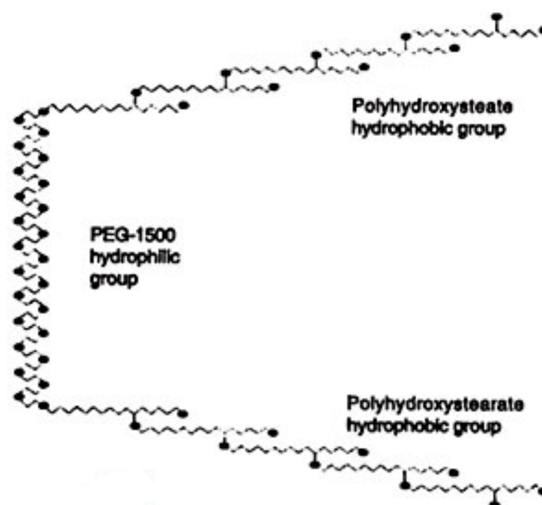
regarded as a substitute for rheological properties. Rheological properties of semisolid emulsions can often be influenced by emulsifying agents (Sherman, 1955). In addition, firmness may also be dependent upon chemical nature of emulsifiers incorporated in an emulsion. In this study, Emulium 22 was used to formulate F#2; this was an oil-in-water nonionic synthetic emulsifier with an HLB value of 10.5. It could form monomolecular film in the interface between oil and water phase. Emulium 22 is Tribehenin PEG-20 esters generated by grafting PEG 20 onto behenin acid, a 22 carbon chain fatty acid. By adjusting Emulium 22 concentration from 2% to 6%, it is possible to formulate oil-in-water emulsions of variable consistency, from fluid lotions to thick creams.

Emulium 22 (6%) is capable of forming a thick cream with high resistance to deformation under external pressure (Brochure Emulium 22, 2010). Martin also pointed out that the higher the concentration of emulsifying agents in a formulation was, the higher the product would demonstrate its resistance ability to deformation (Martin, 1993). Changes in deformation property had often been attributed to increased emulsifier adsorption around the globules (Sherman, 1968). Apifil® used as an emulsifier in F#3 is a natural beeswax modified by PEG-8; its HBL value is 9.4 and it is a powerful emulsifier. Apifil® can also form a variety of oil-in-water emulsions from fluid lotions to thick creams, depending on its percentage in formulations (5%-7%). Apifil® (5%) formed light creams with lower firmness than that of F#2 (Brochure Apifil, 2010). The lower firmness may be attributed to different chemical natures of the emulsifier and lower emulsifier

adsorption in the interfacial film around oil globules. The emulsifier used in F#1 is a surfactant-free emulsifier, commercially known as Emulfree CBG, which includes butylene glycol cocoate, ethyl cellulose, and isostearic alcohol. Ethyl cellulose is capable of forming multilayers on the interface of water and oil phase to stabilize the oil droplets by steric repulsion and to prevent coalescence and flocculation. Butylene glycol cocoate and isostearic alcohol combine with oil phase to increase the rigidity of oil droplets and subsequently reduce the coalescence (Brochure Emulfree, 2010). Unlike synthetic emulsifying agents, ethyl cellulose cannot generate multilayers and form a rigid interfacial film around oil globules. Therefore, this surfactant-free emulsifying agent may not provide a strong molecular barrier surrounding oil globules for resisting a certain amount of external pressure (Sherman, 1963), thereby producing a lower firmness than Emulium 22.

In water-in-oil emulsions, Arlacel P-135 and plurol diisostearique (triglycerol diisostearate) were respectively applied in F#4 and F#5. Arlacel P-135 is a polyethylene glycol diester of polyhydroxystearic acid with an HLB value of 5.5 (**Figure 4.6**). It has two fixed large volume long-chain, polyhydroxysteate hydrophobic groups, which is capable of forming a non-rotating, three-dimensional spatial structure in the interface between oil phase and water phase. The fixed non-rotating spatial structure in Arlacel P-135 cannot freely form the entangled coil in the interfacial film around water globules, which may have induced a low resistance to external press (Martin, 1993). On the other

Figure 4.6. Molecular structure of Arlacel P-135†



†Arlacel P-135 (polyethylene glycol diester of polyhydroxystearic acid) is a water-in-oil emulsifier with HLB 5.5. It has two fixed large volume long-chain, polyhydroxysteate hydrophobic groups, forming a non-entangled structure in the interface film of water globules and inducing the decrease of the resistance to external press in the formulation (UN GOODWAY, 2014).

hand, plurol diisostearique selected for F#5 is triglycerol diisostearate, a PEG-free emulsifier generally used to create creams or lotions with an HLB value of 4.5. Because plurol diisostearique has no fixed three-dimensional spatial structure, flexibly rotating carbon chain may randomly form entangled coils in the interfacial film around water droplets, thereby enhancing the resistance ability to external press in F#5 in comparison to F#4.

The firmness of emulsion-based formulations may also depend on the constituents

of internal phase and external phase. In oil-in-water emulsions, interfacial film surrounding the oil droplets can be affected by the physical and chemical properties of the internal phase. This may produce a significant effect on rheological properties of the emulsion (Sherman, 1963). In this study, two oil-in-water emulsions were composed of 30% internal phase and 70% external phase. Even though components of the external phase in these two emulsions had no difference, the internal oil phases of the formulas were different. As shown in Tables 4.8 and 4.9, F#2 used the same consistency agent Geleol 2% as F#3; the total percentage of the emollient and solubilizer in F#2 was also the same as F#3. The difference in oil phase between F#2 and F#3 was that Precirol Ato 5 was selected as a consistency agent in F#2, but oily vehicle Labrafac PG was used in F#3. Oily vehicle Labrafac PG is a liquid ester with 8 or 10 carbon chain in molecules, while Precirol Ato 5 is a high melting point lipid with C16 or C18 carbon chain in molecules. The long carbon chain in the molecule may form lipid matrix in the internal phase of emulsions and subsequently increase resistance of the globules to external press. Deformation of an emulsion may result from deformation of closely packed globules; a higher resistance of an emulsion to external press may be attributed to these globules within the formulation that possess higher resistance to external press (Sherman, 1968). Regarding water-in-oil emulsions in this study, both F#4 and F#5 included 40% internal water phases and 60% external oil phases. The internal water phases of F#4 and F#5 contained similar components. In external oil phases, the difference was that F#4 used a

higher percentage of Labrafil M1944CS, while F#5 applied a higher percentage of Plurol oleique CC 497 as a co-surfactant. Excessive surfactant molecules may form micelles in the external oil phase; these micelles immobilized the external phase fluid and increased the value of deformation property (firmness) in F#5 (Sherman, 1968).

Presence of active ingredients DEET and OBZ may also influence the deformation property (firmness) in the emulsions. As shown in **Figure 4.5**, firmness of F#1, F#2, and F#3 containing OBZ or combined DEET/OBZ demonstrated a significant increase when compared to F#1, F#2, and F#3 containing DEET, while F#4 and F#5 containing DEET or combined DEET/OBZ showed a significant decrease in firmness when compared to F#4 and F#5 containing OBZ. Because both DEET and OBZ were lipophilic compounds, they would get into oil phases in emulsions. In an oil-in-water emulsion, DEET and OBZ were dissolved in internal oil phase; they were dissolved in external oil phase in a water-in-oil emulsion. Incorporation of OBZ changed physical properties of the internal oil phase, resulting in an increased firmness of the oil-in-water emulsions. OBZ, as a solid component, possessed a good thermal stability with decomposition above 200˚C (Chem, 2005). OBZ molecules packed more closely by greater forces as a powder than DEET molecules. OBZ that was dissolved in the internal oil phase may have formed more compact oil globules and enhanced firmness of the emulsion. Consequently, firmness of oil-in-water emulsions containing OBZ was significantly higher than that without OBZ. In water-in-oil emulsions, presence of DEET

in external oil phase decreased the firmness, as DEET was a liquid component, and possessed weaker intermolecular forces than solid OBZ. Once DEET was incorporated into the external oil phase, it may weaken the continuous oil phase, resulting in decrease of resistance to flow in oil phase. Hence water-in-oil emulsions with DEET displayed significantly lower firmness than that with OBZ in this study.

4.4.2.2. Rheological Behavior

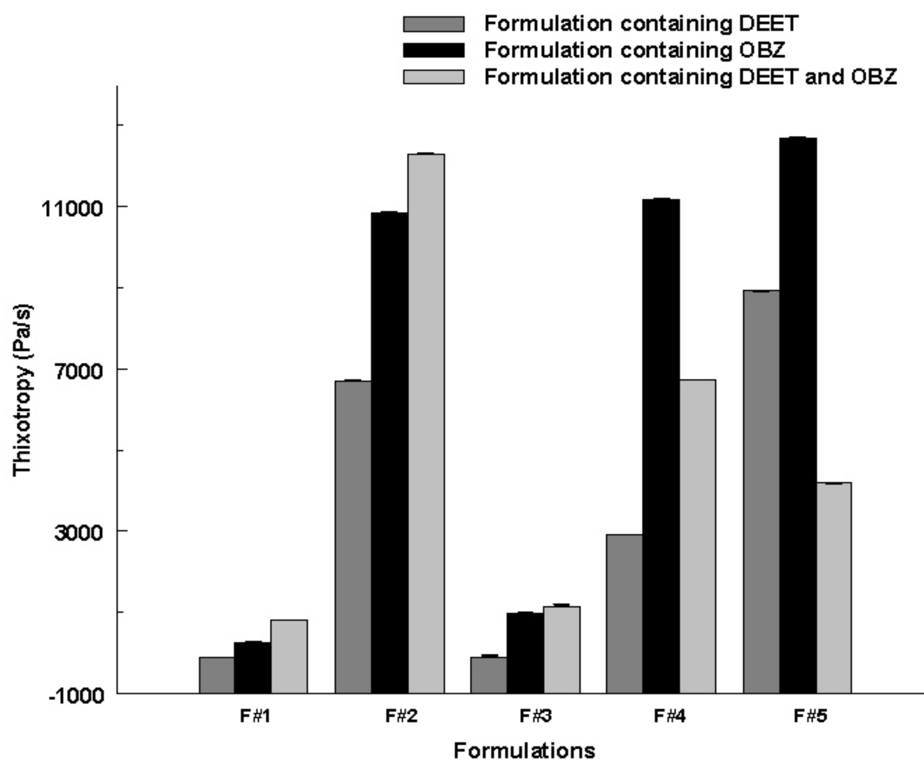
Rheological behaviors are important in the application of semisolid pharmaceutical and cosmetic products, such as spreading an ointment on the skin and squeezing a cream from a tube. Systemic examination of rheological behaviors of liquids and semisolids began only in the early 1910's, mainly through the efforts by Bingham, Hatschek, and Reiner (Martin, 1993). In accordance to flowing types and deformation behaviors, any liquid or semisolid material can be classified into a Newtonian system or a Non-Newtonian system (plastic, pseudoplastic, or dilatant flow body). Emulsions are most likely to demonstrate non-Newtonian flow properties except for those that contain dispersed phase with low proportion. Some emulsions may possess pseudoplastic (shear-thinning) flow; these characteristics are accompanied by thixotropy (hysteresis area). Various rheological parameters of the emulsion-based formulations prepared in this study were calculated from shear rate *vs.* shear stress curves, and are showed in **Table 4.13** and **Figure 4.7**. Zero-rate viscosity is defined as the viscosity at zero shear rate, i.e.,

the viscosity that a product originally possesses at static state or at rest. This parameter is a critical material property in assessing emulsion stability. In oil-in-water emulsions, zero-rate viscosity of F#2 respectively produced an increase of 1.4- and 12.8-fold for DEET, and 28.2- and 1.5-fold for OBZ in comparison to their counterparts of F#1 and F#3. In water-in-oil emulsions, zero-rate viscosity of F#5 containing DEET and OBZ was 2.3 and 3.1 times higher than F#4 counterparts, respectively.

Table 4.13. Rheological parameters of the formulations

Emulsion	**Active ingredients**	**zero-rate viscosity (Pa.s)**	**rate index**	**Standard error**
F#1	DEET	238	0.68	5.5
	OBZ	1329	0.71	8.2
	DEET/OBZ	147	0.62	13.1
F#2	DEET	579	2.24	19.1
	OBZ	38870	1.33	16.2
	DEET/OBZ	5605	2.99	8.0
F#3	DEET	42	2.44	34.3
	OBZ	15530	0.80	12.5
	DEET/OBZ	83	1.40	37.2
F#4	DEET	240	1.59	7.1
	OBZ	1894	2.12	5.2
	DEET/OBZ	470	1.64	3.1
F#5	DEET	800	1.65	3.3
	OBZ	7792	1.24	10.1
	DEET/OBZ	7459	1.12	8.8

Figure 4.7. Thixotropy of formulations†



†All emulsions possessed shear-thinning (thixotropy) behaviors. The thixotropy of F#2 containing DEET or OBZ was higher than counterparts of F#1 and F#3; the thixotropy of F#5 containing DEET or OBZ was higher than F#4 counterparts.

As previously described, viscosity represents the resistance to flow in a system, and viscosity of an emulsion is dependent on chemical natures of emulsifying agents present in the preparation (Sherman, 1955). Emulium 22 selected for F#2 is tribehenin PEG-20 esters. Emulium 22 at 6% is capable of formulating a thick cream of very high viscosity (over 30,000 mPas) (Brochure Emulium 22, 2010). Used in F#2, 6% Emulium

22 produced the highest viscosity at static state among three oil-in-water formulations, which was attributed to chemical nature of this emulsifier and higher adsorption of the emulsifier in the interfacial film. Apifil® is a modified beeswax selected for F#3. It creates a variety of oil-in-water emulsions from fluid lotion to thick cream, depending on its percentage in the formulation. Apifil® at 5% can form light creams with relatively low viscosity (around 5000 mPa·s) (Brochure Apifil, 2010). Used in F#3, 5% Apifil® did form a lotion of lower viscosity than F#2. Emulsfree CBG possesses low viscosity of around 200-500 mPa·s at 46 ºC and 200 S^{-1} (TDS Emulfree, 2008).

Also by varying use level of aqueous gelling agent and/or consistency agent in emulsion formula, it is possible to create very diverse emulsion textures ranging from liquid spray to rich butter cream. For example, low viscosity carbomer and low concentration of xanthan gum were selected in F#1 to create a fluid-texture emulsion; this preparation showed a thinner texture character and lower viscosity than F#2. In water-in-oil emulsion, rheological measurements were consistent with those observed in texture analysis. Plurol diisostearique selected for F#5 increased preparation resistance to flow and viscosity of the emulsion. Compared to plurol diisostearique, arlacel P-135 selected for F#4 possessed a lower resistance to flow than plurol diisostearique, and hence the preparation exhibited a lower preparation viscosity.

Moreover, active ingredients in the prepared emulsions also altered the viscosity. Zero-rate viscosity of oil-in-water emulsions containing OBZ and combined DEET/OBZ

was respectively higher than that of counterparts containing DEET (Table 4.13). OBZ is a more lipophilic compound than DEET; their octanol-water partition coefficients were LogKo/w 3.79 and LogKo/w 2.01 respectively. Droplets with more lipophilic characteristics may increase adsorption of emulsifying agent at the interface and subsequently raise the viscosity (Sherman, 1968). Oil-in-water emulsions containing OBZ did produce higher viscosity than those containing no OBZ. DEET, on the other hand, decreased the resistance of emulsion to flow and lowered the viscosity. Observations obtained from rheometer and texture analyzer were similar, indicating that both parameters demonstrated similar properties of the emulsions.

Rate index calculated from rheologic measurement indicates the degree of dependence of viscosity on shear rate in shear-thinning field. Newtonian flow systems would show a value of zero, while rate index would increase with increasingly shear-thinning behavior (thixotropy) in non-Newtonian flow systems. The rate indexes of the prepared emulsions ranged from 0.62 to 2.99 (Table 4.13), indicating that all emulsions possessed shear-thinning behaviors. The thixotropy was displayed in Figure 4.7. Among oil-in-water emulsions, thixotropy of F#2 containing DEET or OBZ was higher by 60- and 71-folds, and 40- and 10-folds than counterparts of F#1 and F#3, respectively. Between water-in-oil emulsions F#4 and F#5, thixotropy of F#5 containing DEET or OBZ was higher than F#4 counterparts, respectively. These results of thixotropy may be also related to the chemical nature of emulsifying agents applied in

emulsions. Moreover, the thixotropy of oil-in-water emulsions containing OBZ was respectively higher than that of counterparts containing single DEET; the thixotropy of water-in-oil emulsion containing single OBZ was respectively higher than that of counterparts containing DEET. This may be attributed to the different physicochemical properties of the oil phase containing DEET or OBZ in emulsions.

4.5. Conclusion

Formulation design and preparation are very important for the topical drug formulation. Most of topical drug formulations are emulsion-based formulations due to their excellent solubilizing properties for lipophilic and hydrophilic compounds and the good customer acceptability. An appropriate preparation method has to be chosen according to active ingredients and emulsion components. The fusion method and incorporation method were usually applied under the lab condition. In this formulation preparation study of insect repellent DEET and sunscreen OBZ, the fusion method was an optimal preparation method. By using the fusion preparation method, the stable, good appearance emulsion-based formulations were able to be produced. The droplet size distribution was observed by an optical microscope and measured by Malvern Mastersizer. The droplet size distribution of finished preparations depended on the viscosity enhancer, the type of emulsions, and active ingredients through the observation of microscope and laser refraction instrument. Furthermore, the texture properties and

rheological properties of emulsions were also measured in this formulation study. The texture properties and rheological properties depended on the emulsifying agents, the internal phase, the external continuous phase, active ingredients in the emulsion-based formulations.

In five emulsion-based formulations with different emulsifiers, F#2 exhibited the highest firmness, viscosity, and thixotropic property. These physical properties provided many advantages in F#2, such as fine texture, stability in container, easy pick-up, easy-spreadability on the skin. In F#2, the emulsifying agent was tribehenin PEG-20 esters (Emulium 22), which was generated by grafting PEG 20 onto the behenin acid, a 22 carbon chain fatty acid. This long chain fatty acid can stabilize the interfacial film surrounding the oil droplets. The chemical structure of this emulsifying agent generated the proper apparent viscosity and thixotropic property in the emulsion. Moreover, Precirol Ato 5, an oil phase component, provided further stabilization of oil globules in the external phase and produced the higher texture parameters and rheological property in the emulsions. Therefore, F#2 was considered to be a promising emulsion bases applied for the insect repellent DEET and sunscreen OBZ and selected for the further animal study.

CHAPTER 5

In Vitro Diffusion Study

5.1. Introduction

In vitro diffusion experimentation is commonly utilized during topical formulation development to identify potential formulas and to evaluate preparation qualities. The methodology can not only provide useful information in selecting appropriate dosage forms for optimal drug delivery, but also set up standardized testing protocol for regulatory approval and quality assurance. The USP (United State Pharmacopeia) is to publish a new general chapter entitled "Topical and Transdermal Drug Product: Performance Test" describing the application details of *in vitro* diffusion studies. The method hence plays an important role in formulation screening of novel topical and transdermal drug delivery systems.

Insect repellents and sunscreens are over-the-counter consumer care products that have been extensively used by the general public to prevent vector-borne diseases and sunlight-induced skin damages (Scotto, *et al.*, 1983; Sudakin, *et al.*, 2003; US EPA, 2010). Under ideal application conditions, active ingredients such as DEET and OBZ should possess minimal transdermal absorption and systemic disposition. With the preparation of various emulsion-based DEET and OBZ formulations in this thesis, it appeared that *in vitro* diffusion experiment would be a simple and cost-effective method to characterize membrane permeation and retention of DEET and OBZ. Results from *in vitro* diffusion experiments may also be beneficial in finalizing a potential topical formulation that would be further tested in an animal model.

Semisolid emulsion-based preparations are composed of various excipients and additives including oil phase, aqueous phase, emulsifiers and stabilizers. By changing composition of these ingredients, it is possible to produce different emulsion types, modify physical properties of the emulsions, and subsequently adjust transmembrane characteristics of the active components in the formulations. In this study, membrane permeation and retention of DEET and OBZ from the developed emulsion preparations were studied and compared using *in vitro* diffusion experiment, with artificial and biological skin membrane models. The influences of emulsion excipients such as emulsifiers, emollients, and permeation modifiers on overall DEET and OBZ permeation and retention were also assessed in order to decide an optimal emulsion formula that would demonstrate minimal penetration of the active ingredients.

5.2. Materials

Pure DEET (N,N-diethyl-*m*-toluamide) was purchased from Fluka Chemika GmbH (Buchs, Switzerland), and pure OBZ (2-hydroxy-4-methoxybenzophenone) was purchased from Riedel Haer GmbH (Seelze, Germany). Acetonitrile (HPLC grade), glacial acetic acid, potassium phosphate monobasic, sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Polyoxyethylene 20-oleyl ether (Brij® 98) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade deionized water was purified by a Milli-Q® Pure Water System (Millipore, Nepean, ON,

Canada) in the laboratory. Trypsin was purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

5.3. Methods

5.3.1. Membranes

5.3.1.1. Human Skin

Human skin specimens were obtained from St. Boniface General Hospital of Winnipeg and stored in -20°C prior to use. The study protocol was approved by the University of Manitoba Human Research Ethics Board. To prepare human skin samples for diffusion experiments, the skin was taken out from the freezer and thawed at room temperature for approximately 8 hours. The specimens were then dermatomed to a thickness of 380 µm and soaked in saline solution to prevent membrane from dehydration. The undamaged skin section with an even thickness was selected for diffusion experiments, and was cut into small pieces of 2×2 cm^2 before being mounted to diffusion cells.

5.3.1.2. LDPE

Artificial low-density polyethylene (LDPE) membrane was obtained from Key Container, Winnipeg, Manitoba, Canada. This membrane model possessed lipophilic properties. Before the diffusion experiment, LDPE membrane was cut into small pieces

of 2×2 cm^2, and soaked in saline solution prior to being mounted to vertical Franz-style diffusion cells.

5.3.1.3. Piglet Skin

Pig skin samples were obtained from Department of Animal Science, University of Manitoba. The animal use protocol was approved by the University of Manitoba Fort Garry Campus Protocol Management and Review Committee and conducted according to current guidelines published by the Canadian Council on Animal Care. Skin samples were collected and kept at -20°C prior to use. Before each experiment, the sample was thawed, rinsed with water and dried with paper towels. The skin was shaved using a razor to provide a smooth surface; the fat and underlying tissues were removed with a scalpel. The skin was dermatomed to a thickness of 380 μm using an electric dermatome. Integrity of the samples was carefully examined. Only undamaged, evenly-cut skin sections were selected for the diffusion studies.

5.3.2. Diffusion Study from Three Emulsion-based Formulations

For the three emulsion-based formulations (**Table 4.4-4.6**), *in vitro* diffusion experiments were conducted in a transdermal diffusion cell console (Logan Instruments Corporation, Somerset, New Jersey, USA), which was composed of six vertical Franz-style diffusion cells, a circulated water bath, a magnetic stir console and an

automatic sampling collector. Prior to the experiments, a very thin layer of vacuum grease was spread on the connection surface of the receptor and donor cells to avoid leakage of the samples. 7.0 ml pH 7.4 phosphate buffer containing 4% Brij 98 (W/V) was filled into the receptor cell. 2×2 cm^2 LDPE membrane or human skin with stratum corneum facing up was mounted to the connection surface of the receptor cell. The donor cell was put on the membrane; and both receptor and donor cells were tightly fixed by a clamp. Emulsion sample (1 g) was accurately weighed and carefully applied into the donor cell, so that a complete contact between the emulsion and the membrane surface was maintained. The donor cell was covered with a piece of microscope cover glass to prevent evaporation of the vehicle. The water bath and magnetic stir console were turned on and respectively maintained at 37ºC and 300 rpm. The amount of emulsion applied into the donor cell that was in direct contact with the membrane surface and adjacent effective diffusion area was precisely measured to be 0.1 g, which was used to calculate the overall permeation percentage of DEET and OBZ.

An aliquot of receptor fluid was collected hourly for six h, followed by the replenishment of an equal volume of fresh, preheated receptor fluid at each sampling point. Four test replicates were performed in each experiment. Concentration of DEET and OBZ in the receptor fluid was directly analyzed without any further treatment by an HPLC (High Performance Liquid Chromatography) method that had been previously developed and validated. After the diffusion study, each individual LDPE membrane or

human skin was respectively collected into a small glass vial (Fisher Scientific, Fair Lawn, New Jersey, USA), and soaked in 20 ml acetonitrile for 24 hours at room temperature. The acetonitrile solution in each vial was centrifuged for 15 minutes afterwards. The supernatant was transferred to another vial and also analyzed using an HPLC assay.

5.3.3. Diffusion Study from Five Emulsion-based Formulations

For five other emulsion-based formulations (**Table 4.7-4.11**), *in vitro* diffusion experiments were also conducted using the same transdermal diffusion procedures described above. However, pig skin was selected as the membrane model for this study.

An aliquot of receptor fluid was collected at time 1, 2, 3, 4, 5, 6, 9, 12 and 24 hours, followed by replenishment of equal volume of fresh, preheated receptor fluid at each sampling point. Five replicates were used in each experiment. Concentration of DEET and OBZ in the receptor fluid was directly analyzed using an HPLC method without any further treatment. After the diffusion study, each individual pig skin was carefully wiped with gauze to remove residual test sample from the skin surface and then soaked in a small glass vial (Fisher Scientific, Fair Lawn, New Jersey, USA) with 1 ml 0.001% (W/W) trypsin solution for 24 hours at 37°C. 3 ml ethanol was added into each vial afterwards; the skin was soaked for another 24 hours at room temperature. The solution was then collected using a syringe and passed through a filter (0.22 μm, Fisher

Scientific, Fair Lawn, New Jersey, USA) before being analyzed with an HPLC assay.

5.3.4. HPLC Assay

All study samples were directly measured using an HPLC system composed of a 996 Photodiode Array Detector and a Waters Alliance 2690 Solvent Delivery Module with Millennium software (Milford, Massachusetts, USA). The column was Nova-Pak C18, 3.9×150 mm, 4 μm. The mobile phase was a mixture of acetonitrile, methanol and water (pH 2.8 with acidic acid) in the ratio of 65:20:15 (V/V/V), at a flow rate of 1 ml/min. The detection wavelength of DEET was 254 nm and that of OBZ was 287 nm. Under these conditions, the retention time of DEET and OBZ was 1.49 minutes and 1.98 minutes respectively with a detection limitation of 20 ng DEET and 5 ng OBZ. The range of calibration linearity ($r^2 \geq 0.99$) for DEET and OBZ was 50-2000 ng and 8-500 ng, respectively.

5.3.5. Data Analysis

The Fick's Second Law is commonly utilized to quantitate drug diffusion characteristics across skin membrane, which is expressed as the following (Roberts, *et al.*, 1999):

$$\frac{\partial C}{\partial t} = D_m \frac{\partial^2 Cm}{\partial x^2} \qquad \text{Equation 5.1.}$$

Above equation presumes that a) the concentration of a solute in the donor cell remains constant; b) "sink condition" exists in the receptor cell for the duration of the experiment; and c) the initial concentrations in membrane model are $C_m(x,0)=0$, $C_m(0,t)=K_mC_v$, and $C_m(h_m, t)=0$, where C_m: drug concentration in membranes (g/ml), and C_v: saturated drug concentration in vehicle (g/ml).

Under most circumstances, Fick's Second Law is solved in terms of the amount of solute Q(t) exiting from the membrane in time t, and is expressed as the following (Roberts, *et al.*, 1999),

$$\frac{Q(t)}{A} = K_m h_m C_v\left[\frac{D_m t}{{h_m}^2} - \frac{1}{6} - \frac{2}{\pi}\sum_{n=1}^{\infty}\frac{(-1)^n}{n^2}\exp\left(\frac{D_m \pi^2 n^2 t}{{h_m}^2}\right)\right] \qquad \text{Equation 5.2}$$

where Q(t): accumulative drug amount permeating through the membrane (g)

K_m: partition coefficient between membrane and vehicle

C_v: saturated drug concentration in vehicle (g/ml)

h_m: thickness of the membrane (cm)

A: diffusion area (cm^2)

D_m: diffusion coefficient (cm^2/h).

When t→∞, Equation 5.2 is simplified to the following (Roberts, *et al.*, 1999),

$$\frac{Q(t)}{A} = K_m h_m C_v \left(\frac{D_m t}{{h_m}^2} - \frac{1}{6}\right)$$

$$= \frac{K_m C_v D_m}{h_m}\left(t - \frac{{h_m}^2}{6D_m}\right)$$

$$= K_p C_v (t\text{-}lag) \qquad \text{Equation 5.3.}$$

where permeability coefficient K_p of a solute is expressed in,

$$K_p = \frac{K_m D_m}{h_m} \qquad \text{Equation 5.4.}$$

Lag time is given by,

$$Lag = \frac{{h_m}^2}{6D_m} \qquad \text{Equation 5.5.}$$

Permeation percentage can be calculated as the following,

$$\text{Permeation percentage} = \frac{Q(t)}{\text{drug amount applied in the donor cell}}$$

Once a steady-state status is reached in percutaneous penetration, the permeation parameters can also be calculated by Equation 5.2 using linear regression of the

experimental data. In this linear equation,

$$\text{Slope} = \frac{K_m C_v D_m}{h_m}$$

$$\text{Intercept} = \frac{K_m C_m h_m}{6}$$

Subsequently, permeation parameters can be calculated as the following,

$$K_m = \frac{6 * \text{Intercept}}{C_v}$$

$$D_m = \frac{\text{Slope} * h_m}{K_m C_v}$$

$$K_p = \frac{K_m D_m}{h_m}$$

$$\text{Lag} = \frac{{h_m}^2}{6 D_m}$$

Statistical analysis was performed using two-way ANOVA and Tukey's Test (PC-SAS 8.02, SAS Institute Inc., Cary, North Carolina, USA). The statistical analyses were conducted on the data of the overall permeation percentages and permeation parameters (permeation coefficient, lag time, diffusion coefficient, and partition coefficient) of DEET and OBZ through LDPE, human skin, and pig skin. Differences were considered statistically significant at $P \leq 0.05$.

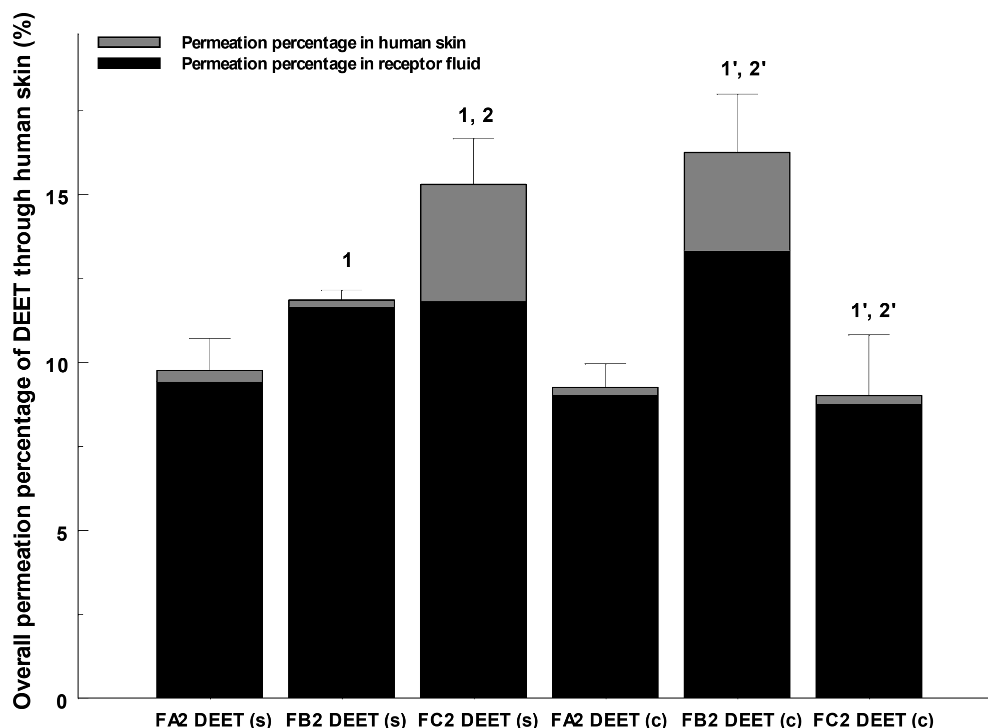
5.4. Results and Discussion

5.4.1. Diffusion Study from Three Emulsion-based Formulations

5.4.1.1. Skin Penetration through Human Skin

The overall permeation percentage of DEET and OBZ in membrane and receptor fluid from Formulation A2 (FA2), Formulation B2 (FB2), and Formulation C2 (FC2) through human skin was showed in **Figures 5.1 and 5.2**. Comparing FB2 to FA2 in single preparation, the overall permeation percentage of DEET and OBZ (including both membrane and receptor fluid) significantly increased by 21% and 150%; the permeation percentage of OBZ in membrane was significantly higher by 400%. The combined preparation of FB2 significantly increased the overall permeation percentage of DEET and OBZ by 36% and 23% comparing to the single preparations; but there was no synergistic percutaneous penetration of DEET and OBZ observed in the combined preparation of FA2. Moreover, the permeation percentage of DEET and OBZ in membrane significantly increased by 1360% and 67% in the combined preparation of FB2 comparing to the single preparation. The higher skin penetration of DEET and OBZ and synergistic percutaneous penetration of two compounds in FB2 may be attributed to the addition of thickening agent in the external phase of the oil-in-water emulsion.

Figure 5.1. Overall DEET permeation percentage through human skin†



n = 4, Mean ± SEM, $p \leq 0.05$

1: Significant difference between FA2 and FB2/FC2 (membrane + receptor fluid)

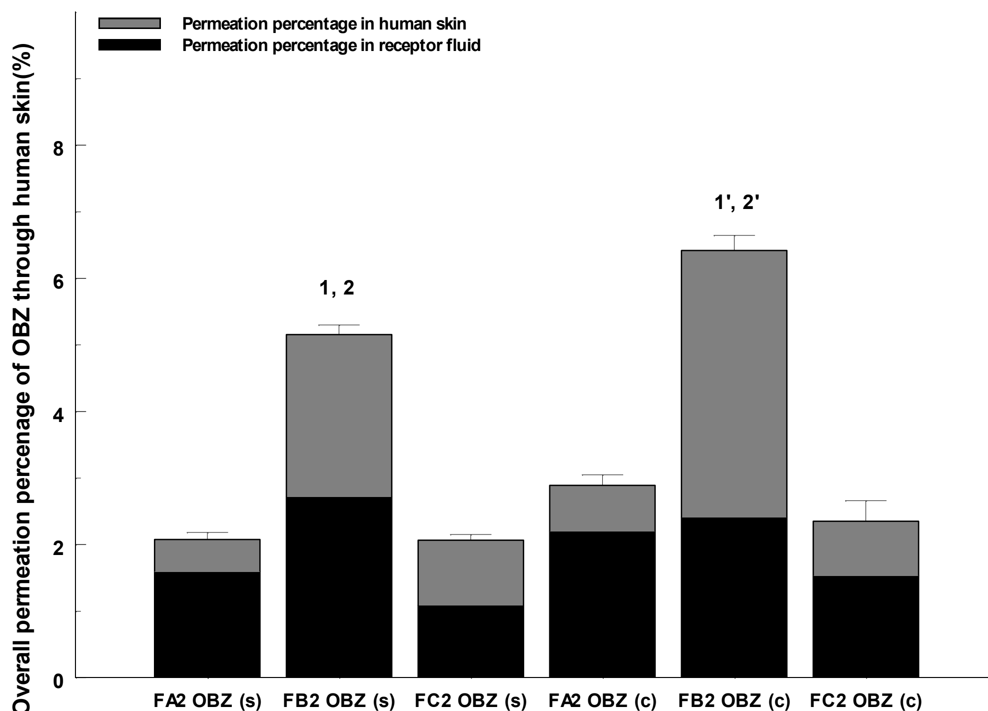
1': Significant different between single preparations and combined preparations (membrane + receptor fluid)

2: Significant difference between FA2 and FB2/FC2 (membrane only)

2': Significant difference between single preparations and combined preparations (membrane only)

†The overall permeation percentage of DEET in membrane human skin (gray bar) and in receptor fluid (black bar) from three emulsion-based formulations, FA2, FB2, and FC2, was obtained by the *in vitro* diffusion experiments through human skin. The overall permeation percentage in FB2 and FC2 was significantly higher than in FA2. The permeation percentage in membrane was significantly higher in FC2 than in FA2. The overall permeation percentage and permeation percentage in membrane were significantly higher in the combined preparation of FB2 than in the counterpart of single preparation; they were significantly lower in the combined preparation of FC2 than in the counterpart of single preparations.

Figure 5.2. Overall OBZ permeation percentage through human skin†



n = 4, Mean ± SEM, $p \leq 0.05$

1: Significant difference between FA2 and FB2/FC2 (membrane + receptor fluid)

1’: Significant differene between single preparations and combined preparations (membrane + receptor fluid)

2: Significant difference between FA2 and FB2/FC2 (membrane only)

2’: Significant difference between single preparations and combined preparations (membrane only)

†The overall permeation percentage of OBZ in membrane human skin (gray bar) and in receptor fluid (black bar) from three emulsion-based formulations, FA2, FB2, and FC2, was obtained by the *in vitro* diffusion experiments through human skin. The overall permeation percentage and permeation percentage in membrane was significantly higher in FB2 than in FA2; there was no significant difference in overall permeation percentage and permeation percentage in membrane between FC2 and FA2. The overall permeation percentage and permeation percentage of membrane was significantly higher in the combined preparation of FB2 than the counterpart of the single preparations.

FB2 was an oil-in-water emulsion with thickening agent, xanthan gum. This thickening agent was added to the dispersing phase to increase homogeneity and stability of the emulsion. The addition of xanthan gum increased stability of the oil droplets and reduced diameter of the oil droplets (Ye, *et al.*, 2004). In FB2, the diameter of oil droplets containing DEET was significantly reduced (**Table 4.12**). The smaller droplet size increased the surface area of oil droplets and enhanced the contact area of oil droplets with skin surface, which may subsequently induce higher skin penetration of the active ingredients, such as DEET, from the emulsion (Ktistis, *et al.*, 1998; Schwarz, *et al.*, 1995). However, the diameter of oil droplets containing OBZ was not decreased, as oil droplets containing OBZ tended to coalesce and formed larger oil droplets. Although FB2 containing OBZ had larger oil droplet size, penetration of OBZ from FB2 still significantly increased comparing to FA2. This implied that addition of xanthan gum may increase OBZ affinity to the membrane and hence facilitate OBZ partition into the skin from oil droplets, thereby increasing membrane retention and permeation of OBZ as shown in **Figure 5.2**. Moreover, the concurrent application of DEET and OBZ in FB2 with xanthan gum induced synergistic skin retention and permeation of two compounds, which may be attributed to the addition of xanthan gum and chemical interactions between two compounds.

FC2 significantly increased the overall permeation percentage of DEET in single preparation by 56% comparing to FA2, while permeation percentage of DEET in

membrane was significantly higher by 9.6-fold in single preparation of FC2 than FA2. There was no significant difference in overall permeation percentage of OBZ between FC2 and FA2. The combined preparation of FC2 produced a decrease of 41% in overall permeation percentage of DEET comparing to its single preparation, but there was no significantly difference in OBZ between combined and single preparations. Skin penetration of active ingredients from a water-in-oil emulsion may be influenced by solubility capacity of a compound in both organic and aqueous media. Both DEET and OBZ are lipophilic compounds with LogKo/w (octanol/water partition coefficient) of 2.01 and 3.79, respectively. They were dissolved in oil phase of the emulsion, which was the external phase in FC2. When a chemical was dissolved in the external phase of an emulsion, it may have a closer contact with interfacial area between membrane and vehicle, and produce higher skin penetration than a chemical dissolved in the internal phase (Wiechers, 2005). DEET, as a lipophilic compound, showed higher skin penetration from the external phase of water-in-oil emulsion, FC2, than the internal phase of oil-in-water emulsion, FA2. And some oil components of the external phase, such as castor oil, may also penetrate into stratum corneum (Butcher, 1953), and increase the solubility of DEET in the skin, resulting in higher skin retention of DEET in FC2. However, OBZ is more lipophilic than DEET with LogKo/w 3.79. In accordance with the law of “like dissolves like”, OBZ may possess stronger affinity with highly lipophilic oil components in the external phase than with stratum corneum of human skin, so that OBZ

may demonstrate lower driven force in FC2 to penetrate through human skin. Therefore, OBZ did not produce a significant increase of skin penetration from FC2 when compared to FA2, even though OBZ was dissolved in the external phase of FC2. Similar results had also been reported by Dal Pozzo & Pastori that skin permeation of lipophilic parabens was enhanced from oil-in-water emulsions compared to water-in-oil emulsions (Dal Pozzo, *et al.*, 1996). Furthermore, presence of OBZ in FC2 may increase the affinity of DEET for the oil components in FC2, thereby decreasing skin penetration of DEET from the preparation.

Release amount of DEET and OBZ from FA2, FB2, and FC2 in 6 hours through human skin was shown in **Figures 5.3 and 5.4**. The release curves of DEET and OBZ were identified as linear. Subsequently permeation coefficient of DEET and OBZ in FA2, FB2, and FC2 through human skin were calculated through these linear curves and listed in **Table 5.1**. Among the three emulsions, permeation coefficients through human skin ranged from 16.06 to 30.07 ($\times 10^{-4}$, cm/h) for DEET and from 2.03 to 4.67 ($\times 10^{-4}$, cm/h) for OBZ. FB2 increased DEET permeation coefficient through human skin by 22% comparing to FA2. Concurrent application of DEET and OBZ in FB2 increased DEET permeation coefficient by 7%. FC2 did not result in an increase of DEET permeation coefficient through human skin comparing to FA2, while presence of OBZ in FC2 decreased DEET permeation coefficient by 27%. There was no significant difference observed in permeation coefficient of OBZ through human skin. Permeation coefficient is

Table 5.1. Permeation coefficient of DEET and OBZ through human skin (cm/h×10^{-4})

Active ingredients	Formulations		
	FA2	**FB2**	**FC2**
DEET (S)	23.31±1.95[1, 2]	27.95±0.76[1, 2, 3]	22.09±2.57[1, 3]
DEET (C)	20.95±2.00[1]	30.07±3.95[1, 3]	16.06±2.47[1, 3]
OBZ (S)	3.09±0.23[1]	3.77±0.50[1]	2.03±0.20[1]
OBZ (C)	4.09±0.31[1]	4.67±0.40[1]	2.16±0.30[1]

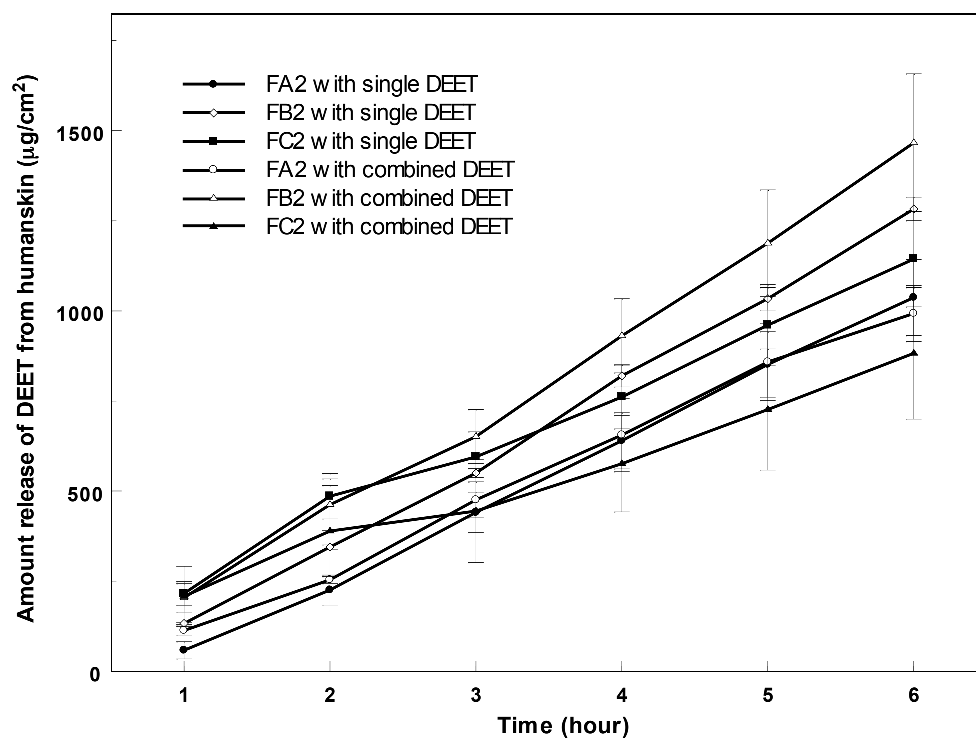
n = 4, Mean ± SEM, $p \leq 0.05$

[1]: Significant difference between LDPE and human skin

[2]: Significant difference in combined DEET between FA2 and FB2/ FC2

[3]: Significant difference between single preparations and combined preparations

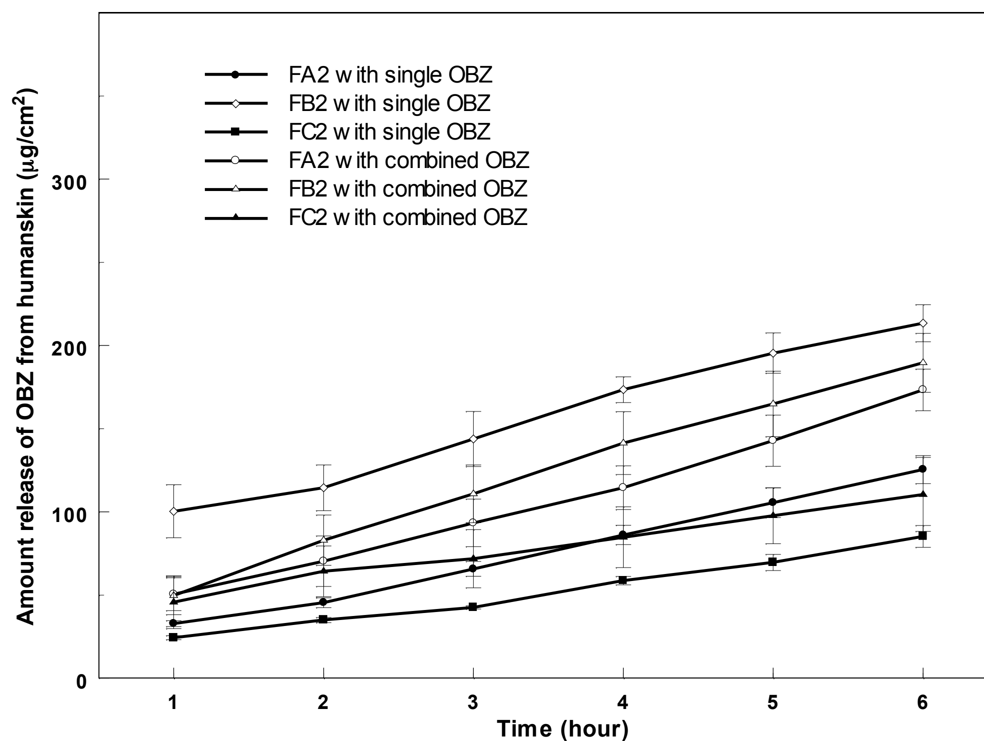
Figure 5.3. Amount release of DEET in 6 h through human skin†



n = 4, Mean ± SEM

†Amount release of DEET from three emulsion-based formulation, FA2, FB2, and FC2 (solid circle: FA2 with single DEET; hollow diamond: FB2 with single DEET; solid rectangular: FC2 with single DEET; hollow circle: FA2 with combined DEET; hollow triangle: FB2 with combined DEET; solid triangle: FB2 with combined DEET), through human skin showed the linear increase by the time in 6 h.

Figure 5.4. Amount release of OBZ in 6 h through human skin†



n = 4, Mean ± SEM

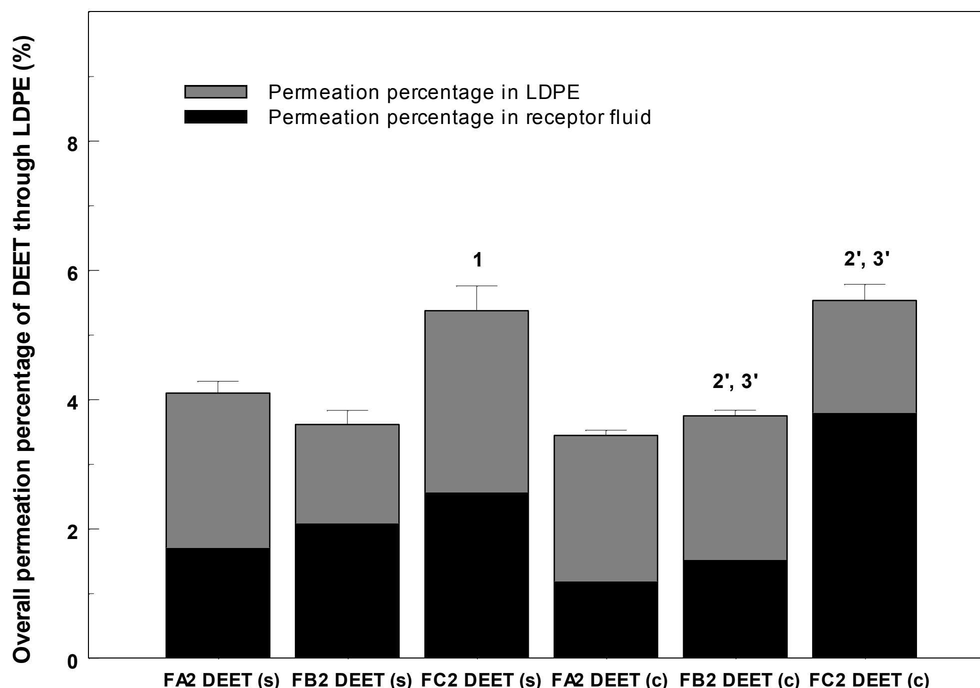
†Amount release of OBZ from three emulsion-based formulation, FA2, FB2, and FC2 (solid circle: FA2 with single OBZ; hollow diamond: FB2 with single OBZ; solid rectangular: FC2 with single OBZ; hollow circle: FA2 with combined OBZ; hollow triangle: FB2 with combined OBZ; solid triangle: FB2 with combined OBZ), through human skin showed the linear increase by the time in 6 h.

an important parameter in skin diffusion and penetration; it describes the velocity a permeant travels across a membrane. Permeation coefficient is often utilized to compare permeation profiles of a substance, which is examined under different experimental conditions. Permeability of an active ingredient can be influenced by carrier vehicle and interaction between the active ingredient and/or vehicle/skin (Otto, *et al.*, 2009). Emulsion type and excipients present in the emulsion frequently play important roles in modifying permeability of the active ingredients through membrane models. Adding thickening agent xanthan gum to emulsion formula was able to decrease droplet size in oil-in-water emulsion containing single DEET, which consequently increased DEET permeability through human skin. Presence of OBZ also increased DEET permeability in oil-in-water emulsion containing xanthan gum, which may be partially attributed to interactions between xanthan gum and the two compounds within both vehicle and membrane. Since OBZ may exhibit higher affinity for oil components in a water-in-oil emulsion, its permeability may be reduced as a result. OBZ may also enhance affinity of DEET for oil phase in a water-in-oil emulsion, and induced a decrease in DEET permeability through human skin. Previous studies have indicated that permeation of DEET and OBZ was dependent upon formulation types and excipients (Aghazarian, *et al.*, 1999; Ross, *et al.*, 2000; Wissing, *et al.*, 2002), which were in agreement with findings from this study.

5.4.1.2. Skin Penetration through LDPE

Figures 5.5 and 5.6 showed the overall permeation percentage of DEET and OBZ in membrane and receptor fluid from FA2, FB2, and FC2 through LDPE. The overall permeation percentage of OBZ through LDPE significantly increased by 26-628% comparing to that of OBZ through human skin. At the same time, the overall permeation percentage of DEET through human skin significantly increased by 64-338% comparing to that of DEET through LDPE. These variable permeation profiles between DEET and OBZ may result from membrane differences and interactions between chemicals and membranes. LDPE is an artificial membrane made of low-density polyethylene resin and possesses lipophilic properties in nature. Human skin is much more structurally complex than LDPE. Stratum cornuem, a principal rate-limiting barrier against percutaneous penetration, is comprised of lipophilic components (intercellular lipid matrix) and hydrophilic components (corneocytes) (Proksch, *et al.*, 2008). DEET may create a proper balanced affinity for both lipid components and hydrophilic keratin parts in stratum corneum due to its appropriate LogKo/w 2.01 and low molecular weight, hence penetrated more through human skin than through LDPE. With a higher LogKo/w of 3.79, OBZ was more lipophilic and possessed a higher affinity for LDPE than for human skin; as such its penetration through LDPE was higher than DEET. It was also noted in **Figures 5.5** and **5.6** that FB2 did not increase the overall permeation percentage of DEET in single preparation comparing to FA2.

Figure 5.5. Overall DEET permeation percentage through LDPE



n = 4, Mean ± SEM, $p \le 0.05$

1: Significant difference between FA2 and FB2/FC2 (membrane + receptor fluid)

1': Significant difference between single preparations and combined preparations (membrane + receptor fluid)

2: Significant difference between FA2 and FB2/FC2 (membrane only)

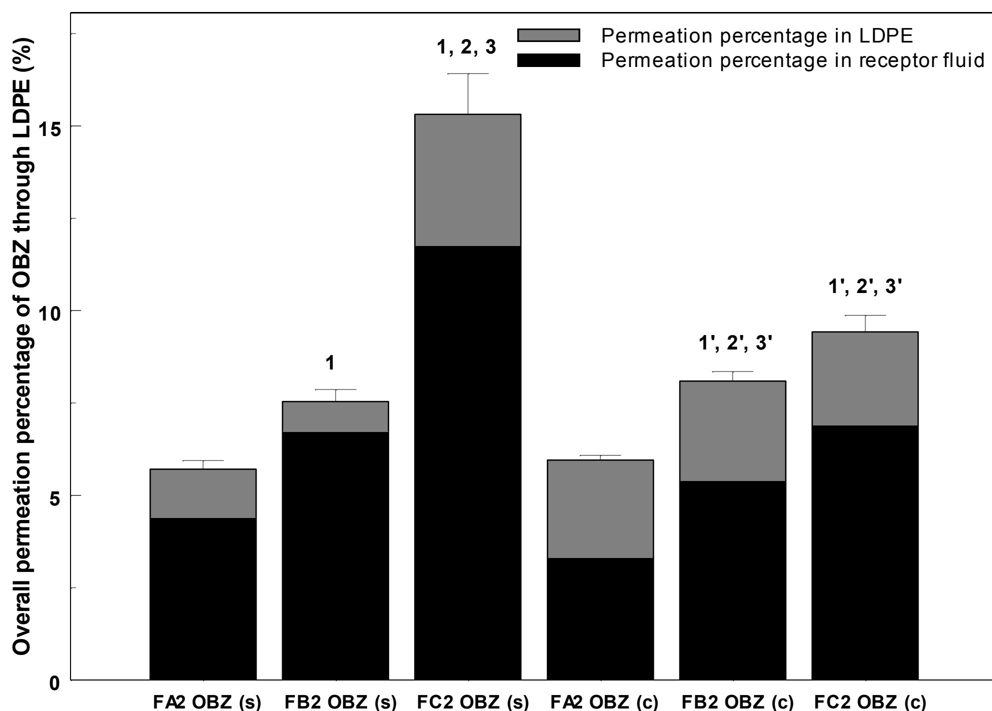
2': Significant difference between single preparations and combined preparations (membrane only)

3: Significant difference between FA2 and FB2/FC2 (receptor fluid only)

3': Significant different between single preparations and combined preparations (receptor fluid only)

†The overall permeation percentage of DEET in membrane LDPE (gray bar) and in receptor fluid (black bar) from three emulsion-based formulations, FA2, FB2, and FC2, was obtained through the *in vitro* diffusion experiments through LDPE. FC2 significantly increased the overall permeation percentage in single preparation comparing to FA2, but there is no significant difference in single preparation between FB2 and FA2. The combined preparation of FB2 increased the permeation percentage in membrane and decreased the permeation percentage in receptor fluid compared to single preparation of FB2, however, the combined preparation of FC2 decreased the permeation percentage in membrane and increased the permeation percentage in receptor fluid compared to single preparation of FC2

Figure 5.6. Overall OBZ permeation percentage through LDPE†



n = 4, Mean ± SEM, $p \leq 0.05$

1: Significant difference between FA2 and FB2/FC2 (membrane + receptor fluid)

1': Significant difference between single preparations and combined preparations (membrane + receptor fluid)

2: Significant difference between FA2 and FB2/FC2 (membrane only)

2': Significant difference between single preparations and combined preparations (membrane only)

3: Significant difference between FA2 and FB2/FC2 (receptor fluid only)

3': Significant different between single preparations and combined preparations (receptor fluid only)

†The overall permeation percentage of OBZ in membrane LDPE (gray bar) and in receptor fluid (black bar) from three emulsion-based formulations, FA2, FB2, and FC2, was obtained through the *in vitro* diffusion experiments through LDPE. FB2 and FC2 significantly increased the overall permeation percentage of OBZ in single preparation comparing to FA2. The combined FB2 preparation significantly increased the overall permeation percentage and permeation percentage in membrane and decreased the permeation percentage in receptor fluid comparing to single preparation. The combined FC2 preparation significantly decreased overall permeation percentage, permeation percentage in membrane, and permeation percentage in receptor fluid

Presence of OBZ in FB2 significantly increased DEET retention within membrane by 120% and decreased its penetration into receptor fluid by 29%, but it did not significantly increase the overall penetration of DEET. Addition of xanthan gum to the emulsion may form smaller droplet size in the preparation containing single DEET, but it may not change DEET affinity for more lipophilic membrane LDPE. The lower affinity of DEET for LDPE membrane became a key factor in modifying its membrane penetration from different formulations. In addition, OBZ may help increase DEET solubility within membrane when xanthan gum was present, so that DEET reserved more in the membrane and diffused less into the receptor fluid. For OBZ, FB2 significantly increased the overall permeation percentage of OBZ in single preparation by 32% comparing to FA2. The combined FB2 preparation significantly increased the overall permeation percentage of OBZ by 8% comparing to single preparation; it also significantly increased OBZ retention in membrane by 240% and significantly decreased OBZ penetration into receptor fluid by 19% compared with single preparation. Presence of DEET together with xanthan gum in FB2 may have increased OBZ affinity for more lipophilic membrane LDPE, leading to higher retention of OBZ within the membrane.

For FC2, the overall permeation percentage of DEET in single preparation significantly increased by 31% comparing to FA2. Presence of OBZ in FC2 significantly increased DEET permeation percentage in the receptor fluid by 46% and decreased its retention in the membrane by 36%. But there was no significant difference observed in

the overall permeation percentage of DEET. In water-in-oil emulsion, DEET was dissolved in the external oil phase. Chemicals that are dissolved in external phase would have higher skin penetration (Wiechers, 2005). Therefore DEET in external phase of a water-in-oil emulsion had higher membrane penetration than in internal phase of an oil-in-water emulsion. In addition, the more lipophilic OBZ possessed higher affinity for lipophilic membrane LDPE than DEET. Due to concurrent presence of DEET and OBZ, OBZ may increase DEET affinity to LDPE and enhance its transmembrane permeation. For OBZ, its overall permeation percentage in single FC2 preparation was significantly higher by 169% than FA2. This was higher by 180% in the membrane and by 52% in the receptor fluid. Presence of DEET significantly decreased overall OBZ permeation percentage by 40% in the membrane and by 36% in the receptor fluid. In water-in-oil emulsion, a lipophilic OBZ was also dissolved in the external phase; it possessed a higher affinity for LDPE. These may contribute to higher driving force for OBZ to release into the membrane and to diffuse into the receptor fluid, thereby increasing its membrane retention and transmembrane permeation. Compared to OBZ, DEET had a lower affinity for lipophilic LDPE. Possible interaction between DEET and OBZ may affect OBZ affinity to LDPE. Thus OBZ membrane retention and transmembrane permeation were reduced.

Release amount of DEET and OBZ from FA2, FB2, and FC2 in 6 hours through LDPE was shown in **Figures 5.7 and 5.8**. The release curves for both DEET and OBZ

were identified as linear. Permeation coefficients of DEET and OBZ in FA2, FB2, and FC2 through LDPE were calculated through these linear curves and listed in **Table 5.2**. Values of permeation coefficient ranged from 3.13 to 8.80 ($\times 10^{-4}$, cm/h) for DEET and from 7.97 to 26.75 ($\times 10^{-4}$, cm/h) for OBZ respectively. OBZ permeability through LDPE was increased compared to human skin, but that of DEET was decreased. This may be attributed to higher affinity of OBZ to lipophilic LDPE and higher affinity of DEET to human skin. When comparing permeation coefficient of DEET and OBZ among the three prepared emulsions, FB2 significantly increased OBZ permeability in single preparation by 39% in comparison to FA2. OBZ permeability in combined FB2 was significantly decreased by 20% in comparison to single FB2. Adding thickening agent xanthan gum apparently led to higher OBZ permeability, while presence of DEET reduced OBZ permeability. These were consistent with results observed in OBZ permeation percentage from FB2 preparations. Permeation coefficient of OBZ through LDPE in single FC2 preparation had a significant increase of 152% in comparison to that of FA2. Permeation coefficient of OBZ through LDPE in combined FC2 preparation was significantly lowered by 51% in comparison to its single preparation. This implied that OBZ dissolved in the external oil phase exhibited higher permeability in FC2. Presence of DEET decreased OBZ permeability due to decreased affinity of OBZ for LDPE membrane. There was no significant difference observed in DEET permeation through LDPE.

Table 5.2. Permeation coefficient of DEET and OBZ through LDPE (cm/h×10^{-4})

Active ingredients	Formulations		
	FA2	**FB2**	**FC2**
DEET(S)	4.45±0.48[1]	5.18±0.56[1]	5.77±0.87[1]
DEET(C)	3.13±0.21[1]	3.93±0.19[1]	8.80±0.59[1]
OBZ (S)	10.61±0.60[1, 2]	14.65±0.64[1, 2, 3]	26.75±2.64[1, 2, 3]
OBZ (C)	7.97±0.30[1]	11.80±0.54[1, 3]	13.20±0.78[1, 3]

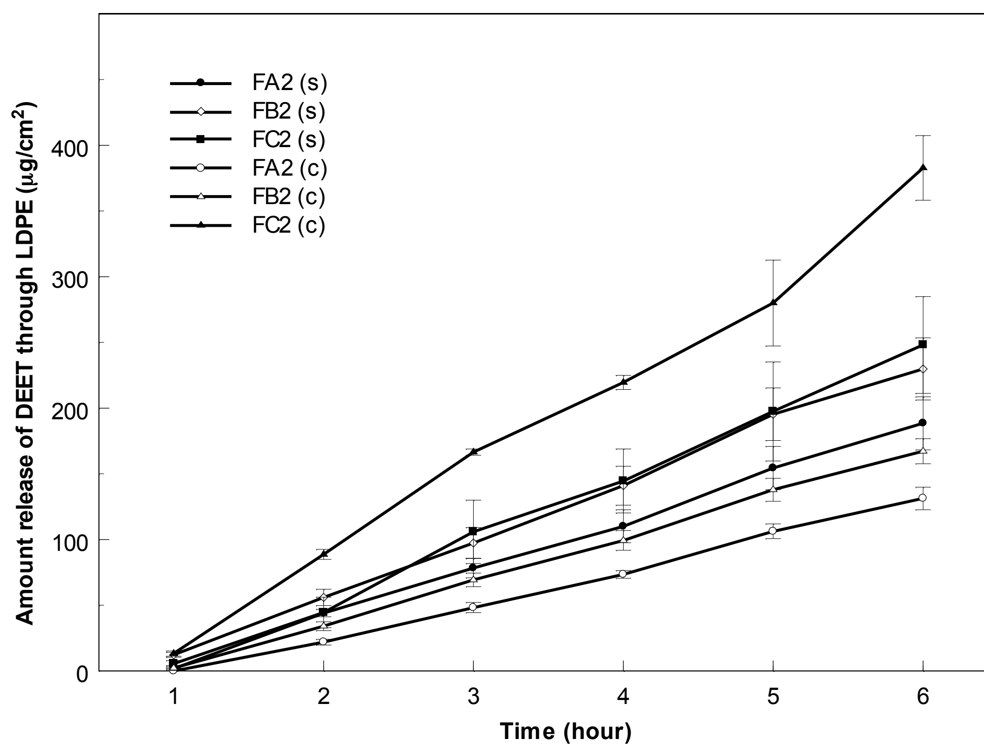
n = 4, Mean ± SEM, $p \leq 0.05$

[1]: Significant difference between LDPE and human skin

[2]: Significant difference in single OBZ between FA2 and FB2/FC2

[3]: Significant difference between single preparations and combined preparations

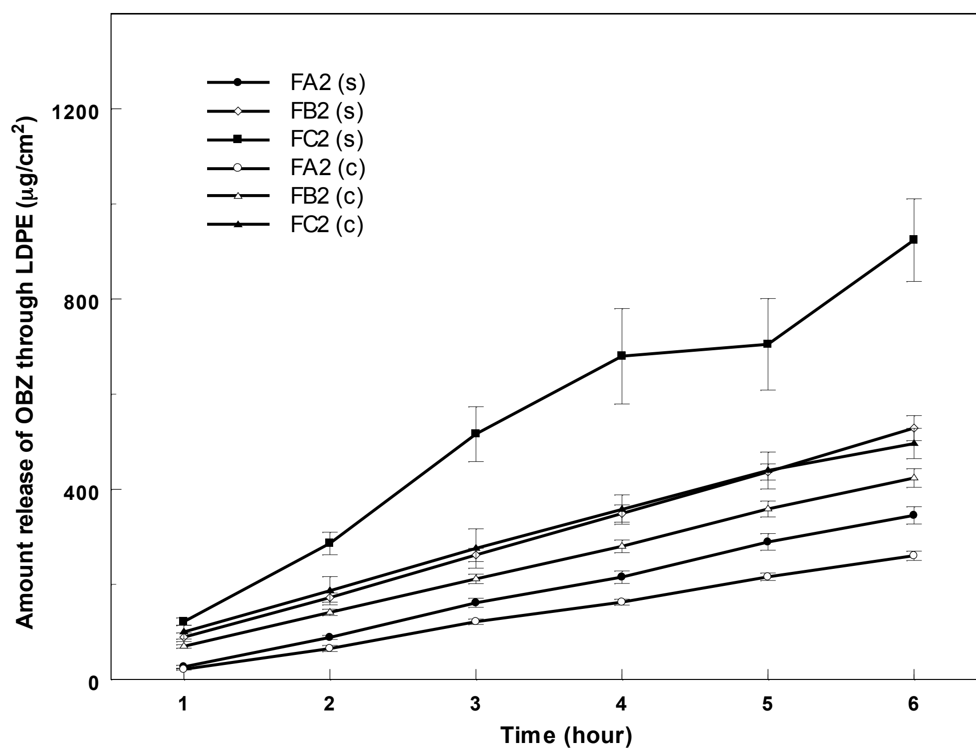
Figure 5.7. Amount release of DEET in 6 h through LDPE



n = 4, Mean ± SEM

†Amount release of DEET from three emulsion-based formulation, FA2, FB2, and FC2 (solid circle: FA2 with single DEET; hollow diamond: FB2 with single DEET; solid rectangular: FC2 with single DEET; hollow circle: FA2 with combined DEET; hollow triangle: FB2 with combined DEET; solid triangle: FB2 with combined DEET), through LDPE showed the linear increase by the time in 6 h.

Figure 5.8. Amount release of OBZ in 6 h through LDPE†



n = 4, Mean ± SEM

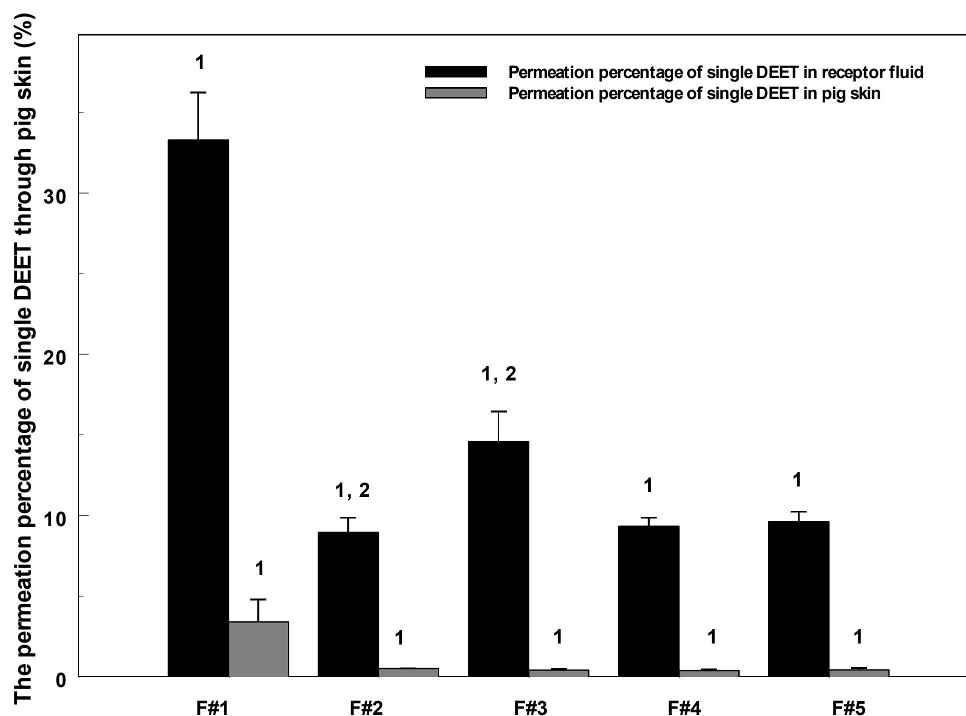
†Amount release of OBZ from three emulsion-based formulation, FA2, FB2, and FC2 (solid circle: FA2 with single OBZ; hollow diamond: FB2 with single OBZ; solid rectangular: FC2 with single OBZ; hollow circle: FA2 with combined OBZ; hollow triangle: FB2 with combined OBZ; solid triangle: FB2 with combined OBZ), through LDPE showed the linear increase by the time in 6 h.

5.4.2. Diffusion Study from Five Emulsion-based Formulations

Permeation percentage of DEET from five emulsion-based formulations through pig skin was shown in **Figures 5.9 and 5.10**. The permeation ranged from 8.72-33.91% in the receptor fluid and from 0.31-3.40% in the membrane. Permeation of single DEET from Formulation #1 (F#1) was significantly higher by 128-272% in the receptor fluid and 569-789% in the membrane than the other formulations. Between oil-in-water emulsions, permeation of single DEET from Formulation #2 (F#2) was significantly lower by 40% in the receptor fluid than Formulation #3 (F#3), while no difference in membrane retention was observed between the two formulas. For water-in-oil emulsions, permeation of single DEET in both receptor fluid and membrane showed no difference between Formulation #4 (F#4) and Formulation #5 (F#5). When comparing permeation between single DEET and combined DEET, permeation of single DEET in F#1 was higher by 113% in the receptor fluid and 992% in the membrane than that of combined DEET respectively. Permeation of combined DEET in F#2 was higher by 57% in the receptor fluid and 438% in the membrane than that of single DEET respectively. There was no difference between single DEET and combined DEET in F#3, F#4 and F#5.

Permeation percentage of OBZ in the same five formulations through pig skin was shown in **Figures 5.11 and 5.12**, which was in the range of 0.31-0.76% in the receptor fluid and 0.19-0.63% in the membrane. No difference was observed among these formulations in OBZ permeation in the receptor fluid or in the membrane.

Figure 5.9. Permeation percentage of single DEET through pigskin†



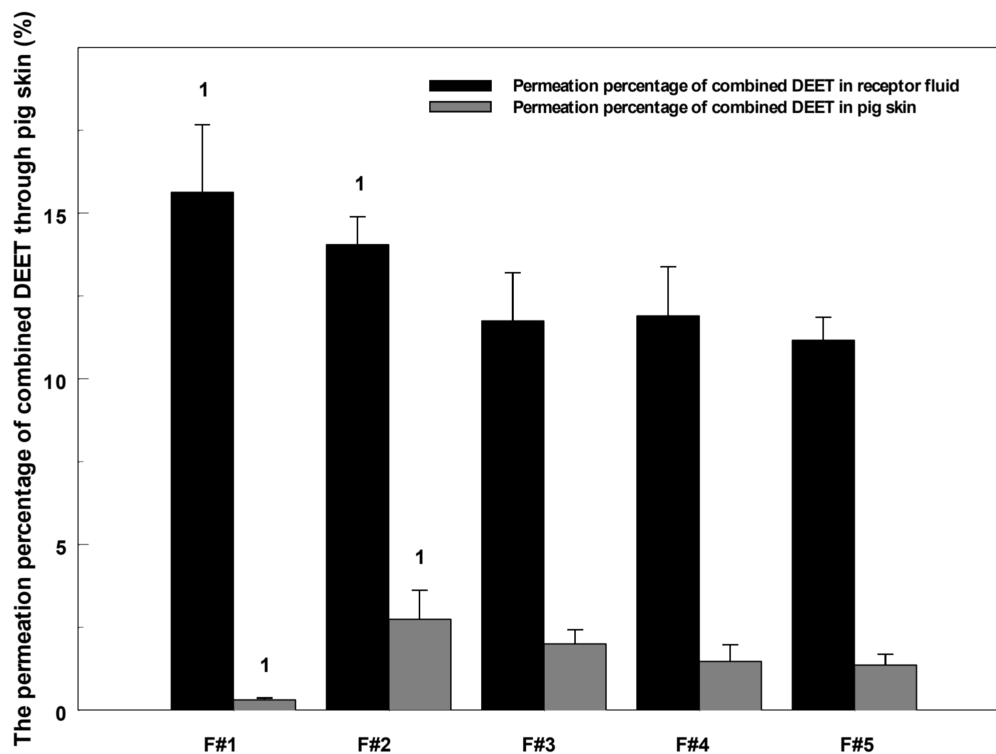
n=5, Mean±SEM, p≤0.05

1: Significant difference between F #1 and other emulsions

2: Significant difference between F#2 and F#3

†The permeation percentage of single DEET in membrane pigskin (gray bar) and in receptor fluid (black bar) from five emulsion-based formulations, F#1, F#2, F#3, F#4, and F#5, was obtained through the *in vitro* diffusion experiments through pigskin. The permeation percentage of single DEET from F#1 was significantly higher in receptor fluid or in membrane than the counterpart of other formulations. The permeation percentage of single DEET from F#2 was significantly lower in the receptor fluid than the other oil-in-water emulsions, F#3; no difference in membrane was observed between the two emulsions. The permeation percentage of single DEET in receptor fluid or in membrane showed no difference between two water-in-oil emulsions, F#4 and F#5.

Figure 5.10. Permeation percentage of combined DEET through pigskin†

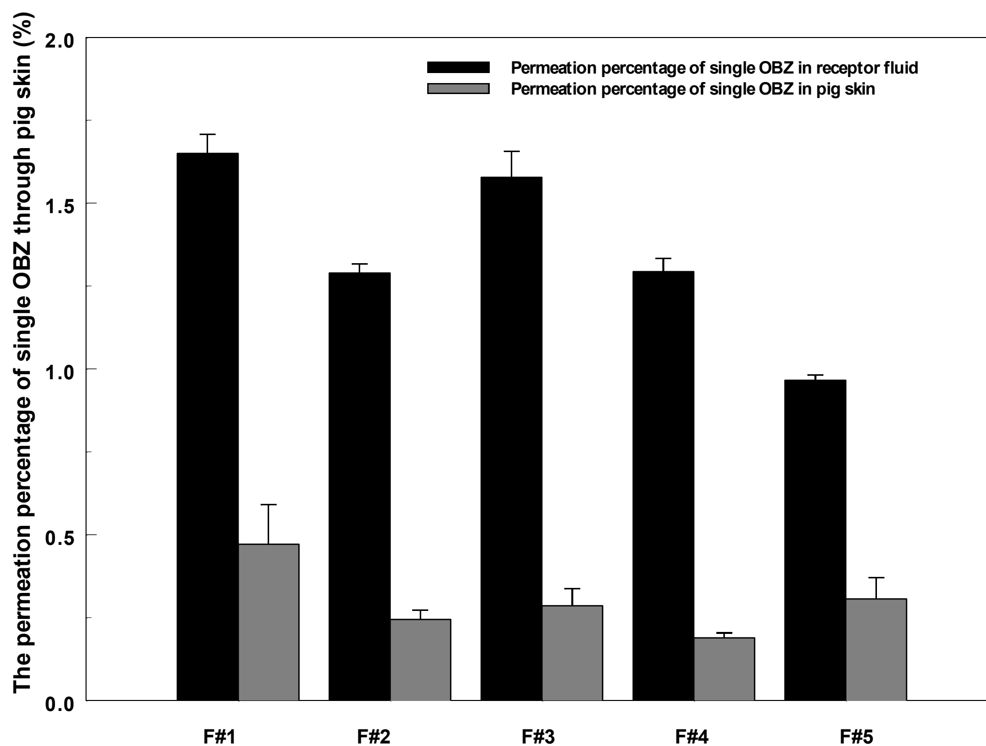


n=5, Mean±SEM, p≤0.05

1: Significant difference between single DEET and combined DEET

†The permeation percentage of combined DEET in membrane pigskin (gray bar) and in receptor fluid (black bar) from five emulsion-based formulations, F#1, F#2, F#3, F#4, and F#5 was obtained through the *in vitro* diffusion experiments through pigskin. The permeation percentage of combined DEET in receptor fluid and membrane from F#1 was lower than that of single DEET; the permeation percentage of combined DEET in the receptor fluid and the pigskin from F#2 was higher than that of single DEET. There was no difference between single DEET and combined DEET in F#3, F#4 and F#5.

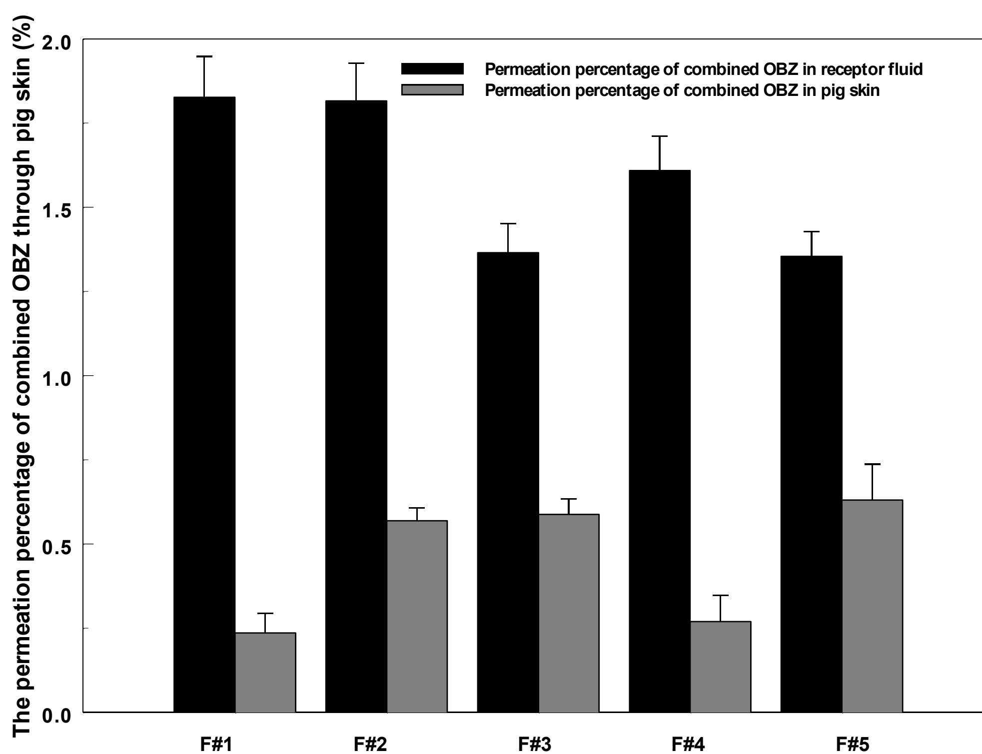
Figure 5.11. Permeation percentage of single OBZ through pigskin†



n=5, Mean±SEM

†The permeation percentage of single OBZ in membrane pigskin (gray bar) and in receptor fluid (black bar) from five emulsion-based formulations, F#1, F#2, F#3, F#4, and F#5, was obtained through the *in vitro* diffusion experiments through pigskin. There was no significant difference observed among these formulations in single OBZ permeation percentage in receptor fluid or in membrane.

Figure 5.12. Permeation percentage of combined OBZ through pigskin†



n=5, Mean±SEM

†The permeation percentage of combined OBZ in membrane pigskin (gray bar) and in receptor fluid (black bar) from five emulsion-based formulations, F#1, F#2, F#3, F#4, and F#5 was obtained through the *in vitro* diffusion experiments through pigskin. There was no significant difference observed among these formulations in combined OBZ permeation percentage in receptor fluid or in membrane.

As previously described, topical preparation vehicle played an important role in skin penetration of the active ingredients. Emulsion, a widely used semisolid topical preparation, particularly exhibited an influence on chemical skin penetration. Within all emulsion constituents, emulsifier and its distribution was a critical factor on drug release and disposition. In this study, five different emulsifiers were selected for each emulsion; they were Emulfree CBG, Emulium 22, Apifil, Arlacel P-135, and Plurol Diisostearique, used in F#1, F#2, F#3, F#4, and F#5 respectively.

Emulfree CBG selected for F#1 was a multicomponent surfactant-free emulsifying agent, which contained butylene glycol cocoate, ethyl cellulose, and isostearic alcohol. Once an oil-in-water emulsion was formed using the emulsifier, oil droplets were dispersed in an aqueous gel; ethyl cellulose would form multilayer film on the interface of water-oil phase to stabilize dispersed oil droplets through steric repulsions. As a result, F#1 could be regarded as a bi-gel multiphase disperse system in which the oil droplets were trapped or embedded into the aqueous phase without the use of a surfactant. With no strong adsorption of surfactants to the interface of oil droplets and lower affinity of active ingredients for oil phase in F#1, DEET and OBZ may show tendency to partition more into stratum corneum from emulsion vehicle. Partition coefficient of DEET in F#1 was indeed significantly higher than that of other emulsions tested (**Table 5.3**). This may also indicate that more DEET would partition from the vehicle into the skin, therefore increasing skin retention and percutaneous permeation of the compound.

Table 5.3. Permeation parameters of DEET and OBZ through pig skin

Formulation	Compounds	$K_m (\times 10^{-2})$	Lag (h)	$K_P (\mu m/h \times 10^{-4})$
F#1	DEET (S)	54.00±8.00[1, 4]	1.54±0.33	23.41±1.90[1, 4]
	DEET (C)	16.00±3.00[4]	1.13±0.30	10.53±1.41[4]
	OBZ (S)	0.20±0.00	0.32±0.07[1, 4]	0.45±0.02
	OBZ (C)	1.00±0.00	1.19±0.28[4]	0.52±0.06
F#2	DEET (S)	12.00±1.00[1]	1.32±0.19	5.95±0.63[1, 2, 4]
	DEET (C)	13.00±2.00	0.90±0.14	9.47±0.62[4]
	OBZ (S)	2.00±0.00	4.22±0.22[1, 2, 4]	0.29±0.02
	OBZ (C)	1.00±0.00	2.15±0.28[4]	0.40±0.04
F#3	DEET (S)	11.00±2.00[1]	0.76±0.17	9.68±1.26[1, 2]
	DEET (C)	8.00±3.00	0.60±0.15	7.83±1.01
	OBZ (S)	1.00±0.00	2.24±0.45[1, 2, 4]	0.38±0.02
	OBZ (C)	0.20±0.1	0.59±0.29[4]	0.21±0.02
F#4	DEET (S)	13.00±1.00[1]	1.39±0.12	6.00±0.37[1]
	DEET (C)	6.00±1.00	0.53±0.10	7.93±0.47
	OBZ (S)	1.00±0.00	2.39±0.57[1, 3, 4]	0.35±0.03
	OBZ (C)	0.60±0.10	0.71±0.27[4]	0.41±0.03
F#5	DEET (S)	17.00±1.00[1]	1.77±0.08	6.18±0.45[1]
	DEET (C)	10.00±0.00	0.87±0.03	7.20±0.46
	OBZ (S)	2.00±0.00	3.84±0.23[1, 3, 4]	0.29±0.03
	OBZ (C)	1.00±0.00	2.15±0.41[4]	0.40±0.03

n=5, Mean ±SEM, $p \leq 0.05$

[1]: Significant difference between F#1 and other formulations

[2]: Significant difference between F#2 and F#3

[3]: Significant difference between F#4 and F#5

[4]: Significant difference between single preparations and combined preparations

Emulium® 22 and Apifil® selected for F#2 and F#3 were both nonionic, synthetic oil-in-water emulsifying agents. Emulium® 22 was tribehenin PEG-20 esters with an HLB of 10.5, while Apifil® was PEG-8 modified beeswax with an HLB of 9.0. Due to a higher HLB, Emulium® 22 possessed a stronger ability to decrease interfacial tension than Apifil®. Consequently oil droplets that dissolved the active ingredients were more stable in F#2 than in F#3. The compact emulsifier adsorption surrounding oil globules may retard the release of active ingredients from oil globules. This may decrease DEET permeation through the skin in F#2 when compared to F#3. However different interfacial films formed around oil globules between F#2 and F#3 showed no influence on skin retention of DEET, which may be attributed to similar partition coefficient of DEET between F#2 and F#3 (**Table 5.3**). For F#4 and F#5, nonionic, synthetic water-in-oil emulsifying agents Arlacel P-135 and Plurol Diisostearique were used respectively. Arlacel P-135 was PEG 30 dipolyhydroxystearate with an HLB of 5.5; plurol diisostearique had an HLB value of 4.5. Since DEET and OBZ were both dissolved in the external oil phase, the effect of emulsifier agents on release of compounds from the emulsion vehicle was minimal. No difference was observed in skin retention and permeation of DEET between F#4 and F#5.

An emollient used in topical skin products is defined as a substance that can soften the skin and protect it from dryness (Otto, *et al.*, 2009). Emollients are usually oily material that prevents water loss from the skin. In 2005, Wiechers *et al.* introduced a

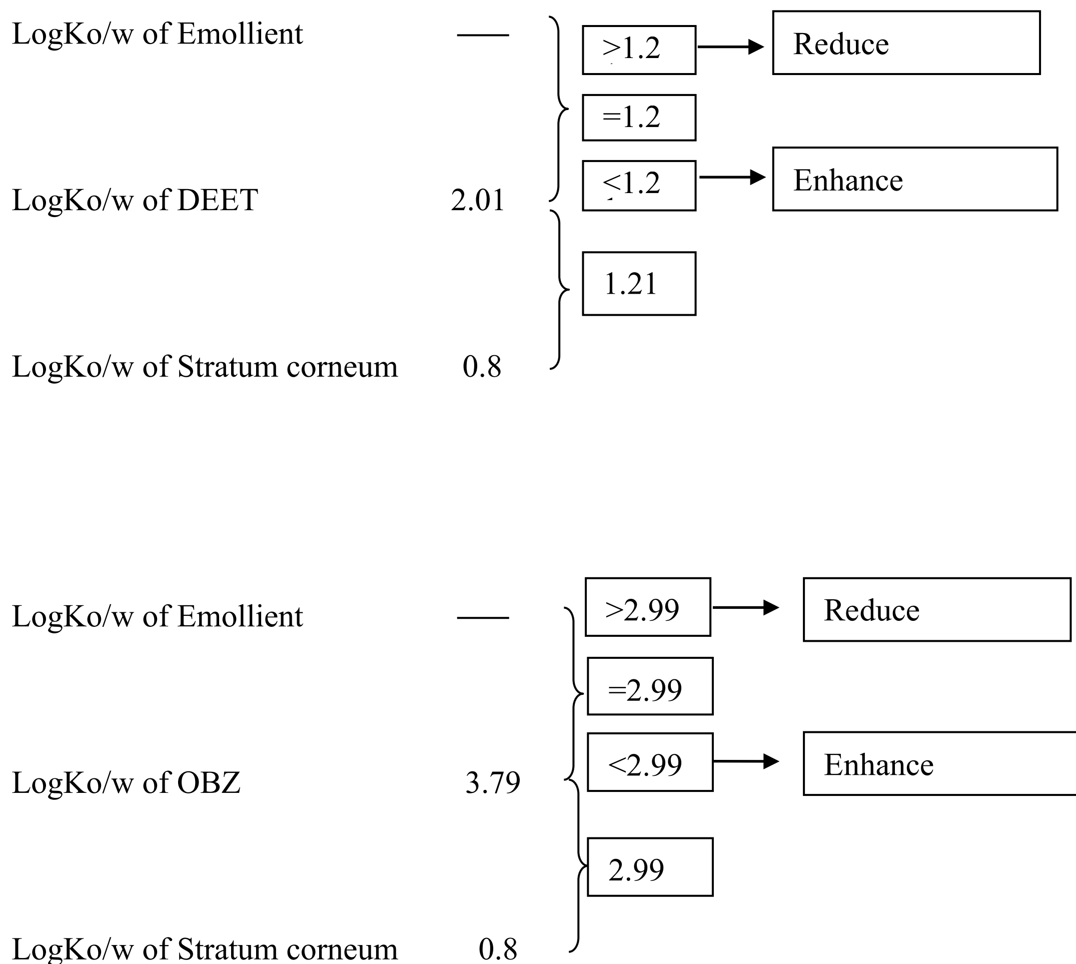
method of "Formulating for Efficacy" to select appropriate emollients for optimizing skin delivery of chemical compounds from emulsions. An emulsion should be designed to incorporate an active ingredient in the preparation at a concentration close to its maximum solubility, but this solubility in the emulsion should be lower than the solubility in the stratum corneum to maximize partition coefficient K_m between stratum corneum and the preparation (Wiechers, 2005). In this study, the primary objective was to reduce overall percutaneous permeation of DEET and OBZ because they were intended for skin surface protection. Hence solubility of DEET and OBZ in the prepared emulsions would rather be higher than that in the stratum corneum, in order to suppress partition coefficient K_m between the stratum corneum and emulsions. To achieve this goal, polarity of the emulsions was considered, and relative polarity index (RPI) was established, which was originally based on the octanol-water partition coefficient. The RPI parameter compared the polarity of the active ingredient relative to the polarity of the stratum corneum and the emollient. Emollients used for the calculation should be in the same phase in which an active ingredient is to be dissolved.

To compare the polarity of an active ingredient with that of the stratum corneum and the emollient components in an emulsion, three LogKo/w values were required, namely the polarity of stratum corneum (0.8) (Bouwstra, *et al.*, 2003), the polarity of the active ingredients (DEET and OBZ), and the polarity of an emollient. DEET and OBZ were lipophilic components with LogKo/w of 2.01 and 3.79 respectively. They were

dissolved in an oil phase that also contained emollients. The difference of polarity between stratum corneum and DEET or OBZ was 1.21 (2.01-0.8) and 2.99 (3.79-0.8), respectively. When the polarity of emollients selected for formulation was larger than 3.22 (2.01+1.21) or 6.78 (3.79+2.99), solubility of DEET and OBZ was enhanced in the formulation and their skin penetration from the formulation would be decreased. On the other hand, should the polarity of emollients selected for formulation was smaller than 3.22 or 6.78, the solubility of DEET and OBZ in the emulsion would be decreased and their skin penetration be enhanced (**Figure 5.13**).

The oil phase of F#1 was composed of emulsifier Emulfree CBG, thickening agent Geleol, and solubilizer/coemulsifier Labrasol; there was no emollient in the preparation. So the polarity of emollient in this emulsion was considered 0. The calculated polarity difference between the oil phase and the active ingredient in F#1 was -2.01 (0-2.01) for DEET and -3.79 (0-3.79) for OBZ, which was lower than 3.22 and 6.78, respectively. As the result, F#1 would possess a high tendency for DEET and OBZ to diffuse and permeate through the skin membrane. This was proven in Figure 5.10, where F#1 demonstrated the highest skin retention and skin penetration of DEET among the five test emulsion formulas.

Figure 5.13. The calculation of the polarity of an emollient in the formulation†



†An appropriate emollient in an emulsion can be selected by comparing the polarity of the active ingredient (DEET and OBZ) relative to the polarity of the stratum corneum and the emollient. When the polarity of emollients selected for the formulation was larger than 3.22 (2.01+1.21) or 6.78 (3.79+2.99), solubility of DEET and OBZ was enhanced in the formulation and their skin penetration from the formulation would be decreased. On the other hand, should the polarity of emollients selected for formulation was smaller than 3.22 or 6.78, the solubility of DEET and OBZ in the emulsion would be decreased and their skin penetration would be enhanced.

MOD (octyldodecyl myristate) and hydrogenated polydencene were utilized as emollients in F#2 and F#3 respectively. Their LogKo/w values were 15.53 for MOD (octyldodecyl myristate) and 4.88 for hydrogenated polydencene, calculated using Meylan's octanol-water partition coefficient method (Meylan, *et al.*, 1995). The difference of polarity between the emollient and the active ingredients in F#2 was 13.52 (15.53-2.01) for DEET and 11.74 (15.53-3.79) for OBZ, which was significantly higher than 3.22 and 6.78. The difference of polarity in F#3 was 2.87 for DEET and 1.09 for OBZ, which was lower than 3.22 and 6.78. In comparison, F#2 produced higher solubility of the active ingredients in the emulsion and lower skin permeation of the compounds than F#3. Emollient selected for F#4 and F#5 was isostearyl isostearate, which had a LogKo/w of 26.98. The calculated polarity difference between this emollient and DEET and OBZ was more than 3.22 and 6.78 respectively. Consequently DEET and OBZ were incorporated in F#4 and F#5 with higher solubility and produced relatively low skin penetration, as evident in lower DEET permeation from F#4 and F#5 in comparison to F#1. Since identical emollient was used in F#4 and F#5, no difference was observed in skin retention and penetration of DEET and OBZ between the two emulsions.

Drug diffusion and permeation though the skin can often be altered by certain chemicals, also known as permeation modifiers. There are two types of permeation modifiers, i.e., permeation enhancers and permeation retarders, which were first presented by Michniak-Kohn at the AAPS meeting 2007 (Michniak-Kohn, 2007).

Permeation modifiers may affect the skin barrier properties in many different ways, including altering solubility of a permeant in the skin, disrupting lipid packing of stratum corneum, and supplying additional driving force to penetrating substances. Some emollients and solvents used to form topical emulsions may modify physical characteristics of the final preparations such as firmness and consistency, which can further alter diffusion and permeation of the active ingredient from the emulsion formulation. Under these circumstances, the excipients may also be considered as permeation modifiers. Transcutol HP (diethylene glycol monoethyl ether) used in F#3 was found to enhance skin permeation by altering permeant solubility in the skin (Harrison, *et al.*, 1996; Irwin, *et al.*, 1990; Ongpipattanakul, *et al.*, 1991). Precirol ato 5 (glyceryl palmitostearate) used in F#2, on the other hand, was able to form lipid matrix and resist thermal activity of a permeant, leading to permeation retardancy. Therefore, both excipients may play a role in influencing penetration of DEET or OBZ observed in F#2 and F#3, for example, higher skin penetration of DEET in F#3 than in F#2.

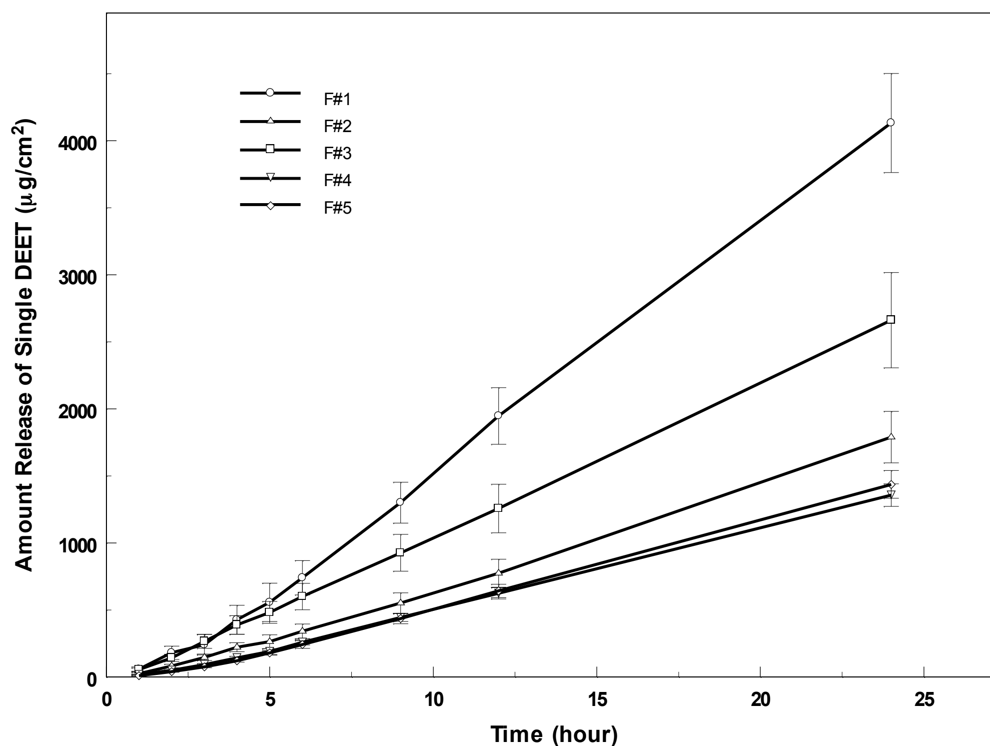
Emulsions may demonstrate psueudoplastic flow properties accompanied by thixotropy, which are desirable for topical administration of the preparations. For example, a topical emulsion should be readily picked up from a container with adequate consistency, spread on the skin surface, and form a coherent film covering the treatment site. This character is a shear-shinning property, also known as a thixotropical property. With such a property, the semisolid emulsion is highly viscous at rest, but readily flows

under a shear force. The generation of thixotropy involves macromolecule polymers that are entangled at rest but disentangled under shearing. A higher thixotropy indicates that macromolecules with entangled long carbon chains need more shear force in order to be disentangled. Emulsions of high thixotropy generally possess high viscosity at static state. The higher the viscosity of a topical formulation is, the lower its drug release rate is (Abd El-Bary, *et al.*, 2001; Mohamed, 2004). Therefore, high thixotropy in an emulsion preparation may represent the resistance of active ingredients to diffuse from the vehicle and decrease their skin permeation from the emulsion. Among the five preparations studied, F#1 possessed the lowest thixotropy, and it produced a significant increase in skin permeation of DEET compared to other four formulations. F#2, on the other hand, possessed higher thixotropy than F#3, and hence produced significantly lower skin permeation of DEET than F#3. But F#5 possessed higher thixotropy than F#4; it did not produce significantly lower skin penetration of DEET than F#4. In this case, polarity of oil phase in an emulsion may become a primary factor influencing skin penetration of DEET. Both F#4 and F#5 contained identical oil components including emollient, which may exert similar permeation of DEET between the two emulsions. It appeared that transmembrane properties of oil-in-water emulsions were more dependent upon thixotropy than those of water-in-oil emulsions. Study of rheological behaviors of the emulsions would certainly benefit selecting the optimal formulations in terms of molecule diffusion and transmembrane permeation.

Concurrent application of DEET and OBZ affected skin penetration of the two compounds from the emulsions. Presence of OBZ in F#1 decreased skin retention and permeation of DEET, which may be attributed to interactions between the oil components and the active ingredients. OBZ as a lipophilic substance may increase DEET affinity for the oil phase, thus lowering DEET diffusion from the emulsion and reducing its skin retention and permeation. However, combined OBZ and DEET in F#2 increased skin retention and permeation of DEET, which may be attributed to different oil phase constitutes between F#1 and F#2. OBZ may increase the solubility of DEET in the skin with the aid of emollients, hence increasing DEET affinity for the skin and enhancing DEET retention within the skin and its permeation through the skin.

Release amount of DEET and OBZ from F#1, F#2, F#3, F#4, and F#5 in 24 h through pig skin were shown in **Figures 5.14-5.17**. The overall release curves of DEET and OBZ were considered in a linear relationship. Various permeation parameters, including partition coefficient, lag time and permeation coefficient, were calculated according to Equation 5.3; results are listed in **Table 5.3**. Permeation coefficient of DEET ranged 6.00-23.41 μg/cm^2 among the five formulations, while that of OBZ ranged 0.21-0.52 μg/cm^2. Permeation coefficient of single DEET in F#1 produced a significant increase ranging 1.4-2.9 folds compared to the other four preparations tested. Permeation coefficient of single DEET in F#2 was significantly lower by 39% than F#3. There was no significant difference between F#4 and F#5 in permeation coefficient of single DEET.

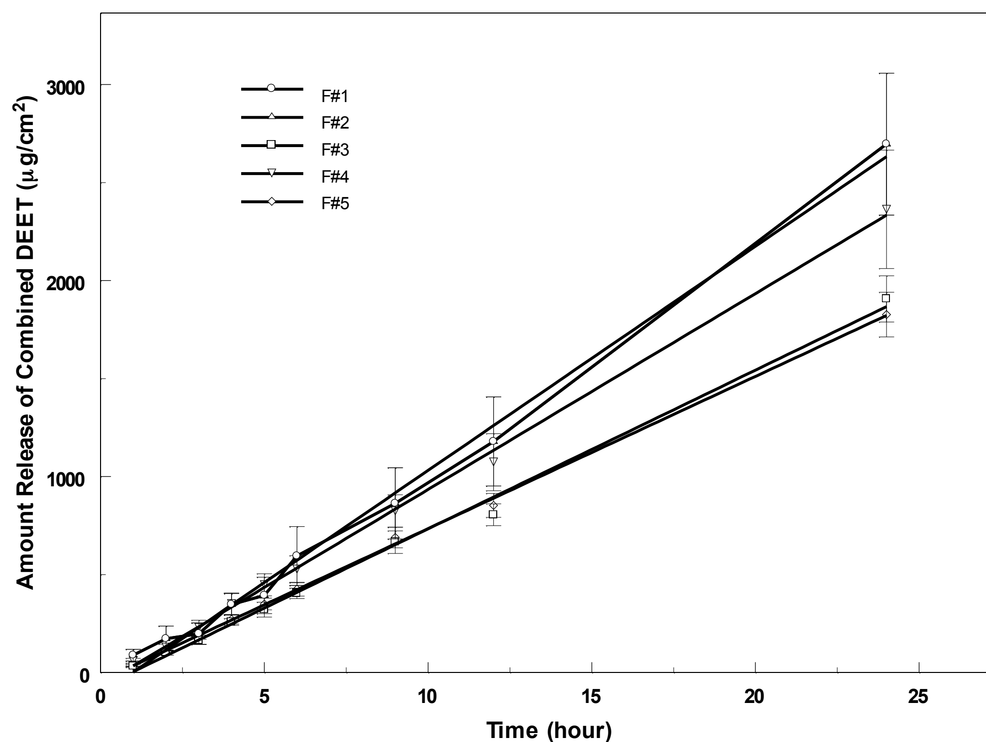
Figure 5.14. Amount release of single DEET in 24 h through pig skin



n=5, Mean±SEM

†Amount release of single DEET from five emulsion-based formulation, F#1, F#2, F#3, F#4, and F#5 (hollow circle: F#1; hollow triangle: F#2; hollow rectangular: F#3; hollow triangle: F#4; hollow diamond: F#5), through pigskin showed the linear increase by the time in 24 h. Various permeation parameters, including partition coefficient, lag time and permeation coefficient, were calculated using this curve.

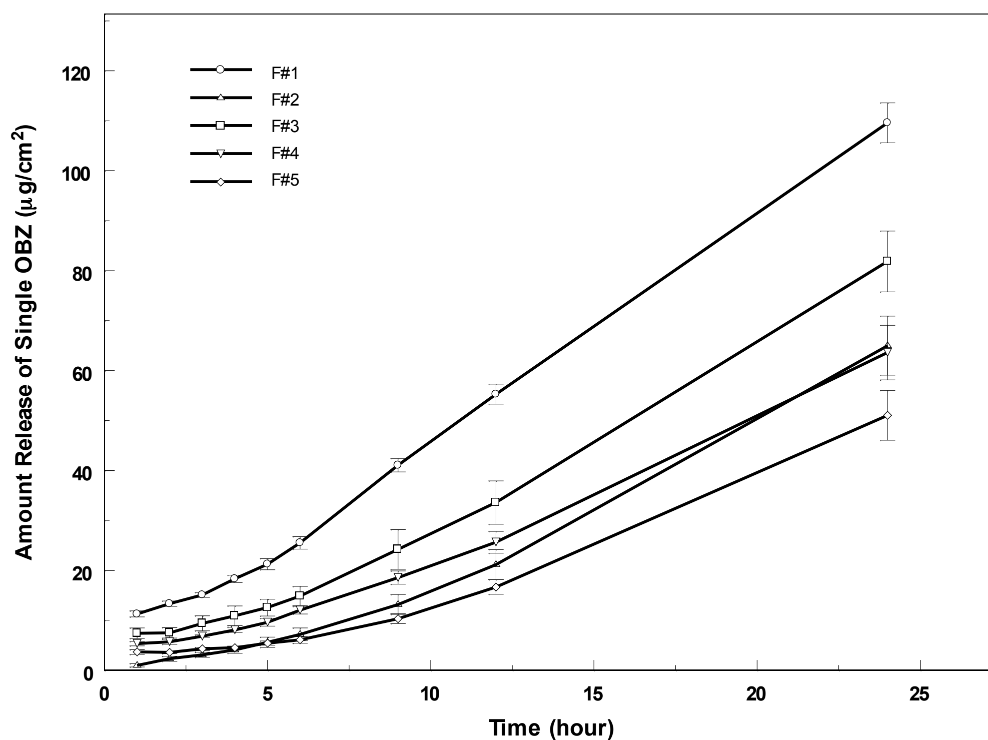
Figure 5.15. Amount release of combined DEET in 24 h through pig skin†



n=5, Mean±SEM

†Amount release of combined DEET from five emulsion-based formulation, F#1, F#2, F#3, F#4, and F#5 (hollow circle: F#1; hollow triangle: F#2; hollow rectangular: F#3; hollow triangle: F#4; hollow diamond: F#5), through pigskin showed the linear increase by the time in 24 h. Various permeation parameters, including partition coefficient, lag time and permeation coefficient, were calculated using this curve.

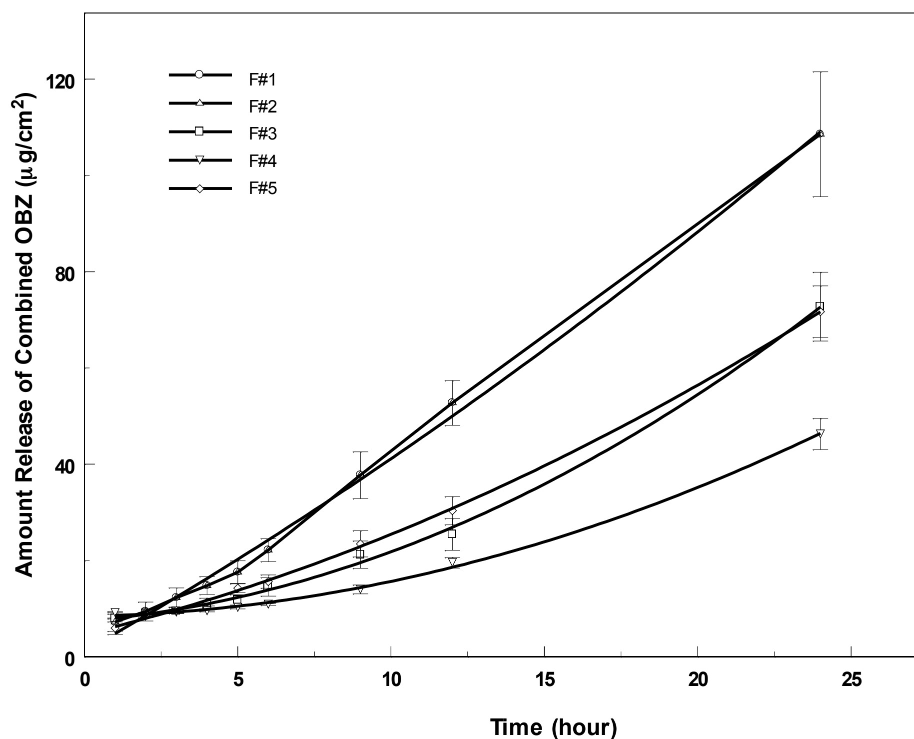
Figure 5.16. Amount release of single OBZ in 24 h through pig skin†



n=5, Mean±SEM

†Amount release of single OBZ from five emulsion-based formulation, F#1, F#2, F#3, F#4, and F#5 (hollow circle: F#1; hollow triangle: F#2; hollow rectangular: F#3; hollow triangle: F#4; hollow diamond: F#5), through pigskin showed the linear increase by the time in 24 h. Various permeation parameters, including partition coefficient, lag time and permeation coefficient, were calculated using this curve.

Figure 5.17. Amount release of combined OBZ in 24 h through pig skin†



n=5, Mean±SEM

†Amount release of combined OBZ from five emulsion-based formulation, F#1, F#2, F#3, F#4, and F#5 (hollow circle: F#1; hollow triangle: F#2; hollow rectangular: F#3; hollow triangle: F#4; hollow diamond: F#5), through pigskin showed the linear increase by the time in 24 h. Various permeation parameters, including partition coefficient, lag time and permeation coefficient, were calculated using this curve.

Permeation coefficient of single DEET in F#1 was significantly higher by 122% than its combined counterpart, while permeation coefficient of combined DEET in F#2 was significantly higher by 60% than its single counterpart. There was no significant difference observed in OBZ permeation coefficient among these five formulations. Permeation profiles of DEET and OBZ in terms of permeability were consistent with the results obtained from permeation percentage.

In **Table 5.3**, the partition coefficient of single DEET in F#1 produced a significant increase ranged from 2-3.9 folds comparing with the counterparts of other formulations. The partition coefficient of single DEET in F#1 was significantly higher by 2.4-fold than combined DEET. There were no significantly different findings in the partition coefficient of OBZ. The partition coefficient of compounds represented the partition ability of compounds from vehicles to membrane. Partition coefficient is an important parameter in skin penetration, and can be utilized to predict dermal absorption and permeability. Usually the higher is the partition coefficient, the higher will the permeability of compounds be (Baynes, *et al.*, 2002). In this study, the highest partition coefficient of DEET in F#1 may be related to the highest permeability of DEET in F#1 among five formulations. The presence of OBZ lowered the partition coefficient of DEET in F#1, which may be related to the lower permeability of DEET in the combined preparation of F#1. These results were consistent with the previous observations (Baynes, *et al.*, 2002) and further indicated the partition coefficient may be related to the

permeability of compounds.

As shown in **Table 5.3**, the lag time of single OBZ in F#1 was significantly lower than that of all other formulations. For oil-in-water emulsions with synthetic emulsifiers, lag time of single OBZ in F#2 was significantly higher by 88% than its F#3 counterpart. For water-in-oil emulsions, lag time of single OBZ in F#5 was significantly higher by 61% than its F#4 counterpart. The lag time of single OBZ in F#2, F#3, F#4, and F#5 was significantly higher than their combined OBZ counterparts, but that of F#1 was significantly lower than combined OBZ counterpart. Lag time is defined as the diffusion time of an active ingredient before it reaches the steady-state flux. A short lag time implies a fast skin penetration rate and a high skin penetration per unit of time for a diffusant in the early period of diffusion process. This parameter may also be used to differentiate penetration profiles of various active ingredients through the skin. Lag time may change distinctively, when permeability is hardly influenced (Rhee, *et al.*, 2007). A short lag time of OBZ meant that OBZ diffused out of the emulsion and permeated through the skin at a fast rate. F#1 possessed the shortest lag time for OBZ; OBZ would have a higher permeation through the skin than all other formulations. This may have resulted from a variety of factors in F#1, including surfactant-free emulsifying agent, lack of emollients, and a lower thixotropical property. For oil-in-water emulsions with synthetic emulsifiers, a higher lag time of OBZ in F#2 than F#3 may be attributed to a higher HLB value of the emulsifying agent, a more lipophilic emollient, presence of a

penetration retarder and a higher thixotropy in F#2. For water-in-oil emulsions, F#5 demonstrated a higher lag time of OBZ than F#4, possibly associated with a higher thixotrypical property in F#5. OBZ, as a solid component, was dissolved in the external oil phase and subsequently produced a shorter distance and greater van de Waals forces between molecules of oil phase. Structure of an emulsifying agent surrounding the oil globules, as well as thixotropical property, may influence overall skin permeation of OBZ, thereby leading to lower penetration rate of OBZ in F#5 during early diffusion period. Incorporation of DEET together with OBZ reduced OBZ penetration rate in F#1. This suggested that presence of DEET might increase OBZ solubility in the oil phase, lowering its diffusion and penetration rate. However, the presence of DEET increased skin penetration rate of OBZ in F#2-F#5, which may be attributed to the high lipophilic emollients in the oil phase of F#2-F#5, but not in F#1. The presence of DEET may increase the solubility of OBZ in the skin with the aid of emollients, which can soften the skin and may change the solubility of diffusants in the skin (Otto, *et al.*, 2009), thereby increasing the skin penetration of OBZ in F#2- F#5. Therefore, the concurrent application of DEET and OBZ enhanced the skin penetration rate of OBZ in the traditional oil-in-water or water-in-oil emulsions and decrease the skin penetration rate of OBZ in F#1 with the surfactant-free emulsifier, which may have been attributed to the different oil phases between the traditional oil-in-water or water-in-oil emulsions and F#1.

5.5. Conclusion

Oil-in-water emulsions demonstrated desirable potential of lowering percutaneous penetration of insect repellent DEET and sunscreen OBZ through human skin in comparison to water-in-oil emulations from *in vitro* diffusion experimentation. Moreover, addition of xanthan gum to oil-in-water emulsion enhanced skin penetration of the compounds and produced a synergistic percutaneous penetration through human skin. Skin penetration of DEET and OBZ was also dependent on type of emulsifiers, emollients, penetration modifiers used to form emulsions, thixotropic properties of the emulsions, and concurrent application of the two compounds. Synthetic oil-in-water emulsifiers tended to produce lower skin permeation of DEET and OBZ; the oil-in-water emulsion using Emulium 22 possessed the lowest skin permeation of DEET and OBZ among the five emulsions tested. Proper selection of emollients enabled to optimize skin delivery of active ingredients from emulsions; oil-in-water emulsion containing emollient MOD generated a lower skin permeation of DEET and OBZ than other oil-in-water emulsions. Penetration enhancer Transcutol HP increased skin permeation of the active ingredients in F#3, while penetration retarder precirol ato 5 formed lipid matrix to inhibit skin permeation of the active ingredients in F#2. Thixotropic properties of an emulsion were related to skin permeation in oil-in-water emulsions, but not in water-in-oil emulsions.

CHAPTER 6

In Vivo Animal Study

6.1. Introduction

In vivo animal studies can be used to evaluate and understand the pharmacokinetics and biodistribution characteristics of chemical substances in topical formulations. Many *in vivo* animal models have been carried out and documented (Wester, *et al.*, 1983; Wester, *et al.*, 1987; van Haaren, *et al.*, 2001); drug dosing could range from single application to multiple administrations. *In vivo* studies using human subjects are the ultimate goal of the research, and they are capable of generating reliable and relevant clinic pharmacological and toxicological data from topical skin applications. Nevertheless, there are many factors in study ethics, study expense, and drug analysis that may hinder decision-making in conducting a clinical trial in humans. Consequently, *in vivo* animal studies are commonly utilized as substitute to collecting fundamental clinical pharmacological and toxicological information for new drug candidates. The data is required by the U.S. Food and Drug Administration (FDA) for approval of New Drug Application (NDA) or Abbreviated New Drug Application (ANDA).

Concurrent application of over-the-counter insect repellent and sunscreen products has been extensively practiced for summer months to prevent vector-borne diseases, such as West Nile Virus and Lyme disease (Brown, *et al.*, 1997; Qiu, *et al.*, 1998; Robbins, *et al.*, 1986; Sudakin, *et al.*, 2003;), and skin cancers induced by the sunlight radiation. Studies have found that concurrent application of repellent DEET and sunscreen OBZ induced a synergistic percutaneous penetration of the two compounds in

a series of *in vitro* diffusion studies using artificial membrane, piglet skin and human skin (Gu, *et al.*, 2005; Wang, *et al.*, 2006). However, few studies had utilized animal models to assess pharmacokinetics, biodistribution and percutaneous mechanisms of DEET and OBZ from concurrent application of the ingredients. There were reports of concurrent use of DEET in combination with pyridostigmine bromide, diisopropylfluorophosphate (DFP), and permethrin (Abou-Donia, *et al.*, 1996; McCain, *et al.*, 1997; Riviere, *et al.*, 2003); or concurrent use of OBZ and herbicide 2,4-dichlorophenoxyacetic acid (Brand, *et al.*, 2007). In this study, we selected a lotion formulation that had been developed in the lab, and then tested it in an animal model. The primary purpose was to investigate deposition characteristics of DEET and OBZ in blood, skin and vital organs following topical application of the preparation for a 60-day period.

6.2. Materials

Pure DEET (N,N-diethyl-*m*-toluamide) was purchased from Fluka Chemika GmbH (Buchs, Switzerland), and OBZ (2-hydroxy-4-methoxybenzophenone) was purchased from Riedel Haer GmbH (Seelze, Germany). Acetonitrile (HPLC grade), methanol (HPLC grade), glacial acetic acid, potassium phosphate monobasic, and sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). HPLC-grade deionized water was purified by a Milli-Q® Pure Water System (Millipore, Nepean, ON, Canada) in the laboratory. RapTrace SPE workstation was purchased from

Zymark Corporation (Hopkinton, MA, USA). Oasis MAX 3μl (60mg) extraction cartridges were bought from Waters Corporation (Milford, MA, USA). 15ml polypropylene centrifuge tubes, flat top microcentrifuge tubes and borosilicate glass disposable culture tubes (12×75 mm and 13×100 mm) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). 4ml clear glass vials with caps were purchased from Waters Corporation (Milford, MA, USA). Syringe-driven filter units (non-sterile) were purchased from Millipore Corporation (Bedford, MA, USA). Microvette CB 300 LH was purchased from Sarstedt, Aktiengesellschaft & Co. (D-51588 Nümbrecht, German). 22G1 needle, 5ml syringes, and 10ml syringes were bought from BD Corporation (Franklin Lakes, NJ, USA). Elma cream (Lidocaine 2.5% and Prilocaine 2.5%) was manufactured by Astra Zeneca Canada Inc. (Mississauga, ON, Canada). Biohomogenizer was purchased from Biospec P roducts INC. (Bartlesville, OK, USA). Ultrasonic cleaner was produced from Cole-Pormer Instrument Company (Chicago, IL, USA). REAX top was manufactured by Rose Scientific LTD (Edmonton, AB, Canada). Standard solutions of DEET metabolites (ET and DHMB) and OBZ metabolites (THB, DHB, and DMB) were donated by Agriculture Canada.

F#2 preparation selected for the animal study was an oil-in-water emulsion (**Table 4.8**); it was prepared according to procedures previously described and divided into four portions. 15% DEET, 10% OBZ, and 15% DEET/10% OBZ were incorporated

into the emulsion respectively, and a placebo preparation control was also used for the study. **Table 6.1** lists major contents of the four testing preparations.

Table 6.1. Applied emulsion-based formulations

	Preparation code			
Ingredients	A	B	C	D
DEET (% w/w)	15%	--	15%	--
OBZ (% w/w)	--	10%	10%	--
Other components (% w/w)	85%	90%	75%	100%

6.3. Methods

6.3.1. Animals

The Animal Use Protocol was approved by the University of Manitoba, and conducted according to current guidelines certified by the Canadian Council on Animal Care. Forty Sprague-Dawley rats (male, three-week age, 150 gram body weight) were housed individually, and provided with food and water ad libitum. The animal room was controlled with room temperature of 21-23°C and a reversed 12-hour dark and light circle. The back of the study animals was shaved to expose a skin surface of 2×2 cm^2 for dosing application.

Forty rats were randomly divided into four groups. The three study doses were 40 mg/kg of DEET in DEET group; 25 mg/kg of OBZ in OBZ group; and 40 mg/kg of DEET and 25 mg/kg of OBZ in DEET/OBZ group respectively. The control group received blank preparation dosing at the same time. The weight of the study animals was recorded periodically during the experiment, and the doses were then adjusted accordingly. Briefly, 40-50mg of emulsion-based formulations containing DEET and/or OBZ was respectively applied to the shaved skin surface once daily for a 60-day period; the control group (placebo group) typically received the blank emulsion sample (40-50mg) for 60 days.

6.3.2. Biological Sample Collections

Blood samples were collected by Microvette from the rear legs of the rats at 1, 2, 3, 4, 5, 6, 7, 9, 11, and 24 hours on the 60th day of the experiment. The blood samples were centrifuged at 10000 g for 30 minutes, and plasma samples were separated and frozen prior to drug analysis.

Study animals were euthanized by carbon dioxide asphyxia after the last blood sample. The skin area where the preparation had been applied was swabbed with a gauze tissue containing 100% ethanol. The gauze was collected and soaked in 5 ml ethanol for 48 hours at 4 °C. Once complete, the solution was transferred into a 15 ml polypropylene centrifuge tube and centrifuged at 10000 g for 30 minutes. Supernatant was separated and

frozen prior to drug analysis.

Biological tissues including liver, kidney, brain, and skin of drug application site ($2 \times 2\ cm^2$) were also collected by technicians. The skin samples were soaked in 5ml ethanol for 48 h at 4 °C. Then they were centrifuged at 10000 g for 30 minutes, and supernatants were collected. Samples of liver, kidney, and brain were accurately weighed upon collection and homogenized individually. An appropriate amount of acetonitrile was added to the homogenized tissues to prepare the samples with 1 g homogenized tissue per ml of acetonitrile. The tissue samples were then centrifuged at 10000 g for 30 minutes; supernatants were separated and transferred to clean disposable culture tubes before drug analysis.

6.3.3. HPLC Assay

Both DEET and OBZ were extracted from the supernatant of individual samples using a Zymark Rapidtrace SPE (Solid Phase Extraction) Workstation, followed by drug analysis with an HPLC assay. Briefly, 100 μl plasma was mixed with 100 μl deionized water, and then loaded to an Oasis MCX 3 cc (60 mg) extraction cartridge (Milford, Mass, USA) that had been preconditioned with acetonitrile and water. The column was rinsed with water; the cannula was purged by water. Then acetonitrile and 0.03 M ammonium acetate (pH 4.5) were used to wash the column. Finally, the column was eluted with 300 μl methanol. The cannula was purged and cleaned again with water. The extracted

solution of DEET and OBZ (300 μl methanol solution) from the plasma was analyzed by HPLC. Tissue samples were treated using the same extraction method.

To prepare the calibration curve, solution of DEET, OBZ, and their metabolites was prepared by spiking 50 μl of blank rat plasma with 150 μl of working solutions. The solution was vortexed for 10 seconds and transferred to Zymark Rapidtrace SPE Workstation for the extraction of DEET, OBZ, and their metabolites. 50 μl of the extracted solution was injected to the HPLC system for drug analysis. The detection limitation for DEET, DMHB and ET was 5 ng, 50 ng, and 50 ng respectively. The range of calibration linearity ($r^2 \geqslant 0.99$) of DEET was 15-50 ng/50 μl, while the ranges of calibration linearity of DMHB and ET were 250-1250 ng/50 μl. The detection limitation was 5 ng for OBZ and 50 ng for DHB, THB, and DMB respectively. The range of calibration linearity ($r^2 \geqslant 0.99$) of OBZ was 15-50 ng/50 μl, while the ranges of calibration linearity of DHB, THB, and DMB were 250-1250 ng/50 μl.

The HPLC system was composed of a 996 Photodiode Array Detector and a Waters Alliance 2690 Solvent Delivery Module with software Millennium (Milford, Massachusetts, USA). The column used was Nova-Pak C18, 3.9×150 mm, 4 μm. The mobile phase was a mixture of acetonitrile, methanol and water (pH 2.8 with acidic acid) in a ratio of 30:50:20 (V/V/V), at a flow rate of 1 ml/min. The detection wavelength for DEET and its metabolites, DHMB and ET was 254 nm; detection wavelength for OBZ and its metabolites, DHB, DMB, and THB was 287 nm. The retention times for DEET,

DMHB and ET were 1.9, 1.4, and 1.5 minutes, while the retention times for OBZ, DHB, DMB, and THB were 2.5, 2.7, 3.9 and 2.1 minutes.

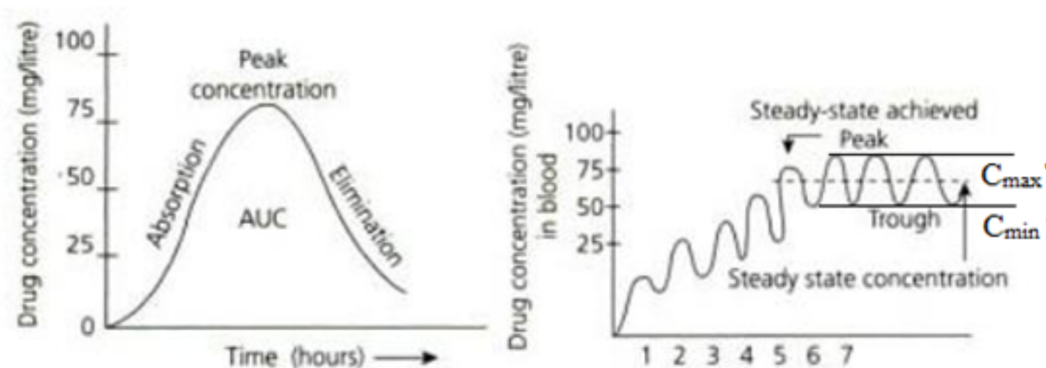
6.3.4. Data Analysis

Different from single dosing regimen, DEET and OBZ would accumulate in the study animals after repeated topical applications for a prolonged period of time. The level of chemicals in plasma and other tissues built up at the beginning of the administration and eventually reached a plateau concentration (steady-state concentration), as demonstrated in **Figure 6.1**. Hence average steady-state concentration of DEET or OBZ could be calculated using the following equation (Gibaldi, 1990),

$$\bar{C}_{ss} = \frac{\text{AUC}}{\text{Interval Administration of Dose}} \qquad \text{Equation 6.1}$$

Plasma concentrations of DEET and OBZ obtained from the study were subjected to pharmacokinetic simulation using non-compartmental modeling. The peak plasma concentration (C_{max}) and the time at which the peak plasma concentration was reached (T_{max}) were determined by visual inspection of plasma concentration-time curves. Area under the plasma concentration *vs.* time curves (AUC) and area under the first moment plasma concentration *vs.* time curves (AUMC) was calculated using the trapezoidal rule. The elimination rate (k_e) was calculated by the first-order elimination kinetic equation.

Figure 6.1. Plasma concentration *vs.* time curves following single and repeated transdermal administration†



†The left side of the graph is the curve of plasma concentration *vs.* time after single transdermal administration; the right side of the graph is the curve of plasma concentration *vs.* time after repeated transdermal administration. Unlike the single dose administration, the chemicals, such as DEET and OBZ, would accumulate in the study animals and eventually reached a plateau concentration after repeated topical applications for a prolonged period of time (Gibaldi, 1990).

The mean residence time (MRT) was calculated by the following equations (Svensson, 2006),

$$MRT = \frac{AUMC}{AUC} \qquad \text{Equation 6.2}$$

In addition, distribution percentages of DEET, OBZ and their metabolites in skin, kidney, liver and brain were calculated based on the last dosing on the 60th day.

Statistical analysis was performed using two-way ANOVA and Tukey's test

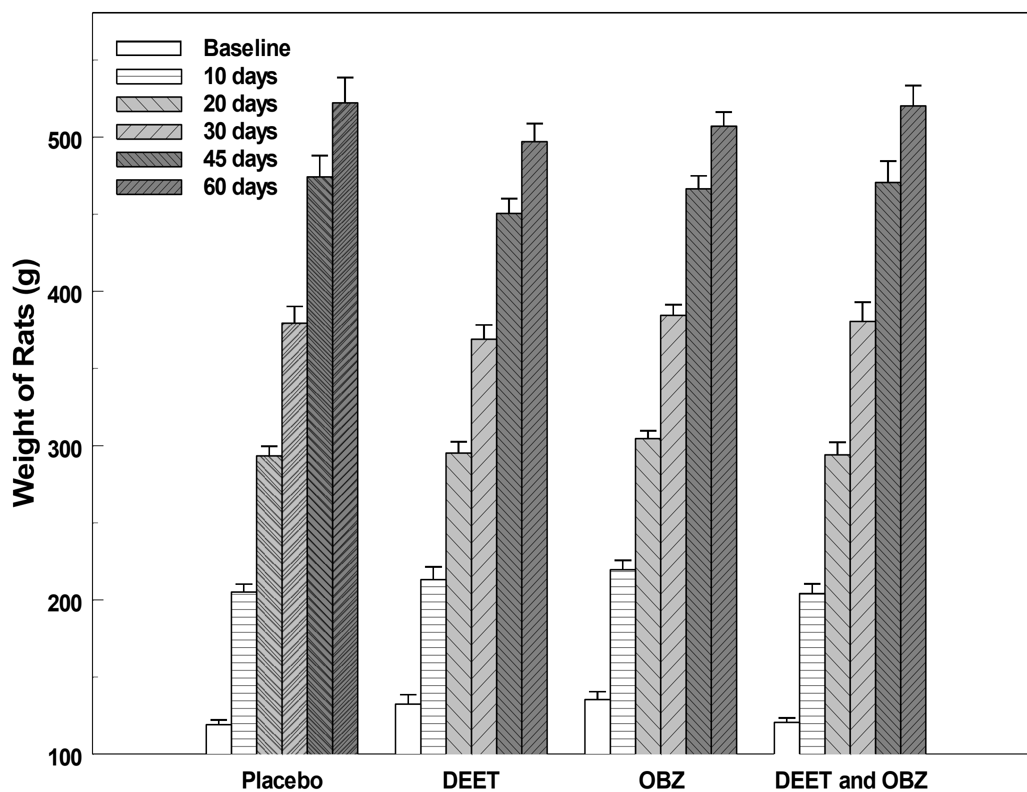
(PC-SAS 8.02, SAS Institute Inc., Cary, North Carolina, USA). The following statistical analyses of the data were conducted, a) peak plasma concentration, time reaching the peak plasma concentration, average steady-state concentration, and area under the plasma concentration-time curve of DEET and OBZ; b) concentration and distribution percentage of DEET and OBZ in swab, skin, kidney, liver, and brain samples; c) distribution percentage of metabolites of DEET and OBZ in kidney, liver, and brain samples. All data was expressed in Mean±SEM, and differences were considered statistically significant at $P \leq 0.05$.

6.4. Results and Discussion

6.4.1. General Observations

The body weight of the study animals naturally increased from 0 to 60 days in all study groups. **Figure 6.2** shows the body weight changes in the four study groups. Repeated topical applications of the testing formulations containing DEET and/or OBZ exerted no effect on body weight increase in comparison to the placebo group. Neither were there abnormal observations on food consumption, incidence of gross lesion, or animal survival among the four study groups. Some animals treated with formulations containing single OBZ and combined OBZ developed scratching wound on application site. These animals were treated with Elma cream (lidocaine 2.5% anesthesia drug and prilocaine 2.5%), and recovered on the following day. This might be related to contact

Figure 6.2. Body weight changes of the animals in different study groups†



Mean ± SEM (n=8)

†The body weight of rats increased from 0 d (baseline) to 60 d in the four study groups. Repeated topical applications of the testing formulations containing DEET and/or OBZ exerted no effect on body weight increase in comparison to the placebo group.

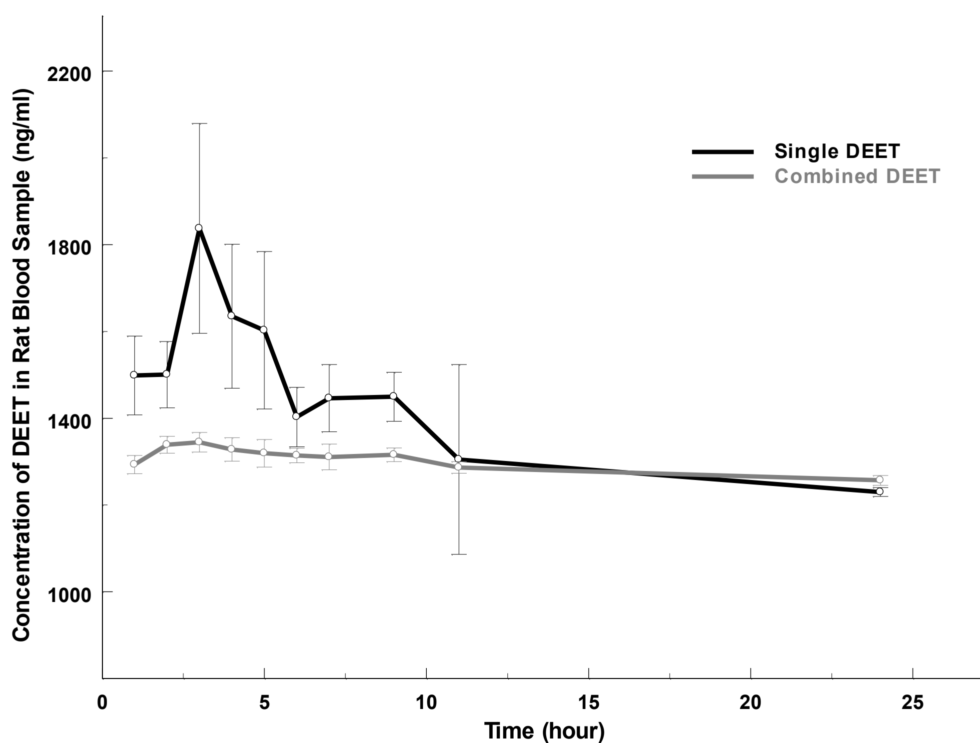
allergic dermatitis induced by topical application of OBZ (Bryden, *et al.*, 2006).

6.4.2. Plasma Concentration of DEET and OBZ

Drug disposition throughout the body observes a complex mechanism. Some body sites such as central nervous system and fat tissues may have lower drug disposition than plasma, whilst other sites such as liver and kidney will reach higher drug concentration than plasma, all due to drug metabolism and elimination. Using a noncompartmental model, drug concentrations in different body parts were presumed to be equal at equilibrium.

Figures 6.3 and 6.4 showed the plasma concentration-time curves of DEET and OBZ following the 60-day repeated topical administration of 40 mg/kg DEET and 25 mg/kg OBZ, in combination or individually. Plasma levels of DEET and OBZ remained detectable 24 hours after the last dermal administration; concentrations fluctuated in the range of 1.23-1.84 μg/ml for DEET and 0.40-0.44 μg/ml for OBZ. Concentration of DEET and OBZ rose rapidly following the topical dosing, indicating a quick percutaneous permeation of the compounds. A slow terminal decline occurred following the C_{max} in the plasma; this terminal decline rate may also be attributed to continuous and steady absorption of the compounds from skin layers following dermal administration (Robert, *et al.*, 1998).

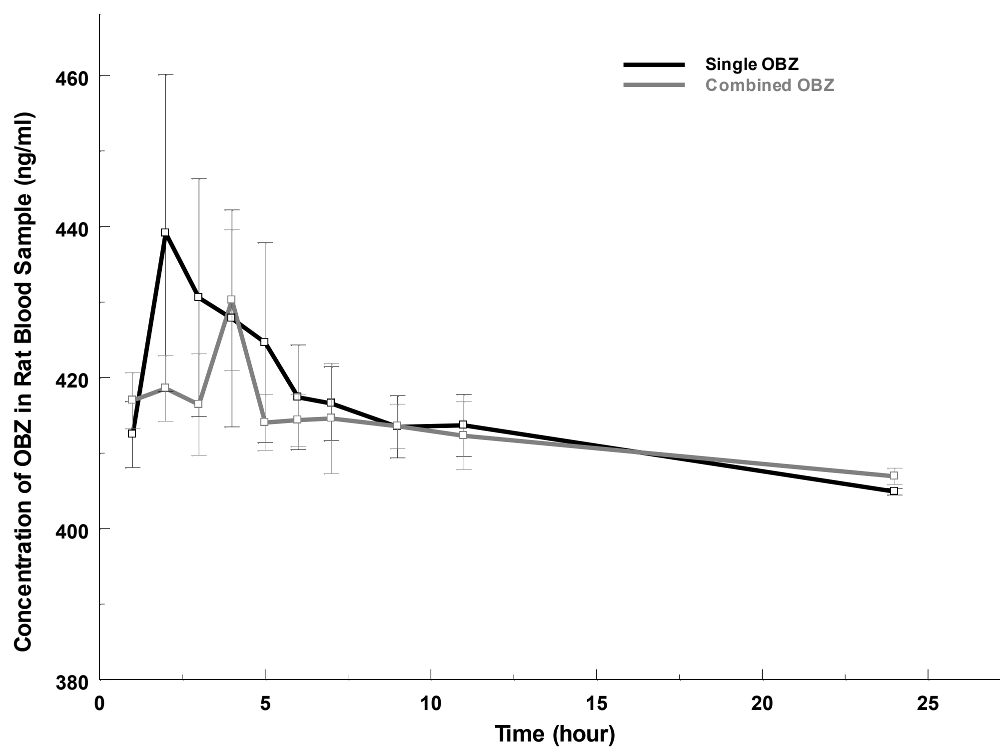
Figure 6.3. Plasma concentration of DEET *vs.* time curve†



Mean±SEM, n=8

†This figure showed the plasma concentration *vs*. time curves of DEET following the 60-day repeated topical administration of 40mg/kg DEET and 25 mg/kg OBZ, in combination or individually. Plasma level of DEET remained detectable 24 h after the last dermal administration. Concentration of DEET rose rapidly following the topical dosing; a slow terminal decline occurred following the C_{max} in the plasma.

Figure 6.4. Plasma concentration of OBZ *vs.* time curve



Mean±SEM, n=8

†This figure showed the plasma concentration *vs*. time curves of OBZ following the 60-day repeated topical administration of 40mg/kg DEET and 25 mg/kg OBZ, in combination or individually. Plasma level of OBZ remained detectable 24 h after the last dermal administration. Concentration of OBZ rose rapidly following the topical dosing; a slow terminal decline occurred following the C_{max} in the plasma.

Table 6.2 lists the primary pharmacokinetic parameters of DEET and OBZ following repeated topical application in rats. DEET and OBZ reached the maximum plasma concentration at the same time; DEET produced a significantly higher C_{max} than OBZ. There was no significant difference observed between the single and combined preparations in C_{max} and T_{max}. In an *in vivo* study of single-dosing, topical application of commercially available insect repellent and sunscreen in piglets (Kasichayanula, *et al.*, 2007), C_{max} of DEET and OBZ was higher by over 10 times than what observed in this study. The discrepancies may be attributed to the two testing preparations; emulsifier (Emulium 22) and other excipients (MOD and Precirol ATO 5®) used for this emulsion formulation could hinder dermal permeation of DEET and OBZ after topical skin application, which was one of the primary objectives of formulation development previously described. In addition, different animal models used may also result in variable skin absorptions. Hair follicular size, density and structure in the skin of different animal species may exert influences on dermal absorption of topical compounds (Zhai, *et al.*, 2008). Pigskin possesses larger follicular diameter of 320 nm (Lademann, *et al.*, 2009) as compared to rat skin follicular size of 100 nm (Yano, *et al.*, 2001), which would allow for higher dermal permeation of DEET and OBZ through pig skin.

Table 6.2. Pharmacokinetic parameters of DEET and OBZ for 24 h after 60-day topical application in rats

Parameters	DEET (S)	DEET (C)	OBZ (S)	OBZ (C)
Dose (mg/kg)	40	40	25	25
T_{max} (hr)	3.2±0.5	3.4±0.5	3.2±0.5	3.5±0.7
C_{max} (μg/ml)	3.03±0.84[2]	2.26±0.84	0.45±0.02[2]	0.44±0.01
$\bar{C}_{ss}$ (μg·ml)	1.48±0.09[2]	1.28±0.04	0.40±0.01[2]	0.41±0.00
AUC (μg·hr/ml)	35.64±2.06[1, 2]	30.77±1.27[1]	9.71±0.17[2]	9.92±0.06
MRT (hr×10^2)	1.05±0.18[1, 2]	4.77±1.27[1]	7.04±1.99[2]	5.97±2.40
k_e (1/hr×10^{-3})	7.00±0.90[1, 2]	2.6±0.60[1]	1.4±0.44[2]	2.40±1.40

Mean±SEM, n=8, P≤0.05
[1]: Significant difference between single preparation and combined preparation
[2]: Significant difference between DEET and OBZ

Average steady-state plasma concentration ($\bar{C}_{ss}$) was a simple way to describe *in vivo* drug behaviors following repeated transdermal drug administrations for a prolonged period of time (Gibaldi, 1990). There was no significant difference between the single and combined preparations in $\bar{C}_{ss}$. The results indicated that concurrent topical application of DEET and OBZ from this emulsion-based lotion exerted no enhancement effect on dermal absorption of DEET or OBZ. On the contrary, a synergistic

percutaneous enhancement between DEET and OBZ was observed 2 hours after topical application of commercially available repellent and sunscreen products in piglets (Kasichayanula, *et al.*, 2007). This difference in transdermal pattern between the developed emulsion and the existing commercial preparations was thought likely resulted from different formulations used for the two experiments. In comparing absorption between DEET and OBZ, dermal absorption of DEET was significantly higher by 283% for the single preparation and by 212% for the combined preparation than OBZ, which was in agreement with previous results found in piglets (Kasichayanula, *et al.*, 2007). Different partition coefficients between DEET (LogKo/w 2.01) and OBZ (LogKo/w 3.79) may have led to higher affinity of OBZ to oil phase of the emulsion and oil components in the skin than DEET; OBZ may release from the preparation at a slower rate and retained in the skin layers with slower permeation (Balmer, *et al.*, 2005; Cecchine, *et al.*, 2006). DEET possessed a tendency to penetrate into the systemic circulation and acquire a higher systemic absorption extent.

Average plasma AUC of DEET was significantly higher than that of OBZ. The combined preparation resulted in significantly lower average plasma AUC of DEET by 14% as compared to the single preparation, but had no influence on the AUC of OBZ. This implied that combined application of DEET and OBZ in this emulsion-based formulation might decrease overall DEET exposure through transdermal permeation, which could show a promising potential in concurrent use of DEET and OBZ. MRT of

DEET and OBZ after repeated applications lasted longer than that of a single dosing (Fediuka, *et al.*, 2011). In general repeating drug administration leads to substance accumulation in the body and extends elimination phase upon cessation of drug use (Jufer, *et al.*, 2000). MRT of OBZ from the single preparation was significantly higher by 5.7 times than that of DEET. The higher retention time of OBZ may be associated with its lower elimination rate in blood circulation. Elimination of OBZ (k_e) from the single preparation was significantly lower by 80% than that of DEET; this may be attributed to slower skin absorption of OBZ following dermal administration. Moreover, MRT of DEET from the combined preparation was significantly higher by 3.5 times than that of the single preparation; k_e of DEET from the combined preparation was significantly lower by 63% than that of the single preparation. This implied that OBZ presence increased the retention time of DEET and decreased the elimination rate of DEET *in vivo*; chemical interaction between the two compounds within the skin and/or in systemic circulation may have played a role in these kinetic patterns.

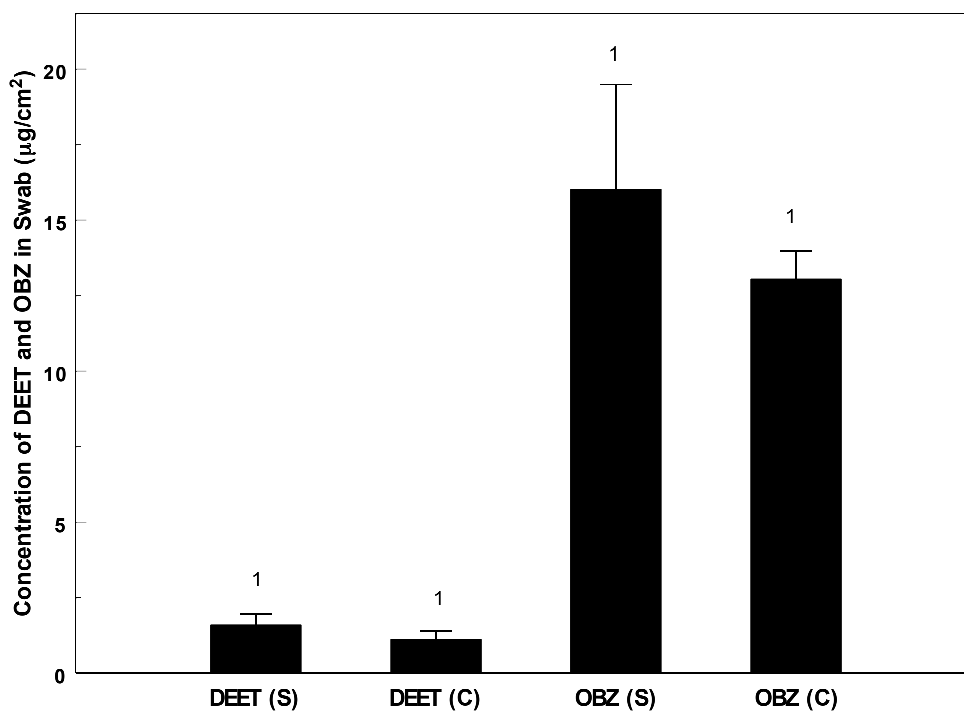
6.4.3. Deposition of DEET and OBZ

Tissue disposition of DEET and OBZ following the repeated skin applications was also quantified and compared in the study. **Figure 6.5** shows the concentration of DEET and OBZ collected in the swab samples, i.e., residual content of the applied dose at 24 hours post-dosing. OBZ concentration in the skin swab was significantly higher by

9.1-folds than DEET counterpart for the single preparation and by 10.7-folds for the combined preparation, indicating that OBZ was still present on the application surface at the conclusion of the study. The combined preparation produced a lower value of the residual content in DEET (30%) and in OBZ (19%), although there was no significant difference. **Figure 6.6** shows the concentration of DEET and OBZ within the skin of application site. Significant differences were observed not only between DEET and OBZ, but also between the single and combined preparations. OBZ concentration within the skin was significantly higher than DEET by 61% in the single preparation and by 165% in the combined preparation, which suggested that OBZ possessed higher deposition within the skin than DEET. Moreover, concurrent application of DEET and OBZ produced significant permeation increase by 26% for DEET and by 108% for OBZ in comparison to their individual counterparts. Concurrent application of DEET and OBZ apparently enhanced the skin absorption and decreased residual amount of testing compounds on the skin surface. This pattern was consistent with that observed in previous *in vivo* study. Concurrent application of DEET and OBZ produced greater recovery of DEET (48-138% increment) and OBZ (12-55% increment) in all skin layers using tape stripping method; combined application of DEET and OBZ showed lower surface recovery of residual substances than their individual counterparts following a single-dosing dermal application in rats (Fediuka, *et al.*, 2011).

Figure 6.7 shows tissue concentration of DEET and OBZ in the liver, kidney and

Figure 6.5. Concentration of DEET and OBZ in swab†

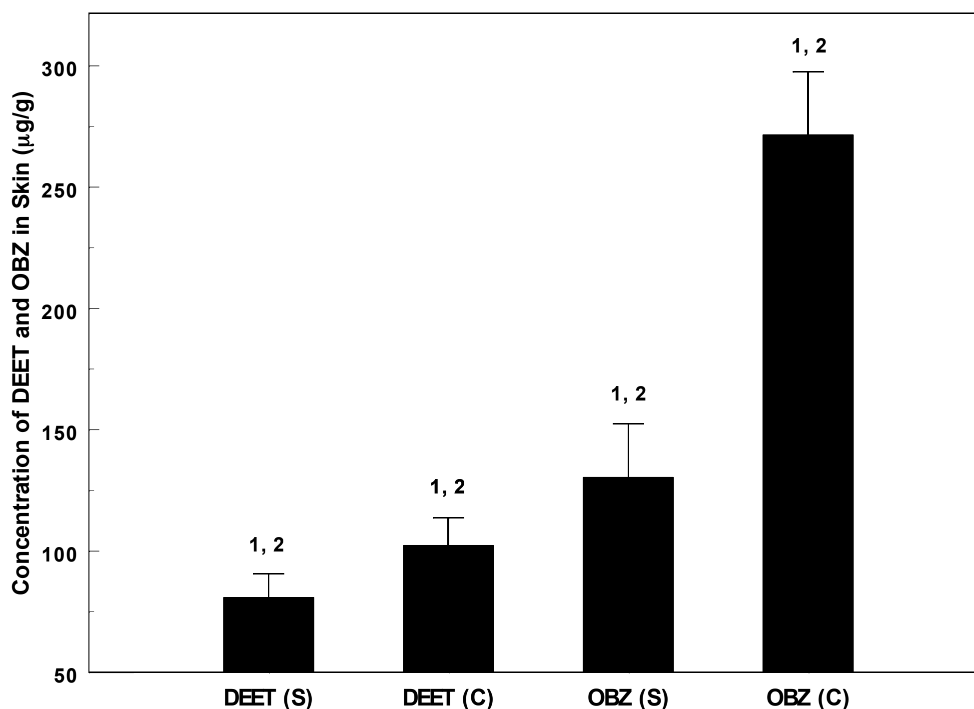


Mean±SEM, n=8, P≤0.05

1: Significant different between DEET and OBZ

†This figure showed the concentration of DEET and OBZ collected in the swab samples. OBZ concentration in the skin swab was significantly higher than DEET counterpart in the single preparation (9.1-folds) and in the combined preparation (10.7-folds). The combined preparation produced a lower value of the residual content in DEET (30%) and in OBZ (19%) than the single one, although there was no significant difference.

Figure 6.6. Concentration of DEET and OBZ in skin†



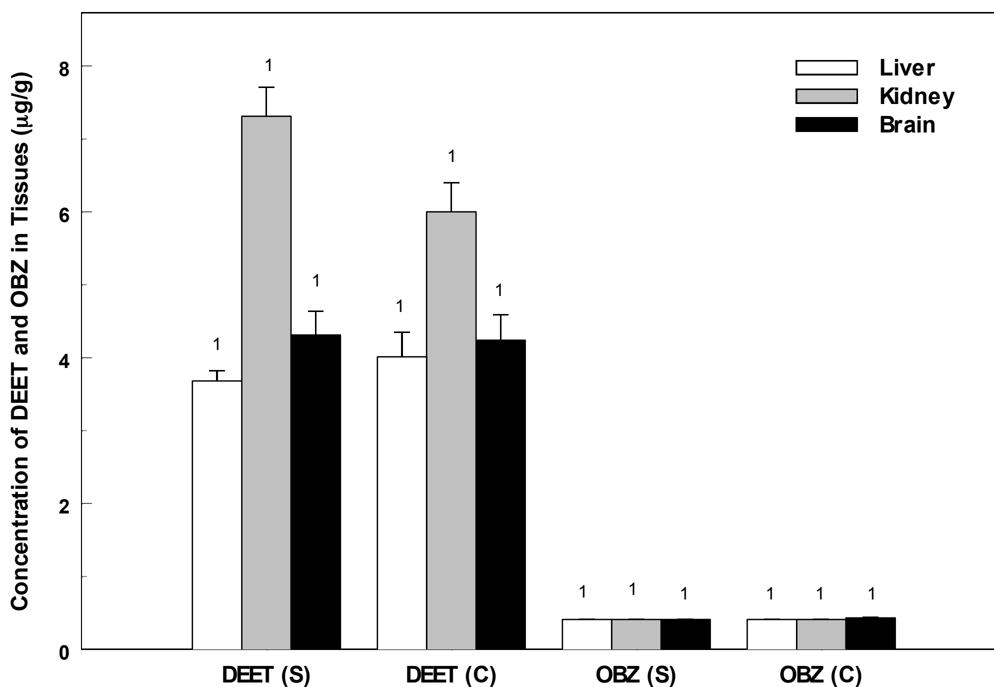
Mean±SEM, n=8, P≤0.05

1: Significant different between DEET and OBZ

2: Significant different between single preparation and combined preparation

†This figure showed the concentration of DEET and OBZ collected in the skin samples. OBZ concentration within the skin was significantly higher than DEET in the single preparation (61%) and in the combined preparation by (165%). The concurrent application of DEET and OBZ produced significant permeation increase for DEET (26%) and for OBZ (108%) in comparison to their individual counterparts.

Figure 6.7. Concentration of DEET and OBZ in tissues†



Mean±SEM, n=8, P≤0.05

1: Significant different between DEET and OBZ

†This figure showed the concentration of DEET and OBZ collected in the tissue samples, liver (white bar), kidney (gray bar), and brain (black bar). DEET tissue concentrations were significantly higher than that of OBZ in the range of 8-17 folds, No significant difference was found between the single and combined preparations for DEET and OBZ. However, the combined preparation produced a lower DEET concentration in kidney by 18% than the single preparation.

brain samples. DEET tissue concentrations were significantly higher than that of OBZ in the range of 8-17 folds, indicating that DEET had a tendency to penetrate into systemic circulation and subsequently to deposit in the organs as compared to OBZ. No significant difference was found between the single and combined preparations for DEET and OBZ. However, the combined preparation produced a lower DEET concentration in kidney by 18% than the single preparation; this may be attributed to higher skin deposition of DEET from the combined preparation.

Table 6.3 summarizes distribution percentage of DEET and OBZ in all biological samples collected from the study animals. Total skin recovery (swab and skin) of OBZ was significantly higher than that of DEET, while total tissue recovery (liver, kidney and brain) of DEET was significantly higher than that of OBZ. This was consistent with the results of DEET or OBZ concentration in skin and vital organs.

Table 6.3. Distribution percentage of DEET and OBZ in rat at 24h post-dose

Percentage	DEET (S)	DEET (C)	OBZ (S)	OBZ (C)
Dose (mg/kg)	40	40	25	25
Liver (%)	0.34±0.01	0.39±0.03	0.06±0.01	0.06±0.00
Kidney (%)	0.11±0.01	0.10±0.01	0.01±0.00	0.01±0.00
Brain (%)	0.04±0.00	0.04±0.00	0.01±0.00	0.01±0.00
Total tissue (%)	0.49±0.01*	0.53±0.03*	0.08±0.01*	0.08±0.00*
Skin (%)	0.84±0.32	0.87±0.20	2.23±0.45	2.38±0.35
Swab (%)	0.04±0.01	0.16±0.10	0.50±0.12	0.40±0.13
Total skin (%)	0.88±0.25*	1.03±0.15*	2.73±0.40*	2.78±0.30*

Mean±SEM, n=8, $P \leq 0.05$
*: Significant difference between DEET and OBZ.

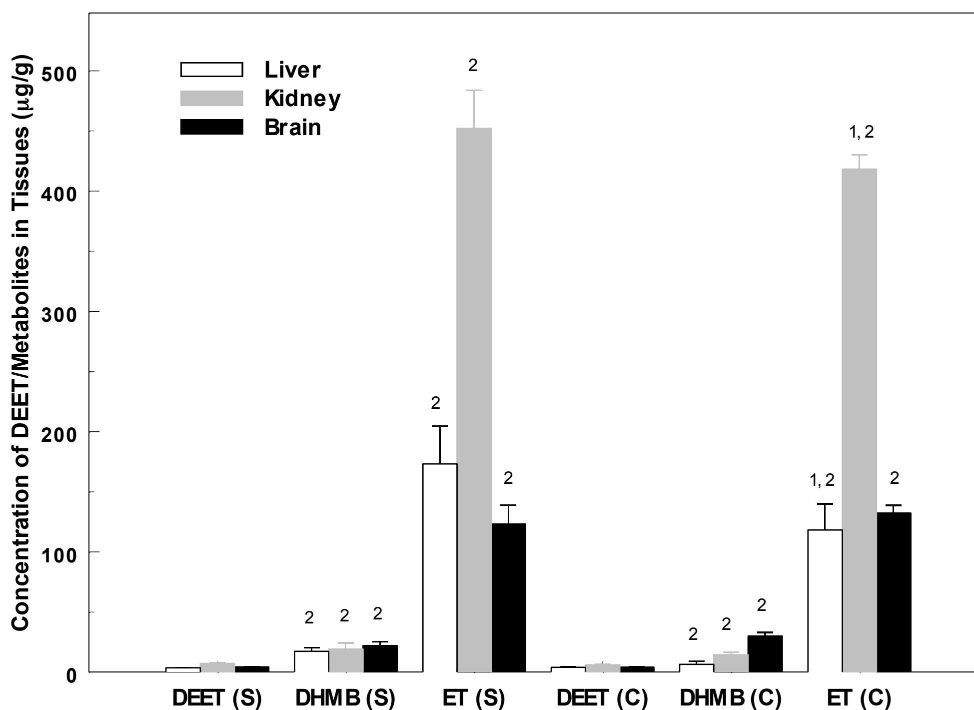
6.4.4. Metabolites of DEET and OBZ

DEET is metabolized in vital organs and eliminated via urine and feces. Two major metabolites of DEET were identified as DHMB (*N,N*-diethyl-*m*-hydroxymethylbenzamide) and ET (*N*-ethyl-*m*-toluamide). They were detected in rat urine (Schoenig, *et al.*, 1993); they were also confirmed in human volunteers topically treated with 12 mg or 15 mg DEET on the forearms (Selim, *et al.*, 1995). The

predominant metabolic routes of DEET were oxidation of the benzylic moiety and hydroxylation of the side chain of DEET molecule (Qiu, *et al.*, 1998). Three OBZ metabolites were detected in animal models and human volunteers, which were THB (2,3,4-trihydroxybenzophenone), DHB (2,4-dihydroxybenzophenone), and DMB (2,2'-dihydroxybenzophenone), respectively. In this study, two metabolites of DEET, DHMB and ET, and three metabolites of OBZ, THB, DHB and DMB, were all detected in rats after repeated topical application of 40 mg/kg DEET and/or 25 mg/kg OBZ once daily for 60 days.

Figures 6.8 and 6.9 show the metabolites concentrations of DEET and OBZ detected in liver, kidney, and brain. ET concentrations in the tissues were significantly higher than DHMB counterparts. Higher tissue concentration of ET may result from higher activity of CYP450 isoforms, CYP2C19, CYP3A4 in male rats, because it had been reported that high activity of CYP2C19, CYP3A4 produced higher amount of ET (Usmani, *et al.*, 2002). Moreover, ET concentration in kidney was significantly higher than that in liver and brain. This finding was in agreement with previous study that rats showed higher [^{14}C] residue levels in kidney than in liver and brain after dermal administration of [^{14}C] DEET at a single dermal dose of 100 mg/kg (Schoeig, *et al.*, 1995). In addition, ET concentration from the single preparation was higher by significantly 8% in kidney and 46% in liver than that from the combined preparation. As previously shown, the combined preparation increased DEET retention in the skin.

Figure 6.8. Concentration of DEET and its metabolites in tissues†



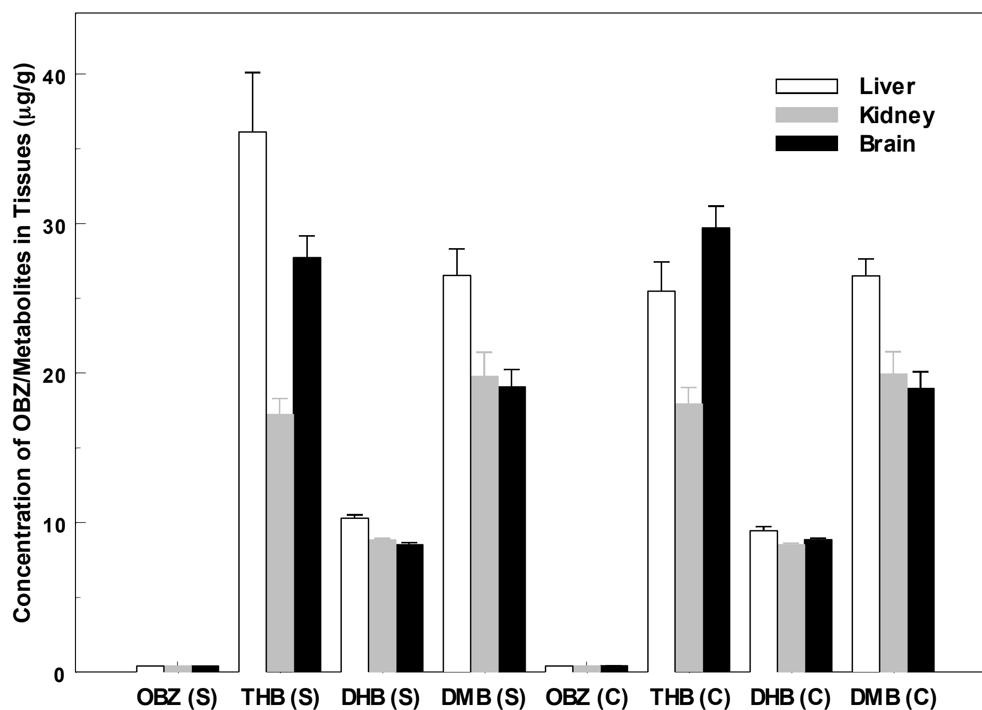
Mean±SEM, n=8, P≤0.05

1: Significant different between single preparation and combined preparation

2: Significant different between ET and DHMB

†This figure showed the concentration of DEET and its metabolites collected in the major tissues, liver (white bar), kidney (gray bar), and brain (black bar). ET concentrations in the tissues were significantly higher than DHMB counterparts. ET concentration in kidney was significantly higher than that in liver and brain. ET concentration from the single preparation was significantly higher by 8% in kidney and 46% in liver than that from the combined preparation.

Figure 6.9. Concentration of OBZ and its metabolites in tissues



Mean±SEM, n=8

†This figure showed the concentration of OBZ and its metabolites collected in the major tissues, liver (white bar), kidney (gray bar), and brain (black bar). There was no significant difference found between any two of the three metabolites, neither was there significant difference found between the single and combined preparations.

Subsequently, DEET metabolites may be suppressed in kidney and liver.

For OBZ metabolites, their tissue concentrations were variable, which may be attributed to different activities of CYP450 isoforms in rats. There was no significant difference found between any two of the three metabolites, neither was there significant difference found between the single and combined preparations. This may indicate that DEET in combined preparation did not sufficiently influence transdermal absorption of OBZ into the circulation. Previous studies also found that enhancement effect of OBZ on DEET absorption exceeded that of DEET on OBZ absorption (Gu, *et al.*, 2005).

Table 6.4 lists distribution percentage of DEET, OBZ and their metabolites in biological samples collected from the study. Total distribution percentage of DEET was approximately 30%, while that of OBZ was about 15%. Therefore around 70% of DEET and 85% of OBZ were either eliminated from the animals through urine and feces or lost from application site by evaporation 24 hours after the dosing. Total skin distribution of OBZ was significantly higher than that of DEET, while total tissue distribution of DEET and its metabolites was significantly higher than OBZ counterparts. Furthermore, the single preparation produced significantly higher DEET metabolites (33%) and OBZ metabolites (10%) than the combined preparation. Concurrent presence of DEET and OBZ in this oil-in-water emulsion apparently decreased deposition of DEET or OBZ metabolites in liver, which may indicate that original DEET and OBZ from the preparation were suppressed in skin permeation. Comparing to parent DEET and OBZ,

concentrations of metabolites were significantly higher; a majority of DEET and OBZ had undergone metabolism 24 hours after topical dosing.

6.5. Conclusion

DEET and OBZ were detected in plasma, skin, and vital organs including liver, kidney and brain after repeated topical application of DEET and/or OBZ from an emulsion-based preparation in rats for 60 days. The emulsion selected for this study produced lower plasma concentration of DEET and OBZ in comparison to commercially available repellent and sunscreen products. Concurrent application of DEET and OBZ in this formulation did not show significant synergistic enhancement of the two compounds in plasma, even though synergistic permeation of DEET and OBZ was observed in the skin. OBZ demonstrated higher deposition in the skin than DEET, while DEET permeated more into the systemic circulation than OBZ. In addition, DEET and OBZ were metabolized rapidly *in vivo* following dermal administration. Concurrent application decreased deposition of DEET, OBZ and their metabolites in plasma and other vital organs in rats.

Repeated topical application of insect repellent DEET and sunscreen OBZ is fairly common for the general public. Overexposure to DEET and OBZ may induce unwanted adverse effects in susceptible individuals. This *in vivo* animal study provided insight into pharmacokinetics and biodistribution characteristics of DEET and OBZ from

an improved semisolid emulsion developed in the laboratory. Studies are ongoing to further improve formulation characteristics so that this emulsion-based preparation could be used to minimize overall transdermal permeation of DEET and OBZ from topical skin application.

Table 6.4. Distribution percentage of DEET, OBZ and their metabolites in rat euthanized at 24h after 60-day topical application

Percentage	DEET (S)	DEET metabolites (S)	DEET (C)	DEET metabolites (C)	OBZ (S)	OBZ metabolites (S)	OBZ (C)	OBZ metabolites (C)
Dose (mg/kg)	40	40	40	40	25	25	25	25
Liver (%)	0.34±0.01	19.18±3.27*	0.39±0.03	14.44±1.83*	0.06±0.01	11.00±0.65*	0.06±0.00	9.97±0.32*
Kidney (%)	0.11±0.01	8.37±0.51	0.10±0.01	8.2±0.37	0.01±0.00	0.97±0.05	0.01±0.00	1.00±0.04
Brain (%)	0.04±0.00	1.37±0.25	0.04±0.00	1.41±0.13	0.01±0.00	0.77±0.04	0.01±0.00	0.81±0.05
Skin (%)	0.84±0.32		0.87±0.20		2.23±0.45		2.38±0.35	
Swab (%)	0.04±0.01		0.16±0.10		0.50±0.12		0.40±0.13	
Total (%)**	30.07±3.50		28.02±3.03		15.67±0.60		14.65±0.51	

Mean±SEM, n=8, $P \leq 0.05$

*: Significant different between single preparation and combined preparation

**: Including the parent molecules and the metabolite

CHAPTER 7

Conclusions

Insect repellents and sunscreens are common consumer care products that are utilized to prevent vector-borne diseases and skin aging and damages. Semisolid emulsion-based preparations have been extensively selected for active repellent and sunscreen ingredients due to excellent solubilizing properties for both lipophilic and hydrophilic components and satisfactory customer acceptance. By designing and adjusting various parameters in emulsion formulation, such as emulsion type, emulsifier, emollient, oil/water proportion and internal droplet size, a final emulsion could exert significant influence on percutaneous permeation of the active ingredients from the preparation. For topical repellent and sunscreen compounds, they should possess maximal skin protection effect with minimal transdermal absorption under ideal use situation. Therefore, formulating an appropriate and elegant emulsion-based lotion through adjusting various parameters in the emulsion would be the first step towards achieving the ultimate goal.

Two types of emulsion containing DEET and/or OBZ were prepared by using the fusion method; and transmembrane permeation of DEET and OBZ was measured using *in vitro* diffusion experiments. In general, percutaneous penetration of an active ingredient would be higher when the compound is dissolved in the external phase of the emulsion. It was found that percutaneous permeation of DEET in the external phase of water-in-oil emulsion was significantly higher than that of DEET in the internal phase of oil-in-water emulsion. On the other hand, percutaneous permeation of more lipophilic

compound OBZ was not enhanced in the external phase of water-in-oil emulsion in comparison to that of OBZ when it was in the internal phase of oil-in-water emulsion.

Several parameters were specifically examined for these emulsion formulations. A thickening agent is usually added in dispersing aqueous phase to improve homogenization and stability of an emulsion. The addition of xanthan gum in this study was able to modify diameter of the oil droplets and enhance contact surface of the oil droplets with skin membrane, subsequently leading to higher skin permeation of DEET and OBZ. In addition, adding xanthan gum to an oil-in-water emulsion produced a synergistic percutaneous penetration of DEET and OBZ through human skin. This work showed that the thickening agent may lead to faster skin penetration of active ingredients from the emulsion. Hence, care should be taken in selecting an appropriate thickening agent in the insect repellents and sunscreens. The thickening agent selected should not only improve homogenization and stability of the formulation, but also have the minimum enhancement on skin penetration of the active ingredients. The screening experiment of thickening agent for insect repellents and sunscreens might be necessary in the further studies.

An emulsifier is the primary component in an emulsion to decide the type of the preparation; it is absorbed to the interface between the oil phase and the water phase to stabilize the emulsion. Its distribution at the interfacial film of an emulsion is also an important factor in material diffusion and percutaneous permeation. Five different

emulsifiers were selected in this study, and they produced different skin permeation profiles for DEET and OBZ. F#1 was an oil-in-water emulsion using surfactant-free emulsifier Emulfree CBG, which kept the natural skin balance without the irritation and dehydration on the skin. But the preparation produced significant increase in permeation of DEET and OBZ among the five emulsions prepared. F#2 was formed with synthetic emulsifier Emulium 22; this oil-in-water emulsion demonstrated a significantly lower skin permeation of DEET and OBZ than the other emulsions. Emulium 22 formed the oil-in-water emulsion, which was water-washable and cosmetically- and aesthetically-acceptable. This emulsifier, with long chain behenic acid in the molecular structure, formed the lotion with a high thixotropic property. Thus, the lotion product was easily-spreadable and stable on the skin surface and led to lower skin permeation of DEET and OBZ compared to other emulsion-based formulations. The synthetic oil-in-water emulsifier with the long chain fatty acid may be the most appropriate emulsifier selection for insect repellents and sunscreens. For water-in-oil emulsions using synthetic emulsifier Arlacel P-135 or Plurol Diisostearique, no difference was observed in skin permeation for DEET, but there was difference in lag time for OBZ.

Various emollients were also incorporated into the developed preparations for the purpose of softening the skin and protecting skin dryness. Appropriate emollients are capable of modifying solubility of an active ingredient in emulsions as well as within the skin, thereby altering skin permeation of the chemical. F#1 contained no emollients, and

it produced a significantly higher permeation of DEET and OBZ compared to other emulsions. F#2 contained a high lipophilic emollient MOD (LogKo/w 13.53). It may increase the solubility and affinity of DEET and OBZ in emulsion, and generate a significantly lower skin permeation of two compounds than other oil-in-water emulsions. There are many lipophilic emollients, like MOD, so more screening work is needed to select a compatible emollient for lowering the skin penetration of active ingredients in insect repellents and sunscreens. When the same emollient was used for both F#4 and F#5, the substance showed no influence on skin permeation of DEET and OBZ, even though other components of the preparations were different. The permeation modifier was another type of chemical that altered skin diffusion and permeation. It was found that Transcutol HP altered DEET and OBZ solubility within the skin to enhance their permeation in F#3, while precirol ato 5 (a high melting point lipid) formed a lipid matrix to resist diffusion activity of DEET and OBZ, thus hindering skin permeation of the two compounds in F#2. The application of a certain amount of a high melting point lipid, like precirol ato 5, in insect repellent and sunscreen products can delay or sustain the topical release of active ingredients from the emulsion formulations.

Rheological characteristics are one of the typical physical properties for liquid and semisolid materials, and play an important role in topical drug dosage forms. Rheological property of an emulsion can be influenced by many formulation factors such as emulsifier, internal/continuous phase, and active ingredients/excipients. In general the

higher the viscosity of a topical formulation is, the lower the drug release rate will be, and subsequently the lower the percutaneous permeation of the active ingredients. F#2 was found to possess the highest thixotropy among the five emulsions studied, and also produced the lowest skin permeation for DEET and OBZ *in vitro*. On the other hand, F#1 demonstrated the lowest thixotropy and the highest skin permeation for DEET and OBZ. Therefore, F#2, with the lower skin permeation of DEET and OBZ, was selected for further study in an animal model.

DEET and OBZ were incorporated into F#2 according to original preparation protocol; the lotion was topically applied to rats once daily for a 60-day period to investigate systemic concentration and tissue deposition of DEET, OBZ, and their respective metabolites. This formulation produced lower systemic absorption of DEET and OBZ than the commercially available products reported in a previous animal study; concurrent application of DEET and OBZ from this study did not show synergistic systemic enhancement between the two compounds either. DEET and its metabolites had a tendency to distribute in blood circulation, in vital organs especially in kidney, however DEET concentration in the skin was low. For OBZ and its metabolites, their concentrations in the blood and vital organs were relatively low, but there was a significantly higher amount of OBZ detected in the skin than DEET. Concurrent application of DEET and OBZ did facilitate synergistic skin retention of the two compounds, but no synergistic systemic absorption of the substances was found in blood

and vital organs.

Based on the *in vitro* and *in vivo* diffusion results in this study, F#2 provided the lowest skin permeation of DEET and OBZ among several emulsion-based formulations, produced no synergistic systemic absorption of two compounds, and displayed the highest thixotropic property. The clinical implication was that this emulsion-based lotion may avoid the potential unwanted adverse effects of DEET and OBZ in susceptible subjects, and decrease health concerns about daily application of insect repellents and sunscreens. Moreover, this lotion is spread easily on the skin, and formed a stable and uniform film on skin surface due to its high thixotropic property. These distinctive characteristics made this lotion a promising emulsion base for DEET and OBZ. The insect repellents and sunscreens prepared with this emulsion base may become products with low health concerns and high customer acceptability. In this emulsion base, the synthetic oil-in-water emulsifier with long chain fatty acid, the high lipophilic emollients, and a certain amount of high melting point lipid are the important factors to modify the skin permeation of active ingredients topically applied. Actually, the influence of the emulsion on the skin penetration of active ingredients is complicated; various emulsion parameters and extraordinarily complex interactions between the emulsion components are involved. The further formulation modification is worthy of focusing on the effect of emulsion on the skin penetration of active ingredients to minimize their potential toxicity and achieve their protection efficacy on the skin surface.

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