INVESTIGATION OF NOVEL PENETRATION MODIFIERS AS ENHANCERS AND RETARDANTS

AND

HUMAN PHARMACOKINETICS AND PHARMACODYNAMICS OF ORALLY ADMINISTERED QUERCETIN

BY

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ABSTRACT OF THE DISSERTATION

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The delivery of the actives through the outermost layer of the skin, the stratum corneum has posed a challenge for scientists for a very long time. There are compound called "enhancers" that have been developed to enhance drug delivery across skin and at the same time there are compounds referred to as "retardants" that prevent or retard the delivery of actives across the skin. Since both enhancers and retardants are believed to act by modifying the properties of the SC barrier, they are collectively referred to as "penetration modifiers". The first part of the study aimed at investigating five penetration modifiers that are analogues of laurocapram and iminosulfurane. These penetration modifiers were evaluated initially for their enhancement/retardation potential by computer modeling and investigated for their physical attributes (solubility, lipophilicity, etc.) and formulation effects. The effect of permeation modifier formulations (prepared in commonly used pharmaceutical vehicles) on permeation of model permeants were evaluated and mechanistic studies were performed using thermal, spectral and

microscopic analyses. The penetration modifiers were also assessed for their in vitro permeation and cytotoxicity using MTS assay. These investigations should lead to a better understanding of the properties, effects and mechanisms of action of penetration modifiers.

The second part of the dissertation covers human pharmacokinetics and pharmacodynamics of quercetin. Quercetin is a naturally occurring flavonoid with antioxidant and anti-inflammatory potential. In order to assess some of benefits of quercetin, this investigation examined the pharmacokinetics and pharmacodynamics of orally administered formulations to human healthy volunteers. Initially, we developed and validated a quercetin assay in human plasma and urine followed by selection of suitable oral carrier for pharmacodynamics study based on pharmacokinetic profiles of three nutritional carrier systems. This was followed by determination of the effects of quercetin supplementation on improving maximal oxygen uptake, enhancing exercise performance during heat stress and relief of muscle soreness. We expect to make conclusions regarding effects of quercetin in overall improvement in work performance of human subjects that would in turn enable the U.S. Department of Defense to consider inclusion of quercetin in daily rations for soldiers to further improve their performance.

Dedication

To Almighty God

To my husband, Ranajoy and our family

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Abbreviations

- TDDS: Transdermal drug delivery system
- SC: Stratum corneum
- DSC: Differential scanning calorimetry

ATR-FTIR: Attenuated total reflectance fourier transform infra-red spectroscopy

PMR: Penetration modifier ratios

ADHD: Attention deficit hyperactivity disorder

DEET: Diethyl-m-toluamide

DMBIS: S,S-Dimethyl-N-(4-bromobenzoyl) iminosulfurane

DMMCBI: S,S-Dimethyl-N-(2-methoxycarbonylbenzenesulfonyl) iminosulfurane

TBDOC: tert-Butyl 1 -dodecyl-2-oxoazepan-3-yl-carbamate

PG: Propylene glycol

PBS: Phosphate buffer saline

PEG 400: Polyethylene glycol 400

Tm: Mean transition temperature

H: Mean enthalpy

H-bond: Hydrogen bond

HSD: Honestly significantly different

SEM: Scanning electron microscopy

MTS: 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-

tetrazolium, inner salt)

DOD: Department of Defense

URTI: Upper respiratory tract Infection

VO_{2max}: Maximal oxygen uptake

SIRT1: Sirtuin

PGC-1α: Peroxisome proliferator-activated receptor-γ (gamma) coactivator

COMT: Catechol-o-methyltransferase

ED: Electrochemical detection

HPLC: High-performance liquid chromatography

MS: Mass spectroscopy

UV: Ultraviolet

DAD: Diode array detector

GIT: Gastrointestinal tract

SGLTI: Sodium-dependent glucose transporter I

UDP-GT: Uridine-5'-diphospho-glucuronosyltransferase

CYP1A: Cytochrome P450 monoxygenase 1A

VLDL: Very low-density lipoproteins

NF- κB: Nuclear factor kappa-light-chain-enhancer of activated B cell

ROS: Reactive oxygen species

CK: Creatine kinase

IL-6: Interleukin-6

CRP: C-reactive protein

GI: Gastrointestinal

PVDF: Polyvinyledene diflouride

MRE: Meals ready to eat

NSRDEC: Natick Soldier Research Development and Engineering Center

RSD: Relative standard deviation

- Lz: Slowest disposition rate constant
- T $_{1/2}$: Half life of elimination, $\ln 2/lz$

T_{max}: Time to each maximum concentration

- C_{max}: Maximum concentration
- AUC_{tot}: Area under curve in concentration vs. time curve
- AUMC_{tot}: Area under moment curve
- MRT: Mean residence time, AUMC_{total}/AUC_{total}

Cl: Clearance

Vss: Apparent volume of distribution in the plasma compartment at steady state

V_z: Apparent volume of distribution during terminal phase

OATP- B: Organic anion transporting protein- B

BMI: Body mass index

V_T: Tidal volume (VT),

RR: Respiratory rate

V_E: Expired volume

RER: Respiratory exchange ratio

HR: Heart rate

RPE: Rating of perceived exertion

DOMS: Delayed onset muscle soreness

 η 2: percentage of variability

USG: Urine specific gravity

W_{max}: maximal workload

AR: Army regulations

RH: Relative humidity

LF: Liner factors

T_{re}: Rectal temperature

Tsk: Skin temperature

RPE: Ratings of perceived exertion

RP: Ratings of pain

RM: Rating of motivation

RTC: Ratings of thermal comfort

Ki: Inhibition constant

DARPA: Defense Advanced Research Projects Agency

SSA: 5-Sulfosalicylic acid

DMSO: Dimethyl sulfoxide

NADPH: Nicotine adenine dinucleotide phosphate (reduced form)

DRI: Dietary reference intake

POMS: Profile of mood states

CRF: Classical risk factors

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Part 1: Investigation of novel penetration modifiers as enhancers and retardants

Chapter 1: Introduction

The transdermal drug delivery system (TDDS) approach is known to have the advantages over conventional drug delivery systems in achievement of steady blood level profiles, reduced systemic side effects, avoidance of first pass effect, user friendly dosing, convenient, pain-free therapy and multi-day dosing with improved patient compliance (1). The successes of the transdermal industry can be estimated from the statistics that more than 35 transdermal patch products have been approved by United States FDA in the last two decades. Twelve million prescriptions are being filled for transdermal products with indications ranging from angina to bladder control. Patches have been developed for indications such as hormone replacement (testosterone, estrogen replacement therapy), angina (nitroglycerin), pain relief (fentanyl), hypertension (clonidine), smoking (nicotine), motion sickness (scopolamine), incontinence (oxybutynin), depression (selegiline), ADHD (Attention Deficit Hyperactivity disorder), (methylphenidate), and others (Table 1.1).

Despite the promise of the TDDS approach, its scope is currently limited to drugs with low molecular weight (less than 500 daltons), log P ranging between 1-3, low melting point (less than 200°C) and high potency (dosage in the range of mg/day) (2) (3). The development is also limited by barrier properties of the skin. Both physical and chemical enhancement techniques have been introduced to overcome the resistance offered by skin that are either used alone or in combination. The physical enhancement approaches that have been examined include iontophoresis (4), phonophoresis (5), electroporation (6) and microneedles (7). However, the use of chemical enhancement techniques dominates due to their simplicity and low cost. Several chemical enhancers have been introduced and commercialized including some recent ones such as SEPA009[®] (Macrochem), NexAct88[®] (NexMed), SR38[®] (Pharmetrix) (8). At the same time, there have been certain candidate enhancers that did not successfully transition from purely research to market, due to skin irritancy issues. Therefore, the pursuit of novel penetration enhancers is still in process. Besides the development of enhancers, compounds termed retardants have emerged that have the potential to condense the lipids of stratum corneum and retard the permeation of the actives. Up to now, published retardants such as N-0915 and *S*,*S*-dimethyl-*N*-(2-methoxycarbonylbenzenesulfonyl) iminosulfurane are structural analogues of known enhancers laurocapram and iminosufuranes. The activity of a penetration modifier as an enhancer or retardant is based on its molecular shape, H-bonding potential, polarity, chemical structure and the accompanying formulation.

The objectives of the present study was to investigate the stratum corneum modifying effects of some relatively potent percutaneous permeation enhancers/retardants including laurocapram, 3-dodecanoyloxazolidin-2-one (N-0915), *S*,*S*-dimethyl-*N*-(4-bromobenzoyl) iminosulfurane (DMBIS), *S*,*S*-dimethyl-*N*-(2-methoxycarbonylbenzenesulfonyl) iminosulfurane (DMMCBI) and *tert*-butyl 1 –dodecyl-2-oxoazepan-3-yl-carbamate (TBDOC). The effects that were investigated included formulation effects on enhancer/retardant activity, mechanism of action of penetration modifiers in corresponding formulations, penetration modifier-ceramide interactions and cytotoxic potential of the penetration modifiers.

Initially, the interaction of penetration modifier with ceramide 6 was investigated using computer modeling to determine the enhancement and retardation potential on permeation of the active. Secondly, the formulation effects of penetration modifiers were investigated by determining enhancement/retardation of the percutaneous permeation of diethyl-*m*-toluamide (DEET) in presence of five percutaneous penetration modifiers (laurocapram, N-0915, DMBIS, DMMCBI and TBDOC). These compounds were formulated in water, propylene glycol (PG), ethanol or polyethylene glycol 400 (PEG 400).

The mechanism of action of each formulation and effect on the barrier properties of skin (particularly of the stratum corneum, SC) was determined by thermal and spectral analyses of treated and untreated SC. This was performed using differential scanning microscopy (DSC) and attenuated total reflectance fourier transform infra-red spectroscopy (ATR-FTIR).

The cytotoxic effects of each penetration modifier was also evaluated using scanning electron microscopy (SEM) and MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)) assay. The MTS assay was performed using keratinocytes and fibroblasts that represent the most abundant cells in epidermis and dermis respectively. The permeation studies of penetration modifiers were performed to determine the extent to which the penetration modifier permeates across skin.

Chapter 2: Background and Significance

2.1 Background

The skin is the largest and most accessible organ of the body that makes it an obvious target as a route for drug delivery. However, delivery of permeants across the skin is limited by the resistance offered by the topmost non-viable layer of the epidermis termed the stratum corneum (SC). The remaining layers of skin, namely viable epidermis (stratum lucidum, stratum granulosum, stratum spinosum, stratum basale) and dermis offer lower barrier properties to the passage of actives (Figure 2.1). Even though several physical methods (9-11) have been introduced to compromise the resistance offered by the SC, the use of chemical penetration modifiers (12, 13) dominates the transdermal drug delivery field. The transdermal delivery of several therapeutic agents such as non steroidal anti-inflammatory drugs (NSAIDS) (14), contraceptives (15), antihypertensives (16), and others has been significantly increased by the use of chemical permeation enhancers, some of which are also formulation vehicles. Several percutaneous permeation enhancers such as surfactants, terpenes, laurocapram, dimethyl sulfoxides, fatty acids, alcohols, water, etc. are known to improve permeability of actives by providing fluidity to lipid bilayers of the SC. Unlike most therapeutic drugs, certain chemicals including pesticides (17), chemical warfare agents (18), some sunscreens (19), and mosquito repellants (20) unfortunately tend to pass through skin in reasonably high concentrations resulting in undesirable side-effects or toxicity. Some of these agents are classified as permeation enhancers themselves (DEET- diethyl *m*-toluamide) and easily penetrate across the skin leading to toxic effects and systemic exposure. The percutaneous

permeation of such agents can be minimized by the use of penetration retardants. The application of these percutaneous penetration retardants extends beyond exposure against agrochemicals (such as pesticides and household chemicals) and cosmetics (such as sunscreens and mosquito repellants) to protection against exposure to lethal chemical warfare agents. Permeation retardants are usually structural analogues of enhancers and are hypothesized to act by tightening and condensing the lipid arrangement of SC. Owing to the modifying action of permeation enhancers and retardants on the barrier properties of stratum corneum, these compounds are collectively termed permeation modifiers.

2.2 Characteristics of ideal penetration modifiers

Penetration modifiers should possess certain attributes for their safe and effective use in transdermal and topical formulations. An ideal permeation modifier should be pharmacologically inert and chemically stable. Modifiers should be potent in low concentrations and compatible with all formulation components. The penetration modifiers should possess non-toxic, non-sensitizing, and non-irritant properties. Modifiers should also have a rapid and reversible onset of action and specific activity (21). Penetration modifiers should ideally work in a unidirectional manner that is, they should control input of actives in the body and at the same time, they should not lead to loss of endogenous material from the body. Also penetration modifiers should be compatibile with different formulation excipients and drugs. Beside compatibility, penetration modifiers should be cosmetically acceptable and have a good aesthetic appeal (22). Up till now, no permeation modifier has been found that possesses all of these characteristics. One of the major concerns associated with modifiers is their significant skin irritancy and in some cases toxicity. In one such study performed to evaluate skin

irritation potential of different saturated fatty alcohols after application in vivo on rat skin revealed that all fatty alcohols increase the transepidermal water loss (TEWL) and skin blood flow significantly. TEWL, skin blood flow and visual scoring method were the criteria used for evaluating skin irritation potential in the study (23). There have been some encouraging reports such as that by Moffat *et al.* that found that silicon analogues of carbon based penetration modifiers have less irritancy potential (24). Besides evaluation of the irritation potential of penetration modifiers, in vitro studies have been performed to determine toxicity associated with these compounds. For instance, Song et al., investigated the cytotoxic effects of iminosulfuranes (penetration enhancers) on epidermal keratinocytes and dermal fibroblasts at several concentrations. The study revealed that iminosulfuranes showed concentration dependent cytotoxicity and in most cases it was negligible below 0.2 M (25). Unlike short term toxicity studies of penetration modifiers in vitro, there are limited reports about evaluation of long term toxicity of modifiers both in vitro and in vivo. One such study investigating long term toxicity potential of laurocapram was performed in mice, rats, guinea pigs, rabbits and monkeys and results indicated there was no systemic or dermal toxicity observed in animals on one month or lifetime dermal exposure to laurocapram (26). There are also a few studies reporting systemic absorption of penetration modifiers (27). A pharmacokinetic study performed in healthy human volunteers to evaluate systemic absorption of laurocapram after dermal application revealed that it is poorly absorbed into the human body and the small quantity that is absorbed is readily metabolized and excreted in the urine (28, 29). Despite of some concerns over irritation and toxicity potential of chemical permeation

modifiers, the interest in their use in formulations is still significant since the approach is economical and relatively simple and in most cases, reversible.

2.3 Theory

As mentioned above, the thin (15-20 µm), tough and relatively impermeable stratum corneum forms the rate controlling barrier for passage of nearly all permeants. The dead, flattened, keratin-rich corneocytes embedded in the ordered lipid bilayer arrays are known to provide barrier properties to stratum corneum. Since stratum corneum is composed of non-viable corneocytes, passage of permeant across it follows passive diffusion governed by physicochemical laws with no involvement of active transport (30). There are three major diffusion pathways involved in passage of permeant across SC namely the appendageal route, the transcellular route, and the intercellular route (31). The intercellular pathway is the most prominent pathway in which the diffusant follows a tortuous route with continuous and repeated partitioning in and out of hydrophilic and lipophilic domains of intercellular lipids (32). The intercellular lipid arrays of stratum corneum are usually complex mixture of ceramides, cholesterol, cholesterol esters, and fatty acids (8, 31, 33). The diffusion via the appendageal route is the least common as it involves diffusion of actives across hair follicles, sebaceous glands and eccrine (sweat) glands that constitute only 0.1% of the absolute skin surface area.

Even though skin is a complex, heterogeneous membrane, simple Fick's laws of diffusion are often used to describe percutaneous absorption of permeants. Since transdermal delivery involves application of drug formulation for long period of time, Fick's first law of diffusion is the most relevant law to describe the steady state of the diffusant across stratum corneum (34).

$$J = \frac{KD}{h} (Co - Ci)$$
 (Equation 1)

Equation 1 is the mathematical representation of Fick's first law of diffusion where J is the flux per unit area, K is the SC-formulation partition coefficient of the permeant, D is the diffusion coefficient in the SC of path length h; Co is concentration of permeant applied to the skin surface and Ci is the concentration of permeant inside skin. Practically Co>>Ci, therefore equation 1 simplifies to:

$$J=k_pCi$$
 (2)

Where k_p (=DK/h) is the permeability coefficient that accounts for both partition and diffusion characteristics of the permeant.

Even though permeability across SC is the inherent property of the molecule (based on its partition coefficient and molecular size), percutaneous diffusion of permeants can be altered by use of permeation modifiers that increase/decrease percutaneous absorption. In case of permeation enhancers, skin permeability is altered via several mechanisms (Figure 2.2) (35). Chemical permeation enhancers especially laurocapram, dimethyl sulfoxide, etc. significantly improve the flux, J (Equation 1) of the permeant by increasing its diffusing coefficient, D (Equation 1) via fluidizing the lipid environment of SC. Similarly enhancers such as surfactants and terpenes tend to increase diffusion coefficient (D) of the permeant by interacting with intracellular proteins present in SC. Other enhancers such as propylene glycol, lower alcohols, Transcutol[®], *N*-methyl pyrollidone increase the permeant flux by increasing partitioning and solubility of the drug in the stratum corneum (32, 36). Unlike permeation enhancers, the exact
mechanism for the action of retardants is not clear. However, it is hypothesized that retardants decrease the diffusion coefficient of the drugs by providing order to the lipid-lipid arrangement in the SC (37).

2.4 Enhancement vs. Retardation

2.4.1 Factors affecting performance of transdermal permeation modifiers

2.4.1.1 Molecular shape and size

The shape of a permeation modifier greatly affects its activity as an enhancer or a retardant. For instance, the graphic molecular representation of laurocapram (Figure 2.3) indicates that it exists in 'soup spoon' or 'bent spoon' conformation (38, 39). In the 'soup spoon' conformation, the carbonyl moiety is oriented in such a way that it moves away from the skin lipid bilayer into the polar region of the skin membrane. Also, laurocapram has a cross sectional area of 60 $Å^2$ which corroborates the bent spoon conformation generated by molecular graphics. When laurocapram is applied on the skin surface, it inserts itself into the intracellular lipid bilayer arrays by pushing apart the head groups of ceramides. This disturbance in lipid arrangement creates free volume in the alkyl chain region of the lipid domain that facilitates the diffusion of drugs whose percutaneous absorption was initially limited by slow intercellular diffusion. On the other hand, when a potent retardant, N-0915 (3-dodecanoyloxazolidin-2-one) is applied topically, it inserts itself easily into the ceramide bilayer structure but due to its linear conformation and relatively smaller size (compared to laurocapram) is unable to push the head groups of ceramide molecules apart leading to no facilitation of diffusion of molecules across the stratum corneum. Furthermore, its insertion into the ceramide arrays

strengthens the intercellular lipid arrangement that subsequently retards the permeation of the diffusant (40, 41).

2.4.1.2 Hydrogen bonding potential and polarity

Though the high energy 'bent spoon' conformation hypothesis justifies the transdermal enhancement resulting from the use of laurocapram, it does not explain the favorability for existence of this high energy conformation in the bilayer structure of the packed ceramide molecules of SC (37). Therefore, an alternative hypothesis for the penetration modifier action of laurocapram and its analogues was proposed. The hypothesis is based on the H-bonding potential of penetration modifiers with stratum corneum cerebrosides and more importantly on the polarity of penetration modifiers. According to this concept, the formation of an H-bond favors the feasibility of the penetration modifier for organization in the intercellular lipid array of SC and its unipolar or bipolar nature decides its activity as an enhancer or retardant. Among the various cerebrosides present in SC, ceramide 6 is most predominant one. Studies show that it possesses bipolarity into its head group region owing to four secondary alcohol groups and one secondary amide group. It is hypothesized that laurocapram on insertion into the intercellular lipid domain forms H-bonds with these ceramides from one side of the molecule. Since its ring structure is uniformly positive in nature (unipolar), it tends to force apart the ceramide molecules present on either side due to electrostatic repulsion. On the other hand, N-0915 which is smaller in size compared to laurocapram and possesses a bipolar ring structure undergoes a favorable electrostatic interaction with ceramide molecules that pulls the intercellular lipids together and the subsequent condensed state leads to a decrease in skin permeability. The two oxygen containing groups on both sides of N-0915 enable it to

form hydrogen bonds with groups present in ceramide molecule and therefore impart order to skin lipid bilayer. In contrast, laurocapram has only one oxygen containing group and is only capable of forming hydrogen bond with ceramide groups from one side and thus provides disorder to disorder to intercellular lipid bilayer (8, 37).

2.4.1.3 Chemical structure

Although chemical structure influences the property of the molecule such as log P and solubility, there are no reports that relate the behavior of the permeation modifier as enhancer or retardant with these physical properties. However, the replacement of one substituent group in the same class of penetration modifiers has been observed to alter their property as enhancer or retardant. For example, Kim et al. reported that S,Sdimethyl-N-(4-bromobenzoyl) iminosulfurane and S,S, dimethyl-N-(4-nitrobenzoyl) iminosulfurane that belong to the class of N-aroyliminosulfuranes, enhanced and retarded respectively the permeation of hydrocortisone across the hairless mouse skin (42). The replacement of bromo group in S,S-dimethyl-N-(4-bromobenzoyl) iminosulfurane by a nitro group lead to retardation of the active permeant by three fold. Similarly Hadgraft et al. reported that replacement of the cycloheptane ring of laurocapram by an oxazolidine-2-one moiety changed its activity from an enhancer to a retardant (37). Also replacement of a more electronegative group by a less electronegative group changed the intensity of penetration modifier activity. For instance, structural activity analysis of laurocapram and its analogues showed that replacing oxygen by sulfur in laurocapram structure, a reduced partial net negative charge developed on the molecule, ultimately leading to loss of its enhancement activity (37).

2.4.1.4. Formulation effect

Formulation plays an important role in affecting the degree of enhancement/retardation caused by a penetration modifier. Recent in vitro studies in our laboratory on human cadaver skin investigated the effect of three permeation modifiers (laurocapram, N0915, *S*,*S*-dimethyl-*N*-(2-methoxycarbonylbenzenesulfonyl) iminosulfurane) that were dissolved/suspended in various vehicles : water, ethanol, propylene glycol (PG) and polyethylene glycol 400 (PEG 400), on skin permeation of diethyl-*m*-toluamide (DEET). The study revealed that solvent systems markedly affected the behavior of permeation modifiers in either enhancing or retarding the permeation of the active across the skin. Results showed that the activity of laurocapram in enhancing DEET permeation decreased from a modifier ratio (MR) of 9.4 in PG to 3.9 in PEG 400. Likewise, N0915 acted as a retardant for diffusion of DEET with ethanol (MR = 0.5) and PEG 400 (MR = 0.5) but not with water (MR = 5.0) or PG (MR = 1.6). Similarly, S,S-dimethyl-N-(2methoxycarbonylbenzenesulfonyl) iminosulfurane was a retardant with ethanol (0.9) and PEG 400 (0.9) but not with water (4.6) or PG (2.2) (43, 44).

Previous studies by Kim *et al.* have shown that the penetration modifier *S*,*S*-dimethyl-*N*-(2-methoxycarbonylbenzenesulfonyl) iminosulfurane behaved as retardant of diffusion of hydrocortisone across hairless mouse skin (42). However, current investigations by Kaushik and Michniak demonstrated both enhancement and retardation effects by these compounds depending on the vehicle in which they were incorporated (43, 44). Also in case of N0915, Hadgraft *et al.* reported it to be a retardant of DEET and metronidazole permeation (37) but its activity changed when it was incorporated in PG and water (44). Therefore, it can be proposed that for a penetration modifier to be regarded as an

enhancer, it should always depict enhancement of the permeant irrespective of the formulation in which it is incorporated and converse also holds true for the retardants.

2.5 Characterization of penetration modifiers:

Most in vitro permeation studies are performed using the Franz diffusion cell technique that is a classic and relevant technique for quantifying the degree of enhancement/retardation of percutaneous diffusion of drugs in presence of penetration modifiers. In addition, there are numerous analytical techniques that are used to characterize the effect of penetration modifiers on intercellular lipids of stratum corneum. A good insight on different analytical methods used for characterizing penetration modifiers is given by Potts and Guy (45). Also Touitou *et al.* (46) showed the quantitation of drugs in different skin layers using techniques such as autoradiography and ATR-FTIR (attenuated total reflectance-fourier transform infrared spectroscopy), remittance, fluorescence and photo-thermal spectroscopy. Some common techniques that are used to characterize effects of permeation modifier in skin are summarized below: **Microscopy**: Microscopy involves direct and indirect visualization of the effects of permeation modifiers in skin cross sections (45, 47-49).

Infrared (IR) spectroscopy (especially ATR-FTIR): IR spectroscopy involves measurements of peak heights and areas of symmetric and asymmetric C-H absorbances of skin that are observed before and after penetration modifier treatment (45, 46, 48, 50-52).

Differential scanning calorimetry (DSC): DSC records thermodynamic parameters of stratum corneum such as heat capacity, phase transition temperature as a function of

temperature (thermograms) before and after penetration modifier treatment (37, 45, 49, 52).

Impedance spectroscopy: This technique involves measurement of electrical resistance (impedance) of different layers of skin as a direct function of modifier effect (45, 53).

Fluorescence Spectroscopy: Fluorescence spectroscopy records fluorescence of selected skin layer (e.g. dermis) in presence of light absorbing drug or the drug itself (in case of fluorescent molecule) that is then quantified with the help of carboxyfluorescein assays using a spectrometer, in presence and absence of permeation modifier (46, 49).

Raman spectroscopy: In this technique, modifications induced by permeation modifiers to lipid layers in skin are observed as a function of stretching, twisting, scissoring -CH, - CH_2 and -C-C vibrations (25, 50, 54).

Cytotoxicity assays: Cytotoxicity assays determine *in vitro* toxicity of chemical permeation modifiers on skin cells (dermal fibroblasts and epidermal keratinocytes) (25, 55).

Molecular modeling: In this technique, modifier effect is predicted on computer simulated skin containing certain skin lipids using mathematical algorithms (37, 40).

2.6 Penetration modifiers in drug delivery

The application of penetration enhancers/retardants extend to all kinds of topical and transdermal formulations that may be in form of lotions, creams, gels, patches, etc. On one hand penetration enhancers aim at improving the systemic absorption of the therapeutic agents across the skin and on the other hand, retardants restrict the permeants

to the uppermost layer of the skin. The enhancers have been used to improve the delivery of wide range of therapeutic agents (56) such as antihypertensives (clonidine), analgesics (fentanyl/lidocaine/epinephrine), birth control agents (ethinyl oestradiol), smoking cessation drugs (nicotine), hormone replacement drugs (17β -oestradiol), antianginals (nitroglycerin), and others. The scope for application of retardants is limited to fields where permeants depict systemic toxicity due to uncontrolled and undesired systemic absorption. Such agents include agrochemicals (17) (pesticides and household chemicals), certain cosmetic (19)/dermal products (20) (sunscreens, insect repellants) and lethal chemical warfare agents (18).

Owing to the potential significance of transdermal drug delivery, various enhancers have been identified ranging from classic alcohols, fatty alcohols/acids, sulfoxides, surfactants, amides/cyclic amides, pyrrolidones to novel enhancers such as iminosulfuranes, DDAIP, etc. (57).

Among the various potential enhancers only few have undergone clinical testing. One such potential enhancer is dimethyl sulfoxide (DMSO), but unfortunately its use has been discontinued as it is effective only at high concentrations and it is unpleasant to use (58). Recently its use has been revived and formulation containing DMSO with diclofenac, called Pennsaid Topical Solution (diclofenac sodium topical solution, 1.5% w/w) has been approved by Food and Drug Administration (FDA) (59).

Laurocapram is another extensively investigated chemical enhancer and is often used in research applications and for comparative studies due to its excellent enhancing abilities. A long-term toxicity study of laurocapram performed in mice, rats, guinea pigs, rabbits and monkeys indicated that there was no systemic or dermal toxicity observed in animals

on 1 month or lifetime dermal exposure to laurocapram (26). A human pharmacokinetic investigation conducted on systemic absorption of laurocapram after dermal application showed poor systemic absorption of laurocapram and the small quantity that was absorbed was readily metabolized and excreted in the urine (28, 29) suggesting minimum systemic toxicity associated with its absorption. However, its commercialization is limited owing to its skin-irritancy potential (60).

Fatty acids and fatty acid esters form another class of penetration enhancers that has not only good enhancing potential but also categorized as Generally Recognized As Safe (GRAS) compounds. Despite of being considered as GRAS, one in vivo study reported irritation caused by fatty acids and their esters (36). Criteria such as TEWL, skin blood flow and a visual scoring method were used to evaluate the skin irritation potential after application of different saturated fatty alcohols on rat skin. Results depicted that all fatty alcohols used in the study were capable of increasing the transepidermal water loss (TEWL) and skin blood flow significantly. In spite of this, some combinations of fatty acids and their esters have also been commercialized. The combination of glyceryl monoleate and lauryl lactate has been used by Theratech Inc., (Madison, TN) for transdermal enhancement of testosterone (Andoderm[®] patch) across nonscrotal skin in hypogonadal males (61). Like other potential enhancers, the use of the above combination is limited by its irritation potential. It has been suggested that a prior application of triamcinolone helps in overcoming the irritancy caused due to the above product (3).

Some novel potential enhancers such as Macrochem's SEPA (2-n-nonyl-1,3dioxolane)(62) and Nexmed's Nex-ACT (*N*,*N*-dialkyl-substituted amino acetates) (52) that show enhancement activities comparable to laurocapram are also being clinically tested. Topical preparations containing SEPA enhancer and ibuprofen/alprostadil are currently undergoing clinical trials. Similarly, topical gel formulation with Nex-ACT enhancer and alprostadil is also being evaluated in Phase 2 clinical trials. Unlike the above mentioned enhancers, there are no reports of clinical testing of any chemical retardants.

While penetration enhancers have been studied extensively, little information is available on penetration retarders and their mechanisms. Penetration modifiers consist of a diverse group of agents with varying physiochemical properties, structure activity relationships and mechanisms of action. Table 2.1 provides the classes of penetration modifiers with representative compounds and their penetration modifying effects on various agents. This list, though not comprehensive, attempts to summarize the most investigated and important dermal penetration modifiers reported in the literature.

2.7 Significance

An extensive review of the published literature has revealed that there have been numerous studies reporting the reduction of the barrier properties of skin using penetration enhancers, however, there are limited reports on retarding penetration of actives. At the same time there is limited information about the specific attributes of penetration enhancers and retardants. This dissertation will report on our aims at determining interactions and effects of penetration modifiers especially retardants on stratum corneum. We hope to report on the interactions of penetration modifiers with the SC barrier which is known to be the layer that provides maximum resistance to drug transport. We will usecomputer modeling as well as investigations of the in vitro permeation of permeants in presence of penetration modifier formulations to substantiate our hypotheses.. We will also determine the mechanistic aspects of penetration modifiers through thermal and spectral techniques. Also, in addition, we aim to shed light on the permeation profile properties of enhancers/retardants used in the study as well as their cytotoxicity potential.

We hope that the information reported in this dissertation will provide a better understanding of the formulation and development of therapeutic products with improved drug delivery using penetration modifiers. This will also be one of the first times that the influence of formulation vehicles will show a change of compound property i.e. an enhancer losing activity or becoming a retardant and vice versa.

Chapter 3: Specific Aims

The aim of this dissertation was to investigate the stratum corneum modifying effects of some relatively potent percutaneous permeation modifiers (enhancers/retardants) including laurocapram, 3-dodecanoyloxazolidin-2-one (N-0915), *S*,*S*-dimethyl-*N*-(4-bromobenzoyl) iminosulfurane (DMBIS), *S*,*S*-dimethyl-*N*-(2-methoxycarbonylbenzenesulfonyl) iminosulfurane (DMMCBI) and *tert*-butyl 1 –dodecyl-2-oxoazepan-3-yl-carbamate (TBDOC). We expect to have a better understanding of the modifying action of not only these enhancers/retardants but chemical modification of the barrier properties of stratum corneum. The effects that were investigated involved formulation effects on enhancer/retardant activity, mechanism of action of penetration modifiers in corresponding formulations, penetration modifier-ceramide interactions and cytotoxic potential of the penetration modifiers.

Specific Aim 1: Investigation of formulation effects of penetration modifiers

In order to determine the potential of each of the penetration modifier as enhancer or retardant, molecular modeling of each of penetration modifiers (laurocapram, N-0915, DMBIS, DMMCBI and TBDOC) and ceramide 6 molecules was performed. At the same time, partition coefficient of each of the penetration modifier was determined. This was followed by preparation of formulation of each of penetration modifiers (laurocapram, N-0915, DMBIS, DMMCBI and TBDOC) in one of vehicles (water, propylene glycol (PG), ethanol and polyethylene glycol 400 (PEG 400) in the study. The formulation effects of penetration modifiers were investigated by determination of enhancement/retardation of percutaneous permeation of diethyl-*m*-toluamide (DEET) in presence of prepared percutaneous penetration modifiers-formulations. Also, available amounts of penetration

modifiers in each formulation were determined which was then compared with the degree of their enhancement/retardation activity.

Specific Aim 2: Investigation of mechanism of action of various formulations of penetration modifiers through thermal and spectral analysis

The mechanism of action of SC modification activity of each formulation of penetration modifier (as described above) was determined by thermal and spectral analyses of treated and untreated skin. The thermal and spectral analysis of SC was performed using differential scanning calorimetry (DSC) and attenuated total reflectance fourier transform infra-red spectroscopy (ATR-FTIR) respectively.

Specific Aim 3: Investigation of percutaneous permeation profile of penetration modifiers and their cytotoxic potential

The cytotoxic effect of each penetration modifier was evaluated using scanning electron microscopy (SEM) and MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)) assay. The MTS assay was performed on keratinocytes and fibroblasts that represent the most abundant cells in SC (non-viable epidermis) and dermis respectively. The permeation studies of penetration modifiers were performed to determine the extent to which penetration modifier permeate across skin.

Chapter 4: Percutaneous penetration modifiers and formulation effects

4.1. Introduction

For decades, the utilization of skin as a route for delivering drugs into the body has attracted interest from pharmaceutical formulators. Several physical (9, 63-65) and chemical approaches (66) have been explored that compromise the barrier of the uppermost layer of the skin, the stratum corneum. The chemical approach has resulted in the introduction and commercialization of several permeation enhancers ranging from classics such as laurocapram (67, 68), terpenes (69), alcohols (70, 71), glycols (72), amides (73, 74), sulfoxides (75), fatty acids (76), surfactants (77), etc. to newer compounds such as SEPA009[®] (Macrochem), NexAct88[®] (NexMed), SR38[®] (Pharmetrix) that mainly act by providing fluidity to the lipids of the stratum corneum. Beside the above mentioned enhancers, a novel subclass of iminosulfuranes has emerged that include compounds such as S,S-dimethyl-N-(4-bromobenzoyl) iminosulfurane, that possess enhancement potential (42). At the same time that there are efforts in progress to enhance the delivery of therapeutic agents using permeation enhancers, there are increasing concerns over toxicity associated with agrochemicals (17), mosquito repellants (20), sunscreens (78) chemical warfare agents (78) and household cleaning chemicals (79). These concerns have led to the investigation of percutaneous permeation retardants. Permeation retardants, unlike enhancers decrease the diffusion of applied actives by strengthening the intercellular lipid organization of the stratum corneum. Despite the contrasting behavior of enhancers and retardants, they are collectively referred to as "penetration modifiers" since they both act by modifying the structure of stratum corneum (57, 80). Even though the concept of percutaneous retardation of permeants is a

decade old, there has been scant research performed in the area. The retardants reported in the literature are often structural analogues of potent enhancers. For instance, N-0915 (3-dodecanoyloxazolidin-2-one) is a structural analogue of laurocapram (81).

Hadgraft *et al.* proposed several theories to explain the mechanism of action as enhancers or retardants. It is believed that the action of enhancers and retardants can be explained through their interaction with ceramides (especially ceramide 6) that form the largest group of lipids in the stratum corneum. The ceramides along with cholesterol, fatty acids, cholesterol esters and cholesterol sulfate form multiple lipid lamellae that ultimately provide diffusional resistance to the permeation of the active across the stratum corneum. Among the various ceramides, Hadgraft and coworkers used ceramide 6 to demonstrate the properties of a penetration modifier as an enhancer or retardant. Ceramide 6 was chosen for modeling studies since Wertz (82) indicated that ceramide 6 possesses the highest hydrogen bonding capability among the various ceramides present in human stratum corneum. Hadgraft reported that one sided H-bonding of permeation modifiers with ceramide 6 suggests its activity as an enhancer, whereas two-sided interactions imply its role as a retardant (81).

The objectives of this current study were to investigate the stratum corneum modifying effects of some relatively potent percutaneous permeation enhancers/retardants including laurocapram, N-0915, *S*,*S*-dimethyl-*N*-(4-bromobenzoyl) iminosulfurane (DMBIS), *S*,*S*-dimethyl-*N*-(2-methoxycarbonylbenzenesulfonyl) iminosulfurane (DMMCBI) and *tert*-butyl 1 –dodecyl-2-oxoazepan-3-yl-carbamate (TBDOC) that were formulated in simple pharmaceutical vehicles. The structures of the penetration modifiers used are depicted in Figure 4.1. The solvents used for formulating the penetration enhancers/retardants were

water, ethanol, propylene glycol and polyethylene glycol 400 (PEG 400). Diethyl-*m*-toluamide (DEET) a commonly used mosquito repellant was selected as the model permeant for the study. The study also involved evaluation of each penetration modifier as an enhancer or retardant, based on its interaction with ceramide 6 molecules (as suggested by Hadgraft) through modeling. Throughout, we have addressed permeation enhancers and retardants collectively as "penetration modifiers".

4.2. Materials and methods

4.2.1 Materials

4.2.1.1 Chemicals

3-dodecanoyloxazolidin-2-one (N-0915), *tert*-butyl 1 –dodecyl-2-oxoazepan-3-ylcarbamate (TBDOC) were obtained as generous gifts from Dr. James Chapman from the University of South Carolina, Columbia, SC. Laurocapram, *S*,*S*-dimethyl-*N*-(4bromobenzoyl) iminosulfurane (DMBIS), *S*,*S*-dimethyl-*N*-(4methoxycarbonylbenezenesulfonyly) iminosulfurane (DMMCBI) were provided by New Jersey Center for Biomaterials (Piscataway, NJ). Diethyl –*m*- toluamide (DEET), propylene glycol, ethanol were purchased from Sigma Aldrich and polyethylene glycol 400 (PEG 400), phosphate buffer saline tablets were obtained from Fisher Chemicals. All other chemicals used were of analytical grade.

4.2.1.2 Skin membranes

All skin membranes were purchased from Allosource (Cincinnati, Ohio) and were dermatomed to approximately 380-500 μ m and derived from male and female individuals aged between 30-60 years. These skin pieces were stored at -80° C until use, but for no longer than 2 months. Prior to each experiment, the skin samples were thawed and

hydrated for 1 hr in PBS by mounting on jacketed Franz diffusion cells, which were maintained at 37°C.

4.2.2 Methods

4.2.2.1 Preparation of formulations

Penetration modifiers (laurocapram, N-0915, TBDOC, DMBIS, DMMCBI) were weighed and each was added to one of four vehicles (water, propylene glycol, ethanol and PEG 400) to prepare 0.4 M solutions or suspensions. The solution/suspension was then vortexed at room temperature for 48 hours. The solution/suspension obtained was centrifuged at 8000 rpm for 5 minutes and the supernatant was collected, filtered and used as the final formulation for in vitro skin permeation experiments. The solubility of each penetration modifier in each vehicle was determined (methods described below).

4.2.2.2 Determination of solubility and available amounts of penetration modifiers in vehicles

Assays for the five penetration modifiers namely laurocapram, N-0915, DMBIS, DMMCBI and TBDOC were developed using HPLC with ultraviolet detection (HP 1100, Agilent Technologies, Inc.). The content of each of penetration modifier in water, propylene glycol, ethanol and PEG 400 was determined. Briefly, this involved taking 0.4 M of each of the penetration modifiers in 100 µl of each of the four solvents followed by vortexing at 32°C for 48 hours. The saturated solution was then centrifuged and supernatant obtained was filtered by Vivaspin 500[™] ultrafiltration centrifugal device (Sartorius Biotech., USA), diluted by mobile phase and injected into HPLC and analyzed.

4.2.2.3 Modeling and partition coefficient determination of penetration modifiers

The molecular modeling of penetration modifiers and ceramide 6 molecules were performed using MOE 2008.09 (Molecular Operating Environment) software from Chemical Computing Group, Toronto Canada. Both the ceramide 6 molecules and penetration modifiers were built using MOE. Their three dimensional (3D) structures were minimized using molecular mechanics force field, MMFF94 (83). Next, the 3D structures of ceramide 6 were kept rigid and the penetration modifiers were brought in close proximity of the ceramide 6 molecules to mimic possible H-bond formations. The maximum distance between donor-acceptor pairs is less than 3.5 Å to allow the H-bond formation. The pictures shown in Table 4.3 were generated using the same MOE software package.

The partition coefficients (AlogP) of each penetration modifier were calculated using Pipeline Pilot software package (Accelrys Software Inc., San Diego, CA). The ALogP component of Pipline Pilot was used to calculate the Ghose/Crippen group-contribution estimate for LogP (84), where P is the relative solubility of a compound in octanol vs. water.

4.2.2.4 In vitro skin permeation study

Human cadaver skin (Allosource, Cincinnati, OH) prehydrated with phosphate buffered saline, pH 7.4 was mounted on vertical Franz diffusion cells (Permegear, Inc., Bethlehem, PA). The in vitro skin permeation studies were performed using Franz diffusion cells with receptor compartment of 5.1 ml volume and donor compartment of 1 ml capacity. Each skin piece was treated with 50 μ l of selected formulations containing laurocapram or N-0915 prepared in ethanol, water, propylene glycol or PEG 400 1 hour prior to application of the permeant, DEET. This was followed by 100 μ l application of

neat solution of DEET (infinite dose approach) over a surface area of 0.64 cm². DEET was chosen as the model permeant. Similarly, skin pieces were treated with 50 μ l of DMBIS, DMMCBI and TBDOC prepared in ethanol, water, propylene glycol or PEG 400 1 hour prior to application of 100 μ l of neat DEET. The donor compartment was covered with Parafilm[®] to minimize the evaporation of the formulation of penetration modifier or the permeant. The receptor compartment stirred at 600 rpm contained phosphate buffered saline at pH 7.4 and was maintained at 37°C using thermostatic water pump (Haake DC10, Karlsruhe, Germany). 300 μ l aliquots were withdrawn from the receptor at 0, 2, 4, 6, 8, 23, 24, 26, 28 and 30 hours and replaced by an equivalent amount of fresh phosphate buffer. Suitable controls were also run in the study, and greater than three replicates were run for each formulation. These controls included no treatment, treatment with laurocapram alone, water alone, propylene glycol alone, ethanol alone and PEG 400 alone followed by application of DEET on the skin surface.

The experimental data (drug concentration values) were corrected for progressive dilution using the following equation:

 $M_t(n) = V_r \cdot C_n + V_s \cdot \Sigma C_m$

where $M_t(n)$ is the cumulative mass of the drug transported across the skin membrane at time t, C_n represents drug concentration in the receiver compartment and V_r corresponds to volume of the receptor compartment; ΣC_m refers the summed total of the previous measured concentrations [m=1 to (n-1)] and V_s denotes to the volume of the sample removed for analysis (Meiden *et al.*, 2003).

All in vitro skin permeation experiments were performed with human cadaver skin obtained from three different donors. The percutaneous permeation parameters obtained

in case of each formulation from each piece of skin was then examined for reproducibility on repetition of the same experiment using skin piece derived from different donor. This was the method used for ensuring the integrity of the skin during the experiment.

4.2.2.5 Analysis of DEET and penetration modifiers.

The analysis of DEET and penetration modifiers was performed using HPLC (HP 1100, Agilent Technologies, Inc.) equipped with degasser (G1379A), autosampler (G1313A), quaternary pump (G1311A), a UV-visible diode array (G1315A) and an Eclipse XDB-C18 RP column (Agilent Technologies, USA) having a pore size of 5 μ m and dimensions equivalent to 4.6 X 150 mm. The details of HPLC methods of DEET and penetration modifiers are listed in Table 4.1. An external standard technique was employed for all the test compounds.

All methods were validated for linearity, precision and accuracy. The correlation coefficients of 0.999 for linearity of plots were observed in case of DEET and all penetration modifiers used in the study. Intraday variability was less than 0.2 % for all methods, and interday variability was also calculated to be less than 3.0 % for all penetration modifiers.

4.2.3 Data and statistical analysis

The amount of active present in the samples was determined using validated assay methods. In vitro permeation studies, several transdermal parameters such as mean flux, cumulative amount permeated after 30 hours and permeability coefficient were calculated. For determination of the mean flux, cumulative amounts of permeant per area $(\mu g/cm^2)$ was plotted against time (hour) and flux was calculated as the slope of the linear

portion of the plot (between 8 and 30 hours) using linear regression (Microsoft Excel) (Batheja *et al.*, 2009). The permeability coefficient (cm/hour) was calculated from the ratio of mean flux and permeant concentration (ug/ml) in the donor compartment.

The degree of enhancement/retardation was evaluated from the modifier ratio, MR, which was calculated as described below:

 $MR_J = J$ in presence of treatment/J in absence of treatment

 $MR_{Q30}=Q_{30}$ in presence of treatment/ Q_{30} in absence of treatment

The vehicles used in the study themselves act as penetration modifiers. Therefore DEET permeation was determined in presence of the penetration modifier and was compared with DEET permeation in presence of vehicles alone using MR* values:

MR*_J=J in presence of penetration modifier-vehicle formulation/J in presence of vehicle alone

MR*_{Q30}=Q₃₀ in presence of penetration modifier-vehicle formulation/Q₃₀ in presence of vehicle alone

The values above unity represented enhancement and values below 1 represented retardation of the permeant.

All results were statistically analyzed using Minitab software version 15 (State College, PA) with multiple comparison tests done using Tukey HSD method.

4.3. Results and discussion

4.3.1 Determination of solubility and available amounts of penetration modifiers in vehicles

Content of the five penetration modifiers (laurocapram, N-0915, DMBIS, DMMCBI and TBDOC) was determined in water, propylene glycol, ethanol and PEG 400. The

solubility in mg/ml is depicted in Table 4.2. It is a well known fact that laurocapram is insoluble in water, however, a detectable amount of laurocapram was recorded in our laurocapram-water formulation. This is probably due to the fact that our study involved formulation of a non-emulsifier stabilized emulsion of laurocapram and water. Laurocapram content determination in other laurocapram formulations such as laurocapram-PG and laurocapram-PEG 400 showed a similar degree of solubility (p>0.05). However, relatively less laurocapram was observed in the laurocapram-ethanol formulation. N-0915 was undetected in N-0915-water formulation, but some N-0915 was detected in both ethanol and propylene glycol. The highest amount of N-0915 was obtained in PEG 400. On the other hand, DMBIS showed little solubility in water but recordable amounts were observed in propylene glycol, ethanol and PEG 400. In contrast, DMMCBI dissolved in water, propylene glycol and PEG 400, but had better solubility in ethanol (p<0.05). TBDOC showed no solubility in water but dissolved to some extent in propylene glycol, ethanol and PEG 400 (p>0.05).

4.3.2 Modeling and partition coefficient determination of penetration modifiers

Software generated partition coefficients showed relatively high lipophilicity of laurocapram, N-0915 and TBDOC, justifying their low or non detectable solubility in water. The results also provided information concerning the relative hydrophilic nature of DMMCBI and DMBIS that is supported by the higher solubility of DMBIS and DMMCBI in water.

Each penetration modifier selected for this study was subjected to modeling studies to examine its potential as an enhancer or retardant. Hadgraft based the definition of enhancers and retardants on the way the compound interacted with ceramide 6. We used the same criterion in the study (Hadgraft *et al.*, 1996).

The interactions of ceramide 6 with penetration modifiers obtained from modeling studies, as well as partition coefficients, are listed in Table 4.3. Modeling studies revealed that laurocapram and DMBIS are capable of forming one sided H-bonding; a finding which suggests that they are potential enhancers, based on Hadgraft's proposed theory. It has been hypothesized (Hadgraft *et al.*, 1996) that one sided H-bonding of enhancers with skin lipids especially ceramides disturbs the inter H-bonding among the ceramide molecules leading to fluidization of the stratum corneum, causing enhancement in active permeation. Both laurocapram (67) and DMBIS (42) have been reported to enhance the permeation of numerous actives; a finding that agrees with results obtained from modeling studies. However, N-0915, DMMCBI and TBDOC are capable of forming multiple two sided H-bonds with ceramide 6 suggesting retardation behavior. The percutaneous retardation activity of N-0915 and DMMCBI has been confirmed by reports from Hadgraft (81) and Song (25) on permeation of DEET and hydrocortisone respectively. Also reports by Purdon indicate TBDOC to be potential retardant of actives (80).

4.3.3 In vitro skin permeation study results

In order to investigate the effect of the modifier compounds in various vehicles, in vitro percutaneous permeation studies were conducted using DEET as the model compound. The penetration modifiers selected were either nitrogen or sulfur containing compounds and included laurocapram, N-0915, DMBIS, DMMCBI and TBDOC. The study involved penetration modifiers that have been reported to act as enhancers (67), (85)) and also

included some compounds that have shown retardation activity for certain actives (81), (42), (80). Solvents used in the study included water, ethanol, PG and PEG 400 that are not only commonly used vehicles in dermal formulations, but some are also good penetration modifiers (86). DEET was chosen as the permeant since it is a liquid under ambient conditions and in this way we avoided the added complication of other vehicles and solubility issues.

The transdermal permeation parameters such as mean flux between 8 and 30 hours, J $(\mu g/cm^2/hr)$, cumulative amount of DEET permeated after 30 hours, Q₃₀ and permeability coefficient, Kp (cm/hr) were determined. The determination of lag times was not performed because steady state flux of permeant was not achieved in various cases. Therefore mean flux of the permeant was calculated between 8 and 30 hours.

4.3.3.1 Effect of various formulations of laurocapram on permeation of DEET

The permeation parameters for DEET in presence of laurocapram are summarized in Table 4.4. It was observed that laurocapram formulated in water, PG or ethanol, enhanced the permeation of DEET by approximately 5, 6 and 2 fold respectively (as indicated by their MR₃₀, MR_J values in Table 4.4). However, laurocapram formulated in PEG 400, resulted in the retardation of DEET with MR_J, MR_{Q30} value of 0.3. All treatments containing laurocapram were statistically different as compared to no treatment (p<0.05). MR*_J and MR*_{Q30} values were also determined to compare the effect of laurocapram formulations with that of treatment with vehicle alone. The cumulative amount of DEET permeated in presence of various laurocapram formulations is depicted in Figure 4.1. It was observed that in the treatment with laurocapram-water, the major contribution to enhancement was due to water, because there was no statistical difference

between laurocapram-water and water treatments (p>0.05). However, permeability coefficient, flux and Q₃₀ values with laurocapram-water were significantly greater than that after laurocapram treatment (p<0.05). Since our study involved pretreatment of SC with an emulsion (not stabilized with an emulsifier) of laurocapram and water, it seems that laurocapram and water contributed independently towards enhancement of DEET permeation. In laurocapram-PG treatment, no statistical difference in terms of DEET permeability coefficient, flux and Q_{30} values was observed between the laurocapram treatment and PG treatments (p>0.05). However, significant statistical difference was obtained between laurocapram and laurocapram-PG treatments (p<0.05). This suggests that the addition of PG in laurocapram-PG formulation leads to enhancement of DEET permeation. This finding was not surprising because synergism between laurocapram and PG in permeation of actives has been reported previously (22). The permeability coefficient, flux and Q₃₀ values after laurocapram-ethanol treatment were similar to ethanol and laurocapram treatments. This finding suggests enhancement was due to presence of ethanol and laurocapram in the formulation (p>0.05). Similarly permeability coefficient, flux and Q₃₀ values after laurocapram-PEG 400 and PEG 400 were similar (p>0.05) and retardation was probably due to activity of PEG 400. Similar results were Wotton et al. who reported extremely slow in vitro permeation of reported by metronidazole across full thickness human skin after application of formulation containing 18 µmol metronidazole dissolved in vehicles containing 1 % V/V of laurocapram and 18 % W/V PEG 400 (87). The authors attributed this observation to no release of metronidazole from the vehicle containing laurocapram and PEG 400.

4.3.3.2 Effect of N-0915 in selected vehicles on permeation of DEET

The permeation parameters for DEET after application of N-0915 in selected vehicles are listed in Table 4.5. All N-0915 treatments were significantly different from controls (no treatment) in terms of permeability coefficient, flux and Q₃₀ values (p<0.05) except N-0915-PG (p>0.05). When the effect of formulations of N-0915 were compared with controls on permeation of DEET, it was observed that N-0915-water enhanced the permeation of DEET, whereas N-0915-PG, N-0915-ethanol, N-0915-PEG 400 retarded the permeation (Figure 4.2). The enhancement of DEET in presence of N-0915-water was probably caused by the low solubility of N-0915 in water. This is evident by MR^{*}_J and $MR^{\ast}_{\ O30}$ values close to unity for N0915-water and similar flux and Q_{30} values of DEET in presence of N-0915-water and water alone (p>0.05). But it does not imply that enhancement of DEET in presence of N-0915-water formulation can be entirely attributed to the presence of water in the formulation. Even though, N-0915 was undetected by our analytical technique in the N-0915-water formulation, there is a possibility that trace amounts of N-0915 were present. These miniscule amounts of N-0915 could have partitioned into the SC lipids via the lipids present on the surface of SC (owing to high log P of N-0915) and in presence of water in the formulation, causing fluidization/disruption of lipid barrier of the SC leading to enhancement of DEET. The permeability coefficient, flux and Q₃₀ values of DEET in presence of N-0915-PG showed significant decrease in DEET permeation as compared to PG alone treatment (p < 0.05). Since N-0915-PG contains N-0915 (a potent retardant) and PG (an enhancer), the resulting antagonistic action of the penetration modifiers resulted in weakening of the retardation activity of N-0915 that was similar to control (p>0.05). Similarly N-0915ethanol showed a significant decrease in permeability coefficient, flux and Q₃₀ values as

compared to PG alone (p<0.05). Unlike N-0915-PG, N-0915-ethanol was significantly different from no treatment because the rapid evaporation of ethanol after addition on application site led to a high concentration gradient for rapid diffusion of N-0915 across the stratum corneum (88). As there was little ethanol left at the site of application, its enhancement action was reduced, and there was less effect on the action of N-0915. Comparing the permeability coefficient, flux and Q_{30} values obtained after treatment with N-0915-PEG 400 and PEG 400 treatments, no difference was observed (p>0.05) and the retardation was mainly due to PEG 400 in the formulation.

4.3.3.3 Effect of DMBIS in selected vehicles on permeation of DEET

The effects of DMBIS and vehicles on DEET permeation were investigated and data are provided in Table 4.6. DEET permeation in presence of the formulations was compared and it was observed its permeation was significantly enhanced in presence of DMBISwater, DMBIS-PG, DMBIS-ethanol and DMBIS-PEG 400 (as compared to controls (no treatment) (p<0.05)). No retardation was observed in presence of any DMBIS formulation. However, differential enhancement of DEET was observed in DMBIS formulations (Table 6) with MR ratio ranging from 3.4 in DMBIS-PG to 1.5 in case of DMBIS-ethanol. With DMBIS-water treatment, enhancement of DEET permeation was similar to water alone (p>0.05). The enhancement seemed to be mainly due to presence of water in the formulation. Unlike DMBIS-water, in DMBIS-PG treatment, permeability coefficient, flux and Q_{30} values of DEET were significantly enhanced as compared to PG alone. This suggests that DMBIS and PG present in DMBIS-PG act synergistically to enhance the permeation of DEET. This suggestion is further supported by the fact that besides PG, DMBIS itself has been reported to be a good enhancer. The enhancement of DEET in presence of DMBIS-ethanol was probably due to ethanol because the permeability coefficient, flux and Q_{30} values after DMBIS-ethanol and ethanol treatment were similar (p>0.05). The permeation of DEET in presence of DMBIS-PEG was markedly different (p<0.05) from that of PEG 400 suggesting the importance of the enhancer DMBIS in improving the permeation of DEET.

4.3.3.4 Effect of DMMCBI in selected vehicles on permeation of DEET

Table 4.7 provides the permeation parameters for the DMMCBI formulations. Comparing DEET permeation in the absence of any treatment, DMMCBI-water (p<0.05) and DMMCBI-PG (p>0.05) enhanced the permeation of DEET and DMMCBI-ethanol (p>0.05) and DMMCBI-PEG 400 (p>0.05) retarded the permeation of DEET. The enhancement of DEET in DMMCBI-water treatment was due to presence of water in the formulation because no statistical difference in permeability coefficient, flux and Q_{30} values was observed between DMMCBI-water and water alone (p>0.05).

However, in DMMCBI-PG treatment, there was a significant decrease in permeability coefficient, flux and Q_{30} values of DEET as compared to PG alone (p<0.05). The antagonistic nature of the two components in DMMCBI-PG (with DMMCBI being a retardant and PG being an enhancer) led to weakening of the retarding action of DMMCBI. This weakening led to statistically insignificant (p>0.05) enhancement of DEET permeation as compared to control (no treatment). In DMMCBI-ethanol, a significant decrease in DEET permeation was observed compared to ethanol alone (p<0.05). The retardation observed in DMMCBI-ethanol was due to a similar phenomenon as described in N-0915-ethanol. Comparing the permeation of DEET in presence of DMMCBI-PEG 400 and PEG 400 no statistical difference was obtained

(p>0.05). Use of DMMCBI-PEG 400 resulted in a slight retardation of DEET permeation in comparison to control (no treatment). Also, slight enhancement of DEET permeation was observed when DMMCBI-PEG 400 was compared to PEG 400 alone. Statistical comparison of permeability coefficient, flux and Q_{30} values of DEET in presence of DMMCBI-PEG 400, PEG 400 alone and controls showed all three treatments to be similar (p>0.05) suggesting no action (enhancement/retardation) by DMMCBI-PEG 400.

4.3.3.5 Effect of TBDOC in selected vehicles on permeation of DEET

It was observed that formulations TBDOC-water, TBDOC-PG and TBDOC-PEG 400 enhanced the permeation of DEET and TBDOC-ethanol retarded permeation (Table 8). The permeability coefficient, flux and Q_{30} values of DEET in presence of all TBDOC formulations were statistically different from permeability coefficient, flux and Q_{30} values in absence of any treatment (p<0.05). The various transdermal parameters in presence of TBDOC are summarized in Table 4.8.

Comparing the permeability coefficient, flux and Q_{30} values of DEET in presence of TBDOC-water with water alone reveals no statistical difference (p>0.05), suggesting enhancement was due to the presence of water. Moreover, no detectable amounts of TBDOC were observed in the TBDOC-water mixture. In TBDOC-PG treatment, permeability coefficient flux and Q_{30} values of DEET were significantly greater than those in absence of any treatment (p<0.05). This suggests that enhancement of DEET in presence of TBDOC-PG, occurs due to contribution of both components of the formulation. Unlike other TBDOC formulations, retardation of DEET was observed in TBDOC-ethanol treatment. The statistical analysis showed significant decrease in permeability coefficient, flux and Q_{30} values as compared to ethanol alone (p<0.05).

TBDOC has been reported to show significant retardation of several actives such as paraoxon, DEET, 2-hydroxy-4- methoxybenzophenone and others by Purdon (80). The reason for retardation of DEET in presence of TBDOC-ethanol as opposed to enhancement by TBDOC-PG will be investigated using thermal and spectral analytical techniques. TBDOC-PEG 400 treatment showed significant enhancement of DEET compared to PEG 400 alone (p<0.05) suggesting the contribution of both components towards enhancement. The exact mechanisms are currently being explored.

4.4. Conclusion

The results in this study were based on determination of mean flux values, permeability coefficient and cumulative amount of permeant absorbed after 30 hours. In this study, percutaneous permeation parameters determined were solely based on in vitro experimentation as compared to the use of mathematical models. There are reports of models of differential complexity that contain several relationships linking the permeant flux across the human skin to the physico-chemical properties of the compound being evaluated (Potts and Guy, 1992, Potts and Guy, 1995, Abraham et al., 1997, Lien and Gao, 1995). These models have the advantage of conveniently and cost-effectively predicting the extent of percutaneous absorption of the molecule without actually performing the in vitro and in vivo measurements. All the known models relate the permeability coefficient to properties such as octanol-water partition coefficient, melting point, molecular weight or aqueous solubility that can readily be obtained or calculated from group contribution approach or Hansch fragment values for partition coefficient (Leo *et al.*, 1971). The equations derived in the mathematical models were best fit of experimentally determined flux values the above mentioned physico-chemical properties.

In spite of sophistication of these models, the results are mere estimates and they still require verification by in vitro and eventually by in vivo experimentation. Therefore in our study, no mathematical model was utilized for predicting permeability parameters.

The results in this study indicate that the effect of a penetration modifier on the permeation of an active can change from vehicle to vehicle irrespective of its potency as an enhancer/retardant, or its potential to form one-sided hydrogen bonds or two-sided hydrogen bonds with lipids present in the stratum corneum. All these results suggest that formulations of penetration modifiers in various vehicles create a series of unique interactions on the surface of stratum corneum that lead to the enhancement/retardation of the permeant. In order to explain the type of interaction occurring in the stratum corneum after such applications, studies using differential scanning microscopy and infra-red spectroscopy are being performed in our laboratory. Our results also showed that the amount of enhancement/retardation was not solely dependent on the concentration of penetration modifier present in the formulation. This was evident in the laurocapram-PEG 400 formulation, where despite sufficient solubility of laurocapram in the vehicle, no enhancement was observed.

Therefore, our results suggest that there is a need for modification/extension for the preexisting theory of H-bond formation with ceramide molecules of the stratum corneum to explain the phenomenon of enhancement/retardation, because our study indicates that enhancers can act as retardants or vice versa with change of the formulation vehicle. Moreover, enhancer/retardants should be collectively termed as penetration modifiers since their activity changes with the formulation.

Chapter 5: Percutaneous penetration modifiers and formulation effects: thermal and spectral analyses

5.1 Introduction

Stratum corneum (SC) is a complex tissue that provides a barrier to most permeants due to its unique composition and organization of lipids and proteins present within it (82). In order to modify the barrier properties of the SC, enhancers and retardants have been developed where enhancers act by disrupting the lipid bilayer within the SC and retardants act by strengthening the lipid organization of the SC (57). Up to now, known enhancer molecules were always thought to cause enhancement of the permeant, and retardants were expected to decrease the permeation of the active irrespective of the formulation components.

However, we recently discovered that an enhancer can become a retardant, or vice versa, for a specific permeant (DEET) depending upon the vehicle in which it is applied to the skin (89). In a previous study, we investigated the effect of five percutaneous penetration modifiers (laurocapram, 3-dodecanoyloxazolidin-2-one (N-0915), *S*,*S*-dimethyl-*N*-(4-bromobenzoyl) iminosulfurane (DMBIS), *S*,*S*-dimethyl-*N*-(2-methoxycarbonylbenzenesulfonyl) iminosulfurane (DMMCBI) and *tert*-butyl 1 –dodecyl-2-oxoazepan-3-yl-carbamate (TBDOC)) on permeation of model permeant diethyl-*m*-toluamide (DEET). The formulations of the above penetration modifiers were formulated in either water, propylene glycol (PG), ethanol or polyethylene glycol 400 (PEG 400). The penetration modifiers selected were either nitrogen-containing (laurocapram, N-0915, TBDOC) or sulphur-containing (DMBIS, DMMCBI) compounds. Another criterion for selection of penetration modifiers was that some of the selected penetration

modifiers such as laurocapram, DMBIS have been known to act as enhancers (67), (85) while rest of the modifiers in the study namely N-0915, DMMCBI and TBDOC are known to have retardant activity (81), (42), (80). Solvents used in the formulations were water, ethanol, PG and PEG 400 that are not only commonly used vehicles in dermal formulations, but some are also good penetration modifiers (86). DEET was chosen as the permeant in the previous study since it is a liquid under ambient conditions and in this way we avoided the added complication of other vehicles and solubility issues. Moreover, DEET has been used as a model permeant in evaluation of numerous skin permeation/retardation studies (90, 91). Results from permeation studies indicated that laurocapram enhanced DEET permeation in PG, but retarded in PEG 400. Similarly, N-0915 was a retardant of DEET with PG, ethanol and PEG 400, but not with water. DMBIS decreased the permeation with ethanol as compared to permeation with water, PEG 400 or PG. Likewise, DMMCBI showed retardation of DEET with ethanol and PEG 400, but not with water or PG. TBDOC formulations showed retardation with ethanol, but behaved as enhancer with water, PG and PEG 400. The mean flux values have been summarized in Table 5.1 (89). Suitable controls were performed to determine whether the enhancement/retardation effect was due to permeant-vehicle effect or permeantpenetration modifier effect (89).

In order to understand the mechanism by which the properties of enhancers/retardants change in a given vehicle, molecular investigation of stratum corneum (SC) was performed using differential scanning calorimetry (DSC) and attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR). Therefore the objective of this paper was to investigate molecular changes in stratum corneum after treatment with

different formulations of laurocapram, N-0915, DMBIS, DMMCBI and TBDOC using the two analytical methods of DSC and ATR-FTIR that provide independent yet complementary information regarding the interaction of enhancer/retardants within the SC (92, 93).

5.2 Materials and methods

5.2.1 Materials

5.2.1.1 Chemicals

3-Dodecanoyloxazolidin-2-one (N-0915), TBDOC were obtained as generous gifts from Dr. James Chapman at the University of South Carolina, Columbia, SC. Laurocapram, DMBIS, DMMCBI were provided by New Jersey Center for Biomaterials (Piscataway, NJ). Propylene glycol, ethanol, trypsin (Type 1) were purchased from Sigma Aldrich and polyethylene glycol 400 (PEG 400), phosphate buffer saline tablets were obtained from Fisher Chemicals. All other chemicals used were of analytical grade.

5.2.1.2 Skin membranes

All human skin samples were purchased from New York Fire Fighters Skin Bank (New York) that were surgically removed within 24 hours of the time of death of individuals and dermatomed to approximately 250-300 μ m. All skin samples obtained were derived from left posterior leg of male and female individuals aged between 30-60 years. These skin pieces were stored at -80° C until use, but for no longer than 2 months.

5.2.2 Methods

5.2.2.1 Preparation of formulations

The preparation of the penetration modifier formulation was as reported by Kaushik *et al.* (89). Briefly, weighed amount of penetration modifiers (laurocapram, N-0915, TBDOC, DMBIS, DMMCBI) were added to one of four vehicles (water, propylene glycol, ethanol and PEG 400) to prepare 0.4 M solutions or suspensions. The solution/suspension was then vortexed at room temperature for 48 hours. In case where suspension was obtained, centrifugation was performed at 8000 rpm for 5 minutes to collect the supernatant and the supernatant was used as the final formulation for in vitro skin treatment. All formulations used in the study had 0.4 M concentrations of penetration modifiers or were saturated solution of penetration modifiers (with comparable thermodynamic activity). The available amounts of penetration modifiers in each vehicle were as reported by Kaushik *et al.*(89).

5.2.2.2 Human stratum corneum isolation and sample preparation

The stratum corneum (SC) was separated from the dermis by placing the human cadaver skin sample over a filter paper soaked in 0.1% trypsin (type I, Sigma Aldrich) in phosphate buffered saline pH 7.4 for 4 hours. Epidermal cells were removed from the overlying SC by digestion with trypsin for additional 1 hour. The resulting SC sheets were rinsed with deionised water, dried at room temperature and stored in desiccator overnight. Next day, the SC sheets were mounted on Franz diffusion cell (Permegear, Inc., Bethlehem, PA) set up with their epidermal side up and covered completely with 50 µl of formulations of penetration modifiers prepared in one of the four vehicles for 30 hours at 37°C. The penetration modifiers used in the study included laurocapram (1-dodecylazepan-2-one), DMBIS, N-0915, TBDOC and DMMCBI. The vehicles used in the study included water, propylene glycol, PEG 400 and ethanol. The receptor

compartment stirred at 600 rpm contained phosphate buffered saline at pH 7.4 and was maintained at 37°C using thermostatic water pump (Haake DC10, Karlsruhe, Germany). After 30 hours, the SC sheets were removed and excess enhancer/retardant solution present on SC sheets was removed using KimWipesTM (Fisher Scientific, PA). All experiments were performed in triplicate at 37 ± 2 °C. SC sheets were then subjected to DSC or ATR-FTIR analysis. Suitable controls experiments were also performed that included untreated SC, treatment with laurocapram alone, water alone, propylene glycol alone, ethanol alone and PEG 400 alone. Among all the penetration modifiers tested, laurocapram was the only one that existed in liquid state; therefore it was used as one of the controls while comparing laurocapram containing formulations.

5.2.2.3 Differential scanning calorimetry (DSC)

For DSC, approximately 10–20 mg of stratum corneum sheets (treated or untreated) were cut in form of discs and placed in 40 µl aluminum standard pans. Thermal analysis was performed using a STARe/Thermal-Analysis-System (Mettler-Toledo, Gießen, Germany, DSC 821/822, Intracooler, Gas-Switch, 200W, FRS-5 sensor). All samples were analyzed between 5 to 160°C at 10°C/ min heating rate under nitrogen flow.

The mean transition temperatures (T_m) and their corresponding enthalpies were noted and results were evaluated statistically using one-way ANOVA through Minitab software (State College, PA). Tukey HSD test at 95 percent confidence interval was used to compare the results.

5.2.2.4 Attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR)

FTIR spectra were recorded with a Bruker Equinox 55 spectrometer (100 scans; 4 cm⁻¹ resolution), equipped with an attenuated total reflection diamond crystal accessory (Pike Technologies, Madison, WI). Spectra were acquired at a resolution of 4 cm⁻¹ and the measurement range was 4000–650 cm⁻¹. All spectra (100 scans) were collected after baseline correction. The spectrometer was linked to a PC equipped with Bruker OPUS software to allow the automated collection of IR spectra. The IR spectra were imported to KnowItAll[®] informatics system (Bio-Rad Lab., USA) for peak area integration. All measurements were performed at ambient temperature, 25 ± 2 °C.

5.3 Results and discussion

DSC analysis: Three endothermic transition peaks at temperatures around 59-63°C (T_{m1}), 75-85° (T_{m2}) and 110-120°C (T_{m3}) were obtained on DSC analysis of untreated skin. The temperature and enthalpy changes in DSC of human SC after different formulation treatments are listed in Table 5.2 and endotherms of human SC from one of the replicates are depicted in Figure 5.1, 5.2, 5.3, 5.4, 5.5 and 5.6.

FTIR analysis: The different spectra depicting band positions and peak intensities after FTIR analysis of untreated human stratum corneum and treated (after application of various penetration modifier-vehicle formulations) stratum corneum are depicted in Figures 5.7, 5.8, 5.9, 5.10, 5.11 and 5.12.

DSC: Since DSC technique is widely used to investigate the effect of percutaneous penetration enhancers and retardants on the thermotropic behavior of SC (by comparing the endotherms and exotherms for mean transition temperature (T_m) and their enthalpies (H)), it was used to understand the molecular mechanism of each formulation on SC. DSC thermograms of SC treated with various formulations were evaluated by comparing
endotherms for ΔT_m and their ΔH . The shift in T_m of both T_{m1} and T_{m2} to lower temperature is interpreted as disruption of the lipid bilayer while reduction in ΔH is ascribed to fluidization of lipid bilayers (93-95). DSC of the stratum corneum membrane sheet produced three endothermic transition peaks at temperatures around 59-63°C (T_{m1}), 75-82° (T_{m2}) and 99.5-120°C (T_{m3}). Three replicates were performed with human cadaver skin obtained from three different individuals; therefore some variability was obtained in the position of three transitions. The temperature transitions and enthalpy changes after several treatments are provided in Table 5.2. We did not observe a commonly reported low transition temperature peak (at 35-40°C) (96) because our SC samples were desiccated before DSC analysis and 15% moisture content is required for observation of low temperature transition (97). Moreover, Golden et al. (95) attributed the variable nature of the endothermic transition at 35-40°C to sebaceous lipids that are not tightly bound with the SC surface and have little influence on the ordering of the SC. T_{m1} corresponds to the lipid transformation from a lamellar to disordered state. Similarly T_{m2} is thought to be due to the melting of lipid-protein (keratin) complex that transition from a gel to a liquid form during DSC analysis (98). T_{m3} is known to occur due to the irreversible denaturation of proteins in the SC (99). DSC was also performed on suitable controls that included SC treated with the following different treatments: water, PG, ethanol, PEG 400 and laurocapram. The DSC data of stratum corneum treated with water showed lowering of mean transition temperature transitions and lowering of heat of enthalpy suggesting enhancement of the active by water, by SC lipid disruption, and by extraction. This observation also implies that water causes transdermal enhancement either by expanding the polar head groups of SC lipid bilayer or by squeezing and

distorting the lipid bilayer by swelling/engorgement of the corneocytes (86). However, these explanations of water enhancement are contradicted by studies performed on hydrated SC using X-ray diffractometry (100) and freeze fracture electron microscopy (101) that reported neither expansion of lipid bilayer, nor distortion of lipid bilayer in hydrated skin. At the same time, Menon *et al.* (102) proposed an aqueous pore pathway for the diffusion of actives across the skin under high stress conditions such as excessive hydration. According to this theory, under conditions such as excessive hydration, preexisting scattered lacunae embedded in the lipid bilayer expand and form continuous water channels that facilitate diffusion of both hydrophilic and lipophilic permeants. This may also suggest that formation of these aqueous pathways disrupt the surrounding lipid bilayer as shown by change in transition temperatures and enthalpy seen in our DSC results. PG is thought to cause enhancement predominantly by disturbance of lipid protein complex and the interaction with lipids shown by lowering of T_{m1} (p<0.05) and T_{m2} (p<0.05). Like PG, the thermal analysis of ethanol treated SC suggested enhancement is mainly due to disturbance of SC lipid-protein complex and SC lipid interactions. In case of PEG 400, thermal analysis showed increase in first and second transition temperatures and increase of enthalpy indicating the strengthening of SC lipids organization and SC lipid protein complex after treatment with PEG 400. This observation explains the retardation of DEET permeation after PEG 400 treatment. The treatment of SC with laurocapram alone led to complete extraction of lipid at T_{m1} and T_{m3} with significant lowering of endothermic transition at T_{m2} . All these observations suggest enhancement by laurocapram via both lipid disruption in addition to protein and lipid extraction. This finding is corroborated by results reported by Ogiso *et al.* (103).

Laurocapram formulations: DSC analysis of SC treated with laurocapram-water showed disappearance of T_{m1} and T_{m2} transitions. At T_{m3} two peaks were observed at positions 90° and 98°C. These results imply that laurocapram and water in the formulation contribute to enhancement by lipid disruption, lipid extraction and protein denaturation. This also explains the increased flux of DEET after treatment with laurocapram-water formulations as compared to controls (3).

In the laurocapram-PG formulation, there was a lowering of T_{m1} (p<0.05) and T_{m3} (p<0.05) peaks and disappearance of T_{m2} indicating that laurocapram-PG causes lipid disruption, lipid and protein extraction. The laurocapram and PG present in laurocapram-PG formulation contributes to lipid and protein disruption in the SC and also lead to complete extraction of lipid-protein complex. Both laurocapram and PG act in synergy to increase the permeability of the SC as shown by increased flux of DEET in presence of laurocapram-PG (22).

As with PG, the DSC results suggested enhancement of active after treatment with laurocapram-ethanol formulation was due to lipid extraction, lipid and protein extraction shown by significant (p<0.05) lowering of temperature at T_{m1} transition and ΔH . However, at T_{m2} elevation in transition temperature was observed suggesting organization of lipid protein complex. This also explains less degree of enhancement of DEET in presence of laurocapram-ethanol (2-fold) formulation as compared to treatment after laurocapram-water (4-fold) and laurocapram-PG (5-fold) formulations (89).

In laurocapram-PEG 400 formulation, DSC data showed decrease in T_{m1} transition temperature, no change in T_{m2} and disappearance of T_{m3} . It seems that laurocapram and PEG 400 in laurocapram-PEG 400 formulation tend to cause opposite effect on SC with

laurocapram causing disruption of lipid and proteins in the SC (T_{m1} and T_{m3}) and PEG 400 causing strengthening of lipid-protein complex evident by no change in T_{m2} . This observation supports the results observed in the in vitro permeation experiment where the laurocapram-PEG 400 formulation retarded the permeation of DEET (89). The in vitro permeation studies comparing flux of DEET in presence of laurocapram-PEG 400 and PEG 400 alone have indicated that incorporation of laurocapram in the formulation increased the flux of DEET as compared to treatment with PEG 400 alone (89). Similarly, on comparison of DEET permeation in presence of laurocapram-PEG 400 and laurocapram alone showed that DEET permeation in presence of laurocapram was higher than in presence of laurocapram-PEG 400 (89). Nevertheless, other analytical techniques such as confocal Raman spectroscopy (104, 105), X-ray diffractometry (106), etc. should be investigated in addition to DSC, that would not only measure the extent of permeation of the actives/penetration modifiers across the SC, but also determine the corresponding effect of the formulations (under study) on SC lipids and proteins. This would also help us to establish whether PEG 400 retards the activity of the permeant physically (by forming a barrier layer) or by its interaction with SC lipids and proteins or both. However, in our study, beside DSC, FTIR was also used to understand the mechanism of action of each formulation.

N-0915 formulations: DSC analysis of N-0915-water showed merger of T_{m1} and T_{m2} at 70°C and multiple peaks at 92, 94 and 100°C suggesting of lipid fluidization of SC lipids. Mean flux determination of DEET after N-0195-water produced enhancement of DEET as compared to control(89), a finding that agrees with conclusions from the DSC results. DSC results in N-0915-PG treatment showed lowering of T_{m1} , T_{m2} but elevation in T_{m3} transition. It seems that retardation of DEET permeation after N-0915-PG application was through structural changes in SC protein (89).

In N-0915-ethanol treatment, flux determination of DEET indicated retardation of DEET as compared to control (89), an observation that agrees with our DSC results. N-0915ethanol formulation seems to cause retardation of active by organization of lipid structure evident by increase in ΔT_{m1} (p<0.05), strengthening the lipid protein complex shown by higher shift of T_{m2} (p<0.05) and organization of proteins to a higher temperature (evident by higher ΔT_{m3}). However, lowering of ΔH was seen at most transition temperatures.

Likewise, N-0915-PEG 400 showed retardation of DEET (89) by organization of lipids (evident by higher shift in T_{m1}) and strengthening of the lipid protein envelope (suggested by increase in T_{m2}) of SC. There was no enthalpy change at all the corresponding transition temperatures.

DMBIS formulations: DMBIS-water treated SC showed lowering of shifts at T_{m1} (p<0.05), T_{m2} (p<0.05), T_{m3} (p<0.05) indicating that enhancement is probably caused by lipid disruption and protein interactions. Slight effects of DMBIS-water were seen on lipid extraction as evidenced by insignificant changes in enthalpy of lipids at each transition. DSC results support the enhancement in permeation of DEET in the presence of DMBIS-water (89).

DMBIS-PG formulation seemed to cause significant lowering of T_m transitions (p<0.05) that suggests enhancement caused by lipid and protein disruption. These results may explain the approximate 3 fold enhancement of DEET flux as compared to controls (89).

DSC analysis of DMBIS ethanol treated SC depicted no change in T_{m1} and T_{m2} transition but disappearance of T_{m3} endotherm explaining slight enhancement in DEET permeation after treatment with DMBIS-ethanol in in vitro experiments (89). The enhancement activity by DMBIS ethanol treatment seems to be due to extraction of proteins.

Like other DMBIS formulations, DMBIS-PEG 400 showed enhancement of DEET permeation (89). However, DSC analysis of DMBIS-PEG 400 treated SC showed higher ΔT at all three temperature transitions. Owing to the inconsistent results from in vitro permeation study and DSC, FTIR analysis was performed to understand the mechanism of action of DMBIS-PEG 400.

DMMCBI formulations: DMMCBI-water formulation showed lowering of mean transition temperature at T_{m1} , T_{m2} and T_{m3} with some lowering of enthalpy at T_{m3} . These results imply enhancement in presence of DMMCBI-water formulation which explains the enhancement of DEET in presence of DMMCBI-water formulation. DMMCBI-water formulation seems to cause enhancement by lipid disruption and protein fluidization.

In DMMCBI-PG, we observed significant lowering of mean transition temperature (p<0.05) at all transitions with lowering of enthalpy as compared to untreated SC at T_{m2} . These results suggest enhancement by DMMCBI-PG formulation mainly by lipid disruption and partially by lipid and protein fluidization.

Thermal analysis of DMMCBI-ethanol treated SC showed lowering of transition temperature at T_{m1} (p>0.05), two peaks at 84°C and 88°C at T_{m2} with disappearance of T_{m3} transition. In vitro permeation studies showed significant retardation in presence of DMMCBI-ethanol (p<0.05) (3) suggesting retardation action of DMMCBI-ethanol formulation by interaction at lipid protein complex.

In vitro permeation studies of DEET in presence of DMMCBI-PEG 400 indicated slight retardation of the permeant (89). However, DSC analysis of SC treated with DMMCBI-PEG 400 showed significant increase in temperature shifts at T_{m1} , T_{m2} and disappearance of peak at T_{m3} . This suggests organization of lipid structure as well as strengthening of lipid-protein complex in presence of DMMCBI-PEG 400.

TBDOC formulations

In TBDOC-water treated SC, DSC showed lowering of T_{m1} and T_{m2} (p<0.05) with no change in T_{m3} . At the same time, in vitro flux determination of DEET in presence of TBDOC-water formulation showed a 2 fold increase in flux as compared to no treatment (89). The enhancement of DEET in presence of TBDOC-water seems to be due to disruption of the lipid with little effect on lipid fluidization.

TBDOC-PG application on human cadaver skin led to 5 fold increase in DEET flux (89). DSC analysis of SC treated with TBDOC-PG showed merger of T_{m1} and T_{m2} transition at 70°C and disappearance of peak at T_{m3} . These results suggest enhancement in presence of TBDOC-PG was caused by disruption of the lipid protein complex with fluidization and extraction of certain SC lipids and proteins.

Analysis of TBDOC-ethanol formulation showed significant increase (p<0.05) in shifts at T_{m1} , T_{m2} and T_{m3} indicating strengthening of SC lipid bilayer suggesting the role of TBDOC in the ethanol formulation as a retardant. This finding is corroborated by in vitro permeation study where significant retardation of DEET was observed in the TBDOC-ethanol formulation.

In TBDOC-PEG 400 treated SC, no change was obtained in temperature transitions at T_{m1} and T_{m2} . However, decrease in ΔH at T_{m2} and disappearance of endotherm at T_{m3}

suggested enhancement by extraction of SC lipid protein complex and SC protein extraction suggesting The in vitro permeation study indicated 2 fold enhancement in flux of DEET after TBDOC-PEG 400 formulation (89).

It appeared that enhancement/retardation of formulations under investigation could not be explained by DSC alone in certain cases, therefore SC treated with all formulations were also assessed by FTIR analysis.

FTIR: The FTIR analysis of SC provides bands at different wavenumbers, which are attributed to lipid and protein molecular vibrations in the SC (107), (108). In our study, untreated human SC showed bands at 3270.7, 2917.8, 2850.3, 1735.6, 1637, 1538.9 and 1455.9 cm⁻¹. Among these bands, the signal around 3270 cm⁻¹ represents symmetric H-O-H stretching and overlaps with an amide A band located at 3300 cm⁻¹ position. Generally, the bands observed in range of 3000-3600 cm⁻¹ represent the N-H and O-H stretching from lipid, protein and water. The prominent peaks obtained near 2920 and 2850 cm⁻¹ represented respectively asymmetric and symmetric stretching modes of the terminal methylene groups of the lipids (ceramides, phospholipids, etc.) that provided specific information about the interior composition of the lipid bilayer. The ceramides along with cholesterol, fatty acids, cholesterol esters and cholesterol sulfate constitute the multiple lipid lamellae that impart barrier properties to SC. The SC also contains corneocytes that contain keratin. The band positions for symmetric and asymmetric peak stretching represents trans form (stable form); change in the band position from trans to gauche conformation indicates the fluidization of the lipid bilayer (109). A small band obtained at 1738 cm⁻¹ position represented lipid ester carbonyl stretching in the SC. Spectral deconvolution techniques have shown that the band shape at 1738 cm⁻¹ can be simulated with two band components at 1740 and 1710 cm⁻¹ with the former, indicating the presence of carbonyl moiety as in triglycerides /phospholipids and the latter suggesting the presence of carbonyl group of fatty acids (110, 111). The bands observed near 1637 and 1550 cm⁻¹ represented amide 1 (C=O stretching) and amide 2 (C-N stretching) linkages of the helical secondary structure found in epidermal keratin (112). Besides absorbance due to protein structure, amide linkages in ceramides and other related components in the SC also contribute to amide 1 band (113, 114). However, the presence of protein contributes more to the appearance of signal at this band position. Along with amide 1 band, another peak is reported around 1645 cm⁻¹ that corresponds to H-O-H bending. The amide bands along with 1645 cm⁻¹ band are known to be sensitive to Hbond change in the SC. In our experiment, there was overlapping of the 1645 cm⁻¹ and 1655 cm⁻¹ peaks in untreated human SC sample that led to appearance of a single intense peak at 1637 cm⁻¹ that was regarded as amide 1 linkage of the proteins and lipids. The SC lipids can be examined by the investigation of the band representing lipid alkyl backbone through the CH₂ scissoring (1462-1474 cm⁻¹) that are indicators of lateral packing within the lamellae. A single methylene band at ~ 1468 cm^{-1} represents hexagonal acyl chain packing of the lipids whereas orthorhombic chain packing is indicated by two components at 1472 cm⁻¹ and 1464 cm⁻¹. Triclinic chain packing is known to give a single band $\sim 1473 \text{ cm}^{-1}$ (115).

In our study, SC sheets were treated with various formulation of penetration modifiers for a period of 30 hours at 37 °C followed by spectral analysis by ATR-FTIR. Spectral analysis involved examination of change in peak positions and their intensities at around 3270.7, 2917.8, 2850.3, 1735.6, 1637, 1538.9 and 1455.9 cm⁻¹ with respect to the control (untreated) SC (Figure 5.7). The spectral data were used to understand the enhancement/retardation mechanism of the penetration modifiers (laurocapram, N-0915, DMBIS, DMMCBI and TBDOC) formulated in commonly used vehicles (water, PG, ethanol and PEG 400). The rationale for use of FTIR technique to understand mechanism of penetration modifier was that the treatment with enhancers/retardants, sometimes yield a shift in specific band position to higher or lower wavenumber or lead to change in the intensity of the signal observed at that band position. If the shift is to higher wavenumber (blue shift) it signifies SC membrane (lipid bilayer) fluidization that in turn leads to disruption of the barrier properties that thereby causes enhancement of the permeation of the active across the SC (116, 117). On the other hand, a shift to lower wavenumber (red shift) indicates reorientation of lipid groups leading to strengthening of the SC barrier properties and therefore leads to retardation of the entry of permeant across the skin (118, 119). If the penetration modifier acts by affecting lipoidal pathway, the phase transition of the lipids is represented by increase/decrease in the band position (wavenumber) of the signals at 2920, 2850 and 1738 cm⁻¹. Similarly, when penetration modifiers affect the ceramides of the SC at the amide 1 signal, a decrease in wavenumber (red shift) is indicative of strong H-bonding with the ceramide 2 molecules (120, 121). On the other hand, an increase in the wavenumber at 1537 cm⁻¹ position (blue shift) is indicative of strong hydrogen bonding interactions within the stratum corneum ceramides (121). The change in protein conformation is also reflected by splitting of amide 1 linkage where there is change from alpha helix secondary structure to beta helix structure of keratin and vice versa (113).

Since the height or area of the bands represents the amount of lipids/proteins in the SC, any change in the peak intensity suggests the extraction or strengthening of the lipid present in the SC. The treatment with penetration modifiers may lead to extraction of the membrane lipids from the SC as in the case of enhancers, or might increase the intensity at particular band representing retardation in the case of retardants.

Laurocapram formulation effects: The spectral analysis of laurocapram-water treated SC suggests that water component of the formulation caused enhancement by increased water partitioning into the hydrated keratin fibrils of SC (evident by 3270 and 1637 cm⁻¹ blue shifts) and laurocapram contributed to the phase transition of the ceramides of the SC (blue shift at 1637 cm⁻¹) (121). We observed that laurocapram alone actually caused shift in the wavenumber of the symmetric (2917.8 cm⁻¹) and the asymmetric (2850 cm⁻¹) bands (109), but there was no effect of on these bands in the laurocapram-water formulation. The results suggest that laurocapram alone caused enhancement via lipid fluidization and lipid extraction indicated by a marked decrease in lipid content in SC at all bands shown by blue shifts observed at certain frequencies. These spectral results explain the 4 fold flux enhancement of DEET in presence of laurocapram-water formulation (89).

The spectra of laurocapram-PG treated SC suggests that PG present in laurocapram-PG formulation contributes to enhancement by changing the conformation of proteins (formation of distorted beta structures) or lipids (breaking of H-bonds of the ceramides) of the SC (121). Moreover, laurocapram causes phase transition of the lipids (122). There was significant decrease in peak height at 3270, 2917, 2850, 1538 cm⁻¹ in the laurocapram-PG formulation, suggesting lipid extraction by the laurocapram-PG. These

observations also support synergistic effects of PG and laurocapram combinations in disturbing the SC barrier properties and thereby causing enhancement of the active. This phenomenon has been reported by other researchers (22).

The ethanol in the laurocapram-ethanol formulation contributed to enhancement mainly by SC lipid extraction, whereas laurocapram present in the formulation caused enhancement by SC lipid extraction, lipid phase transition and fluidization. Ethanol alone treatment showed red shift at 2850 cm⁻¹ position suggesting lipid ordering effect of the solvent that is consistent with observations by Bommannan *et al.* (96). However, in presence of laurocapram-ethanol, we observed no red shift indicating role of laurocapram in preventing the lipid ordering effect of ethanol. The spectral results support the enhancement of DEET by laurocapram-ethanol observed in vitro permeation experiments.

Laurocapram-PEG 400 formulation showed blue shift at 2917 cm⁻¹, 2850 cm⁻¹ and 1735.6 cm⁻¹ band positions on spectral analysis. These band shifts suggest enhancement of permeant as opposed to the retardation of DEET observed in our in vitro permeation studies. No change in band intensity or band position was observed at any other band position. This observation is not consistent with our DSC data that suggested retardation of the permeant after treatment with laurocapram-PEG 400 formulation. This emphasizes the need for use of additional analytical techniques that will not only determine penetration modifier uptake, but also the interactions with SC lipids and proteins. The retardation of DEET in presence of laurocapram-PEG 400 was presumably due to the fact that laurocapram-PEG 400 prevented the partitioning of DEET into the skin resulting in reduced permeation. Wotton *et al.* made a similar observation, a test solution of 1 %

laurocapram and 18 % PEG 400 decreased the permeation of metronidazole across the SC (87).

N-0915 formulations: The spectral analysis of N-0915-water treated SC (Figure 5.9), suggest enhancement may be due to increased partitioning of water from the N-0915 formulation into the SC with formation of aqueous pores (102) in the SC affecting the alpha helical keratin structure along with lipid interactions (ceramides/triglycerides) shown by a band shift at 1641 cm⁻¹ (121). This is suggestive of increased enhancement of DEET in vitro permeation in presence of N-0915-water formulation (3).

In N-0915-PG treated SC, a blue shift was observed at 1735 cm⁻¹ band position with marked increment in the peak intensity representing the increase organization of triglycerides molecules or other lipid components (112) (121) present in the SC. The increase in peak intensity was due to the presence of N-0195 in the formulation since the increase was absent in the spectrum obtained from PG alone treated SC. The blue shift in the amide 2 band suggests the formation of a strong H-bond within the ceramide molecules (121), and hence strengthening of the barrier function of SC. The spectral changes observed are in agreement with retardation of DEET observed in presence of N-0915-PG in the in vitro permeation experiment (89). Moreover, ATR-FTIR technique proves to be a suitable alternative as compared to DSC to explain the mechanism of surface modification of SC by enhancers/retardants.

In N-0915-ethanol, SC spectral analysis showed a red shift at 3270 cm⁻¹ position with no change in peak intensity at this position. The red shift indicates possible dehydration of SC. Also N-0915-ethanol treated SC showed two intense bands at 1724 and 1764 cm⁻¹ suggesting reorganization of the head groups of triglycerides or ceramides in the SC to

form a tighter structure (123). Furthermore in comparison to 1736 cm⁻¹ band, a relative red shift was observed at 1724 cm⁻¹ indicating formation of strong hydrogen bonds within the lipid molecules. Band splitting at 1637 and 1538 cm⁻¹ was also observed that may be due to interlamellar vibrational coupling between amide groups of ceramide 2 molecules of the SC (120). Moreover, reduced peak intensity at 2850 cm⁻¹ was observed that may be related to extraction of lipid due to presence of ethanol in the formulation. It appears that the dehydration effect (124), along with the organization of SC lipids following application of N-0915-ethanol caused more impact than lipid extraction, an effect that ultimately led to the retardation action of the formulation. Levang reported that dehydration causes keratins of the SC to shrink, which ultimately decreases skin permeability (125). Thus, the above spectral changes in the SC support the retardation action observed in our study of N-0915-ethanol.

Similar to the N-0915-ethanol and N-0915-PG formulations, spectral analysis of SC in presence of N-0915-PEG 400 showed an intense peak at 1760 and 1787 cm⁻¹ representing a possible increase in lipid content (112). On the other hand, a blue shift was observed at 1637 cm⁻¹ after N-0915-PEG 400 suggesting the weakening of the hydrogen bonds between the amide linkages of the ceramides. No other change in peak intensity or band shift was observed. Despite of the weakening of H-bonds at amide 1 linkage, it seems the appearance of two peaks near the 1735 cm⁻¹ band (123) after N-0915-PEG 400 treatment were due to the retardation effect on SC.

DMBIS formulations: The spectral analysis of DMBIS-water formulation (Figure 10) showed blue shifts at 3270 and 2917 cm⁻¹ indicating lipid bilayer fluidization, increased SC-hydration and lipid extraction by DMBIS treatment. Splitting of peaks around 1637

cm⁻¹ was observed indicating change in protein conformation (formation of beta pleated sheets) that suggested permeation enhancement. At the same time, the blue shift at 1538 cm⁻¹ suggested formation of strong H-bonds at the amide 2 band. The mechanism of action of DMBIS is similar to that of dimethyl sulfoxide (DMSO) (25). DMBIS initially binds to the free water from the formulation and if there is not sufficient free water present in the surroundings, it is thought to remove the protein-bound water molecules. As a result, structural changes occur in the SC-proteins (evident by band splitting at amide 1 linkage and blue shift at amide 2 band) in order to improve intermolecular hydrogen bonding to compensate for the water loss. Like DMSO, the removal of protein bound water is the reason for enhancement of the permeant in presence of DMBIS-water formulation (126).

The infrared spectrum of DMBIS-PG treated SC showed a red shift around 3270 and 1538 cm⁻¹. The red shift at 3270 cm⁻¹ is because of removal of water by DMBIS (as described earlier) and shift at 1538 cm⁻¹ is indicative of weakening of hydrogen bonds at the amide linkage. Also, the red shifts at the above band positions were accompanied by decrease in peak height, suggesting protein and lipid extraction. On the other hand, a significant blue shift occurred at 1637 cm⁻¹ that indicated the enhancement action via weakening of H-bonds among the protein/ceramide molecules. The shifts at 1538 and 1637 cm⁻¹ may also be due to the interaction of DMBIS-PG with skin proteins (25, 113). Since the DMBIS-PG formulation contained no water, DMBIS removed SC-protein bound water leading to significant structural changes in the protein structure and therefore led to enhancement of the permeant. No other change in peak shifts or intensity was observed. All the above data suggest enhancement in presence of DMBIS-PG. Our

results corroborate results of mechanistic testing performed by Song using confocal Raman microscopy technique, where significant interactions of DMBIS-PG formulation were observed at amide 1 linkage (25).

The FTIR analysis of SC after DMBIS-ethanol formulation application showed a red band shift at 3270 cm⁻¹. At the same band positions, a decrease in intensities of the signals was observed suggesting lipid extraction. At the same time, significant band splitting was observed at positions 1637 and 1538 cm⁻¹ with significant decrease in corresponding signal intensities that suggests enhancement via protein or lipid interactions, or both. The mechanism of enhancement of permeant followed by application of DMBIS-ethanol formulation (like other DMBIS containing formulations) was due to removal of SC protein bound water (126), which increased in the presence of ethanol in the formulation (evident by significant band splitting at amide 1 and amide linkages). Other bands did not indicate any shift or change in signal intensity.

The DMBIS-PEG 400 formulation when applied to SC showed red shift at 3270 cm⁻¹ (observed in most DMBIS formulation due to removal of water from SC) with reduced peak intensity at the signal. At the same time the DMBIS-PEG 400 formulation showed blue shifts at 1735 and 1637 cm⁻¹ accompanied by decrease in peak intensity. In addition splitting of amide 2 linkage was observed. All these observations suggest enhancement in presence of DMBIS-PEG 400 (also seen in in vitro permeation of DEET) by structural change in protein structure due to loss of water from SC protein (127).

DMMCBI formulations: Unlike DMBIS, DMMCBI do not possess similarity to DMSO in modification of SC. The FTIR analysis of SC following DMMCBI-water application (Figure 5.11) showed a blue shift at band positions of 3270 and 1637 cm⁻¹ and a red shift

at 1735 cm⁻¹ position. The in vitro permeation of DEET after application of DMMCBIwater showed enhancement as compared to no treatment but retardation as compared to water alone treatment. It suggests that enhancement is due to presence of water in DMMCBI-water formulation (as evidenced by the blue shift) that leads to formation of an aqueous pathway (102) which indirectly leads to state of disorder in the SC lipid bilayer. The retardation was due to DMMCBI in the formulation (as evidenced by the red shift). The contrasting effect of DMMCBI and water in the formulation led to an insignificant effect on permeation of DEET in the in vitro experiments (89).

In spectral analysis of SC after application of DMMCBI-water, blue shifts (121) and decrease in peak intensities (112) at different bands explain the enhancement of permeant (DEET) in presence of DMMCBI-PG formulation.

The stratum corneum spectral analysis following treatment with DMMCBI-ethanol formulation showed red shifts at 1735, 1637, 1538 and 1455 cm⁻¹ band positions. This observation (except red shift at 1538 cm⁻¹) implies the development of an ordered state within the SC (118). Also, intense peaks at 1724 and 1779 cm⁻¹ were observed suggesting the formation of H-bonds in the triglycerides in the region. All these changes support the establishment of stable organization of lipids and proteins in the SC, which is further supported by retardation of DEET permeation, as observed in the DMMCBI-ethanol formulation (89). Moreover, a blue shift was observed at symmetric band indicating that retardation activity of DMMCBI-ethanol formulation does not occur through the symmetric methylene stretching of the SC lipids.

When SC treated with DMMCBI-PEG formulation was analyzed by FTIR, a blue shift was observed at 1637 cm⁻¹ and a red shift at 1538 cm⁻¹ (121). Even though the observed

shifts suggest enhancement in presence of DMMCBI-PEG 400 formulation, in vitro permeation studies indicated retardation of DEET. The observed retardation of DEET seemed to be due to PEG 400 in the formulation that acted as a physical barrier preventing the permeation of permeant across the SC.

TBDOC formulations: The spectral analysis of SC treated with TBDOC-water formulation (Figure 5.12) depicted blue shifts at 3270, 1637 and 1538 cm⁻¹ band positions. Even though the blue shift in case of amide 2 linkage (1518 cm⁻¹) is indicative of strong hydrogen bonds in the ceramide molecules; it may be possible that these interactions occurred between the water molecules present in the formulation and ceramide molecules in the stratum corneum leading to fluidization of lipid bilayer of SC. These shifts were also accompanied by decrease in peak heights suggesting that TBDOC-water formulation caused disorder in the stratum corneum along with lipid extraction and interactions with proteins. TBDOC-water formulation might have also led to increased hydration of SC (evident by blue shifts at 3270 and 1637 cm⁻¹) that might have created aqueous pore pathways in the SC that facilitated the diffusion of permeant (102). This hypothesis is supported by the evidence that in vitro permeation enhancement of DEET was observed in presence TBDOC-water (89).

In spectral analysis of TBDOC-PG treated SC, the observation of blue shifts at 3270, 2917, 2850 cm⁻¹ suggest enhancement due to disorder in the lipid arrangement by the TBDOC-PG formulation by its impact on alkyl chain organization (109, 121). The red shift at 1735 cm⁻¹ and blue shift at 1544 cm⁻¹ suggest formation of strong H-bonds (121) that may be due to the presence of TBDOC in the formulation (a potential retardant). There was splitting of band at 1637 cm⁻¹ position to yield peaks at 1639, 1627 and 1691

cm-1 suggesting reorientation (hydrogen bond) of the ceramide head groups with propylene glycol in the formulation leading to loosening of the organization of lipid bilayer. The amide 1 splitting also suggests formation of distorted beta pleated structure of keratin that contributed to enhancement of the permeant in presence of TBDOC-PG. In spectral analysis of SC treated with TBDOC-ethanol, blue shifts were observed at 2850 and 1544 cm⁻¹ positions along with appearance of peak at 1710 cm⁻¹ (red shift). The blue shift at 1544 cm⁻¹ suggests the formation of strong hydrogen bonds among the ceramides (121). Furthermore, peak splitting at 1637 cm⁻¹ band was also observed. However, there was no shift seen at the 3270 and 2917 cm⁻¹. The above observation suggests that TBDOC-ethanol formulation act as retardant by its action on amide 2 linkage and lipid ester linkage.

TBDOC-PEG 400 formulation treated SC showed similar spectral bands like TBDOCethanol, except it showed blue shift at 3270 cm⁻¹ (128) leading to its enhancement action due to increased hydration of SC (22).

5.5 Conclusion

In this study, DSC and IR techniques showed that each penetration modifier formulation caused unique changes in SC lipid organization that in turn led to modification of their inherent enhancer/retardant activity. We observed that penetration modifier formulations that enhanced the permeation of active acted mainly by disruption and fluidization of the lipid bilayer as evidenced by characteristic lowering of mean transition temperature and occurrence of blue shifts at characteristic frequencies with additional decline in peak intensity. On the other hand, thermal and spectral analyses of penetration modifier formulations that enhances showing retardation depicted elevated T_{m2} , certain red shifts and formation

of multiple peaks around 1738 cm⁻¹ transition suggesting retardation by strengthening of lipid protein complex and organization of SC lipids by increased H-bonding. However, thermal and spectral analyses did not reveal the reason for the intensity of enhancement/retardation in presence of each formulation. More investigations in future are warranted to give advanced insight or to provide an exact explanation for degree of enhancement or retardation in complex systems.

Chapter 6: Percutaneous permeation profile of penetration modifiers and their cytotoxic potential

6.1 Introduction

The skin being the largest organ is an ideal route for systemic drug delivery. However, delivery across skin is often limited by its barrier properties. In order to overcome the SC barrier, scientists has developed several physical and chemical techniques to deliver actives across the skin. Among the two major approaches, the chemical enhancement technique is simple, cost effective and non-invasive method of enhancement. Its application in transdermal and dermal products is often limited by skin irritation usually due to the formulation ingredients such as the enhancer compounds. The determination of the cytotoxicity of chemical permeation modifiers is important and aids in screening potential penetration modifiers (25). The cytotoxicity testing of penetration modifiers is usually performed using human dermal fibroblasts or keratinocytes and the MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium, inner salt) assay. The MTS assay which is a simple colorimetric assay, is used to measure the viable cells surviving after test treatment. The cells contain mitochondrial dehydrogenase enzyme that cleaves the tetrazolium ring of MTS reagent which in presence of phenazine methosulfate (a reagent) forms a water soluble formazan product that can be detected colorimetrically at 490 nm. The intensity of the color developed gives an indication of percent of viable cells present in the sample.

The objective of the current study was to determine the cytotoxicity of five penetration modifiers namely laurocapram, N-0915, DMBIS, DMMCBI and TBDOC using MTS assay. Since keratinocytes and dermal fibroblasts are the abundant cells found in

epidermis and dermis respectively, they were selected for the experiments to determine toxicity of penetration modifiers.

The surface erosion potential and cytotoxicity was also measured through scanning electron microscopy (SEM). The permeation profile of penetration modifiers across epidermal and dermal membranes was also evaluated.

6.2 Materials and methods

6.2.1 Materials

3-Dodecanoyloxazolidin-2-one (N-0915) and TBDOC were obtained as generous gifts from Dr. James Chapman at the University of South Carolina, Columbia, SC. Laurocapram, DMBIS, DMMCBI were provided by New Jersey Center for Biomaterials (Piscataway, NJ). Human dermal fibroblasts, human keratinocytes and Epilife[®] medium were obtained from Invitrogen Corporation (Carlsbad, CA). Dulbecco's Modified Eagles Medium (DMEM) and 10% fetal bovine serum (FBS) were purchased from Gibco-Invitrogen Corporation (Carlsbad, CA) and Sigma (St. Louis, MO) respectively. MTS reagent was purchased from Promega (Madison, WI).

6.2.2 Skin membranes

All human skin samples were purchased from Allosource (Cincinnati, Ohio) and were dermatomed to approximately 380-500 μ m and derived from male and female individuals aged between 30-60 years. These skin pieces were stored at -80° C until use, but for no longer than 2 months.

6.2.3 Methods

6.2.3.1 Preparation penetration modifier formulations

Penetration modifiers (laurocapram, N-0915, TBDOC, DMBIS, DMMCBI) were weighed and each was added to propylene glycol to prepare 0.04 M solutions. The solution was then vortexed at room temperature for 48 hours, diluted appropriately and used for cytotoxicity, microscopy and permeation studies.

6.2.3.2 Toxicity testing of penetration modifiers in human dermal fibroblasts and keratinocytes

6.2.3.2.1 Cell culture

Secondary cultures of fibroblasts were established with media containing Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Secondary cultures of keratinocytes were established with supplemented Epilife[®] medium containing 0.06 mM calcium chloride, bovine pituitary extract (0.2% v/v), bovine insulin (5 µg/ml), hydrocortisone (0.18 µg/ml), bovine transferrin (5 µg/ml), and human epidermal growth factor (0.2 ng/ml).

6.2.3.2.2 Preparation of standard plots

For preparation of standard plot of keratinocytes/fibroblasts, various dilutions (n=6) in the range of 1000 cells/ul to 10,000 cells/ul were prepared by adding 10, 20, 30, 40, 50, 60, 70, 80 and 100 ul from 100 cells/ml stock solution. The well plate was then incubated for 24 hours at 37°C at 5%CO₂. After 24 hours, the standard plot well plate was removed from incubator and 100 ul of the culture media was removed from it, followed by washing with PBS and replacement by 20 ul of the MTS reagent and 90 μ l of culture medium. The plate was incubated for 2 hour. After 2 hours the absorbance of standard plot well plate was measured in the plate reader (Biotek Instruments Inc., Winooski, VT) at 490 nm. Absorbance of media alone was also noted and subtracted from the absorbance of each well. Absorbance Vs cell concentration was then plotted.

6.2.3.2.3 Cytotoxicity assay

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium, inner salt) (MTS) assay was used to determine the toxic effects of penetration modifiers on dermal fibroblasts and epidermal keratinocytes in vitro. The penetration modifiers tested included laurocapram, N-0915, DMBIS, DMMCBI and TBDOC. Skin cells (fibroblasts or keratinocytes) were transferred to the 96-well plate at concentration of 8000 cells/µl in 100 ml medium per well. After 24 h, the cells were treated with varying concentrations of penetration modifier solution prepared in PG (vehicle) in culture medium and incubated for 24 h at 37°C. The cells that were treated with culture medium containing 1% PG were used as control.

The medium was then replaced by fresh medium with 20 μ l MTS solution and the cells were incubated for 2 h. After 2 hours, plate was removed from the incubator and intensity of color developed in each well was measured using a plate reader (Biotek Instruments Inc., Winooski, VT) at 490 nm.

6.2.3.3 Scanning Electron Microscopy (SEM)

Human skin before or after penetration modifier treatment was fixed with 5% glutaradehyde aqueous solution at 4°C for 1.5 h and then washed with water at 4°C overnight. The fixed skin was dehydrated with 30%, 50%, 75%, and 95% ethanol in water, for 2 h each, at room temperature and then with two changes of absolute ethanol for 2 h/change at room temperature. The dehydrated skin was dried with a critical-point drying machine (Balzers CPD 020 Critical Point Drier, Germany) and coated with gold

(Balzers SCD 004 Sputter Coating Unit, Germany). The Scanning electron microscopy (Amray 1830 I) of dermatomed skin treated with different formulation of penetration modifiers was performed to detect their effect on surface of SC.

6.2.3.4 Skin permeation studies

6.2.3.4.1 Franz cell procedures

Vertical Franz cells (receptor volume 4.3 ml-Permegear, Inc., Bethlehem, PA) were used with a donor area of 0.196 cm². Prior to the experiment, the dermatomed piece of human cadaver skin was removed from -80 deg C freezer and thawed. This was followed by immersion of the skin piece at 60 deg C for 1 minute. This was followed by teasing out of epidermis from the dermis. Then the separated epidermis and dermis were incised into appropriate sized pieces, and mounted on Franz diffusion cell and hydrated for one hour. Each cell contained 15% V/V of ethanol-normal saline solution (ethanol was used as a solubilizer). The receptor fluid was maintained at 37°C and stirred continuously at 600 rpm using a magnetic stirrer. 0.04 M solution of penetration modifiers were prepared in propylene glycol and 50 μ l of each of these solutions of penetration modifiers were added to the donor compartment of the Franz cell. 300 μ l receptor aliquots were withdrawn at 2, 4, 6, 8, 12, 24, 28, 36 and 60 hr. Analysis by HPLC and subsequent calculations yielded penetration parameters which included flux (J), lag time and amount of penetration modifier per unit area of drug penetrated into the receptor in 60 hours (Q₆₀).

6.2.3.4.2 Analysis of penetration modifiers

The analysis of penetration modifiers was performed using HPLC (HP 1100, Agilent Technologies, Inc.) equipped with degasser (G1379A), autosampler (G1313A), quaternary pump (G1311A), a UV-visible diode array (G1315A) and an Eclipse XDB-

C18 RP column (Agilent Technologies, USA) having a pore size of 5 μ m and dimensions equivalent to 4.6 X 150 mm. The details of HPLC methods of penetration modifiers are listed in Table 4.1. An external standard technique was employed for all the test compounds.

All methods were validated for linearity, precision and accuracy. The correlation coefficients of 0.999 for linearity of plots were observed in case of all penetration modifiers used in the study. Intraday variability was less than 0.2 % for all methods, and interday variability was also calculated to be less than 3.0 % for all penetration modifiers.

6.3 Results and discussion

6.3.1 Toxicity testing of penetration modifiers in human dermal fibroblasts/keratinocytes

The standard plot of absorbance Vs. number of dermal fibroblasts and keratinocytes was plotted (Figure 6.1 and 6.2) to estimate the number of number of viable cells after penetration modifiers treatment on dermal fibroblasts and keratinocytes. At the same time cells were seeded in separate well plates and treated with various concentrations of five penetration modifiers namely laurocapram N-0915, DMBIS, DMMCBI and TBDOC as shown in the Tables 6.1 and 6.2. The dilutions of penetration modifiers were prepared in propylene glycol as it the most commonly used solvent in transdermal/dermal formulations. Moreover, all penetration modifiers showed suitable degree of solubility in propylene glycol. N-0915 was not soluble in propylene glycol at 400 μ M; therefore the cytotoxicity testing was not at that concentration. However, cytotoxicity testing of N-0915 was performed at concentrations 40, 4, 0.4 and 0.04 μ M concentrations. Percent

viability of keratinocytes and dermal fibroblasts following treatment with different formulations of penetration modifiers is listed in Table 6.1 and 6.2 respectively. All penetration modifiers showed concentration dependent toxicity for keratinocytes as well as fibroblasts. The data indicates that keratinocytes were more sensitive to penetration modifier treatment at higher concentrations as compared to fibroblasts. This is because of the fact that fibroblasts are more robust cells in terms of growth than keratinocytes (129). Among the various penetration modifiers, iminosulfurane based penetration modifiers (DMBIS and DMMCBI) were well tolerated by both keratinocytes and fibroblast even at higher concentrations suggesting their advantage over laurocapram based modifiers.

6.3.2 Scanning Electron Microscopy (SEM)

The surface of human cadaver skin was treated with different formulations of penetration modifiers each containing 40 μ M of penetration modifier (laurocapram, N-0915, DMBIS, DMMCBI and TBDOC) for period of 30 hours and then examined via SEM. The images after various treatments are depicted in Figure 6.3. No major differences on skin surface were observed following various treatments except in the case of laurocapram treatment where some erosion of the corneocytes was observed. This observation also corroborates our cytotoxicity assay where no cell growth was observed after 40 μ M laurocapram treatment as compared to other penetration modifier treatments.

6.3.3 Skin permeation studies

The permeation profiles for five penetration modifiers were recorded using human cadaver epidermis and dermis. The reason for determining the permeation profile in epidermis and dermis separately was to investigate the residence time and retention of the above penetration modifiers in each of the layers of the skin. Five penetration modifiers of diverse log P values, solubilities, molecular weights were chosen that were either laurocapram derivatives or iminosulfurane derived (Table 6.3). The epidermal and dermal membranes separated by a heat separation method were mounted on Franz diffusion cells followed the measurement of permeation profile of the penetration modifiers over a period of 60 hours. Epidermis used in the experiment was 60-70 μ m thick and thickness of dermis ranged from 100-150 μ m. Ethanol (15% V/V) was used in the receptor fluid as the solubilizer. Permeation profile of 4–bromobenzamide was also measured as DMBIS converts into 4-bromobenzamide with time in aqueous environment and also undergoes biotransformation into 4-bromobenzamide in the skin (85).

Epidermis: It was noted that across epidermis TBDOC showed the no permeation and DMBIS showed maximum permeation. The permeation of DMBIS was measured as a function of 4-bromobenzamide as DMBIS converts into 4-bromobenzamide in aqueous medium. This behavior can be explained by the difference in the partition coefficient of these compounds with TBDOC having a partition coefficient of 6.2 and DMBIS having the log P value of 2.6. However, DMMCBI that has a log P value of 1.3 showed little permeation only after 36 hours. Mass balance studies with DMMCBI showed that that high percentage of DMMCBI (99.85 percent) was present on the top of the epidermis at the end of the study with only 0.1 percent of the compound in the skin and only 0.05 percent permeated across the skin. It seems that DMMCBI being relatively hydrophilic in nature had less tendency to partition from propylene glycol into the epidermis thus explaining the lower permeation. TBDOC that showed no transdermal permeation, however, showed a percentage of 22.5 percent with the epidermis with the remaining amount on top of the epidermis. High Log P value promoted the partitioning of the

molecule into epidermis, however its relatively large size and Log P of 6.2 prevented it from partitioning from the epidermis into the receptor compartment. N-0915 and laurocapram with log P values of 5.7 and 4.6 respectively showed slow permeation profiles that were not significantly different from one another. Unlike TBDOC, they showed permeation across the epidermis during the coarse of the study perhaps owing their appropriate log P value that helped its partitioning from the formulation into epidermis followed by its partitioning from epidermis into receptor fluid.

Dermis: The five permeation modifiers were investigated across dermis with receptor aliquots collected over the time range from 0-60 hours. Laurocapram and N-0915 showed minimum permeation and DMBIS showed the highest permeation. Across dermis, TBDOC showed improved partition as compared to epidermis. Owing to its hydrophobic nature, no retention of TBDOC was observed within the dermis. DMMCBI showed improved partitioning into the dermis from the applied formulation and out of the dermis into the receptor fluid. It was observed that in case of profile of all penetration modifiers, a plateau was reached within the course of the study. This can be explained due to back diffusion of receptor fluid into the dermis that led to equilibrium of the diffusion.

6.4 Conclusions

The main goal of this study was to obtain better understanding about the selected penetration modifiers by means of cytotoxicity testing, microscopy and in vitro permeation studies. The cytotoxicity testing revealed that iminosulfurane based penetration modifiers (DMBIS and DMMCBI) were well tolerated by both keratinocytes and fibroblasts even at higher concentrations suggesting their advantage over laurocapram based modifiers. Surface microscopy of penetration modifiers suggested no surface effect on skin in terms of erosion of corneocytes after treatment except in case of laurocapram. The permeation studies suggested that permeation of modifiers across epidermis and dermis is dependent on their partition coefficient, solubility, molecular weight and the formulation in which they were prepared.

Part 2: Human pharmacokinetics and pharmacodynamics of orally administered quercetin

Chapter 7: Introduction

A diet rich in fresh fruits and vegetables has always been considered a key to good health. For a long time, nutritional research has focused on levels of carbohydrates, proteins, fats, vitamins and minerals present in a regular diet. Currently, research is getting focused on the secondary plant metabolites present in diet that are not only present in small amounts in plant food sources but have significant effect on humans. Though these secondary plant metabolites are not vital for maintaining life, researchers are beginning to recognize them for their potential role in protection against inflammation and free radical production in the body.

Several secondary plant metabolites have been isolated that include flavonoids, carotenoids, terpenes, saponins, phytosterols, etc. (130). Among these, flavonoids represent one of the largest and widely distributed groups of secondary plant metabolites (131).

Flavonoids are a group of low-molecular weight polyphenolic substances that are present in most edible fruits and vegetables. There are approximately 4,000 different flavonoids known which have been categorized as flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids (132). Quercetin is one of the main representatives of the flavonoid subclass of flavonols (133).

Quercetin is the most investigated flavonoid and several in vitro and in vivo studies in animals have indicated its potential role as antioxidant (134, 135), anticarcinogenic agent (136), anti-inflammatory agent (137), cognitive enhancement agent(138) and exercise performance agent. But few studies have been performed in humans that investigate effect of quercetin supplementation on exercise induced inflammation and oxidative stress (139) (140).

In this investigation, method for analysis of quercetin in biological matrices (plasma and urine) were developed and validated. In addition, pharmacokinetics of quercetin in humans on supplementation via three oral carrier systems: quercetin fortified Tang[®] suspension, First Strike[™] Bar and Q-chews[®] was determined. This was followed by human pharmacodynamics studies of quercetin after single and multiple dosing of quercetin from one of the selected carrier systems. This work is part of a project investigating the effects of quercetin supplementation in humans supervised by US Department of Defense (DOD) on exercise performance in heat stress, aerobic exercise and oxidative stress and muscle soreness in humans. The results from this research will assist Natick Soldier Research, Development and Engineering Center, a division of US Department of Defense (DOD) to incorporate quercetin in the military food rations.

Chapter 8: Background and Significance

8.1 Chemistry and Structure

Flavonoids consist of three phenolic rings referred to as A, B and C rings where the benzene ring A is fused with oxygen containing pyran ring C. The six-member ring C carries a phenyl benzene ring (B) as substituent in the 2-position. The whole structure is represented as flavonoid nucleus and is represented in Structure 8.1.

Flavonoids containing a hydroxyl group at the 2 position are classified at 3hydroxyflavonoids (flavonols, anthocyanidins, leucoanthocyanidins and catechins) and those lacking it as desoxyflavonoids (flavanones, flavones). Within the 3hydroxyflavonoids and desoxyflavonoids group, classification is based on position and introduction of methyl or hydroxyl groups. Isoflavonoids differ from flavonoids in placement of B ring that is bound to 3- position of C- ring instead of C-2 position. Anthocyanidins and catechins differ from other groups in lacking the carbonyl group on C-4. The various classes of flavonoids and their examples are represented in Table 8.1.

Up till now, 6000 flavonoids have been identified in plants that mainly exist as glycosides. The flavonoids lacking sugar moieties or aglycones occur rarely in nature. The flavonoid aglycone is capable of binding to at least 8 monosaccharides or can bind with di- or trisaccharides at different hydroxyl groups present in the molecule. The sugar moieties usually present include D-glucose and L-rhamnose and glycosides formed are mainly O-glycosides where glycone is bound to the hydroxyl group at the C-3 or C-7 position (141).

Among the various flavonoids, quercetin is the most abundant and investigated one (134, 142). Quercetin belongs to the flavonol subclass of flavonoids and is chemically 3,5,7,3',4'-pentahydroxyflavone (Structure 8.2). Quercetin occurring in edible fruits and vegetables mainly exists in form of glycosides (glycone plus aglycone) with quercetin-3-rutinoside (or quercetin-3-rhamnoglucoside or rutin) being the most abundant. Quercetin represents the aglycone part of the glycoside. Other forms of quercetin glycosides include quercetin-4'-glucoside and quercetin-3, 4'-glucoside that are abundantly present in onions. Glycosides such as quercetin glactosides and quercetin arabinosides also exist that are found in apples and berries respectively. Major dietary quercetin glycosides are listed in Table 8.2.

8.2 Dietary sources of quercetin

Quercetin is present in numerous edible fruits, vegetables and beverages but it is predominantly found in onions, tea, apples, berries and red wine (Table 8.3). Hertog *et al.* (143) reported flavonol intake (including quercetin) from the Seven Countries Study and reported that tea was the major source of quercetin in the Netherlands and Japan, wine was the predominant source of quercetin in Italy, while onion and apples were the chief source of quercetin in United states, Finland, Greece and former Yugoslavia. The daily intake of flavonoids reported by Hertog *et al.* ranged between 3-38 mg in the Seven Countries Study. In another study, the estimated intake of flavonois and flavones in United States was reported to be 20-22 mg/day of which 73-79.6 % was quercetin (144). Nowadays, products in form of tablets, capsules, chews and bars are also available that provide quercetin supplementation in diet.

Although quercetin is widely distributed in numerous plant products but their distribution and synthesis within a plant species is controlled by various intrinsic and extrinsic There is intricate system of genetically controlled enzymes regulating their factors. synthesis and distribution in the plant organism. Extrinsic factors such as plant distribution and variety, growth, season, climate, degree of ripeness, food preparation and processing all contribute to quercetin content (142). Quercetin as the glycosideform is observed to be more concentrated in epidermal cells as compared to mesophyll cells. This explains the reason for higher quercetin content in skin of red grapes, Spanish cherry tomatoes and apples as compared to interior of the fruit. Major difference in quercetin contents has been observed in different varieties of same plants species such as apples, lettuce and tomatoes. Seasonal variations also result in differences in quercetin content in several plant species such as tomatoes and berries. It has also been observed that accumulation of flavonoids increases in response to increased light especially ultraviolet-B rays and high temperature (sunnier climates). Seasonal, spatial and interspecific variations in two traditional varieties of herbal teas showed that the content of quercetin in both varieties of tea reached peak levels in summer and lowest levels in autumn (145). Significant differences in quercetin content have been observed in different geographical locations with higher accumulation of quercetin seen in cooler climates. The degree of ripeness also influences flavonol content in plant product with higher flavonol production in ripe vegetable/fruits.

Reports suggest that food preparation, processing and storage affect quercetin content in food. Dietary sources of quercetin subjected to boiling and frying showed lower quercetin content compared to their raw forms because of thermal degradation and leaching of
quercetin into boiling water (142). However, dietary sources containing quercetin conjugates retain their stability up to temperatures of 100°C. Storage conditions also affect quercetin content. For example, onions lose 25-33 % of their quercetin content in the first twelve days of storage (146). Unlike onions, quercetin content in strawberries increase by approximately 32 percent when stored at -20°C for 9 months. Though flavonol content tend to significantly lower in processed food as compared to fresh foods, but in certain cases processing tend to increase flavonol content. Increased accumulation of quercetin has been observed in processed foods due to enzymatic hydrolysis of quercetin conjugates during pasteurization and fermentation. Similarly higher concentrations of quercetin have also been detected in wine as compared to grapes and berries because extraction procedures during wine processing release quercetin from rind that thereby result in higher quercetin content in wines.

A separate study on the common yellow onion (*Allium cepa*) investigated numerous additional variables on flavonol synthesis, including the effect of nitrogen fertilizer level, lifting time, and field curing time (147). This research showed no effect on quercetin production with variation of nitrogen levels, minor effects due to the lifting times (referring to removing the onions from the ground at different developmental stages), and significant increases in the amount of quercetin post field-curing.

8.3 Biological effects/benefits

Quercetin is a strong antioxidant that is capable of inhibiting lipid peroxidation by scavenging free radicals and binding transition metal ions (132, 148). Owing to its antioxidant properties, quercetin possesses potential benefits in preventing pathological conditions related to free radical production such as atherosclerosis, cancer and chronic

inflammation. Quercetin is not only capable of inhibiting lipid peroxidation, but also increases glutathione (GSH) levels (149). GSH is part of the neuron's defense against oxidative damage that converts harmful superoxide or hydrogen peroxide radicals to oxygen and water by means of superoxide dismutase and thus helps neurons in scavenging free radicals (150).

Quercetin also reduces inflammation and thus helps in preventing chronic inflammatory diseases (151). Among the various inflammatory conditions, chronic prostatitis is one such condition that involves inflammation of the genital tract. Quercetin showed a 25 % improvement in symptoms of chronic prostatitis in 67 % of subjects receiving 500 mg of quercetin twice a day for one month. The effect of quercetin in treatment of chronic prostatitis improved with co-administration of Prosta-Q (bromelain and papain) that increased absorption of quercetin leading to at least 25 % improvement in ameliorating symptoms of chronic prostate in 82 % subjects.

Diabetes is another condition that is believed to develop partially as a result of mitochondrial dysfunction, inflammation and reactive oxygen species. Several animal studies have indicated beneficial effects of quercetin on blood glucose levels and lipid levels in doses ranging from 10-50 mg/kg (152, 153). However, certain epidemiological studies in humans investigating relationship between dietary flavonoid intake and diabetes have yielded mixed results (154, 155).

Neurodegeneration is yet another inflammatory condition that involves progressive loss of nervous system function associated with aging and has been seen in many diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, epilepsy and stroke. Oxidative damage, inflammation and mitochondrial dysfunction that accompany aging are usually associated with neurodegeneration. In vitro studies performed have shown neuroprotective effect of quercetin which is likely due to its antioxidant and anti-inflammatory actions. Although no human experimental study has been reported in the area, there is evidence that consumption of flavonoids such as quercetin limits neurodegeneration and prevents or reverses age dependent losses in cognitive performance (156).

Similarly quercetin has been investigated for its potential role in reducing cardiovascular disease. Preclinical and clinical data suggest that quercetin reduces several risk factors for cardiovascular diseases such as hypertension (157), elevated levels of C-reactive protein (158). Quercetin also possess certain cardio-protective attributes such as vasodilatation in coronary vessels (158) and protection from oxidative stress induced cell injury (159). In vitro studies have indicated that good amounts of quercetin (equivalent to two glasses of red wine) possess 'clot-busting' activity (160). A study performed by Graf and coworkers showed a 21 % reduction in cardiovascular disease mortality when the intake of quercetin was greater than 4 mg/d (161). Chopra et al. (162) suggested that quercetin also play a role in reduction of LDL cholesterol in hyperlipidemic patients. Besides quercetin, its conjugate, quercetin-3-O- β -D-glucuronide also possesses antioxidant ability on human plasma LDL (163). Phase III clinical trials are being conducted in order to determine proper status of quercetin in prevention and treatment of cardiovascular diseases in humans (164). Like other benefits, quercetin has demonstrated anticarcinogenic effect in cell culture models due to its anti-mutagenic, antioxidant and anti-inflammatory activity. Quercetin appears to act by modulating signal transduction pathway such as apoptosis, cell cycle regulation and angiogenesis (164). However, animals studies investigating anticarcinogenic role of quercetin have yielded inconsistent results which is probably due differences in cancer models and dose of quercetin used in the study. Similarly, limited human epidemiological studies conducted to investigate anticancer potential of quercetin have provided mixed results with few studies reporting some link between higher intake of quercetin and lower risk of the disease (155).

Quercetin has also been investigated for its effects on immunity and infection. In vitro cell culture studies have revealed this compound's role as an anti-infective agent that is capable of reducing replication of variety of respiratory viruses (165-168). Although the mechanism of antiviral action of quercetin is unknown, it is thought that quercetin either acts by inhibiting viral replication at early stage of development or by suppressing viral enzymes that influence viral's ability to cause disease, or by binding to the viral capsid protein that surrounds the virus core. Another hypothesis to explain quercetin's antiviral activity is by its action on interferon and other components of immune response that guard body's immune system(169, 170). Although, many in vitro studies have been conducted to investigate the antiviral effect of quercetin, limited animal and human data is currently available. In a study performed by Nieman and colleagues in a group of cyclists to investigate quercetin's role in lessening immune dysfunction and counteracting risk of upper respiratory tract infection (URTI) following repeated and sustained exercise showed that 1 g/day of quercetin reduced the incidence of URTI following three days of exhaustive cycling, although there were no accompanying effects of quercetin on several measures of immune function (140). However, in another study of ultra marathoners participating in the Western States Endurance Run, no benefits of quercetin on illness rates (i.e., self-reported illness symptoms) and similar immune changes were observed

between quercetin and placebo groups during intense exercise (171). Owing to the mixed effects of quercetin on immunity in previous two studies, Davis *et al* (139)conducted a study in untrained mice that were administered seven days of quercetin feedings, then subjected to a treadmill run for three days and inoculated with a standardized dose of influenza virus. In the study, susceptibility was measured by time to sickness, symptom severity, and time to death. Davis *et al* observed a strong trend toward a decrease in susceptibility to infection in mice that were given quercetin. Results revealed that mice that had been given the quercetin were significantly less susceptible to the influenza virus following stressful exercise (P<0.05).

Quercetin is capable of positively affecting physical and mental performance of subjects during intense aerobic exercise (164, 172, 173). This attribute is not only due to its antiinflammatory and anti-oxidant activities but also its ability to cause effects such as increased mitochondrial biogenesis in the muscle and brain (164), COMT inhibition (172), adenosine antagonistic activity (174) etc.. As mitochondria are responsible for energy production, increased mitochondrial biogenesis leads to increased aerobic

capacity (measured as maximal oxygen uptake, \dot{VO}_{2max}) of subject which is indicator of cardio-respiratory fitness. Mitochondrial increase also helps in slowing muscle glycogen and glucose utilization with a greater reliance on fat oxidation for energy, and a decrease in lactate production in the muscles (175). Until recently, exercise was the only means of increasing mitochondrial biogenesis in muscles but now, evidence is that natural flavonoids such as quercetin can increase mitochondrial biogenesis via intracellular signaling pathways involving peroxisome proliferator-activated receptor- γ coactivator (PGC-1 α) and sirtuin (SIRT1). This effect of quercetin has been linked to its potential in improving endurance and health in mice (176, 177). Studies performed by Davis et al. involved feeding of sedentary mice with quercetin for seven days followed by measurement markers of mitochondrial biogenesis in the muscle and brain, as well as endurance exercise tolerance. Results indicated statistically significant increases in PCG- 1α and SIRT1 gene expression, mitochondrial DNA, and cytochrome c concentration in mice suggesting quercetin causes mitochondrial biogenesis in brain and muscle of mice. It was also found that an increase in mitochondrial biogenesis was associated with significant increases in endurance capacity (treadmill running to fatigue), as well as voluntary wheel-running activity (willingness to be physically active) (164). In addition to the animal studies, investigations have been performed in humans to investigate quercetin's potential as an agent that increases mitochondrial biogenesis but have yielded mixed results. In one study on 26 untrained men, investigators found consistent trends toward increase in several different markers of mitochondrial biogenesis in subjects consuming quercetin (140). However, in another study in highly trained athletes no effect on mitochondrial biogenesis was observed following quercetin administration (173). This was due to the fact that highly trained athletes already reached capacity for muscle mitochondrial density as a result of their high level of aerobic training.

Another attribute of quercetin by which it enhances endurance is its inhibitory effect on the breakdown of catechol-O-methyltransferase (COMT), an enzyme that degrades norepinephrine (178),(179). It is believed inhibition of COMT automatically extends the body's adrenaline, allowing for a more pronounced catecholamine (norepinephrine) effect during exercise and triggering the release of glucose from energy stores, as well as increasing skeletal muscle readiness (172). Another characteristic of quercetin that may be responsible for its ability to enhance physical and mental performance is its caffeine-like psycho-stimulant effect. Like caffeine, quercetin has also shown adenosine A_1 receptor antagonist activity in vitro (180), (174).

8.4 Analysis of quercetin in human plasma and urine matrix

In past, analysis of flavonoids including quercetin was mainly performed using paper chromatographic methods (131). Nowadays, with the advent of new technology, the extraction of quercetin from plant material typically involves acid hydrolysis, followed by high-performance liquid chromatography (HPLC) (181, 182). Beside, extraction and analysis of quercetin from plants, pharmacokinetics interest in quercetin has led to development of bioanalytical assays of quercetin in biological matrices such as plasma(183), urine(184), tissue(183) and saliva(185). However, analysis of quercetin from human plasma or tissues is much more difficult because the compounds are usually bound to proteins and are present in much lower concentrations. It has been observed that when concentrations of quercetin are very low in biological matrices, extraction methods cause degradation of the quercetin molecule leading to low recoveries of the desired molecule. Moreover, binding to metals and silica poses a problem when analyzing low levels of the compounds with chromatographic methods. Therefore, several solid phase extractions methods for the analysis of quercetin from human plasma and urine have been developed to improve extraction efficiency of quercetin (184, 186). Ishii et al reported 96 percent extraction efficiency of quercetin in plasma and urine matrices using OasisTM HLB solid phase extraction cartridges. Similarly Erlund *et al.* found 71 percent recovery of quercetin on extraction from plasma using Bond Elut C 18 solid phase extraction

column. Several groups have also used liquid-liquid extraction methods for quercetin extraction (187). Though separation of quercetin following extraction has been performed exclusively using reverse phase chromatography (184, 186), detection has been performed using several techniques such as such as mass spectroscopy (MS) (188), electrochemical detection (186), UV/diode array detection (189), fluorescence detection (187) etc. Better sensitivity of quercetin assay was obtained by HPLC methods with mass spectroscopy detection, fluorometric detection coupled with post-column derivatization or with electrochemical detection (ED) techniques. Moon et al developed LC-MS assay of quercetin in plasma with LOD as low as 100 pg/ml. Hollman et al. (187) developed a very sensitive bio-assay of quercetin (LOD of 2 μ g/l) that was based on acid hydrolysis and HPLC with post-column derivatization. The method involved heating quercetin containing plasma samples with a mixture of hydrochloric acid and methanol followed by separation of quercetin aglycone using HPLC-fluorescence method after complexation with aluminum ions (187). Another sensitive assay of quercetin has been reported by Erlund *et al.* that utilized HPLC coupled with electrochemical detection to yield LOQ of quercetin as low as 0.63 ng/ml in plasma matrix. Researchers such as Bongaetz and Hesse (190)] developed other sensitive assays such as boronic acid affinity chromatography to determine urinary quercetin in a concentration range of 0.10–30.0 μ g/ml. In addition, to the above sensitive techniques, UV detection is also a commonly available, cost-effective and simpler detection technique for quercetin analysis. On one hand HPLC–UV methods developed by some workers reported poor sensitivities for determining low levels of plasma quercetin (LOD, 80–100 ng/ml) (191, 192). But on the other hand, reports by Ishii *et al.* showed a detection limit of quercetin as low as ~ 0.35

ng/ml in plasma or urine through HPLC-UV methods (184). The sensitivity of HPLC– UV method (LOD, 5 ng/ml urine) reported by Ishii *et al.* was similar to HPLC assay with fluorometric detection (187) and HPLC–ED (186). Other researchers such as Nielsen *et al.* (193) improved HPLC-UV detection technique by developing column-switching HPLC method for quantifying quercetin in human urine.

8.3 Absorption Distribution Metabolism Excretion (ADME) and Bioavailability

The benefits of quercetin are largely guided by its bioavailability this being the rate and extent of absorption of an active in the systemic circulation after administration. Bioavailability in turn is dependent on the absorption, distribution, metabolism and excretion of the active in the body. As dietary quercetin is present mainly in form of glycosides, the first stage in absorption of quercetin is deglycosylation of quercetin glycosides (hydrolysis) to quercetin by bacteria present in the large intestine (colon). Though the process of deglycosylation mainly occurs in colon, some glucosidase activity occurs throughout the intestinal tract including small intestine, liver, etc. (194). There are reports of activity of β -glycosidases and lactase phlorizin hydrolase that aid in deglycoxylation of quercetin glycoseides in the small intestine (195). The release of aglycone from glycosides is also accompanied by heterocyclic fission of quercetin to phenolic acids and CO_2 by the colonic microflora. It is not clear to what extent the released aglycone resulting from the hydrolysis is absorbed by small intestine prior to degradation to phenolic acids and carbon dioxide along the gastrointestinal tract (GIT) (195, 196). Though small intestine is major site for absorption of quercetin aglycone, some absorption also occurs through colon wall. Quercetin aglycone upon deglycoxylation is mainly absorbed passively by small intestine; however, there are

reports about interaction of quercetin with sodium-dependent glucose transporter (SGLT1) in intestine making it a potential transport mechanism for quercetin absorption in intestine. In the small intestine, quercetin either undergoes O-methylation via Catechol ortho Methyl Transferase (COMT) or glucuronidation by uridine-5'-diphosphoglucuronosyltransferase (UDP-GT) or both. Quercetin aglycone, methylated quercetin derivatives and phenolic acid in the intestine then pass into the blood stream where the hepatic portal vein carries quercetin and all its derivatives to liver. Liver is the main site for quercetin metabolism, but some quercetin can also be metabolized in the absorptive cells of intestine (163), kidneys and any other tissues containing cytochrome P450 monooxygenase, methyl transferase and conjugative enzymes (197). Quercetin metabolism involves both Phase I and Phase II enzymes. Cytochrome P450 monoxygenase (CYP1A) is primarily responsible for Phase 1 reactions that includes hydroxylation and demethylation. Liver also possess COMT and β -glycosidases activity. Phase II reactions of quercetin in liver involve conjugation with glucuronide (by UDP-GT) or sulfate (sulfur transferase). Sulfation detoxification pathway occurs at low concentrations of quercetin because it is prone to get saturated easily. At the same time, glucuronidation is important for increasing molecular weight but it easily get saturated at higher concentrations. Conjugation of quercetin with glucuronidation and sulfation is an important metabolic pathway to increase quercetin's solubility and molecular weight so that it can be easily excreted in the bile. Bile is the major mechanism for excretion of quercetin. Some of the quercetin glucuronides undergo enterohepatic circulation where quercetin glucuronides or methyl quercetin glucuronides are excreted via bile into small intestine where they are deconjugated via β -glucosidase. The resultant aglycone is either

reabsorbed or degraded to phenolic acids and finally excreted in the feces as unmetabolized quercetin or bacterially degraded quercetin derivative or as quercetin metabolite. Some quercetin is also exhaled through the pulmonary route as carbon dioxide. Some amounts of quercetin and its derivatives after metabolism in the liver are routed to the kidney where they are excreted in the urine as unmetabolized, metabolized or as bacterial-degraded derivatives. Metabolites of quercetin excreted in the human urine have been identified as hydroxyphenylacetic acids, 3,4-dihydroxyphenylacetic acid, 3hydroxyphenyl acetic acid, 4-hydroxy-3-hydroxy-3-methoxyphenyl acetic acid and dimethoxyphenylacetic acid (198-203).

Quercetin possess high affinity for serum albumin with 99.4 % bound to albumin and 39 and <0.5 % quercetin bound to α_1 -acid glycoprotein and very low-density lipoproteins (VLDL), respectively (204). This is also evident from the biphasic disposition of quercetin following absorption as reported by Hollman *et al.*(187). The fact that quercetin shows very slow elimination from the body is due to its enterohepatic circulation and high affinity for plasma proteins (187). Quercetin affects the absorption of several xenobiotics as it is potent inhibitor of CYP3A4 and CYP2C9 that are enzymes that break down most drugs in the body.

Quercetin being a low solubility and low permeability compound shows poor bioavailability. Studies performed by Ader *et al.* on four pigs that received 50 mg quercetin/kg body weight showed oral bioavailability of free quercetin, conjugated quercetin, total quercetin and metabolites to be 0.5 %, 8.6% and 17 % respectively (205).

8.4 Safety concerns

Harwood *et al.* (206) provides a comprehensive review of safety of quercetin and explains the mechanisms for the observed mutagenicity of quercetin in vitro and absence of carcinogenicity of quercetin in vivo.

A number of in vitro studies were performed with quercetin under several experimental conditions to evaluate its role as an anti-oxidant or pro-oxidant. Experiments conducted in mouse thymocytes incubated with 50 µM of quercetin, exhibited anti-oxidant activity of quercetin with no induction of cytotoxicity (207). Conversely, in another set of experiments, when quercetin was tested at concentrations up to 300μ M in human lung embryonic fibroblasts, cytotoxicity was observed with increased levels of reactive oxygen species (ROS) (208). Similarly, a study conducted in human red blood cells showed that quercetin caused oxidation of oxyhemoglobin to produce methemoglobin in presence of extracellular horseradish peroxidase suggesting its pro-oxidant nature (209). Likewise, Musonda et al. (210) investigating the anti-oxidant/pro-oxidant effects of quercetin at 10 μ M concentration on nuclear factor κ B (NF- κ B), observed that the antioxidant attribute of quercetin surpasses its oxidative potential in a cell as quercetin instead of breaking of the DNA strand, inhibits hydrogen peroxide mediated DNA strand breakage and nuclear factor kappa-light-chain-enhancer of activated B cell (NF- κ B) activity. The in vitro mutagenic effect has been attributed to the pro-oxidant activity of quercetin. Reports suggest that quercetin in presence of oxidative conditions (such as hydrogen peroxide, horseradish peroxidase) degrades to form ortho-semiquinone intermediate followed by subsequent transformation into ortho-quinone which is accompanied by formation of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide, etc. The ortho-quinine then undergoes tautomerization to form orthoquinone/quinine methides that bind to DNA/proteins in presence of hydrogen peroxide/peroxidase to cause mutagenicity (211).

Despite the discouraging in vitro mutagenicity data of quercetin, quercetin occurs ubiquitously in the human diet with a wide distribution in berries, citrus fruits, leafy vegetables, roots, tubers, bulbs, herbs, leaves, legumes and cereal grains. National dietary record-based cohort assessments (from United States, Australia, the Netherland, Finland, Italy, Croatia and Japan) suggest that the mean consumption levels of quercetin are in the range of less than 5-40 mg/day. However, daily levels of quercetin by high–end consumers of fruits and vegetables especially in cases where the individuals consume the peel portion of quercetin- rich fruits and vegetables, is as high as 200-500 mg (206). Moreover, this type of diet has been consumed by generations suggesting no carcinogenic effects in human on oral administration.

Harwood *et al.* gave several explanations to explain the behavior of quercetin as a mutagenic agent in vitro as opposed to its non-mutagenic effects in vivo. According to the authors, the metabolic fate of quercetin in vivo especially the first-pass effect significantly reduces the potential for quercetin-mediated adverse effects. The authors state that that the O-methylation of quercetin in vivo eliminates the potential for formation of potential mutagens in vivo. The authors also claim that numerous protective mechanisms exist in vivo to limit the pro-oxidant activity of small quantities of systemically available unmetabolized quercetin. Also the poor bioavailability of the quercetin aglycone on oral administration prevents occurrence of saturation of the systems that provide protective mechanisms on high exposure to quercetin.

Chapter 9: Specific Aims

This part of the dissertation will describe the pharmacokinetics of quercetin in humans followed by determination of effect of quercetin in human subjects on exercise performance under various stress conditions following quercetin oral supplementation. This work is part of a project investigating the effects of quercetin supplementation in humans supervised by U.S. Department of Defense (DOD) on exercise performance in heat stress, aerobic exercise, oxidative stress, and muscle soreness. The aim of these studies is to provide sufficient evidence to the Natick Soldier Research, Development and Engineering Center, a division of U.S. Department of Defense (DOD) whether quercetin should be added to military food rations.

Specific Aim 1: Comparison of quercetin pharmacokinetics following oral and buccal supplementation in humans.

The main aim was to determine pharmacokinetics of quercetin in humans on supplementation via three oral carrier systems: Quercetin fortified Tang[®] suspension, First StrikeTM Bar and Q-chews[®]. In order to achieve aforementioned goal, HPLC assay of quercetin was developed in biological (plasma) and non biological (methanol, water, phosphate buffer saline) matrices. Thereafter quercetin content in all three carrier systems was determined. A controlled human clinical study was then designed with appropriate dosing and sampling regimen. This was followed by determination of quercetin in plasma samples from the clinical study and pharmacokinetic modeling of the obtained raw data using KineticaTM software. Finally comparison of pharmacokinetic parameters obtained from various groups that administered quercetin via 3 different carrier systems was

performed. This study was performed in collaboration with University of Massachusetts, Amherst, MA where human clinical study was performed. The validated analytical/bioanalytical assay development of quercetin and pharmacokinetic modeling were performed at Rutgers University.

Specific Aim 2: Effect of quercetin supplementation on maximal oxygen uptake in healthy, untrained, sedentary individuals.

The aim of this investigation was to test the effects of five days (six mornings) of quercetin supplementation (1 g/day) as compared to placebo in healthy, untrained and sedentary individuals on maximal oxygen uptake ($\dot{V}O_{2max}$). In order to achieve our goal, placebo controlled clinical study was designed and performed at University of Connecticut, Storrs, CT. Quercetin blood measurements were performed at Rutgers University and maximal aerobic capacity of subjects was performed at University of Connecticut, Storrs, CT.

Specific Aim 3: Effect of quercetin on exercise performance of human subjects in heat stress.

The main goal of this study was to determine the effect of quercetin in human subjects on exercise performance in the heat stress. In order to achieve this goal, a double blind placebo controlled study was designed where subjects were provided 2 g of quercetin and subjected to exercise in heat (40°C, 20-30% RH) while carefully controlling for dehydration, critical hyperthermia, and exercise duration. Quercetin measurement in blood samples of subjects were performed at Rutgers University and physiological (heart rate, rectal, mean skin, and mean body temperatures), perceptual (ratings of perceived exertion, pain, thermal comfort, motivation), and performance (total work and

pacing strategy) measurements were carried at U.S. Army Research Institute of Environmental Medicine, Natick, MA.

Specific Aim 4: Effect of quercetin supplementation on muscle soreness and recovery in humans.

The overall objective of the muscle soreness study was to examine the efficacy of quercetin in reducing muscle soreness from strenuous exercise and facilitating recovery. The specific objective was to evaluate whether quercetin administration or supplementation into a military food ration would reduce soreness, swelling, antioxidant and inflammatory markers in leukocytes and in the blood, losses of strength and range of motion, and improve recovery after exercise. This study was in collaboration with University of Massachusetts, Amherst, MA where clinical study and in vitro assays for plasma creatine kinase (CK) activity, interleukin-6 (IL-6) and C-reactive protein (CRP) were performed. Plasma quercetin content was determined at Rutgers University.

Chapter 10: Comparison of quercetin pharmacokinetics following oral and buccal supplementation in humans

10.1 Introduction

Bioflavonoids are a diverse group of naturally occurring phenolic compounds found in a wide variety of fruits, vegetables, berries, and seeds. Quercetin (3,3',4',5,7-pentahydroxyflavone) (Structure 8.2), a primary flavonoid-source in fruits and vegetables in the human diet, mainly occurs in the form of glycosides (glycone plus aglycone) with quercetin-3-rutinoside (or quercetin-3-rhamnoglucoside) being the most abundant (212). Quercetin represents the aglycone part of the glycoside or glucoside (212) molecule. Other forms of quercetin glycosides include quercetin-4'-glucoside and quercetin-3, 4'-glucoside found in onions and quercetin galactosides and arabinosides found in apples and berries, respectively (142, 212).

Quercetin has been widely studied for its anti-inflammatory, antioxidant, antiviral and anticarcinogenic properties in animals and humans (132, 139, 151, 173, 213-217). Owing to the potential therapeutic benefits of quercetin, it is important to understand the optimal dosage and carrier system that maximize quercetin uptake via buccal and gastrointestinal (GI) routes in humans. Several previous studies have examined quercetin uptake and pharmacokinetics in humans at dietary levels (218, 219); however, little is known about the bioavailability and pharmacokinetics of quercetin when delivered in the form of nutritional food products at dosage capable of causing physiological response (139, 173, 220-222). Therefore, the purpose of this study was to examine the pharmacokinetic parameters of three quercetin-fortified carrier systems with different dosage and nutrient

forms. Real FX Q-Plus[®] Soft Chews (Nutravail Inc., Chantilly, VA) and a nutrition bar (First Strike[™] bar, Natick Soldier RDEC, Natick, MA) were chosen as the carrier systems for GI/ buccal routes. A beverage formulation of quercetin mixed with Tang[®] (Kraft Foods Inc, Northfield, IL) suspended in spring water was chosen to represent GIT delivery.

10.2 Materials and methods

10.2.1 Materials

Food grade quercetin (\geq 99.5% purity) (Merck S.A. Rio De Janiero, Brazil. QU995), Tang[®] dry mix (Kraft Foods Inc., Northfield, IL), First StrikeTM Bar (NSRDEC, Natick Soldier Research, Development and Engineering Center, Natick, MA) and RealFXTM Q-PlusTM Chews/Q-chews (Nutravail Technologies, Chantilly, VA) were provided by Natick Soldier Research Development and Engineering Center (NSRDEC), Natick, MA. The compositions of the quercetin-fortified formulations are listed in Table 10.1. Fisetin (\geq 99.9% purity), β -glucuronidase (Type HP-2 from *Helix pomatia*) were obtained from Sigma Aldrich. The extraction procedure was done using a 20 Position Vacuum Extraction manifold (Waters, Milford, MA) using an Oasis HLB cartridge (Waters, Milford, MA). All other reagents and chemicals were of analytical grade.

10.2.2 Methods

10.2.2.1 In vitro testing of quercetin

HPLC analysis of quercetin: Quercetin concentrations were determined using a High Performance Liquid Chromatography (HPLC) method (Agilent Technologies, CA) with ultraviolet detection at 370 nm with 20 μ l injection volume at 1.0 ml flow rate using methanol:water (80:20) mobile phase adjusted to pH 3.72. The Phalanx C₁₈ reversed

phase column (5 μ m and 250 x 1.6 mm) (Nest Group Inc., MA) was used for chromatographic separation and maintained at 30°C. The method was tested for linearity, precision, and accuracy.

Solubility of quercetin in various solvents: The solubility of quercetin was determined in various solvents, namely phosphate buffered saline (7.4), deionized water adjusted to acidic pH with acetic acid (4.0), spring water (pH 6.3) and methanol. The procedure involved dissolving excess amount of quercetin in 20 ml of the solvents and shaking in water bath (Julabo, Fisher, PA) maintained at 37° C for 1 week until no change in solubility measurement (peak area of quercetin at 370 nm) was observed. For determination of quercetin in methanol, excess amount of methanol was shaken continuously in a shaker (Fisher Genie vortex mixer, PA) at room temperature (25° C). After one week, the supernatant from each solvent was taken, filtered using PVDF (Polyvinyledene diflouride) syringe filter of 0.45 μ size (Fisher Scientific, PA), diluted with mobile phase and analyzed by HPLC. In case of quercetin determination in aqueous medium, a two- fold dilution was made and for methanol, a 100 fold dilution was made.

*Quercetin content in RealFX*TM *Q-Plus*TM *Chews and First Strike*TM *Bars:* The procedure for determination of quercetin content in bars and chews involved weighing 1 g of the sample from each bar or chew and dissolving in 10 ml of methanol. The mixture was then shaken in a vortex mixer (Fisher Genie Vortex Mixer, Fisher Scientific, PA) for 30 minutes followed by homogenization of mixture of bar/chew and methanol using Polytron homogenizer (Kinematica, Fisher, PA). The suspension was then incubated at - 20° C overnight. Each suspension after incubation was then vortexed for 15 minutes and sonicated for 5 minutes. From each suspension, a 1 ml aliquot was shaken and

microcentrifuged (Microcentrifuge, capacity 40 ml (20X1.5/2.0 ml) (Fisher Scientific, PA) for 3 minutes. The resulting supernatant from each 1 ml aliquout was filtered using a 0.45 µm PVDF syringe filter (Fisher Scientific, PA), diluted 100 times and analyzed by HPLC as described above. Three replicates were performed for determination of quercetin in each bar or chew. The average weight of quercetin fortified bars and chews were also determined and tested for content uniformity. In order to determine content uniformity, from each bar or chew, 1 gram of sample was weighed from diagonally opposite corners and the center of a representative bar or chew and dissolved in methanol. Extraction of the quercetin from chews and bars was performed as described above.

10.2.2.2 In vivo pharmacokinetics in humans

Analysis and validation of assay of quercetin in plasma matrix: Analysis of quercetin in plasma samples was conducted using an Agilent 1100 HPLC system (Agilent Technologies, USA) with Phalanx C_{18} (5 µm and 250 x 1.6 mm) reversed phase column (Nest Group Inc., MA,). The injection volume was maintained at 20 µl with detection wavelength of 370 nm. Methanol:water (80:20, pH 3.72) was used as the mobile phase that was pumped at 0.5 ml/min. The column temperature was maintained at 30°C during the analysis. The method was tested for linearity, precision, repeatability of injection, intra-day, inter-day variability, intermediate precision, recovery and limit of detection. For linearity, a calibration plot of plasma samples spiked with various concentrations of quercetin was prepared. Blank human plasma samples (provided by University of

Massachusetts, MA) were spiked with quercetin to obtain concentrations 1, 3, 5, 50, 100,

500, 2000 and 4000 μ g/l. Blank samples once spiked were subjected to the extraction procedures as described below.

Quercetin extraction from plasma matrix: The quercetin extraction procedure was derived and modified from procedures reported in the literature (186, 223). Briefly, to plasma samples containing 10 % W/V ascorbic acid, 0.78 M sodium acetate buffer (pH 4.8), β -glucuronidase/sulfatase and 100 µg/ml of fisetin (HPLC internal standard) were added. The mixture was then incubated for 17 hours at 37°C in a shaking water bath (Julabo, CA) for the completion of the hydrolysis of quercetin glucuronides and sulfate conjugates to quercetin aglycone. The above mixture was then subsequently diluted with 0.5 M phosphoric acid solution and centrifuged at 8000 rpm for 20 minutes for clarification of the samples. The supernatant obtained was subjected to extraction on a preconditioned Oasis HLB cartridge mounted on a vacuum manifold. After addition of the supernatant, 3 ml of methanol was added to cartridge to elute quercetin retained on the cartridge. The solution containing quercetin was then evaporated to dryness under nitrogen. The residue obtained was reconstituted in 300 µl of methanol with vortex mixing for 30 sec and then subjected to HPLC analysis.

Subjects: Eighteen healthy men and women aged 18-25 years were recruited from the Amherst, Massachusetts community. The study was approved by the University of Massachusetts Amherst Institutional Review Board and Department of Defense Institutional Review Boards and written informed consent was obtained from each subject prior to participation. Subject characteristics (Table 10.2) were gathered at the morning visit on Day 1. All subjects were instructed to engage in their normal physical activity behaviors up to 72 hours prior to Day 1 and then refrain from activities other than

those required for daily living. Health status was determined by a brief health history questionnaire, and a 7-day physical activity recall was recorded on an International Physical Activity Questionnaire (IPAQ). Subjects were healthy, sedentary to recreationally active, and refrained from the use of drugs, over-the-counter medications, quercetin containing dietary supplements, and foods rich in quercetin 7-days prior to and during the study. Use of oral contraceptives was permitted.

Experimental Clinical Study Design: The study duration was two consecutive days in which subjects reported to the laboratory in a fasted state each morning. This testing took place at the University of Massachusetts Amherst campus. On Day 1, subjects were given a single 500 mg dose of quercetin, and blood levels were monitored at specific time points over a 12 hour period. On Day 2, subjects made three visits to the laboratory (once every four hours) for blood sampling. Strict control over macronutrient intake and meal timing was maintained each day by providing subjects with Meals Ready to Eat (MRETM NSRDEC, Natick Soldier Research Development and Engineering Center, Natick, MA) and requiring them to refrain from any other self-selected caloric intake until completing the study.

Supplementation: Subjects were randomized to ingest one of three quercetin formulations: 1) a beverage formulation containing 500 mg quercetin QU995 powder (Merck S.A., Brazil) mixed in 26.9 g Tang[®] dry mix (Kraft Foods Inc., Northfield, IL) suspended in 250 ml spring water (6 subjects), 2) 500 mg quercetin QU995 in a First Strike[™] bar (NSRDEC, Natick Solider Research Development and Engineering Center, MA) (6 subjects), or 3) 500 mg quercetin QU995 powder in two RealFX[™] Q-PLUS[™] chews (Nutravail Technologies, Chantilly, VA) (6 subjects).

Blood Collection: Subjects reported to the laboratory having fasted for a minimum of 12 hours and were required to remain in the laboratory for the first 12 hour period (Day 1) of data collection. A venous catheter was inserted into a forearm vein distal to the antecubital space of the elbow joint. Blood samples (~12 ml per sample) were collected into K2 EDTA vacuum containers (BD, Franklin Lakes, NJ) at baseline, 15, 30, and 45 minutes after ingestion; and 1, 2, 3, 4, 6, 8, 12, 24, 28, and 32 hours after ingestion. Samples were immediately centrifuged for 15 minutes at 1000 x g at room temperature. Plasma was separated from collection tubes and 1 ml aliquots were transferred to Nunc® cryotube vials (Fisher Scientific, USA) containing 100 μ l 10% ascorbic acid solution (mixed with distilled, deionized water) to maintain quercetin stability (188). Samples were then vortexed for 30 seconds and stored at -80 °C prior to shipment (overnight on dry ice) to Rutgers University for subsequent extraction and analysis. The samples were stable during the shipment on dry ice, extraction and analysis periods as per specifications reported by Moon *et al.* (188).

Non compartmental Pharmacokinetic Modeling: The pharmacokinetic modeling was performed using Kinetica software version 4.4.1. In absence of any intravenous data, a non compartmental, extravascular model was used to calculate the pharmacokinetic parameters. The model inputs involved 500 mg of quercetin (dose of quercetin in oral carrier systems) and the time and concentration values for each subject.

The software generated the following outputs:

L_z: Smallest (slowest disposition rate constant), slope of the elimination phase of the PK profile.

 $T_{1/2}$: Half life of elimination, ln2/lz

T_{max}: Time to each maximum concentration

C_{max}: Maximum concentration

AUC_{tot}: Area under curve in Concentration Vs Time curve, AUC_{tot} = AUC_{0-t} + C_t/L_z

The AUC was computed using the Log Linear Method, trapezoidal when $C_n > C_{n-1}$.

AUMC_{tot}: Area under moment curve, AUMC_{total} = AUMC_{0-t} + $(C_t * T_t)/L_z + C_t/(L_z)$

MRT: Mean residence time, AUMC_{total}/AUC_{total}

Cl: Clearance, (Dose/AUC_{total})

 V_{ss} : Apparent volume of distribution in the plasma compartment at steady state, (Dose.MRT)/AUC total

Vz: Apparent volume of distribution during terminal phase, Dose/(AUCtotal..Lz)

10.2.2.3 Statistical analysis

All results were statistically analyzed using SPSS software (SPSS 16, SPSS, Inc.). Oneway ANOVA was performed to compare pharmacokinetic parameters (AUC, AUMC, Clearance, C_{max} , L_z , MRT, $T_{1/2}$, T_{lag} , T_{max} , V_{ss} , V_z) of quercetin following administration of quercetin through three different vehicles followed by multiple comparison tests using Tukey HSD (Honestly Significantly Different) post hoc method at 95 % confidence interval (CI) using SPSS software (SPSS 16, SPSS, Inc.).

10.3 Results & discussion

In vitro testing of quercetin

HPLC analysis of quercetin: The HPLC assay of quercetin was developed and validated. For linearity, a calibration plot for quercetin was prepared in the range of 100 μ g/ml to 0.003 μ g/ml in triplicate and linearity was recorded in the range of 100 μ g/ml to 0.390 μ g/ml with R² value greater than 0.998 (Figure 10.1). The limit of detection (LOD) was 0.02 μ g/ml and limit of quantification (LOQ) was 0.390 μ g/ml. The run time was maintained at 8 minutes and retention time for quercetin was 4.1 minutes. The precision percent RSD (Relative Standard Deviation) was less than 0.1 percent for intraday analysis and less than 1 percent for interday analysis. The accuracy of the method was tested by analyzing different samples of quercetin at various concentration levels in pure solution and results were expressed as percent recovery. The percent recovery of quercetin in pure solutions was 98-99% with an RSD of 0.6 percent.

Solubility of quercetin in various solvents: The solubility of quercetin was determined in various solvents (Table 10.3). There was no statistical difference (p>0.05) in solubility in phosphate buffer saline, pH 7.4, water adjusted to pH 4 and spring water (pH 6.3). However, significantly greater amounts of quercetin were soluble in methanol (p<0.05).

Quercetin content in oral carrier systems: The average weight of the quercetin fortified Real FXTM Q-PLUS chews were 5.2 ± 0.02 g and First StrikeTM Bars was 35.0 ± 0.1 g. The quercetin content in Real FXTM Q-PLUSTM and quercetin fortified First StrikeTM Bar is listed in Table 10.4. We observed higher levels of quercetin present in First StrikeTM Bar (531.2 ± 32.1 mg) and Q-chews[®] (265.1 ± 11.1 mg) than expected 500 mg and 250 mg respectively. On determination of content uniformity, the variability in quercetin content within a chew and bar showed RSD (Relative Standard Deviation) of not more than 4.2 and 6.0 percent respectively. In case of quercetin Tang[®] suspension, each beverage formulation contained a premeasured amount of quercetin (500±0.04 mg).

In vivo pharmacokinetics in humans

Analysis and validation of assay of quercetin in plasma matrix: The calibration plot of quercetin in plasma matrix was prepared in triplicates (at three different times) in the range of 5-4000 μ g/l. The calibration plot (n=3) showed linearity with R² of 0.987 in the range of 5 μ g/l-4000 μ g/l (Figure 10.2). The LOQ was 5 μ g/l and LOD was 3 μ g/l. The RSD values for intraday and interday analysis were less than 2 and 12 percent respectively. The percent recovery of quercetin at all concentrations ranged from 80-85 percent. At the same time, the percent recovery of internal standard, fisetin used in the analysis was 98-99 percent. The retention times for fisetin and quercetin were found to be 7.4 and 8.5 minutes respectively in the run time of 15 minutes.

Non compartmental pharmacokinetic modeling: The concentration-time profiles of mean total quercetin in healthy subjects following administration of quercetin among the three oral carrier systems is presented in Figure 10.3. The pharmacokinetic parameters generated by non-compartmental modeling using Kinetica software are listed in Table 10.5. In descending order, the data indicate that the greatest C_{max} (~1052 µg/l) was attained within 3 hours in subjects administered RealFXTM Q-PlusTM Chews, followed by a C_{max} of ~698 µg/l within 2 hours in subjects administered quercetin-fortified First StrikeTM Bars, and lowest C_{max} (~354 µg/l) was attained with quercetin fortified Tang[®] suspension within 5 hours. On comparison of AUC_{total}, in descending order, maximum AUC was obtained in subjects that administered First StrikeTM Bars (5314.8±1432.4 µg/l.h²) followed by group receiving RealFXTM Q-PlusTM Chews (4147.1±671.8 µg/l.h²)

followed by subjects receiving quercetin fortified Tang[®] suspension (3845.9±689.8 μ g/l.h²). The half life of 8 hr was observed in First StrikeTM Bars and quercetin fortified Tang[®] suspension. However, a relatively low half life was observed in RealFXTM Q-PlusTM Chews (5.5 h). However, significant differences in the pharmacokinetic parameters were not observed between supplement groups at the 95 % confidence interval (p>0.05).

The main objective of this study was to investigate the effects of quercetin fortified nutritional supplements on quercetin pharmacokinetics at dosage capable of causing pharmacological effects(139, 173). Rim and colleagues (224) reported dietary flavonoid intake of 20 mg/day in American adult males. In our study, the dose of quercetin was 25 fold higher than that available from regular diet and was capable of causing physiological effect (173, 221). The quantification of quercetin present in each carrier system and quercetin solubility measurements were performed using a validated HPLC assay method. Quercetin fortified supplements, namely First Strike[™] Bars and Real FX[™] Q-PLUS[™] chews, were evaluated for quercetin content and were found to have certain amount of overage beyond their labeling of 500 mg and 250 mg of quercetin, respectively. In the quercetin fortified Tang[®] suspension, a known quantity of 500±0.04 mg quercetin was added to each Tang[®] beverage and overages were negligible.

The pharmacokinetic profile of quercetin delivered through various carrier systems, showed low uptake of quercetin via Tang[®] as compared to First StrikeTM and Q-ChewTM delivery carriers. Solubility of quercetin in various carrier systems is a key source of differences in quercetin uptake in humans (225, 226). Solubility of quercetin along a range of pH (4, 6.3 and 7.4) was determined that were representative of the pH of Tang[®]

suspension, spring water and blood respectively. No statistical difference (p>0.05) in quercetin solubility was observed in aqueous media maintained at those pH values. Quercetin was found to be sparingly soluble in water at neutral and acidic pH. This indicates the reason for relatively low uptake of quercetin via Tang[®] as compared to First StrikeTM and Q-ChewTM vehicles. Our finding is supported by experiments performed by Piskula *et al.*(226) that reported that quercetin's solubility in carrier systems enhances its absorption but at the same time is not a absorption limiting factor. This explains the reason for some quercetin uptake via Tang[®] in spite of sparing solubility of quercetin in Tang[®] vehicle.

Since the aim of the study was to compare concentration of total quercetin absorbed from across three nutritional supplements of interest (and not individual isolation and determination of concentrations of quercetin aglycone and quercetin conjugates), an enzymatic hydrolytic method was used to convert quercetin sulfate and the glucouronide conjugates to quercetin aglycone using β -glucuronidase derived from *Helix pomatia* extract. Subsequently the assay for quercetin was developed to determine quercetin aglycone. The quercetin extraction procedure was performed using solid phase extraction and elution of quercetin was achieved from the extraction cartridges with methanol owing to its high solubility in this solvent (~8.6 mg/ml).

The clinical study involved 18 healthy subjects that were divided into 3 groups each group receiving a 500 mg dose of quercetin in one of the formulations. The dose for each subject was not normalized according to their body weight as it was not feasible to modify the quercetin present in each formulation (as quercetin content uniformity testing in Q-ChewTM and First StrikeTM showed variability of 4.2 and 6.0 percent respectively).

However, to control for inter-individual variability due to meal timing, potential druginteractions, and exogenous sources of quercetin, subjects were provided standardized meals, refrained from the use of drugs, over-the-counter medications, quercetin containing dietary supplements, and foods rich in quercetin 7-days prior to and during the study. Nevertheless, variability was observed in the PK parameters due to high/low responders within groups receiving same treatments (Figure 10.4, Figure 10.5). Owing to inter-individual variability within a group receiving quercetin fortified formulations especially RealFXTM Q-PlusTM Chews and First StrikeTM Bars, statistical analysis at 95 percent confidence interval showed no significant difference (p>0.05) in the PK parameters among the groups receiving various quercetin fortified supplements.

Maximum variability in terms of quercetin absorption were observed in case of RealFXTM Q-PlusTM Chews and First StrikeTM Bars as compared to Tang[®]. Interindividual variability is not a rare phenomenon and has been reported in many studies (197, 227, 228). Within a group, certain individuals are better absorbers than others. For instance, in group receiving First StrikeTM Bars (Figure 10.4), UM 3 is better absorber of quercetin than UM 12. Similarly, in group receiving Q-PlusTM Chews (Figure 10.5), UM 2 is better absorber of quercetin than UM 14. The inter-individual variability is often linked with polymorphism existing in intestinal enzymes or transporters of subjects within a group (229). Quercetin is known to be absorbed by passive diffusion and a pH-dependent mechanism mediated by the organic anion transporting protein B (OATP-B)(230). There are reports of polymorphism in OATP-B transporters in studies performed in Japanese population (231). Another possibility that emerges, points to buccal absorption of quercetin that contributed to quercetin absorption more in certain subjects than others. Various bioactives such as nicotine, aspirin, etc. are known to be absorbed through the buccal route via a chewable dosage forms such as tablets, chewing gums, etc. (232). It is suggested that in high responders receiving RealFXTM Q-PlusTM Chews and First StrikeTM Bars, quercetin absorption was increased through the buccal route as compared to those volunteers receiving Tang[®]. However, further investigation is required to prove this hypothesis. Another factor for high variability in this group was the quantity of saliva in oral cavity of subjects. The amount of saliva produced while chewing increases premature swallowing thus preventing absorption from the buccal cavity (233). In individuals where less saliva is produced while chewing, there is maximum probability of absorption via the buccal route with chews (233). Therefore it is suggested that the differential mode of administration of RealFXTM Q-PlusTM Chews and First StrikeTM Bars in terms of chewing/biting/swallowing of the formulation by each individual likely contributed to the variability of quercetin absorption through buccal/GIT route. More future studies are currently being planned to test the aforementioned hypothesis.

In spite of intra-subject variability, RealFXTM Q-PlusTM chews and First StrikeTM Bars showed higher overall quercetin absorption than quercetin-Tang[®] suspension. Similar findings have been reported by Lucker *et al.* (234) who showed higher bioavailability of acetyl salicylic acid from acetyl salicylic acid chewable tablets as compared to the oral conventional tablet in humans. Our finding suggests that the dosage form/delivery form plays a vital role in the absorption of quercetin. Our findings are further corroborated by Piskula *et al.*(226) who compared quercetin solubility in solvents (water, propylene glycol and 25 % propylene glycol in water V/V) with their respective absorption profiles using rat plasma and reported that the vehicle was one of the significant factors

influencing the degree of absorption of quercetin. Another interesting finding was the quercetin fortified Tang[®] suspension showed minimum quercetin bioavailability (AUC of 3846 µg/lh) as compared to AUC for First Strike[™] Bars (5314.8 µg/lh) and RealFX[™] Q-Plus[™] Chews (AUC of 4147 µg/lh). Li et al. (235) also reported poor biovailability of quercetin via suspensions than corresponding solid lipid nanoparticle carrier systems. In principle, oral suspensions improve the rate and extent of the absorption of the active because the viscosity of the suspending agent present in the suspension increases with time and the absorption of the active is increased by improved wetting, particle deaggregation, particle surface area, improved GIT transit time and greater dispersability (236, 237). However, when a nutraceutical/bioactive such as quercetin that is sparingly soluble in water (~1.9 µg/l) is suspended in an aqueous carrier in absence of any suspending agent, rate and extent of GIT transit time is significantly decreased. Similar results were observed by Hackett and Griffiths (238) who showed that flavonoid (quercetin and catechin) absorption in rat plasma increased with co-administration with an emulsifying agent (3-palmitoyl-(+)-catechin) (226).

In carrier systems such as Tang[®], buccal absorption of quercetin is not expected because Tang[®] suspension had very low soluble amounts of quercetin available for buccal absorption. This explanation is further supported by observation of substantial amount of quercetin on the lingual tissue of subjects (yellow color observed on the tongue) receiving quercetin via Tang[®] carrier. Another observation that supports absorption of quercetin is primarily through GIT is that Tang[®] carrier showed a higher T_{max} of 4.7 hrs as compared to 2.3 hrs and 3.3 hrs observed with First StrikeTM Bars and RealFXTM Q-PlusTM Chews respectively. The relatively earlier T_{max} observed in First StrikeTM Bars

and RealFXTM Q-PlusTM Chews was due to buccal absorption accompanying its GIT absorption. Since quercetin absorption via Tang[®] carrier was primarily through GIT it showed less bioavailability as compared to First StrikeTM Bars and RealFXTM Q-PlusTM Chews. It should also be noted that PK profile of quercetin via Tang[®] carrier showed less intersubject variability (Figure 10.3 and Figure 10.6). Since quercetin absorption was mainly through GIT via the Tang[®] carrier with minimal involvement of the buccal component, different chewing and swallowing habits of individuals did not affect subject to subject quercetin absorption profile. This finding also supports our hypothesis that groups receiving First StrikeTM Bars and RealFXTM Q-PlusTM Chews showed intersubject variability within a group in quercetin absorption owing to their different swallowing and chewing habits and subsequent differential buccal and GIT absorption.

The terminal half life in the study ranged from 5-8 hrs with $T_{1/2} \sim 5$ hours being for Real FXTM Q-PLUSTM and ~8 hrs for the First StrikeTM Bars and quercetin-Tang[®] suspension. There were no significant differences between the terminal half life (or slope of elimination phase) across different groups which was expected as these parameters are pharmacokinetic characteristics of the molecule (quercetin). Similarly factors such as AUMC_{Total}, L_Z, Cl, MRT, V_{SS} and V_Z did not show significant differences across the group as they represented characteristics of quercetin pharmacokinetics. The pharmacokinetics (PK) parameters estimated from preliminary pharmacokinetic modeling studies are of physiological importance as they aid in selection of appropriate dosage regimens and carrier systems while designing quercetin efficacy studies. PK parameters also aid in estimating the extent to which bioactives are either retained or cleared by the body with time.

10.4 Conclusion

Our study is the first reporting comparisons among different quercetin-fortified nutritional supplements and the pharmacokinetics of total quercetin following their administration in human volunteers. Unlike other studies that investigated the pharmacokinetics of quercetin at dietary levels (218, 219), this study for the first time investigated the pharmacokinetics at dosage levels that are capable of causing physiological effects (220-222, 239). The data suggest that quercetin administration following RealFXTM Q-PlusTM chews and First StrikeTM Bars resulted in the high absorption as compared to quercetin fortified Tang[®] suspension due to quercetin absorption by both the GIT and the buccal route via chews and bars. The non-significant differences in the PK parameters among groups are attributed to high inter-individual variability in absorption amongst the volunteers and differences in the composition of the food matrix and solubility properties of each delivery vehicle. Finally, it can be concluded that Real FXTM Q-PLUSTM and First StrikeTM Bars are better carrier systems for clinical studies than Tang[®] as they yield high quercetin absorption in the short time intervals. But at the same time, use of Tang[®] in study design has the advantage of less inter-subject variability. Nevertheless, each oral carrier system significantly increased total plasma quercetin levels in human volunteers compared with that obtained through regular dietary intake.

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Chapter 11: Effect of quercetin supplementation on maximal oxygen uptake in healthy, untrained, sedentary individuals

11.1 Introduction

The daily consumption of quercetin in a regular U.S. diet is approximately 15-26 mg/day (140)that is usually derived from food sources such as apples, onions, berries, leafy green vegetables, hot peppers, red grapes, and black tea (140). The consumption of quercetin in diet is important as it may reduce incidence of upper respiratory tract infection (140), influenza (139), and certain forms of cancer (240). Although role of quercetin as an antioxidant and anti-inflammatory agent (241) is a well known fact, there are relatively few studies that have examined the effect of quercetin ingestion on exercise-induced inflammation, oxidative stress, exercise performance and endurance performance (139, 140, 171, 242). Report such as one by Neiman *et al* (139, 140, 171, 242) states that chronic quercetin supplementation (1000 mg/day for 3 wk) do not affect pre- to postexercise changes on markers of inflammation and immune function. However, at the same time, quercetin has shown to decrease the incidence of illness in human subjects (140), Davis, 2008 #95}. Also reports from Davis *et al.* in a mouse model have shown that short term quercetin feedings may offset the susceptibility to respiratory illness that occurs as a result of stressful exercise (139. The few studies that have been performed to test quercetin's effect on exercise performance have either shown no effect (140, 171, 243, 244) or statistically insignificant effect (172) as compared to placebo. Therefore it is difficult to make conclusions about the effect of quercetin on endurance performance. It has been observed that aerobic performance of an individual is highly correlated with one's maximal oxygen uptake $(VO_{2max})(245)$. VO_{2max} is defined as the highest rate of oxygen consumption attainable during maximal or exhaustive exercise (246). As exercise intensity is increased oxygen consumption of the body also rises. However, a stage is reached where exercise intensity can continue to increase without the associated rise in

oxygen consumption. The point at which oxygen consumption plateaus defines the V O_{2max} or an individual's maximal aerobic capacity. It is generally considered the best indicator of cardio-respiratory endurance and aerobic fitness of an individual. Aerobic power, aerobic capacity and maximal oxygen uptake are all terms used interchangeably with \dot{VO}_{2max} . \dot{VO}_{2max} is usually expressed relative to bodyweight because oxygen and energy needs differ relative to size. If quercetin improves performance, it may act by affecting one of these components especially VO_{2max} . VO_{2max} is known to improve exercise performance by increasing mitochondrial biogenesis in the brain (139). Considering the importance of maximal oxygen uptake in exercise performance, the purpose of this study was to investigate the effects of five days (six mornings) of quercetin supplementation (as compared to placebo) on \dot{VO}_{2max} in healthy, untrained and sedentary individuals. Changes in endurance performance were measured by testing V O_{2max} prior to and following dose of quercetin (1000 mg once/day for 5 days) or placebo (same number of nutritional bars with identical appearance and taste but containing no quercetin). We hypothesized that short-term (five days) quercetin supplementation would

improve $\dot{V}O_{2max}$ when compared to five days of placebo ingestion.

11.2 Materials and methods

11.2.1 Materials

Food grade quercetin (\geq 99.5% purity) (Merck S.A. Rio De Janiero, Brazil. QU995), First StrikeTM Bars (NSRDEC, Natick Soldier Research, Development and Engineering Center, Natick, MA) were provided by Natick Soldier Research Development and Engineering Center (NSRDEC), Natick, MA. The compositions of the quercetin-fortified and placebo First StrikeTM Bar are listed in Table 10.1. Fisetin (\geq 99.9% purity), β -glucuronidase (Type HP-2 from *Helix pomatia*) were obtained from Sigma Aldrich. The extraction procedure was done using a 20 Position Vacuum Extraction manifold (Waters, Milford, MA) using an Oasis HLB cartridge (Waters, Milford, MA). All other reagents and chemicals were of analytical grade.

11.2.2: Methods

11.2.2.1 Clinical study (tasks performed at University of Connecticut)

Subjects: Twelve healthy subjects (males and females), aged between 18 - 34 years, were recruited on the basis of their present physical fitness (i.e., sedentary and untrained), body dimensions (i.e., body mass index, BMI <30), and health status (no chronic or exclusionary diseases). The criterion for enrolling subjects was based on their \dot{VO}_{2max} on day 1 of the study. The required cut off for \dot{VO}_{2max} on day 1 in subjects was <50 ml/kg/min. Beside signing an informed consent document that was approved by the Institutional Review Board for Studies Involving Human Subjects, subjects were also required to complete a physical activity and medical history form. Subjects were also evaluated for absence of contraindications to maximal exercise. The following calculation was used to check whether sample size of subjects in the study was sufficient to yield good statistical study design. Assuming subjects had a baseline \dot{VO}_{2max} of
approximately 40 ml/kg/min, a final sample size of 10 subjects was sufficient to detect a

~6.5% increase in $\dot{V}O_{2max}$. Using 2 ml/kg/min as the SD of $\dot{V}O_2$ measurements (247), these calculations provide a statistical power of 0.8 with a two-tailed t test (alpha = 0.05) http://stat.ubc.ca/~rollin/stats/ssize/n2.html).

Prior to experimental testing (Day 0), subjects were familiarized with the testing procedures involved in the study. Body mass ± 50 g (Healthometer® model 349KL, Healthometer, Inc., Bridgeview, IL), height ± 0.1 cm, and 3-site skinfolds of all subjects before the commencement of testing procedures (248) were measured. Percent body fat was estimated from standard equations that were appropriate to the test subject sample (248-250). Subjects were required to reveal all use of nutritional supplements, alcohol, drugs, medications, and stimulants during the 30 days prior to testing and during testing. Subjects were instructed to avoid consumption of food and drink rich in quercetin

throughout enrollment. Most importantly, each subject was then familiarized with VO_{2max} testing procedures by walking and running (10 and 5 min, respectively) on a treadmill while breathing through a mouthpiece and wearing a nose clip that was utilized during subsequent visits.

Study design: A randomized, double-blind, counter-balanced cross-over research design was employed. The study involved 3 phases. In the first phase subjects either administered one of the two kinds of First StrikeTM bar (quercetin fortified or placebo). The first phase lasted from Day 0 to Day 6. This was followed by the wash out phase that was from Day 7 to Day 28. The third phase involved treatment opposite of first phase

testing (i.e., if placebo was ingested in the first phase, quercetin-supplementation occurred in the second phase and vice versa) and lasted from Day 29 to Day 34.

After baseline testing (Day 1), subjects were randomly selected to ingest either a quercetin-supplemented First StrikeTM Bar (NSRDEC, Natick Soldier Research, Development and Engineering Center, Natick, MA) (1000 mg/day for 5 days) or a placebo (no quercetin) First StrikeTM Bar (NSRDEC, Natick Soldier Research Development and Engineering Center, Natick, MA). Since each quercetin fortified First StrikeTM Bar contained 500 mg of quercetin so each subject was randomly assigned 2 First StrikeTM Bars that was either fortified with quercetin or contained no quercetin (placebo First StrikeTM Bar). Subjects administered two assigned bars after baseline testing (Day 1) and for five consecutive mornings (Days 2-6). Subjects were required to ingest the assigned bars only in the presence of investigators each morning from Days 2 to Day 6.

To evaluate the effect of quercetin supplementation, subjects, on day 6, repeated baseline

 \dot{VO}_{2max} testing. The subjects then completed a 22-day "washout" period (Days 7-28) in which food bar ingestion did not occur.

Females enrolled in the study, began each phase of testing in approximately the same phase of their menstrual cycle (follicular phase). On the day of familiarization (Day 0) females were required to fill out an additional form called the self-reported menstrual history questionnaire. Females began phase two of testing approximately 22 days after the beginning of phase 1 (Day 29). In order to standardize testing between genders, males also completed a 22-day "washout".

Subjects reported to the laboratory on day 29 to repeat baseline \dot{VO}_{2max} testing and begin phase two of the cross-over design. They repeated the procedures of food bar ingestion for 5 days (6 mornings, Days 29-34), but were assigned the treatment opposite of first phase testing (i.e., if placebo was ingested in the first phase, quercetin-supplementation occurred in the second phase and vice versa).

Subjects then reported on day 34 to repeat \dot{VO}_{2max} testing. Treatments were randomly assigned but counter-balanced so that an equal number of subjects received placebo and quercetin-supplementation in each phase of testing.

Measurement of \dot{VO}_{2max} : Prior to reporting for \dot{VO}_{2max} testing (Day 1, 6, 29, and 34), each subject consumed a prescribed amount of fluid (in excess of usual fluid intake), to aid in euhydration. The fluid regimen involved drinking 16 ounces (470 ml) of water after dinner (night before testing) and 16 ounces (470 ml) of water immediately upon waking (on the morning of testing). Subjects performed only light exercise during the 24 hours prior to each testing day. Upon arrival to the laboratory for \dot{VO}_{2max} testing (Day 1, 6, 29, and 34), subjects provided a small urine sample for assessment of hydration status (via a hand-held refractometer -Model A300CL, Spartan, Japan) and urinary quercetin levels. A urine pregnancy test was administered twice during this study for females (Days 0 and 29). On Days 1, 6, 29 and 34, subjects were fitted with a heart rate monitor (Model S150, Polar Instruments Inc., Woodbury, NY, HR) and began a treadmill test to measure maximal aerobic capacity (\dot{VO}_{2max} test). Throughout the \dot{VO}_{2max} test, expired air was collected via a Hans-Rudolph mouthpiece integrated with a calibrated metabolic cart

(ParvoMedics, Sandy, Utah). To ensure all expired air was collected, subjects also wore a

noseclip. The \dot{VO}_{2max} test started with the subjects walking on a treadmill (Model C9561, Precor, Inc., Woodinville, WA) at 3 mph at 0% grade for 3 minutes. Each successive incremental bout was 2 minutes in duration. After walking, the subject began a 2 minute run (5 mph for women, 6 mph for men). If the subject's HR rose above 150 bpm during the level jog, the treadmill speed remained the same (5mph/6mph) for the remainder of the test (n = 5 males, 5 females). If the HR remained below 150 bpm, the treadmill speed increased 1-5 mph, based on our evaluation of HR (n = 1, female). Once the treadmill speed was chosen, it remained the same for the duration of the test. The subject then ran at continuous 2 minute bouts at grades of 5%, 7.5%, 10%, and 12.5% until a plateau in oxygen consumption (< 150ml with increase in grade) was reached or volitional exhaustion occurred. Exercise tests were terminated if clinical signs and symptoms indicate that a participant's health or safety was compromised. After a 20-minute rest, the

subject again ran on the treadmill, to verify his or her \dot{VO}_{2max} . This involved running for about two minutes, at a treadmill grade 2.5% greater than the final treadmill setting (same speed) at which he or she finished previously. The procedure used was a modification of

the methods described by Taylor *et al.* (251) and Mitchell *et al.* (252). \dot{VO}_{2max} was defined as the average of the two highest 20-sec values measured.

In addition to oxygen consumption, tidal volume (V_T), respiratory rate (RR), expired volume (V_E), and respiratory exchange ratio (RER) were measured. Every 2 min of exercise and at exhaustion, HR, rating of perceived exertion (RPE) (253) and perceived leg pain (254) were measured. At exhaustion a small blood sample (< 2 ml) was collected

via a finger-stick to assess blood lactate with a YSI 2300 Stat Plus Analyzer (Yellow Springs Instruments, Inc., Yellow Springs, OH). All exercises occurred in a mild environment (approximately 21°C).

On days 1-4, 6-9, 29-32, and 34-37, (at 10 min, 24-, 48-, and 72-hours after each \dot{VO}_{2max} test), delayed onset muscle soreness (DOMS) was assessed by using a pencil-paper scale with a 10 cm line anchored with "No Soreness" and "Unbearable Pain" on the left and right, respectively.

Upon arrival on Days 1, 3, 6, 29, 31, 34, a 12 ml blood sample was collected via venipuncture by a trained, experienced phlebotomist, utilizing sterile technique. Each blood sample was collected prior to food bar ingestion with the exception of Day 6 and

34. On days 6 and 34 (\dot{VO}_{2max} number 2 and 4), subjects consumed two assigned food bars two hours prior to arrival. Venipuncture blood samples were collected into EDTA coated blood tubes and immediately centrifuged. One milliliter plasma samples were then aliquoted into Eppendorf[®] tubes, each with 100µl of 10% ascorbic acid as a preservative. One milliliter urine samples, collected on the same days as blood, were aliquoted into Eppendorf[®] tubes, each with 100µl of 10% ascorbic acid and 500µl of acetic acid as preservatives. Plasma and urine samples were labeled with a unique numeric code (whose identity is known only to investigators) and frozen at -80°C until analysis (within 6 months of acquisition).

*Nutritional First Strike*TM *Bars Ingestion*: On Days 1 and 29, subjects ingested two nutritional First StrikeTM Bars after completing the $\dot{V}O_{2max}$ test. But on Days 6 and 34, subjects consumed two nutritional First StrikeTM Bars 2 hours prior to the $\dot{V}O_{2max}$ test.

For the four days after each baseline VO_{2max} test in each supplementation phase (Days 2-5, 30-33) subjects reported to the laboratory, and in the presence of investigators ingested two nutritional First StrikeTM Bars each 35 g in weight. Depending on assignment (see above), subjects ingested either two First StrikeTM Bars without quercetin (placebo group) or two nutritional First StrikeTM Bars with 500 mg of quercetin added per bar (1,000mg total per day, supplement group). This level of supplementation has been used in similar studies (140, 171). Bars, indistinguishable in taste and appearance, were only identifiable with an alphabetical code that was unknown to investigators and subjects (double blinded study). This code was broken only after all experiments were completed. Quercetin was administered via First Strike Bar since this is able to deliver relatively higher concentrations of quercetin in a shorter duration of time via buccal and GIT routes (Chapter 10). The placebo First StrikeTM bars were identical to the quercetin supplemented First StrikeTM bar except for presence of quercetin (Quercegen, Inc.).

11.2.3 Analysis of quercetin in blood and urine (tasks performed at Dr. Michniak-Kohn laboratory at Rutgers University)

Preparation of calibration plot: Blank plasma samples were spiked with 20 and 100 µg/ml quercetin stock solution to obtain plasma samples with quercetin concentrations of 5 to 8000 ng/ml. Similarly blank urine samples were spiked with quercetin to obtain concentrations of 3.9 to 2000 ng/ml. Blank samples were extracted and analyzed in the same fashion as unknown samples (described below). The calibration plot was tested for linearity, precision, repeatability of injection, intra-day, inter-day variability, intermediate precision and limit of detection.

Processing of unknowns: To each plasma and urine sample (1ml), 110µl of 0.78M sodium acetate (Sigma, USA) buffer (pH 4.8) was added. This was followed by addition of 40µl of β -glucuronidase/sulfatase derived from Helix pomatia, HP-2 and 20µl of 100µg/ml of fisetin (Biochemika, Fluka) used as an internal standard. The internal standard stock solution contained 5 mg of fisetin in 50 ml of methanol. The mixture was then incubated for 17 hours at 37°C. Each test tube was then subsequently diluted with 2ml of 0.5M phosphoric acid solution and centrifuged at 8000 rpm for 40 minutes. From each sample 2.7 ml was extracted on a preconditioned Oasis HLB cartridge. The cartridge was placed on vacuum manifold before loading the samples. The plasma samples were drawn at a flow rate of 0.2ml/min. The cartridge was then washed by adding and drawing 1 ml of 5% methanol in 0.5M phosphoric acid solution. Then the rack containing collection vessels was inserted and vacuum was applied. Three ml of methanol was added to the cartridge and elutes were collected. The flow rate of the elution from the cartridge was maintained at 0.2 ml/min. The solution was then evaporated to dryness under nitrogen; the residue obtained was subsequently dissolved in 1 ml of methanol. After reconstitution of the residue in methanol, the sample was again evaporated under nitrogen. The residue obtained in the second stage was dissolved in 300µl of methanol with vortex mixing for 30 sec. This solution was then analyzed via high-performance liquid chromatography (HPLC). High-performance liquid chromatography separation was performed using methanol: water (80:20 v/v, pH at 3.72) and a C_{18} column (particle size: 5 µm and 250X1.6 mm) with a 20-µl injection volume at a flow rate of 0.5 ml/min at 30°C.

11.2.4 Statistical analysis (tasks performed at University of Connecticut)

Changes in physiological variables measured pre- and post-supplementation (Day 1, 6, 29

and 34) (e.g., \dot{VO}_{2max} , RER) were evaluated with a two-way repeated-measures two-way (condition × time) analysis of variance (ANOVA). Plasma and urine quercetin changes also were evaluated with a repeated-measures two-way (condition × time) ANOVA. Although direct comparisons between male and females lacked statistical power (male n=5, female n=6), exploratory between-subject factor-analysis was performed on select variables. Greenhouse–Geisser corrections were made when the assumption of sphericity was violated. Bonferroni corrections with post hoc t-tests were used to determine pairwise differences in the event of a significant F-ratio. Alpha was set at 0.05 for all statistical comparisons. All values are reported as mean \pm standard deviation (SD).

11.3 Results and discussion

Subjects: One male subject dropped out of the study due to time constraints and therefore data analysis was performed on 11 subjects (n = 5 males; n = 6 females). None of the subjects exhibited or self-reported side effects due to nutritional bar consumption. Average age, height, BMI, and % body fat of the subjects recorded were 19.8 ± 3.8 y, 171.4 ± 9.5 cm, 23.0 ± 3.1 kg/m², and 19.4 ± 8.1 %, respectively.

Subjects recruited in the study had similar mass with no statistically significant difference among the individual masses (average mass: 68.30 ± 11.15 kg, P = 0.747). Body masses on the day of each \dot{VO}_{2max} were not significantly different (P>0.05) and were independent of condition (i.e., non-significant condition × time interaction, P = 0.633). Mean urine specific gravity prior to each \dot{VO}_{2max} test was 1.018 ± 0.008. Although there was a significant interaction of condition × time for urine specific gravity (P = 0.030), followup tests revealed non-significant differences between condition (P = 0.246) and over time (P = 0.185).

Physiological responses (Table 11.1): Order-effect analysis revealed that irrespective of the condition, $\dot{V}O_{2max}$ did not change over time when expressed in absolute (P = 0.069) or relative (P = 0.059) terms. This absolute $\dot{V}O_{2max}$ value does not take into account differences in body size, however, relative $\dot{V}O_{2max}$ takes body size into account for calculation. The interaction between condition and time for $\dot{V}O_{2max}$ was non-significant when expressed in absolute (L/min, P = 0.929) and relative (ml/kg/min, P = 0.778) terms (Table 11.1, Figure 11.1). These findings were similar when taking gender of the subject

into account (L/min, P = 0.584; ml/kg/min, P = 0.524). The mean differences in \dot{VO}_{2max} change from pre to post between groups (quercetin – placebo) was 0.139 ml/kg/min (p = 0.780, $\eta^2 = 0.008$, 95% CI: -0.939 to 1.217). Specifically, the pre to post change in the quercetin group was -0.01 ± 0.07 L/min (p = 0.422, $\eta^2 = 0.065$, 95% CI: -0.040 to 0.018). The pre to post change in the placebo group was -0.01 ± 0.04 L/min (p = 0.547, $\eta^2 = 0.037$, 95% CI: -0.062 to 0.035).

In order to check for the achievement of maximal aerobic capacity, different cardiopulmonary tests such as tidal volume (V_T), respiratory rate (RR), expired volume (\dot{V}_E), respiratory exchange ratio (RER), HR and rating of perceived exertion (RPE). There was a lack of significant interaction (condition × time) for V_T, RR, and \dot{V}_E at \dot{V} O_{2max} (P = 0.285, 0.134, and 0.727, respectively, Table 1). RER at $\dot{V}O_{2max}$ was not significantly different over time or between condition (i.e., non-significant interaction, P = 0.580). Specifically, there were no significant differences in RER for condition or time (pre- vs. post supplementation; P = 0.294 and 0.675, respectively). These findings were similar when taking gender into account (P = 0.452).

Blood lactate at $\dot{V}O_{2max}$ was not significantly different over time or between conditions (i.e., non-significant interaction, P = 0.417, η^2 = 0.067, Table 1). There were no significant differences in blood lactate for condition or time (pre vs. post supplementation; P = 0.849 and 0.826, respectively). HR_{max} was not significant between trials (P = 0.752, η^2 = 0.010).

The duration of each $\dot{V}O_{2max}$ test after supplementation with quercetin fortified First StrikeTM Bar was not significantly different after administration with placebo bar (P = 0.723). Specifically, there were no differences for condition or time period (pre- vs. post supplementation) (P = 0.589 and 0.526, respectively). These findings were similar when taking gender of the subjects into account (P = 0.493).

Perceptual responses (Table 11.1): There was a lack of significant interaction (condition × time) for perceived muscle pain at \dot{VO}_{2max} (P = 0.661, η^2 = 0.020). Similarly,

RPE at $\dot{V}O_{2max}$ was not significantly different over time or between conditions (i.e., nonsignificant interaction, P = 0.432, $\eta^2 = 0.063$).

Delayed onset muscle soreness (DOMS) (Table 11.2): The ingestion of placebo nutrition bars did not influence ratings of DOMS after each $\dot{V}O_{2max}$ test (i.e., similar ratings for pre- and post 5-day ingestion, P = 0.929). Before and after placebo bar

ingestion, DOMS ratings were similar 10 min and 24 hour post \dot{VO}_{2max} test (P = 0.078).

However 24hr to 48hr post VO_{2max} test, DOMS decreased (P = 0.017), and remained depressed through 72hr post (P = 0.073).

A paired-samples t-test revealed that 5-days of quercetin supplementation did not influence DOMS 10 min post $\dot{V}O_{2max}$ testing (P = 0.888). After rating the 10-min DOMS on day 1, subjects began quercetin-supplementation. Supplementation did not influence the decrease in DOMS over the next 72 hours (i.e., non-significant interaction of condition × time, P = 0.876).

After 5 days of supplementation, subjects repeated \dot{VO}_{2max} testing and rated DOMS over the next 3 days while not consuming nutritional bars. Five days of prior supplementation did not influence DOMS ratings for the 10 min to 72 hr post \dot{VO}_{2max} testing (i.e., nonsignificant interaction of condition × time, P = 0.088).

Plasma and urine quercetin concentrations (Tables 11.3 & 11.4): Quercetin present in plasma and urine samples were extracted by solid phase extraction and analyzed by HPLC. The assay of quercetin in plasma and urine was validated and checked for linearity, accuracy and precision. The calibration plot in blank plasma spiked with quercetin (n=3) showed linearity with R^2 of 0.99 in the range of 5ng/ml-8000ng/ml (Figure 11.2). The calibration plot of quercetin in blank urine spiked with quercetin in the range 3.9-2000ng/ml was also prepared in triplicate. The calibration plot (n=3) showed linearity with R^2 of 0.99 ml (Figure 11.3). For plasma samples, the LOQ was 5 ng/ml and LOD was 3 ng/ml. For urine samples, LOQ was

found to be 3 ng/ml and LOD was 2 ng/ml. The RSD values for intraday and interday plasma and urine analysis were less than 4 and 9 percent respectively. The percent recovery of quercetin at all concentrations in plasma and urine ranged from 80-85 percent. At the same time, the percent recovery of internal standard, fisetin used in the analysis was 98-99 percent. The retention times for fisetin and quercetin were found to be 7.4 and 8.5 minutes respectively in the run time of 15 minutes.

There was a significant interaction in quercetin plasma concentration (p < 0.001). Followup tests revealed that there was no significant difference between placebo and quercetin supplementation on day 3 (p = 0.341), but plasma quercetin levels were greater with quercetin versus placebo supplementation (p < 0.001) on day 6. Plasma quercetin levels significantly increased over time (P < 0.01, η^2 (percentage of variability) = 0.813); the increase from day 1 to day 3 was non-significant (p = 1.000), but the increase from Day 3 to Day 6 was significant (P < 0.001, 95% CI). There were no gender differences in plasma quercetin levels (P = 0.543).

There were no overall differences in urine concentrations of quercetin between genders (P = 0.964). There was a significant interaction between condition and time (P = 0.004). Follow-up tests revealed that there urine quercetin levels on day 3 and 6 were greater with supplementation versus placebo (P = 0.042, 95% CI and P = 0.002, 95% CI for day 3 and day 6, respectively). There was significant change in urine quercetin levels over time with supplementation (P = 0.006), but a non-significant change in urine quercetin levels over time with supplementation (P = 0.006), but a non-significant change in urine quercetin levels from day 1 to day 3 (P = 0.983). There was a significant increase from day 3 to day 6 (P = 0.020).

The purpose of this investigation was to test the effect of five days (six mornings) of quercetin supplementation (as compared to placebo) on $\dot{V}O_{2max}$ in healthy, untrained, sedentary individuals. We found that there was a lack of condition × time interaction, non-significant differences in the pre- to post-changes between conditions, and non-significant changes within each group. Further, these results were consistent across genders. Therefore, our data do not support the hypothesis that short–term (five days)

quercetin supplementation improves \dot{VO}_{2max} .

Subjects, in each condition, either reached a plateau in oxygen consumption with increase in grade and/or exercise until volitional exhaustion. The achievement of $\dot{V}O_{2max}$ was confirmed by high measures of RER, blood lactate, HR, and RPE at $\dot{V}O_{2max}$ (Table 11.1) (255). In order to confirm a plateau in oxygen consumption, subjects performed a followup test to exhaustion (~20 min after the initial test) at a treadmill grade which was 2.5% greater than previously achieved. The lack of change in $\dot{V}O_{2max}$ with placebo consumption and non-significant order-effect further confirm that subjects were able to

reach \dot{VO}_{2max} in each testing session.

Immediately after each VO_{2max} test, and for the following three days, subjects rated DOMS. Quercetin supplementation over that same time period may reduce the inflammatory response observed after intense exercise and lower perceptual ratings of muscle soreness (140). Our data show that although DOMS ratings decreased over the three day period, quercetin did not influence these ratings (Table 11.2). Further, five days

of prior quercetin supplementation had no effect on DOMS ratings after the postsupplementation $\dot{V}O_{2max}$ test. Using a similar scale Nieman *et al.* (140) observed a similar lack-of-response for quercetin supplementation. DOMS ratings decreased in a similar fashion (independent of condition) after an arduous foot race.

Subjects were tested after 5 days of nutritional bar consumption. Post-testing occurred on the 6th morning 2 hours after food-bar ingestion, when quercetin plasma levels were high (Table 11.3). Therefore we tested the acute effect of quercetin consumption preceded by 5 days of chronic consumption. Although quercetin may accumulate in several different organs (i.e., lungs, testes, kidney, liver, thymus and muscle) (256), its half-life in the blood can range from 4-28 h (188, 257). In the present study, blood collected on day 3 (approximately 24 hours after the previous food bar ingestion) had very low levels of quercetin, indicating clearance from the blood. Plasma quercetin levels measured two hours post consumption on day 6 were peak levels (188). These values are higher than previously reported (140), but this may be due to consumption of nutritional bars in one

sitting (versus splitting into multiple sittings). Regardless, these high values during V O_{2max} testing would likely only aid in quercetin exerting any possible acute, positive effects (140).

Our data show that any improvement in endurance performance due to quercetin supplementation is not due to increases in $\dot{V}O_{2max}$. MacRae and Mefferd (172) hypothesized that improvements in cycling performance were result of quercetin exerting anti-inflammatory properties and thus protecting skeletal muscle protein and facilitating motor-unit recruitment. Others have hypothesized that quercetin may increase $\dot{V}O_{2max}$ by increasing mitochondrial gene expression in the brain (139). Any increases in brain gene expression have not been shown to influence muscle mitochondria. Regardless, if there were any increase in muscle mitochondrial number or enzyme activity, it did not result in

increment of \dot{VO}_{2max} .

11.4 Conclusions

We demonstrated in a double-blind crossover study design with a washout period that 5

days (6 mornings) of quercetin supplementation (1000mg/day) did not influence $\dot{V}O_{2max}$, ratings of muscle soreness, or other related physiological variables. It is suggested that future studies should investigate the effect of quercetin in a variety of exercise settings and subject populations.

Chapter 12: Effect of quercetin on exercise performance of human subjects in heat stress

12.1 Introduction

Endurance exercise performance is known to get impaired with increase in ambient temperature (258, 259). Exercise in hot environments poses challenges not only to the cardiorespiratory and locomotive systems of the body but also to the brain (260). CNS fatigue is one such challenge that is provoked by excessive exercise in heat (260). Pharmacological studies have provided evidence about the involvement of dopamine in causing central nervous system fatigue. It is known that enhanced dopaminergic activity counteracts central fatigue and therefore improves the performance in heat (260). Dopamine release in the CNS is regulated by various dopamine receptors including adenosine receptors (261). Adenosine receptors in presence of adenosine antagonists cause dopamine release in the brain by inhibition of A_1 receptors (261, 262). Since adenosine receptor antagonism can increase dopamine release (263) and may reduce heat strain (264), one can enhance endurance exercise performance in the heat by dopamine antagonism. The simplest, convenient, safe and efficacious manner to achieve adenosine antagonism is via dietary plant derived compounds. Adenosine receptors are the molecular targets for a variety of the dietary plant derived compounds such as methyl xanthines (e.g. caffeine, theophylline) and flavonoids (e.g. quercetin)(174).

Caffeine (1, 3, 7 – trimethylxanthine) is a nutritional adenosine antagonist (263) that has shown efficacy in enhancing endurance exercise performance in humans when consumed over a wide range of doses (265, 266). Like caffeine, quercetin is another nutritional adenosine antagonist that belongs to flavonoid class of compounds (174). Quercetin's in vitro affinity for adenosine receptor subtypes has been reported to be of the same or greater magnitude than caffeine (174, 267). This suggests that quercetin could mimic or surpass the effects of caffeine on performance via an adenosine antagonism. Moreover, quercetin possesses numerous health benefits including acting as an antioxidant (134), an anti-inflammatory agent (151) and also acting as agent for reducing viral illness(140). This makes quercetin an attractive alternative to caffeine provided that it conveys similar performance benefits. Although quercetin has been investigated for it effects on exercise performance(140), there are no reports about evaluation of quercetin for endurance exercise in heat in humans under controlled conditions (administration of quercetin alone as the bioactive in the formulation with well defined duration and type of exercise regimen).

The purpose of this study was to determine the effects of acute quercetin ingestion in human subjects on endurance exercise performance in the heat. Our hypothesis was quercetin would improve performance. The exercise task and duration were selected with the purpose of testing the aerobic energy system of the subjects while carefully preventing dehydration of the subjects and minimizing the influences of energy depletion and critical hyperthermia on performance.

12.2 Materials and methods

12.2.1 Materials

Food grade quercetin (\geq 99.5% purity) (Merck S.A. Rio De Janiero, Brazil. QU995) and First StrikeTM Bars were provided by Natick Soldier Research Development and Engineering Center (NSRDEC), Natick, MA. The compositions of the quercetin-fortified and placebo First StrikeTM Bars are listed in Table 10.1. Fisetin (\geq 99.9% purity), β - glucuronidase (Type HP-2 from *Helix pomatia*) were obtained from Sigma Aldrich. The extraction procedure was performed using a 20 Position Vacuum Extraction manifold (Waters, Milford, MA) using an Oasis HLB cartridge (Waters, Milford, MA). All other reagents and chemicals were of analytical grade.

12.2.2 Methods

Subjects: Ten healthy, non-heat acclimated male volunteers [age 23 \pm 6 yr, body mass 77.5 \pm 9.1 kg, height 177.9 \pm 6.6 cm, body fat 12 \pm 3%] were enrolled in this study and completed all phases of experimentation. Subjects recruited were physically active and moderately fit with mean maximal oxygen uptake (\dot{VO}_{2max}) of 45.2 \pm 4.6 ml/kg/min. The subjects were refrained from quercetin containing dietary supplements, and foods rich in quercetin 4-days prior to and during the study. Volunteers were also asked to abstain from physical activity and alcohol consumption 24 hours before testing. The study design was approved by Department of Defense Institutional Review Board. Subjects were provided informational briefings and gave voluntary, informed written consent to participate in the study. Investigators adhered to Army Regulation (AR) 70-25 and U.S. Army Medical Research and Materiel Command Regulation 70-25 on the use of volunteers in research.

Preliminary Procedures: Two-weeks of preliminary testing preceded the clinical study.

Maximal oxygen uptake (\dot{VO}_{2max}) and maximal workload (Wmax) was measured in all subjects using an incremental cycle ergometer protocol with continuous gas-exchange measurements (True-Max, ParvoMedics, Sandy, UT). These preliminary experiments were used to calculate workload at 50% \dot{VO}_{2max} . The ergometer used (Lode Excalibur

Sport, Lode, Groningen, The Netherlands) was equipped with pedal-rate-independent (hyperbolic) and -dependent (linear) cycling modes. Initially, linear factors (LF) were calculated [W = LF x (rpm)²] for each volunteer (where W is workload in Watts). L was based on each subject's Wmax and calculated, so that 50 % Wmax was produced at a pedaling rate of 60 rpm. This LF setting was used to calculate workload during the time trial at ~100 rpm. Volunteers performed an average of three (range of 2 to 5) familiarization trials, including one while fully instrumented, in order to reduce training and learning effects (268, 269). Familiarization trials began with 30-min of steady-state

cycle ergometry at 50% of \dot{VO}_{2max} (hyperbolic mode). This was followed by a brief 5 minute rest period, after which a 15-min time trial (linear mode) was completed. These sessions mimicked experimental trials in every way except for dietary supplement intervention and environmental conditions (20-22° C). During the time trial, elapsed time was given at standardized times of 5, 10, 13, 14 minutes, 30-seconds, and the final 10-seconds. Once completed, volunteers were provided with feedback on their performance, defined as the total amount work (kJ) completed in 15 min, as motivation to improve with each subsequent training bout. During this 2-wk period, volunteers were also introduced to and practiced using the perceptual scales during the steady-state portion of the familiarization trials.

During the two-week training period, volunteers reported each morning after an overnight ~8 hour fast for first-void urine specific gravity (USG) and nude body mass measurements. To ensure proper hydration, 30ml/kg of fluid electrolyte beverage was provided supplemental to *ad libitum* fluid intakes and was directed for consumption the

evening before each training day. An average of the 5-10 days of nude body mass measurements were calculated and used as a baseline reference for euhydrated body mass determinations.

Experimental procedures: Volunteers were randomly assigned to complete two experimental trials [quercetin (Q), placebo (P)] separated by 5-7 days each. All experiments were conducted at the same time of day to control for circadian fluctuations in body temperature and other biological variables (270). The elapsed time between trials was also considered adequate to prevent heat acclimation (271). Volunteers drank 30 ml/kg of fluid electrolyte beverage the night before each test and arrived the next morning after an overnight fast for nude body mass and first-void USG analysis. Volunteers with a combination of any two USG < 1.02, nude body mass within 1% of the two week average, or plasma osmolality < 290 mOsmol/kg H₂O were considered euhydrated (272). A fasting blood sample was drawn (baseline), after which volunteers were given a standardized breakfast including four First StrikeTM Bar (placebo or quercetin fortified) and water. A second blood sample was drawn one hour post-breakfast (pre-exercise). Volunteers were then instrumented in the antechamber (~10 min) and weighed immediately upon entering the hot test environment (40°C, 20-30% RH). Following a 20 minute stabilization period, 30 minutes of steady-state cycle ergometry

(pedal rate independent) was completed at exercise intensity equivalent to 50% VO_{2max}. The combination of environmental and exercise heat loads for this phase of the study was, by design, calculated to keep rectal temperature (T_{re}) below laboratory safety cut-off of 39.5°C. This minimized the potential for having to stop the ensuing time trial as a

result of reaching T_{re} safety limits. Drinking was not permitted during exercise due to esophageal temperature measurements, but a 5-7 minute break followed wherein subjects were weighed and re-hydrated to within 1% of the instrumented body mass measured previously upon chamber entry. Volunteers then completed a 15-min performance time trial (pedal rate dependent, pedal cadence set to 100 rpm) as previously described for familiarization sessions. Pedal cadence and workload were blinded so that only elapsed time was known during the time trial and no motivation was provided. A third blood sample was drawn immediately upon time trial completion (post-exercise) that 2.5 hours post ingestion of quercetin. Performance was assessed as the total work (kJ) completed in 15 minutes and calculated using the formula [W = LF x (rpm)²] where LF values of individual subjects were calculated in the preliminary procedures.

Body mass was measured nude with an electronic precision balance scale (WSI-600, Mettler Toledo, Columbus, OH, accuracy \pm 50g) before and after breakfast, as well as after the exercise session. Fully-instrumented body mass was measured in the chamber before and after steady-state exercise to determine fluid needs in the rest period preceding the time trial. Gas-exchange measurements were made once after the initial 15 minutes of steady-state exercise using an automated system (TrueMax, ParvoMedics, Sandy, UT). Heart rate (HR) (Polar a₃, Polar Electro Inc, Woodbury, NY) was recorded at 5-minute intervals, as was rectal temperature (T_{re}) obtained from a telemetric temperature sensor (JonahTM core body temperature capsule, Mini Mitter Inc, Bend, OR) inserted 8-10 cm beyond the anal sphincter. Simultaneous pilot testing of this approach against a conventional rectal probe yielded excellent agreement ($\leq 0.05^{\circ}$ C difference; n = 3) and the techniques were considered equivalent. Mean weighted skin temperature (T_{sk}) was calculated according to Ramanathan (273). Ratings of perceived exertion (RPE) and pain (RP) (274) were measured serially using the appropriate numerical scales anchored by verbal descriptors. Rating of motivation (RM) (275) and thermal comfort (RTC) (276) were also measured using a continuum between verbal anchors, having volunteers mark along a line, and measuring the distance in mm. Skin temperature was monitored continuously at the left chest, arm, calf, and thigh using thermistors (YSI, Yellow Springs, OH) linked to a data acquisition system.

The breakfast each day consisted of four cranberry-flavored energy bars (total energy = 557 kcal; 78% carbohydrates, 18% fats, 4% proteins), similar to commercially available sports bars. Other ingredients (sodium, potassium, chloride, calcium, vitamin C, and vitamin E) were well below ordinary U.S. Dietary Reference Intake levels. The quercetin fortified First StrikeTM Bar was the carrier for food grade quercetin powder (QU995, Quercegen Pharma, Newton, MA) equal to 500 mg per bar (2,000 mg total). The placebo First StrikeTM Bar contained no quercetin. Volunteers received the placebo First StrikeTM Bars and quercetin fortified First StrikeTM Bars (Q) that were indistinguishable by flavor and by ordinary inspection. Treatments were double-blinded and trial order was determined using a Latin square assignment. The dose for quercetin was estimated to elicit blood concentrations in excess of the (inhibition constant) K_i for adenosine receptor antagonism (quercetin ~2.5 μ M) (267).

Blood and urine Analysis: Venous blood samples were collected from a superficial antecubital vein. Baseline 10 ml samples were drawn in the fasted state (~8 hours) after volunteers had been quietly seated for a 15-min stabilization period with arm position standardized. This sample was drawn immediately after the first-morning weight and

urinalysis. The second 10 ml sample was drawn one hour after the breakfast and supplements were consumed, with subjects again seated quietly for 15-min before sample collection. The third 10 ml sample was drawn immediately post-exercise, with the volunteers still seated on the cycle ergometer.

Blood from heparinized tubes was centrifuged for 10 min at 4°C, and plasma aliquoted for the measurement of glucose and lactate (YSI 2300 STAT). Plasma from EDTA tubes was aliquoted and analyzed for plasma osmolality by freezing point depression (Fiske Micro-Osmometer, Model 210, Norwood, MA) or treated and stored frozen (-80°C) for glycerol and quercetin analyses. Glycerol, which was selected as a minimally confounded estimate of lipolytic activity, was measured using standard enzymatic fluorometric methods (Boehringer Mannheim Kit 148270). Quercetin was measured from plasma that was immediately centrifuged for 15 minutes at room temperature, after which 100 μ l of 10% ascorbic acid (Sigma Aldrich, USA) was added. The mixture was vortexed for 30 seconds and immediately frozen until analyzed by HPLC at the Michniak-Kohn laboratory, Rutgers University, NJ. USG was measured by refractometry (1110400A TS Meter, AO Reichert Scientific Instruments).

Quercetin extraction from plasma matrix (tasks performed at Dr. Michniak-Kohn laboratory at Rutgers University): The quercetin extraction procedure was derived and modified from procedures reported in the literature (186, 223). Briefly, to plasma samples containing 10 % W/V ascorbic acid, 0.78 M sodium acetate buffer (pH 4.8), βglucuronidase/sulfatase and 100 μ g/ml of fisetin (HPLC internal standard) were added. The mixture was then incubated for 17 hours at 37°C in a shaking water bath (Julabo, CA) for the completion of the hydrolysis of quercetin glucuronides and sulfate conjugates to quercetin aglycone. The above mixture was then subsequently diluted with 0.5 M phosphoric acid solution and centrifuged at 8000 rpm for 20 minutes for clarification of the samples. The supernatant obtained was subjected to extraction on a preconditioned Oasis HLB cartridge mounted on a vacuum manifold. After addition of the supernatant, 3 ml of methanol was added to cartridge to elute quercetin retained on the cartridge. The solution containing quercetin was then evaporated to dryness under nitrogen. The residue obtained was reconstituted in 300 μ l of methanol with vortex mixing for 30 sec and then subjected to HPLC analysis.

Analysis and validation of assay of quercetin in plasma matrix (tasks performed at Michniak-Kohn laboratory at Rutgers University): Analysis of quercetin in plasma samples was conducted using an Agilent 1100 HPLC system (Agilent Technologies, USA) with Phalanx C_{18} (5 µm and 250 x 1.6 mm) reversed phase column (Nest Group Inc., MA,). The injection volume was maintained at 20 µl with detection wavelength of 370 nm. Methanol:water (80:20, pH 3.72) was used as the mobile phase that was pumped at 0.5 ml/min. The column temperature was maintained at 30°C during the analysis. The method was tested for linearity, precision, repeatability of injection, intra-day, inter-day variability, intermediate precision, recovery and limit of detection.

Statistics: The effects of treatment (quercetin, placebo) on outcome variables of interest was assessed using a one-way (trial) or two-way (trial x time) repeated measures ANOVA. Where the assumption of sphericity was violated, F-values were adjusted using Greenhouse-Geisser or Huynh-Feldt corrections as appropriate. Tukey's HSD procedure was used to identify differences among means following significant main and/or interaction effects. Where indicated, the error uniformity of some variables was tested

using regression analysis. The primary outcome variable of interest in this experiment was time trial performance. A minimum sample size of 6-7 (269, 277) was calculated (α =0.05, β = 0.20) as sufficient to detect a 5% change (~10 kJ) in time trial performance from placebo in the ANOVA. This number was estimated using the peak total work (186 ± 32 kJ) measured during the initial two-weeks of performance familiarization training and the within-subjects coefficient of variation expressed as a percentage of the mean (4.5%) after removing outliers using Grubb's test (2 of 33 training sessions removed). An effect size >1.0 was selected based on the likelihood of experimental perturbations producing unique performance infidelity (269), which would decrease the observed signal-to-noise ratio. A sample size of 10 volunteers was tested to allow detection of desired differences with an effect size as small as 0.7 (277), to guard against possible attrition, and to afford more confidence that our volunteers were representative of their wider population. All data are presented as means ± SD except where indicated.

12.3 Results & discussion

Hydration: Hydration status was assessed on the morning of each trial. There were no significant differences (P>0.05) among trials for first morning measures of body mass, USG, or plasma osmolality. First morning body mass averaged $0.6 \pm 0.1\%$ from baseline, USG was $1.016 \pm .006$, and plasma osmolality was 288 ± 4 mOsmol/kg H₂O. Means included individual volunteers who were above euhydration thresholds at the first morning measurement before being given a mandatory 500 ml of water to drink with breakfast. Volunteers were therefore considered equally and normally hydrated at the start of each trial (272). The level of dehydration incurred during 30-minutes of steady-

state cycle ergometry was also not different among trials (P>0.05), averaging $0.7 \pm 0.1 \%$ and subjects were given an average of 240 ± 7 ml of water to replace the losses.

Physiological Responses: Metabolic rates during 30-minute steady-state exercise were calculated from the average of a 3-minute gas sample made 15-minutes into exercise. Measurements among the two trials were similar (P>0.05) at $51.8 \pm 3.1 \%$ (Q), and 51.2

 \pm 2.0 % (P) of VO_{2max}, thus exercise intensity preceding the time trial was properly matched among treatments. Only a main effect of time was observed for HR, whereby exercise produced expected increases in HR from rest and again in response to the time trial. At the completion of the 15-minute time trial, HR's were at 97.1 ± 5.2 % (Q), 98.3 ± 5.6 % (P). T_{re} rose progressively (P<0.05) at all time points measured (main effect of time). T_{sk} increased likewise at all time points especially at the end of time trial, but only a main effect of time was observed. Sweating rates, calculated from the change in body mass after 30-minutes of steady-state exercise, were not different among trials (P = 0.66 ± 0.11 L/h; Q = 0.67 ± 0.12 L/h) (P>0.05).

Blood: The quantification of quercetin in blood samples was performed using validated assay of quercetin. The developed assay showed linearity with R^2 of 0.9971 in the range of concentration tested (5 ng/ml-1500ng/ml). The LOQ was 5 ng/ml and LOD was 3 ng/ml. The RSD values for intraday and interday analysis were less than 2 and 12 percent respectively. The percent recovery of quercetin at all concentrations ranged from 80-85 percent. At the same time, the percent recovery of internal standard, fisetin used in the analysis was 98-99 percent. The retention times for fisetin and quercetin were found to be 7.4 and 8.5 minutes respectively in the run time of 15 minutes.

The fasting baseline blood levels of quercetin in trials P and Q, were near or below trace amounts, confirming the low level of quercetin in the ordinary Western diet(267)Blood variables (glucose, lactate and glycerol) tended to increase in response to exercise in both P and Q groups.

Perceptual responses: Results from the battery of perceptual scales are presented in Table 12.2. There were no differences observed among trials on RPE, RP, RTC, or RM between trials P and Q. There were, however, higher RPE and RP when comparing steady-state (SS) and time trial (TT) time points in all trials. RTC was also reported higher in TT versus SS for trial Q, but not P. No effects of time were reported for RM (Table 12.2).

Exercise performance: Table 12.3 presents individual and mean time trial performance data. Data indicates that supplementation with quercetin had no impact on the total work performed in the 15-minute time trial. Relative to trial P, the mean percentage change in performance for Q (-1.2%; 95% CI = -9.4 to 7.0%) was small but highly variable. The uniformity of the percent change in performance from P was examined by inspection of the residuals from the regression analysis of percent change in performance (y-axis) against kJ of work completed (x-axis). This analysis indicated greater individual response variability for volunteers who completed < 150 kJ of work in response to Q supplementation (11 of 20 trials). Performance variability at 40°C (trials P and Q) was also larger (CV = 7%) than for training at 22°C (CV = 4.5%). Interestingly, when compared to the peak performances achieved during training at 22°C, all performances in the heat were significantly lower (P<0.05) by -18.7 ± 9.2% (P), and -18.3 ± 9.2% (Q), respectively.

The principle aim of this study was to determine whether the acute administration of nutritional adenosine antagonist (quercetin) could enhance endurance exercise performance in the heat. Based on previous findings of others (278, 279), dehydration was carefully prevented and the exercise task and duration were selected with the purpose of minimizing the influences of energy depletion and critical hyperthermia on performance. Ordinary elements of control over time of day and antecedent diet (AM meal) and exercise were also employed. The principle findings of this study is that acute supplementation with quercetin afforded no advantages over placebo on performance in the heat despite of the fact that actions of quercetin on adenosine receptors are well described (174, 263, 267, 280)and the blood concentrations of quercetin achieved in this study were in excess of the K_i for adenosine receptors (263, 267) (Table 12.1). One possibility for the results obtained is that adenosine antagonism is not responsible for the enhanced exercise performance in heat. Our hypothesis to use adenosine antagonist in improving exercise performance in heat was based on the several evidences (180):

1) Impaired exercise performance has been previously observed in rats that administered an adenosine drug agonist

2) The reversal of impaired exercise performance with adenosine antagonist (caffeine) in rats, and

3) Improved run time to exhaustion with adenosine antagonist (caffeine) only.

Both 2 and 3 were achieved with a small (0.6 mg/kg) intracerebroventricular dose of caffeine, while the same intraperitoneal dose had no effects. Like caffeine, it seems that much larger intraperitoneal dose of quercetin that unfortunately is incompatible with

human ingestion (> 20 mg/kg) (172, 180), might be required to elicit similar observations. It is also possible that ergogenic action of quercetin occurs with chronic, rather than acute, dosing (263).

If the performance outcome reported in this study is the likely range of the true effect of the treatment for the population studied (mean, 95% CI), then quercetin has small-to-no effect on endurance exercise performance in the heat. Although only a very large study sample size can improve the precision of the population estimate, narrow the confidence limits, and add clarity toward extrapolating study outcomes (269), the present interpretation is consistent with the small body of like-literature (278, 279).

Null findings have been reported previously after double-blinded study of wellrecognized ergogenic supplements (e.g., carbohydrate). This is true even in large (n > 40) studies with trained athletes (281) and has been observed in both temperate (281) and warm environments (282).

12.4 Conclusion

This double-blinded study reports no effect of quercetin on endurance exercise performance in the heat. It is possible that individual differences in response to treatments in subjects may have influenced these results. These conclusions were made considering the fact that quercetin supplementation in the study was able to achieve quercetin-blood concentrations capable of producing adenosine antagonism. Moreover, potential study confounders for the heat such as critical hyperthermia, dehydration, and exercise duration were also controlled in this study. Although we report no effect on endurance exercise performance in heat following quercetin supplementation, it is the only study of its kind to examine effects of quercetin on endurance exercise performance in the heat stress.

Acknowledgement

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13.1 Introduction

It is a well known fact that all forms of exercise when performed vigorously and extensively causes pain. However, exercises such as eccentric ones that involve overall lengthening of the muscle in response to external resistance, usually cause soreness and stiffness in unaccustomed individuals. In eccentric exercise there is no onset of pain after immediate completion of the exercise but the pain sets in several hours later and peaks at about 48 h. The pain is believed to be the result of muscle damage produced by the eccentric exercise (283). Eccentric exercise at the same time is also known to produce delayed onset muscle soreness (DOMS) and prolonged loss in strength and range of motion (228, 284-288). These manifestations of eccentric exercise are often the result of inflammation and free radicals generated from oxidative stress (242). Therefore, it is possible that quercetin supplementation could reduce effects of strenuous exercise by reducing harmful effects of free radicals and inflammation and thereby reduce muscle function decrements and improve recovery. Recent studies in humans have demonstrated potent anti-oxidant and anti-inflammatory benefits of quercetin supplementation (140, 289). The studies report that quercetin supplementation not only reduced inflammation after eccentric exercise (289) but also reduced the rate of incidence of upper respiratory tract infections (140). The overall objective of the current study was to investigate the efficacy of quercetin in reducing delayed onset of muscle soreness and decrements in muscle strength after strenuous exercise and therefore facilitating recovery. The significance of this study is that by evaluating the benefits of quercetin supplementation

in reducing muscle soreness and facilitating recovery a case could be made for incorporation of this compound into military food rations. This will assist military personnel in improving their fitness and potentially enhancing their performance in situations of oxidative stress.

This study was conducted in collaboration with University of Massachusetts, Amherst. The tasks such as the clinical study and assay of biomarkers of inflammation were performed at the University of Massachusetts. At the same time, analysis of quercetin in plasma samples was performed at Dr. Michniak-Kohn's laboratory at Rutgers University.

13.2 Materials and methods

13.2.1 Materials

Food grade quercetin (\geq 99.5% purity) (Merck S.A. Rio De Janiero, Brazil. QU995), First StrikeTM Bars were provided by Natick Soldier Research Development and Engineering Center (NSRDEC), Natick, MA. The compositions of the quercetin-fortified formulations are listed in Table 10.1. Fisetin (\geq 99.9% purity) and β -glucuronidase (Type HP-2 from *Helix pomatia*) were obtained from Sigma Aldrich. The extraction procedure was performed using a 20 Position Vacuum Extraction manifold (Waters, Milford, MA) using an Oasis HLB cartridge (Waters, Milford, MA). All other reagents and chemicals used were of analytical grade.

13.2.2 Methods

13.2.2.1 Study design

Subjects: Thirty healthy men and women aged between 18 and 25 years were recruited and signed an informed consent document approved by the University of Massachusetts's and Department of Defense Human Subjects Review Boards. Upon enrollment, subjects were randomized (refer Randomization) to one of two groups: Group 1 received quercetin (500mg) via the First StrikeTM bar carrier system and Group 2 received a placebo First StrikeTM bar not containing quercetin. Subjects enrolled in the study were in good health, were not competing in sport activities, were not taking dietary supplements (other than vitamin/mineral supplements with 100% or less than the Dietary Reference Intakes, DRI), were not following a fad diet plan, were not pregnant or nursing, and were not anemic. Subjects were willing to refrain from the use of dietary supplements and excessive nutrient intake of quercetin containing foods.

Randomization: Fifteen subjects were randomly assigned to: 1) exercise their dominant or non-dominant arm first, and 2) take quercetin or placebo. After subjects had completed Phase 1 of the study, they began either quercetin or placebo supplementation during Phase 2, then exercised the contralateral arm and continued supplementation during Phase 3 of the study.

Supplementation: Supplementation involved ingestion of two First StrikeTM bars containing 250 mg quercetin (or placebo) at about 8 am and again at about 8 pm each day. Subjects ingested a total of 4 First StrikeTM bars per day for 7 days.

Eccentric exercise: The exercise consisted of 24 maximal eccentric contractions of the elbow flexors. It was observed that this eccentric exercise model consistently produced muscle soreness. Each action was approximately 5 seconds in duration and was repeated every 15 seconds. The investigator observed the exercises and confirmed on the Classical Risk Factors (CRF) that they were done correctly as described (290). To perform the exercise, the subjects were seated in a modified preacher's bench with the non-dominant arm resting on a padded support, the wrist fixed between two padded

rollers of the exercise lever and the forearm in the fully flexed position. For each eccentric action, the investigator's staff pulled down on the lever, forced the subject's forearm into a fully extended position as the subject exerted maximum resistance. The mechanical advantage of the lever system allowed the investigator to provide maximal resistance to the subject throughout the entire range of motion. Immediately following the exercise, muscle strength was reassessed. It has been previously reported that 50 maximal contractions of the elbow flexors are safe (291). However, in this study we used a more moderate exercise of 24 contractions.

Study schedule: The study consisted of 3 phases. Table 13.1 provides detailed schematic of the testing schedule:

Phase 1 – Subjects wore an activity monitor, completed daily POMS (Profile of mood states) survey, exercise one arm, and criterion measures were assessed. Phase 1 consisted of visit 1 through visit 7 (Table 13.1) and it represented the pre-supplementation phase. Blood samples were withdrawn in visits 3-7. Exercise was performed in visit 3 and criterion measurements were performed pre and post exercise.

Phase 2 – It represented supplementation phase. Subjects took the quercetin supplement/placebo twice daily for 7 days, wore an activity monitor, and completed daily POMS survey. Phase 2 involved visit 8. Blood samples were withdrawn in visit 8.

Phase 3 - Subjects exercised and continued to take supplement/placebo twice daily for 4 days post exercise (11 days in total), wore an activity monitor, and completed POMS survey. Phase 3 involved visit 9 to 15. Blood samples were assessed between visits 11 and 15. . Exercises were performed in visit 10 and criterion measurements were taken pre and post exercise.

Comparison between Phase 1 and Phase 3 were used to test the effects of the supplement on muscle soreness, inflammation, and recovery. Criterion measures were isometric strength, isokinetic strength, range of motion, circumference of the upper arm, blood sample (Blood samples were not assessed on Visit 2 or 9), POMS survey and soreness.

Isometric strength: A measure of isometric strength of the elbow flexor muscles was assessed on a preacher curl bench. Three (3 second) trials with one minute rest between trials were assessed. For this test, subjects were asked to pull against a force transducer, which measured the amount of force the elbow flexor could exert.

Isokinetic Strength: Isokinetic torque of the elbow flexor and extensor were assessed on a Biodex dynamometer (Biomedical Systems, Inc., Shirley, NY). 12 consecutive isokinetic contractions at 60°.sec⁻¹ and 180°.sec⁻¹ were assessed. For this test, subjects were asked to pull and push against the Biodex arm apparatus (Biomedical Systems, Inc., Shirley, NY) (24 contractions in total) which measured the amount of torque the elbow flexor and extensor can exert.

Range of Motion: The range of motion was determined by measuring the angle about the elbow as the arm hung at rest by the side and when the subject fully contracted the biceps muscle. Arm angle, an index of muscle-tendon unit contracture, was determined by measuring the angle about the elbow with a goniometer as the arm hangs at rest by the side. Marks were made at the distal radio-ulnar joint of the wrist, the lateral epicondyle of the humerus (at the elbow joint), and the greater tubercle of the humerus (at the shoulder) to ensure reproducibility in subsequent measurements. The middle of the goniometer was placed over the mark on the elbow and the proximal and distal ends were aligned with the

marks on the shoulder and wrist, respectively. The resting joint angle was then measured and recorded by the investigator.

Arm Circumference of the upper arm mid biceps region was assessed. The site was marked with indelible ink for consistent measurement day to day. Circumference was measured with a spring loaded anthropometric tape measures.

Physical Activity Monitoring: Activity monitors (Actical®, Phillps Respironics, Inc.) were used to assess physical activity levels of the arm between quercetin and placebo groups and ensure subject compliance with physical activity criteria during the study. Subjects wore small activity monitors on the wrist during Phase I and III (15 days in total) to assess general use of the arm in response to 1) the eccentric exercise protocol, and 2) quercetin/placebo supplementation. Activity monitor data was then downloaded and analyzed with computer software.

Profile of Mood States (POMS) Survey: The POMS Standard assessment is a validated, factor-analytically derived inventory that measures six identifiable mood or affective states (174). The POMS survey was used to evaluate the mood states of subjects throughout the study. Recent evidence suggests quercetin may have caffeine-like effects (174), which may alter their perception of muscle soreness during quercetin supplementation. Thus, the POMS survey was used to examine the effects of quercetin on mood states and perceived muscle soreness. Thus, the POMS survey was used to examine the effects of quercetin on mood states and perceived muscle soreness. The POMS survey consisted of a battery of 65 key words and phrases (e.g. "friendly" and "uncertain about things") The subject rates each key word or phrase on scale of 0-4 (i.e. 0= not at all, 1= A little, 2= Moderately, 3= Quite a bit, 4= Extremely) to describe which number best relates
to the specific key word. Subjects were provided a list of definitions for each key word or phrase on the POMS survey and were instructed to refer to those definitions only to avoid introducing bias or misinterpretation by the investigators. During the first 10 minutes of each visit (during Phases 1&3), subjects completed a POMS survey prior to any other criterion measures. This order was designed to capture subjects' mood state under standard conditions each morning of data collection. During Phase 2, subjects were provided a packet of POMS surveys and a definition sheet and were asked to complete the survey each morning at the same time and under the same conditions as they did the laboratory (i.e. fasted, while resting, at the same time each morning). When subjects returned to the laboratory for Phase 3 of testing, they returned their completed POMS surveys to the investigators.

Soreness: The soreness assessment using a VAS (visual analog scale) (292) were completed. The left side (0 mm) of the VAS scale was the side of the scale that indicated "no soreness," while on the right side (100 mm) of the scale the indication was "unbearable soreness." The subject were asked to provide an initial, pre-exercise evaluation for soreness using a visual analog scale (VAS) after conducting two (293) full range of motion biceps curls holding a 1-pound (for subjects whose weight is <130 pounds) or 2-pound (for subjects whose body weight is >130 pounds) dumbbell on the non-dominant arm. The VAS score was recorded by the subject directly on the case report forms. The VAS score were performed by drawing a vertical line through a 100 mm horizontal line indicating the degree of muscle soreness which was measured.

If the VAS score measurement measured by the investigator at the start for Visit 2 for a subject was determined to be 10 mm or less in each arm on the 100 mm VAS scale, the

subject continued to the exercise program. If the soreness was greater than 10 mm the subject was asked to return 2 or more days later to allow any soreness to dissipate. Throughout the study, all subjects refrained from strenuous physical activity of the arms prior to the baseline visits and it was not necessary to have subjects postpone baseline visits to diminish residual soreness from a non-related event.

13.2.2.2 Blood analysis

13.2.2.1 Quercetin

Analysis and validation of assay of quercetin in plasma matrix: Analysis of quercetin in plasma samples was conducted using an Agilent 1100 HPLC system (Agilent Technologies, USA) with Phalanx C_{18} (5 µm and 250 x 4.6 mm) reversed phase column (Nest Group Inc., MA,). The injection volume was maintained at 20 µl with detection wavelength of 370 nm. Methanol:water (80:20, pH 3.72) was used as the mobile phase that was pumped at 0.5 ml/min. The column temperature was maintained at 30°C during the analysis. The method was tested for linearity, precision, repeatability of injection, intra-day, inter-day variability, intermediate precision, recovery and limit of detection.

For linearity, a calibration plot of plasma samples spiked with various concentrations of quercetin was prepared. Blank human plasma samples (provided by University of Massachusetts, MA) was spiked with quercetin to obtain concentrations 1, 3, 5, 50, 100, 250, 500 and 1000 ng/ml. Blank samples once spiked were subjected to the extraction procedures as described below.

Quercetin extraction from plasma matrix: The quercetin extraction procedure used was modified from procedures reported in the literature (186, 223). Briefly, to plasma samples containing 10 % W/V ascorbic acid, 0.78 M sodium acetate buffer (pH 4.8), β -

glucuronidase/sulfatase and 100 μ g/ml of fisetin (HPLC internal standard) was added. The mixture was then incubated for 17 hours at 37°C in a shaking water bath (Julabo, CA) for the completion of the hydrolysis of quercetin glucuronides and sulfate conjugates to quercetin aglycone. The above mixture was then subsequently diluted with 0.5 M phosphoric acid solution and centrifuged at 8000 rpm for 20 minutes for clarification of the samples. The supernatant obtained was subjected to extraction on a preconditioned Oasis HLB cartridge mounted on a vacuum manifold. After addition of the supernatant, 3 ml of methanol was added to cartridge to elute quercetin retained on the cartridge. The solution containing quercetin was then evaporated to dryness under nitrogen. The residue obtained was reconstituted in 300 μ l of methanol and analyzed by HPLC.

13.2.2.2 Determination of various biomarkers

Serum creatine kinase: Serum creatine kinase (CK) activity was assessed as an indirect indicator of muscle damage. Blood samples were collected in serum vacutainers (BD Biosciences, USA) to clot blood at room temperature for 15 minutes. Samples were then centrifuged at 3000X g for 15 minutes at room temperature. Aliquots were then collected in 1 ml cryovials and stored at -80°C for subsequent analysis. Serum samples were then shipped on dry ice to Holyoke Hospital, Massachusetts for determination of CK enzyme activity.

Inlerleukin 6 (IL-6), interleukin-1 (IL-1) and C- reactive protein (CRP): IL-6, IL-1 and CRP are inflammatory indicators previously found to increase after eccentric exercise of the arm and can be reduced by dietary supplement (286). Blood samples were collected in K₂EDTA vacutainers (BD Biosciences, USA) and immediately centrifuged at 3000 X g for 15 minutes at 4°C. IL-6, IL-1 and CRP were assessed in plasma samples via a

commercially available ELISA kit (R&D Systems) that has widely been used in muscle damage research.

13.2.3 Statistical analyses

Statistical analyses were performed using repeated measures ANOVA by Statistical Analysis System (SAS) Software (SAS Institute Inc., Cary, NC) with a grouping factor (placebo vs. treatment) to compare the following measures before and after supplementation (Phase 1 vs. Phase 3): 1) strength loss and recovery; 2) loss of range of motion about the elbow and recovery; 3) circumference of the upper arm (to assess swelling); 4) plasma CK activity, IL-6, and CRP 5). For muscle soreness, non-parametric statistics was used. 'p' value was set at 0.05 for all statistical comparisons.

13.4 Results and discussion

Isometric strength: Isometric strength of the elbow flexor muscles was assessed to measure muscle force generation on a BiodexTM dynamometer. During the test subjects contracted the biceps isometrically against the dynamometer and peak torque in Newton-meters (N-m) was recorded. The data for isometric strength of the elbow flexors for the Placebo (13.1A) and Quercetin (13.1B) groups are depicted in Figure 13.1. The statistical comparison showed that there was no significant interaction of group (p=0.49) or phase (p=0.27), indicating that the Placebo and Quercetin groups responded similarly. Thus, there was no benefit of the quercetin supplement over that of the placebo.

Isokinetic strength: Isokinetic strength of the elbow flexors and extensors were assessed to determine muscle force generation through the full range of motion on a BiodexTM dynamometer. For this determination, subjects were asked to pull and push against the arm apparatus (24 contractions in total) that subsequently measured the amount of torque

the elbow flexors and extensors could exert. Figure 13.2 (A-D) represents the data for isokinetic strength of the elbow flexors at $60^{\circ} \cdot \sec^{-1}$ and $180^{\circ} \cdot \sec^{-1}$ for the Placebo (13.2 A, C) and Quercetin (13.2 B, D) groups in the study. Like isometric strength, there were no significant main effects of group (p=0.43) or phase (p=0.57) indicating that the Placebo and Quercetin groups responded similarly. Thus, there was no benefit of the quercetin supplement over that of the placebo on isokinetic strength recovery.

Range of motion: Arm angle, an index of muscle-tendon unit contracture, was determined by measuring the angle about the elbow with a goniometer as the arm hangs at rest by the side. Figure13.3 (A-B) presents the data for resting arm angle in degrees for the Placebo (13.3A) and Quercetin (13.3B) groups. A significant effect of time was observed p<0.0001, indicating a decrease in arm angle immediately after the exercise and returned to baseline at 120 hours. However, there were no significant main effects of group (p=0.66) or phase (p=0.34) on arm angle, nor were any significant interactions observed among the main effects, indicating that the Placebo and Quercetin groups responded similarly. Thus, there was no benefit of the quercetin supplement over that of the placebo on reducing muscle contracture of the elbow flexors in response to muscle damage.

Arm Circumference: Circumference of the mid biceps region was measured to assess swelling of the upper arm. Figure 13.4 (A-B) depicts the data for arm circumference. The data are shown as the change in circumference over time in millimeters (mm) for the Placebo (13.4A) and Quercetin (13.4B) groups. It was observed that with time a significant increase in swelling (p<0.0001) occurred within 24 hours after the exercise that reached peak values at ~120 hours. But, there were no significant effects of group (p=0.83) or phase (p=0.12) on arm circumference, nor were any significant interactions

observed, indicating that the Placebo and Quercetin groups responded similarly. Although it seems that quercetin may have a mild effect on reducing swelling at 72-120 hours, the difference pre-post supplementation was not significant. Thus, there was no benefit of the quercetin supplement over that of the placebo on reducing swelling. However, there appeared to be a trend toward a significant attenuation in swelling in the quercetin group at 120 hours, which would suggest that quercetin supplementation had an effect on swelling at this late time point. As an exploratory measure, a Student's paired T-Test was performed at the 120 hour point only, and it was discovered that quercetin significantly reduced swelling (p=0.005) at this time point.

Physical activity monitoring: There was no significant difference (p>0.05) in physical activity on quercetin/placebo supplementation.

Profile of mood states: The POMS survey was used to evaluate the mood states of subjects throughout the study in response to strenuous exercise and quercetin supplementation. No significant difference was observed in POMS (p>0.05) on quercetin/placebo supplementation.

Muscle Soreness: Soreness was evaluated as described previously using a visual analog scale (VAS). Figure 13.5 (A-B) presents the data for muscle soreness in millimeters (mm) along the VAS for the Placebo (13.5A) and Quercetin (13.5B) groups. The statistical analysis showed that a significant effect of time (p<0.0001), indicating an increase in muscle soreness 24-48 hours after the exercise that returned toward baseline values by 120 hours. But there were no significant main effects of group (p=0.92) or phase (p=0.35), nor were any significant interactions observed, indicating that the

Placebo and Quercetin groups responded similarly. Therefore there was no advantage of the quercetin supplement over that of the placebo on reducing muscle soreness.

Quercetin levels: Quercetin present in plasma samples was extracted by solid phase extraction and analyzed by HPLC. The assay of quercetin in plasma and urine was validated and checked for linearity, accuracy and precision. The calibration plot in blank plasma spiked with quercetin (n=3) showed linearity with R^2 of 0.99 in the range of 5ng/ml-1000ng/ml. For plasma samples, the LOQ was 5 ng/ml and LOD was 3 ng/ml. Mean post-supplement plasma quercetin levels within 12 subjects in the Quercetin group are depicted in Figure 13.6. Plasma quercetin (ng/ml) levels within each subject were averaged over 6 days during Phase 3 of the study (Y axis). On the X axis, UMXX indicate individual subject identification numbers (i.e. UM 1, 2, 4, 5, 6, 13, 14, 21, 22, 29, 30, and 33). The data indicates that significantly elevated plasma quercetin levels (from subjects fasted for >10 hours) in the Quercetin group (18-130 ng/ml) relative to the Placebo group (5-12 ng/ml) were obtained after 4 days of supplementation with quercetin containing First StrikeTM bar.

Serum Creatine Kinase (CK): The CK activity in units/liter (U/L) for the Placebo (13.7A) and Quercetin (13.7B) groups respectively is depicted in Figure 13.7 (A-B). The statistical analysis was performed on log transformed data. A significant effect of time (p<0.0001) was observed, indicating an increase in serum CK activity within 24 hours that remained elevated up to 120 hours post-exercise. However, there were no significant effects of group (p=0.10) or phase (p=0.43) on CK, nor were any significant interactions observed among the main effects, indicating that the Placebo and Quercetin groups responded similarly. Therefore it was inferred that there was no benefit of the quercetin

supplement over that of the placebo on weakening the rise in CK levels.

Plasma C-reactive protein: Plasma C-reactive protein (CRP) is a bio-marker of lowgrade, systemic inflammation and is used as an indicator of the inflammatory response to muscle damage. The CRP levels in ng/ml for the Placebo (13.8A) and Quercetin (13.8B) groups are depicted in Figure 13.8. On statistical analysis, no significant effects of group (p=0.96), phase (p=0.33), or time (p=0.34) were observed on plasma CRP levels, suggesting that the Placebo and Quercetin groups responded similarly.

Plasma Interleukin 6: Interleukin 6 (IL-6) is a cytokine that is produced by skeletal muscle tissues and white blood cells as an inflammatory response to muscle damage. In order to assess effect of quercetin supplementation on muscle damage, IL-6 levels were measured and data is depicted in Figure 13.9 (A-B). The statistical analysis was performed on log transformed data. The results showed no significant group (p=0.13), phase (p=0.40), or time effects (p=0.2) on plasma IL-6 nor were any significant interactions observed, implying that the Placebo and Quercetin groups responded similarly. Thus, there was no benefit of the quercetin supplement over that of the placebo on plasma IL-6 levels.

13.5 Conclusions

The objective of the study was to evaluate whether quercetin supplementation would reduce soreness and swelling in muscles, lead to decline in inflammatory markers in the blood and improve the overall profile of recovery after strenuous exercise. However, no benefits of quercetin supplementation were observed on various muscles attributes (isometric strength, isokinetic strength, soreness, arm circumference, range of motion), mood states, physical activity and inflammatory markers (serum creatine kinase, C- reactive protein, IL-6). The quercetin containing First Strike[™] supplement and the dosing regimen effectively increased plasma quercetin levels over baseline values. However, despite the increased blood levels of quercetin, the supplement had no effect on the variables measured. The only exception may be a trend for the quercetin supplement to reduce delayed swelling. Based on the results, it may be concluded that quercetin supplementation in the form of a military ration component does not improve the overall response to exercise-induced muscle damage in the investigated subject population.

Active	Trade name	Indication	Company
ingredient			
Clonidine	-Catapres-	Hypertension	-Boehringer Ingelheim
Estradial	Alora®	Hormona raplacament	Theretech Inc.
Listiaciói	Vivelle Dot [®]	therapy	Novartis Consumer Health
	- Vivene-Dot	therapy	Inc
	-Ciiiiara		-Bayer Healthcare
			Pharmaceuticals
Ethinvl estradiol	-Ortho-Evra®	Birth control	Ortho-McNeil-Janssen
w/Norelgestromin			Pharmaceuticals (Johnson
C			& Johnson)
Fentanyl	-Duragesic	Pain management	-Ortho-McNeil-Janssen
-	Transdermal		Pharmaceuticals (Johnson
	System [®]		& Johnson)
	-Fentanyl		-Teva Pharmaceuticals
	Transdermal		
	System®		
Lidocaine	-Lidoderm [®]	post-herpetic neuralgia	-Endo Pharmaceuticals
Nicotine	-Nicoderm	Nicotine replacement therapy	-Johnson & Johnson
	CQ		-Cygnus/Smithline
	-Nicotrol [®]		Beecham
Nitroglycerine	-Nitro-Dur	Angina	-Keys Pharmaceuticals;
	-Nitrodisc [®]		-Searle Pharmaceuticals,
			Inc.
Scopalamine	-Transderm-	Motion sickness	-Novartis Consumer Health,
	Scop®		Inc.
Testosterone	-Androderm®	Male hypogonadism	-Theratech, Inc.
Selegeline	-Emsam [®]	Depression	-Bristol Myers Squibb
Methylphenidate	-Daytrana [®]	Attention deficit	-Shire US, Inc.
		Hyperactivity Disorder	
Oxybutynin	- Oxybutynin	Urinary incontinence	-Watson Pharmaceuticals
	patch		

Table 1.1: FDA approved transdermal products in U.S.

Table 2.1: Penetration modifiers with their postulated mechanisms of action andPenetration Modifier Ratios (PMR) (57).

Penetration modifiers used	Suggested mechanism of	Drugs tested (enhancer/retardant,
	action	$PMR^{\#}$)
(Category and examples)		
ENHANCERS		
Alcohols, fatty alcohols and glycols		
Alcohols and fatty alcohols:	Alcohols with carbon chains	Mefanamic Acid (Ethanol 10%, 2.42)
(ethanol, butanol, propanol, octanol,	from C1-C6 act as solvents	(70)
lauryl alcohol)	or alter other	
	thermodynamic properties of	Melatonin (Octanol 5%, 4.22) (71)
	the molecule	Melatonin (Lauryl alcohol 5% 3.82)
		(71)
	Alcohols with carbon chains	
	> C6 oot by increasing	
	diffusion due to extraction	
	diffusion due to extraction	
	of lipids	
Glycols: Propylene Glycol	Solvate the keratins in SC	Bupranolol (Propylene Glycol 10%, 2.5)
	resulting in occupation of	(72)
	hydrogen binding sites.	
	Alter the thermodynamic	
	After the thermodynamic	
	activity of drug in the	
	vehicle increasing diffusion	
Sulfoxides		
Dimethylsulfovide (DMSO)	DMSO: Changes karstin	Diclofense sodium (DMSO 10% 1.02)
Daniemyisunoxide (DMSO),	apprises the list	(75)
Decylmethylsulfoxide (DCMS)	confirmation from α helical	(73)
	to β sheet, interacts with	
	lipid domains in SC to	

	distort the packing geometry	
	and also acts as a solvent	
	within skin tissue	
DMSO related compounds		Hydrocortisone (<i>S</i> , <i>S</i> -Dimethyl- <i>N</i> -(4-
(Iminosulfuranes)		chlorbenzenesulfonyl)iminosulfurane,
		21.03) (42)
S,S-Dimethyl-N-(4-		
chlorbenzenesulfonyl)iminosulfurane		
S,S-Dimethyl-N-(5-nitro-2-		
pyridyl)iminosulfurane		
Fatty acids		
Oleic, linoleic, linolenic, lauric,	Interact with the lipid	Melatonin (Oleic Acid 5%, 5.35)
myristic, stearic, undecanoic acids	domains leading to	(Linoleic acid 5%, 6.58) (Linolenic acid
	perturbation of the bilayers	5%, 7.54) (Lauric acid 5% 6.11)
		(Undecanoic acid 5% 8.57) (76)
	Oleic acid also increases	
	lipid fluidity by decreasing	
	their phase transition	
	temperatures	
	Higher concentrations of	
	Oleic Acid are postulated to	
	exist as a separate phase	
	within the lipid bilayers	
	leading to permeability	
	defects	
Surfactants		

Anionic (sodium lauryl sulfate)	Penetrate into the skin and	Lorazepam (SLS 5%, 11.66) (77)
(SLS)	extract water soluble agents	
	that act as plasticizers	
	Interact with and hind to	
	apidermal proteins	
	epidermai proteins	
	Cause damage to the skin	
	leading to high irritation	
	potential	
Cetionic (set of this start and services	Tertained and the sum to be	L
Cationic (cetyl trimetnyl ammonium	interact with the proteins,	Lorazepam (CTAB 5%, 10.16) (77)
bromide (CIAB), benzalkonium	lipid lamellae and other	
chloride)	components of the SC	
	Cause more damage to the	
	skin than anionic surfactants	
Zwitterionic (dodecyl botanies)		
Nonionic (Polysorbates 20, 60, 80),	Solubilize and extract	Hydrocortisone (Polysorbate 60, 2.52)
Tween (20, 40, 60, 80), Polyxamer	membrane components	(294)
(231, 182), Brij (93, 96)	I	L
	Increase membrane fluidity	Lorazepam (1 ween $80\ 0.5\%\ 2.33$) (77)
	and reduce diffusional	
	resistance	
	Emulsify sebum in the skin	
	enhancing the	
	thermodynamic activity of	
	drugs	
_		
Terpenes		
1,8-Cineole, carvone, menthol,	Modify the solvent nature of	Tamoxifen (1,8-Cineole 20%, 7.03) (32)

limonene	the SC, improving drug	Caffeine (carvone 10%, 12.4) (69)
	partitioning into tissue	
	r c	
	Disrupt SC bilayer lipids	
	increasing diffusivity	
	mercusing unrusivity	
	Open polar pathways in and	
	across SC	
	Act as solvents, altering the	
	Act as solvents, altering the	
	thermodynamic activity of	
	the drug	
	Act on the polar head	(295)
	groups of lipids leading to	
	subsequent disruption of the	
	interlamellar and	
	intralamellar hydrogen	
	bonding networks	
Amides		
Urea, dimethylformamide (DMF),	Urea functions as a	Progesterone (Urea 5%, 2.7) (73)
dimethylacetamide (DMA),	hydrating agent and has	
dodecylisobutyramide dodecyl(2-	keratolytic properties	Hydrocortisone (dodecyl (2-
		methoxyethyl)acetamide, 35.2) (74)
methoxyethyl)acetamide	thereby creating diffusion	
	channels	
	DMA and DMF disrupt the	
	lipid packing and increase	
	the fluidity of lipids	
	• <u>+</u>	

Cyclic Amides		
Azone (1-dodecylazacycloheptane-2- one) and analogs	Azone partitions into the lipid bilayers, disrupts their	Hydrocortisone (Azone 19.5) (74) 5-Fluorouracil (Azone 24.21) (296)
	membrane fluidity	
	Azone has been shown to be	
	an enhancer for both	
	hydrophilic and hydrophobic drugs	
Pyrrolidone and derivatives		
N-methyl-2-pyrrolidone (NMP), 2-	Increase permeation by	Hydrocortisone (N-dodecyl-2-
pyrrolidone (2P),	interacting with keratins and lipids in skin	pyrrolidone, 42*) (297)
N-dodecyl-2-pyrrolidone	L	
	Pyrrolidones also form	
	reservoirs within skin	
	membranes leading to a	
	potential sustained release	
	effect on drug release	
Miscellaneous enhancers		
DDAA (Dodecyl	DDAIP interacts with the	Indomethacin (DDAIP) (298)
dimethylaminoacetate)	polar region of the	
DDAIP (Dodecyl-2-(N,N-	phospholipid bilayer	
dimethylamino)propionate)	Increases the motional freedom of lipid	
	<u>r</u>	

	hydrocarbon chains.	
RETARDANTS		
Iminosulfurane analogues:	Postulated to decrease drug	Hydrocortisone (R-1, 0.12, R-2, 0.5, R-
<i>S,S</i> -dimethyl- <i>N</i> -(2- methoxycarbonylbenzenesulfonyl)im inosulfurane (R-1)	partitioning into skin by imparting order to skin lipids	3, 0.5) (42)
S,S-dimethyl-N-		
benzenesulfonyliminosulfurane (R-2)		
<i>S,S</i> -dimethyl-N-(4- chlorobenzenesulfonyl)iminosulfuran e (R-3)	Modification of hydrogen	Metronidazole (0.2)**(37)
Laurocaprani anaiogue:	bonding within the SC linids	
N-0915	producing condensation of	
	the lipid lamellae	

#Penetration modifier ratios (PMR) are listed in italics in the table.

Penetration modifier ratio (PMR) = $\frac{\text{flux of agent in presence of penetration modifier}}{\text{flux of agent in absence of penetration modifier}}$

- * (PMR) = <u>Kp of agent in presence of penetration modifier</u> Kp of agent in absence of penetration modifier (Kp = Permeability coefficient)

*** (PMR) = <u>Amount of penetrated agent in presence of penetration modifier</u> Amount of penetrated agent in absence of penetration modifier

Active/Penetration modifier	Mobile Phase	Flow rate (ml/min), Column temperature (°C)	Injection volume (μl)	Detection wavelength (nm), retention time (min)	Limit of Quantification (LOQ) (µg/ml)
DEET	methanol: water (80:20V/V)	0.7, 25	20	240, 3.4	1.9
Laurocapram	acetonitrile:methanol:water (88:2:10 V/V/V)	1.5, 40	25	210, 4.4	0.03
N-0915	acetonitrile:water (80:20V/V)	1.5, 40	25	210, 4.2	0.14
DMBIS	acetonitrile:methanol:water (65:15:35 V/V/V)	1, 40	20	245, 3.9	0.12
DMMCBI	acetonitrile:methanol:ammonium phosphate buffer (20:5:75 V/V/V), pH 3.0	1,40	10	270, 2.9	4.72
TBDOC	acetonitrile:water (95:5 V/V)	1.5, 40	25	210, 4.9	0.79

Table 4.1: HPLC methods of DEET and penetration modifiers

Table 4.2: Determination of solubility and	available amounts of penetration
modifiers in vehicles	

Penetration modifier↓	Solubility (mg/ml) ±SD (n=3)			
Solvent→	Water	Propylene glycol	Ethanol	PEG 400
Laurocapram	16.9±4.0	104.4±0.5	64.4±5.3	109.9±7.8
N-0915	Below LOQ	5.76±0.6	0.7±0.01	25.8±9.2
DMBIS	2.7±1.0	26.7±4.46	45.30±2.8	47.9±12.8
DMMCBI	48.2±1.3	27.6±4.0	119.6±10.1	43.8±10.5
TBDOC	Below LOQ	43.5±2.7	17.8±2.5	34.1±3.8

SD represents standard deviation

Penetration Modifier	Penetration modifier-ceramide 6 interaction	Log P
Laurocapram		4.6
N-0915		5.7
DMBIS		2.6

 Table 4.3: Partition coefficient of penetration modifiers and computer generated
 potential interactions of ceramide 6 molecules with penetration modifiers



*The colored annotations are representation of certain atoms in modeling software, where red represents oxygen, yellow indicates sulfur, grey refers to carbons, blue represents nitrogen, maroon indicates bromine and black dots represents possible H-bonds.

Permeability Mean flux, J ±SD ³MR*_J **Parameters**→ Cumulative amount of ¹MR_J ²MR₃₀ ⁴MR*₀₃₀ coefficient, $(\mu g/cm^2/hr)$ DEET after 30 hours, **Formulations** Q₃₀±SD K _p X10⁻⁵ (n=5) $(\mu g/cm^2)$ ±SD (cm/hr) No treatment, C 6.0±2.0 58.0±19.4 1413.9±315.6 1.0 1.0 _ -Water, W 17.3±2.36 168.2±22.9 4243.4±364.2 2.9 3.0 1.0 1.0 Propylene glycol, PG 256.0±61.7 5894.6±1458.6 4.4 4.2 1.0 1.0 26.4±6.4 Ethanol, E 11.6±2.4 112.6±23.2 2040.5±1266.3 1.9 1.4 1.0 1.0 **PEG 400, PEG** 2.6±1.2 25.6±12.2 701.7±312.0 0.4 0.5 1.0 1.0 Laurocapram 12.6±2.8 122.3±26.8 2984.3±741.3 2.1 2.1 _ -Laurocapram in 27.7±3.8 268.3±37.4 7546.6±1161.1 4.6 5.3 1.6 1.8 water, LW Laurocapram in PG, 30.0±1.9 291.2±18.1 8310.2±669.2 5.1 5.9 1.1 1.4 LPG 11.5±0.8 111.5±7.9 3060.4±130.4 1.9 2.2 0.9 1.5 Laurocapram in ethanol, LE Laurocapram in 1.9±0.5 18.4±4.9 478.2±118.9 0.3 0.3 0.7 0.6 PEG 400, LPEG

Table 4.4: Permeation parameters of DEET in presence of laurocapram in selected vehicles

6 🖬 MRJ 5 MR30 4 ☑ MR*J Modifier ratio 3 ☑ MR*Q30 2 1 С W PG Е PEG L LW LPG LE **LP**EG 0 Various Treatments

SD represents standard deviation

3. Ratio of J of DEET in presence of penetration modifier formulation in a solvent to J of DEET in presence of a solvent alone.

^{1.} Ratio of J of DEET in presence of treatment to J of DEET in absence of treatment.

^{2.} Ratio of Q_{30} of DEET in presence of treatment to ratio of Q_{30} of DEET in absence of any treatment

4. Ratio of Q_{30} of DEET in presence of penetration modifier formulation in a solvent to Q_{30} of DEET in presence of a solvent alone.

Permeabilit Mean flux, J ¹MR_I $^{2}MR_{30}$ ³MR*_J ⁴MR*₀₃₀ **Parameters**→ Cumulative $(\mu g/cm^2/hr)$ amount of DEET у **Formulations** coefficient, after 30 hours, Q₃₀ (n=5) K _p X10⁻⁵ $(\mu g/cm^2)$ ±SD (cm/hr) 6.0±2.0 58.0±19.4 1413.9±315.6 1.0 1.0 No treatment --Water 17.3±2.36 168.2±22.9 4243.4±364.2 2.9 3.0 1.0 1.0 PG 256.0±61.7 5894.6±1458.6 4.4 4.2 1.0 26.4±6.4 1.0 Ethanol 11.6±2.4 112.6±23.2 2040.5±1266.3 1.9 1.4 1.0 1.0 **PEG 400** 2.6±1.2 25.6±12.2 701.7±312.0 0.4 0.5 1.0 1.0 N-0915 in water 4211.9±573.7 2.7 3.0 0.9 154.4±28.6 1.0 15.9±2.9 N-0915 in PG 5.0±1.6 48.8±15.8 1116.7±354.8 0.8 0.8 0.2 0.2 N-0915 in ethanol 1.7±0.4 16.3±4.1 402.8±112.3 0.3 0.3 0.1 0.2 N-0915 in PEG 400 1.5±0.5 14.7±4.7 224.4±91.7 0.2 0.1 0.6 0.3 5 4 3 **Modifier Ratio** 🛚 MRJ ■ MR30 ◘ MR*J ☑ MR*Q30 0 -С W PG Е PEG NW NPG NE NPEG Various treatments

 Table 4.5: Permeation parameters of DEET in presence of N-0915 in selected vehicles

SD represents standard deviation

Ratio of J of DEET in presence of treatment to J of DEET in absence of treatment.
 Ratio of Q₃₀ of DEET in presence of treatment to ratio of Q₃₀ of DEET in absence of any treatment
 Ratio of J of DEET in presence of penetration modifier formulation in a solvent to J of DEET in presence of a solvent alone.
 Ratio of Q₃₀ of DEET in presence of penetration modifier formulation in a solvent to Q₃₀ of DEET in presence of a solvent alone.

Permeability Mean flux, J ¹MR_J ²MR₃₀ ³MR*_J ⁴MR* Parameters→ Cumulative coefficient, ±SD amount of DEET Q30 (µg/cm²/hr) **Formulations** after 30 hours, K _p X10⁻⁵ Q₃₀±SD (n=5) $(\mu g/cm^2)$ ±SD (cm/hr) No treatment, C 10.1±2.9 97.8±27.8 2736.9±695.0 1.0 1.0 --Water, W 15.9±1.9 154.5±18.7 4513.1±671.4 1.6 1.6 1.0 1.0 Propylene glycol, PG 18.9±3.4 183.7±33.4 4590.7±728.5 1.9 1.7 1.0 1.0 Ethanol, E 3667.5±1120.9 11.3±4.6 109.8±44.9 1.1 1.3 1.0 1.0 PEG 400, PEG 2.6±1.2 25.6±12.2 701.7±312.0 0.3 0.2 1.0 1.0 DMBIS in water, 18.4±0.7 178.0±7.1 5707.5±503.6 2.1 1.3 1.8 1.1 DBW DMBIS in PG, 34.5±2.3 335.0±22.0 9414.0±896.8 3.4 3.4 2.0 1.8 DBPG DMBIS in ethanol, 15.5±1.5 150.1±14.9 4232.3±1.5 1.5 1.5 1.4 1.1 DBE DMBIS in PEG 400, 19.9±0.8 192.8±8.2 5344.5±349.8 1.9 2.0 7.5 7.6 DBPEG 8 7 6 5 Modifier ratic 🛚 MRJ ■ MR30 4 ∎ MR*J ☑ MR*Q30 3 2 1 0 С W PG Е PEG DBW DBPG DBE DBPEG Various treatments

 Table 4.6: Permeation parameters of DEET in presence of DMBIS in selected vehicles

SD represents standard deviation

1. Ratio of J of DEET in presence of treatment to J of DEET in absence of treatment.

2. Ratio of Q_{30} of DEET in presence of treatment to ratio of Q_{30} of DEET in absence of any treatment 3. Ratio of J of DEET in presence of penetration modifier formulation in a solvent to J of DEET in presence of a solvent alone. 4. Ratio of Q₃₀ of DEET in presence of penetration modifier formulation in a solvent to Q₃₀ of DEET in presence of a solvent alone.

Permeabilit Mean flux, J ¹MR_J ²MR₃₀ ³MR*_J ⁴MR*₀₃₀ **Parameters**→ Cumulative amount of DEET after 30 ±SD y **Formulations** coefficient, $(\mu g/cm^2/hr)$ hours, Q₃₀±SD PCX10-5 (n=5) $(\mu g/cm^2)$ ±SD (cm/hr) No treatment, C 6.0±2.0 58.0±19.4 1413.9±315.6 1.0 1.0 -_ Water, W 17.3±2.36 168.2±22.9 4243.4±364.2 2.9 3.0 1.0 1.0 Propylene glycol, 26.4±6.4 256.0±61.7 5894.6±1458.6 4.4 4.2 1.0 1.0 PG Ethanol, E 11.6±2.4 112.6±23.2 2040.5±1266.3 1.9 1.4 1.0 1.0 **PEG 400, PEG** 2.6±1.2 25.6±12.2 701.7±312.0 0.4 0.5 1.0 1.0 DMMCBI in water, 14.8±4.6 143.1±44.7 3718.3±1093.3 2.5 2.6 0.8 0.9 DCW DMMCBI in PG, 7.0±2.7 67.7±25.9 2042.0±824.2 1.2 1.4 0.3 0.3 DCPG DMMCBI in 3.8±0.02 37.3±0.2 1100.4±51.3 0.6 0.6 0.3 0.5 ethanol, DCE DMMCBI in PEG 3.7±0.3 36.0±2.5 993.3±93.5 0.6 0.8 1.4 1.4 400, DCPEG 5 4 Modifier ratio 🛢 MRJ MRQ30 ✓ MR*J ×MR*Q30 1 0 E PEG DCW Various treatments С W PG DCPG DCE DCPEG

 Table 4.7: Permeation parameters of DEET in presence of DMMCBI in selected

 vehicles

SD represents standard deviation

^{1.} Ratio of J of DEET in presence of treatment to J of DEET in absence of treatment

^{2.} Ratio of Q₃₀ of DEET in presence of treatment to ratio of Q₃₀ of DEET in absence of any treatment

^{3.} Ratio of J of DEET in presence of penetration modifier formulation in a solvent to J of DEET in presence of a solvent alone

4. Ratio of Q_{30} of DEET in presence of penetration modifier formulation in a solvent to Q_{30} of DEET in presence of a solvent alone

Permeability ³MR*_J **Parameters**→ Mean flux, J ±SD **Cumulative amount** ¹MR₁ $^{2}MR_{30}$ ⁴MR*₀₃₀ coefficient, $(\mu g/cm^2/hr)$ of DEET after 30 **Formulations** hours, Q₃₀±SD K _p X10⁻⁵ (n=5) $(\mu g/cm^2)$ ±SD (cm/hr) No treatment, C 10.1±2.9 97.8±27.8 2736.9±695.0 1.0 1.0 _ Water, W 15.9±1.9 154.5±18.7 4513.1±671.4 1.6 1.6 1.0 1.0 Propylene glycol, 18.9±3.4 183.7±33.4 4590.7±728.5 1.9 1.0 1.0 1.7 PG Ethanol, E 11.3±4.6 109.8±44.9 3667.5±1120.9 1.1 1.3 1.0 1.0 PEG 400, PEG 2.6±1.2 701.7±312.0 0.3 0.2 1.0 25.6±12.2 1.0 TBDOC in water, 22.4±0.5 216.9±5.1 6151.6±215.9 2.2 2.2 1.4 1.4 TW **TBDOC in PG, TPG** 50.3±4.3 487.6±41.6 12423.6±1163.4 5.0 4.5 2.6 2.7 TBDOC in ethanol, 6.3±0.8 44.91±8.4 192.0±7.2 0.4 0.4 0.4 0.3 ΤЕ **TBDOC in PEG** 19.8±0.7 192.0±7.2 5520.3±240.5 2.0 2.0 7.5 7.8 400, TPEG 9 8 7 **Modifier** ratio 6 🗱 MRJ 5 MR30 4 NMR*J 3 \times MR*Q30 2 1 Ň 0 С W PG Е PEG TW TPG ТЕ TPEG Various treatments

 Table 4.8: Permeation parameters of DEET in presence of TBDOC in selected

 vehicles

SD represents standard deviation

1. Ratio of J of DEET in presence of treatment to J of DEET in absence of treatment

2. Ratio of Q₃₀ of DEET in presence of treatment to ratio of Q₃₀ of DEET in absence of any treatment

3. Ratio of J of DEET in presence of penetration modifier formulation in a solvent to J of DEET in presence of a solvent alon. 4. Ratio of Q_{30} of DEET in presence of penetration modifier formulation in a solvent to Q_{30} of DEET in presence of a solvent alone

Treatments	Mean flux, J (µg/cm².hr)	^a MR _J
No treatment	58.0±19.4	1.0
Water	168.2±22.9 ^b	2.9
PG	256.0±61.7 ^b	4.4
Ethanol	112.6±23.2 ^b	1.9
PEG 400	25.6 ± 12.2^{b}	0.4
Laurocapram	122.3±26.8 ^b	2.1
Laurocapram-water	268.3±37.4 ^b	4.6
Laurocapram-PG	291.2±18.1 ^b	5.1
Laurocapram-ethanol	111.5±7.9 ^b	1.9
Laurocapram-PEG 400	18.4 ± 4.9^{b}	0.3
N-0915-water	154.4±28.6 ^b	2.7
N-0915-PG	48.8±15.8	0.8
N-0915-ethanol	16.3 ± 4.1^{b}	0.3
N-0915-PEG 400	14.7 ± 4.7^{b}	0.2
DMBIS-water	178.0±7.1 ^b	1.8
DMBIS-PG	335.0±22.0 ^b	3.4
DMBIS-ethanol	150.1 ± 14.9^{b}	1.5
DMBIS-PEG 400	192.8±8.2 ^b	1.9
DMMCBI-water	143.1±44.7 ^b	2.5
DMMCBI-PG	67.7±25.9	1.2
DMMCBI-ethanol	37.3±0.2	0.6
DMMCBI-PEG 400	36.0±2.5	0.6
TBDOC-water	216.9±5.1 ^b	2.2
TBDOC-PG	487.6±41.6 ^b	5.0
TBDOC-ethanol	44.5±8.4 ^b	0.4
TBDOC-PEG 400	192.0±7.2 ^b	2.0

Table 5.1: Mean flux of DEET in presence of laurocapram and iminosulfurane analogues in selected vehicles (89)

^a Ratio of J of DEET in presence of treatment to J of DEET in absence of treatment ^b Statistically different compared to no treatment at 95 percent confidence interval (p<0.05)

Treatments	Mean temperature shift (°C) ±SD (n=3)			Mean enthalpy change (J/g) ±SD (n=3)		
	ΔT_{m1}	ΔT_{m2}	ΔT_{m3}	ΔH_1	ΔH_2	ΔH_3
No treatment	-	-	-	-	-	-
Water	-3.0±0.8	-6.2±1.7	-1.5±0.14	- 0.01±0.001	-0.03±0.02	- 0.008±0.003
PG	-2.0±0.9	-6.0±0.5	*	0.4±0.01	0.5±0.01	*
Ethanol	- 14.0±0.01	-6.0±4.0	8.5±0.04	0.4±0.04	0.4±0.09	0.02±0.001
PEG 400	+7.0±2.2	+2.0±5.5	*	0.1±0.03	0.1±0.01	*
Laurocapram	*	-13.1±0.9	*	*	0.1±0.02	*
Laurocapram-water	*	*	Two peaks at 90 ° & 98°C	*	*	-
Laurocapram-PG	-9.0±4.2	*	-7.5±4.4	0.05±0.001	*	0.5±0.04
Laurocapram- ethanol	-5.0±1.9	+2.0±0.8	Two peaks at 90 ° & 98°C	-0.6±0.05	-0.8±0.3	-
Laurocapram-PEG 400	-5.0±1.6	No change	*	0.7±0.05	-0.1±0.01	*
N-0915-water	*	Merger of $T_{m1} \& T_{m2}$ at 70°C	Multiple peaks at 92, 94 &100°C	*	-	-
N-0915-PG	-3.0±1.6	-7.0±0.5	+6.5±1.4	0.7±0.04	-0.1±0.03	1.4±0.4
N-0915-ethanol	+9.0±1.3	+8.0±0.03	+8.5±0.9	-0.4±0.02	-1.0±0.2	No change
N-0915-PEG 400	+6.0±0.4	+1.0±0.3	*	No change	No change	*
DMBIS-water	-3.0±1.5	-8.0±3.7	-9.9±0.09	-0.9±0.3	-0.7±0.03	0.02±0.008
DMBIS-PG	-4.3±1.2	-10.0±0.8	-13.5±4.9	1.0±0.6	0.01±0.01	0.01±0.06
DMBIS-ethanol	No change	No change	*	No change	No change	*
DMBIS-PEG 400	+3.0±1.4	+2.0±0.3	+22.5±4.0	0.29±0.04	-0.06±0.01	0.3±0.01
DMMCBI-water	-1.0±0.05	-8.0±3.0	-3.5±.5	0.05±0.01	0.2±0.01	-0.01±0.002
DMMCBI-PG	-3.0±0.5	-7.0±2.0	*	0.25±0.06	-0.24±0.02	*
DMMCBI-ethanol	-9.0±0.04	Two peaks at 84° & 88°C	*	0.7±0.3	No change	*
DMMCBI-PEG 400	+6.0±0.5	+2.0±0.6	*	0.4±0.03	-0.07±0.01	*
TBDOC-water	-9.0±1.6	-10.0±2.0	No change	No change	0.01±0.001	No change
TBDOC-PG	*	Merger of $T_{m1} \& T_{m2}$ at $70^{\circ}C$	*	*	No change	*
TBDOC-ethanol	+9.0±0.8	+12.0±0.4	+4.5±0.08	0.3±0.09	-0.8±0.1	0.2±0.05
TBDOC-PEG 400	No change	No change	*	0.5±0.03	- 0.01±0.005	*

Table 5.2: Mean temperature shifts and enthalpy change of SC after treatment with laurocapram, laurocapram analogues and iminosulfurane formulations

* Missing peak SD: Standard deviation

Penetration modifier→	Laurocapram	N-0915	DMBIS	DMMCBI	TBDOC	
Concentration (µM)↓	Concentration (µM)↓ Percent viability (%) of keratinocytes (Mean±SD)					
400	0	n/a	0	9.1±0.01	10.9±0.1	
40	0	44.3±0.01	51.2±0.02	70.2±0.1	32.0±0.1	
4	0	44.01±0.01	64.5±0.1	81.3±0.2	65.0±0.05	
0.4	93.6±0.1	44.6±0.02	85.01±0.1	99.9±0.3	71.0±0.03	
0.04	91.8±0.2	52.7±0.02	99.1±0.2	99.9±0.2	72.8±0.1	
0	100	100	100	100	100	
• SD : standard deviation						

Table 6.1: Viability of keratinocytes following treatment with selected formulations of penetration modifiers

Penetration modifier \rightarrow	Laurocapram	N-0915	DMBIS	DMMCBI	TBDOC
Concentration (M)	Damaan	4 mahilitar	(07) of down	al fibrablast	
Concentration (µm)	rercen	it viability	(%) of defin	iai iidrodiasi	.5
		(1	(lean±SD)		
400	0	n/a	58.1±0.1	69.9±0.2	10.9±0.1
40	14.3±0.2	2.6±0.1	60.2±0.1	81.9±0.2	48.5±0.2
4	66.4±0.1	86.4±0.2	65.2±0.1	89.1±0.2	67.8±0.1
0.4	80.8±0.2	94.6±0.2	76.5±0.04	91.1±0.1	74.4±0.2
0.04	81.5±0.1	96.9±0.3	80.0±0.1	95.5±0.2	75.7±0.2
0	100	100	100	100	100

Table 6.2: Viability of dermal fibroblasts following treatment with selected formulations of penetration modifiers

SD: standard deviation

Penetration modifier	Solubility in propylene glycol mg/ml Mean±SD (n=3)	Log P	Molecular weight
Laurocapram	104.45±0.45	4.60	281.47
N-0915	5.76±0.62	5.72	269.38
DMBIS	26.71±4.46	2.58	260.15
DMMCBI	27.64±4.0	1.30	275.34
TBDOC	43.49±2.73	6.24	396.61

 Table 6.3: Physicochemical properties of penetration modifiers

SD: standard deviation

Parameter	Laurocapram	N-0915	TBDOC	DMMCBI	DMBIS
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
	(n=4)	(n=6)	(n=8)	(n=4)	(n=4)
Lag time (hrs)	8.49±5.9	4.17±2.27	No release till 60	36±0	9.34±0.1
			hours		
Flux (µg/	0.11±0.02	0.25±0.05	No release till 60	0.35±0.11	76.07±18.6
cm ² .hr)			hours		
$Q_{60}(\mu g/cm^2)$	6.2±1.3	14.21±6.01	No release till 60	7.1±2.60	4652.5±1043.
			hours		8

Table 6.4: Permeation parameters of penetration modifiers across epidermis

Lag time is time taken for drug to reach steady state n is the number of replicates SD: standard deviation

Paramet	Laurocapram	N-0915	TBDOC	DMMCBI	DMBIS	4-Bromobenzamide
er	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean±SD	Mean±SD
	(n=4)	(n=4)	(n=4)	(n=4)	(n=4)	(n=4)
Lag time	0±0	0±0	0±0	0±0	0±0	0±0
(hrs)						
Q60	2.54±1.01	11.2±2.4	323.1±160.0	7658.2±1663.1	14771.8±1433.2	11182.45±1386.58
					8	
$(\mu g/cm^2)$						

Table 6.5: Permeation parameters of penetration modifiers across dermis

Lag time is time taken for drug to reach steady state n is the number of replicates SD: standard deviation
Table 8.1: Various subclasses of flavonoids

Class	Examples
Flavonol	Quercetin, kaempferol, morin, rutin, fisetin
Flavones	Hesperitin, naringin, naringenin
Flavanones	Luteolin, chrysin, apigenin
Flavan-3-ols	Catechin
Isoflavones	Genistein, diadzin
Anthocyanidins	Cyanidin, delphinidin

Quercetin glycoside	Source	Content of quercetin (mg/kg)	Reference
Quercetin-3,4'- glucoside	Onion	284-486	(299)
Quercetin-3-glucoside			
Quercetin-3-rhamnoglucoside (rutin)	Black tea	10-25	(299)
Quercetin-3-galactoside	Apple	21-72	(299)
Quercetin-3-rhamnoside			
Quercetin-3-arabinoside			
Quercetin-3-glucoside			
Quercetin-3-rhamnoglucoside	Black currant	44	(300)
Quercetin-3-rhamnoside			
Quercetin-3-galactoside			

 Table 8.2: Main dietary quercetin glycosides and their sources in the diet

Source in fruits and vegetables	Quercetin content (mg/100 g)
Apple	2-26
Apricot	2.5-5.3
Blueberry	10.5-16
Black currant	3.3-6.8
Broad bean	2-134
Broccoli	0.6-3.7
Cabbage, red	0.19-0.62
Cabbage, white	0.01-0.1
Cauliflower	0.1-3.1
Chives	10.4-30
Cranberry	149
Elderberry	10.5-24
Endive	0.1-2.6
Onion, white	18-54
French bean	3.2-4.5
Grape, red	1.5-3.7
Grape, whitw	0.2-1.2
Kale	1.2-11
Leek	1-2.5
Lettuce	0.2-47
Pear	0.3-4.5
Plum	0.9-1.5
Rasberry	0.5-2.9
Red currant	0.2-2.7

 Table 8.3 Quercetin content in fruits, vegetables and beverages, modified from (142)

Sour cherry	2.3-8
Strawberry	0.6-1
Sweet cherry	0.6-2.4
Tomato, cherry	0.4-7.1
Tomato red	0.16-43
White currant	0.3-2.8
Beverage	Quercetin content (mg/100 ml)
Apple juice	0.25
Grape juice	0.44
Grapefruit juice	0.49
Lemon juice	0.74
Orange juice	0.34-0.57
Tomato juice	1.1-1.3
Tea, black bags	1.7-2.5
Tea, black loose	1.0-1.6
Tea, green	0.11-2.3
Tea, oolong	1.3
Wine red	0.2-1.6

Quercetin fortified Tang [®] dry mix	Quercetin
	-Tang dry mix
	Sugar, Fructose, Citric Acid, Calcium Phosphate, Contains Less Than 2% of Orange Juice Solids, Natural Flavor; Ascorbic Acid (Vitamin C), Vitamin E Acetate, Niacinamide, Vitamin B6, Vitamin A Palmitate, Riboflavin (Vitamin B2), Beta Carotene; Maltodextrin, Sucralose, Acesulfame Potassium and Neotame (Sweeteners), Guar and Xanthan Gums, Artificial Color, Yellow 5, Yellow 6, Butyl hydroxyl anisole
	-Water
Quercetin fortified First Strike [™] Bar	-Quercetin
	-First Strike™ Bar
	Raspberry filling (fructose, maltodextrin, water, raspberry concentrate, food starch, modified carrageenan, natural flavors, malic acid), maltodextrin, corn syrup, dried cranberries, crisp corn (degermed yellow corn meals, sugar, malt extract, salt, calcium carbonate, mono and diglycerides), apple nuggets (dried apple pieces, flavor, red 40, blue 1), partially hydrogenated cottonseed/soyabean oil, whey protein concentrate, apple powder, rice bran concentrate, glycerin, fructose, natural and artificial flavors, lecithin, vitamin premix (ascorbic acid, DL- alpha-tocopherol acetate, niacinamide, pyridoxine hydrochloride, riboflavin, thiamine mononitrate, folic acid, cholecalciferol, cyanocobalamin, zinc oxide), ascorbylpalmitate, natural mixed tocopherols, red 40, blue 1

Table 10.1: Composition of various quercetin fortified supplements

RealFXTM Q-PlusTM Chews	- Quercetin
	- Sugar, corn syrup, ascorbic acid, soy lecithin, natural and artificial flavors, palm oil, corn starch, glycerin, xylitol, carrageenan, niacinamide, sucralose, sunflower oil, FD&C yellow 6, FD&C yellow 5, folic acid.

Variable*	RealFX TM Q-Plus TM Chews (N=6)	Quercetin fortified Tang [®] suspension(N=6)	First Strike™ Bar(N=6)
Height (m)	1.7±0.07	1.7±0.1	1.8±0.1
Weight (kg)	60.8±10.9	65.7±4.9	71.7±10.7
Age (y)	20.3±1.4	19.5±1.1	20.5±1.1

Table 10.2: Subject Characteristics

* Data are shown as means \pm standard deviation

Solvent (n=3)	Solubility (µg/ml)±SD [*]
Phosphate buffer saline, pH 7.4	1.4±0.03
Water adjusted to pH 4	1.9±0.07
Spring water, pH 6.3	1.9±0.03
Methanol	8564.0±75.4

Table 10.3: Solubility of quercetin in various solvents.

*SD: standard deviation

Type of Formulation (n=3)	Quercetin content (mg) ±SD	Claim (mg)	
RealFX TM Q-Plus TM Chews	265.1±11.1	250.0	
Quercetin fortified First	531.2±32.1	500.0	
Strike™ Bar			

Table 10.4: Quercetin content in Q-chews[®] and First StrikeTM Bar

*SD: standard deviation

Parameters	Tang [®] (N=6)	First Strike TM (N=6)	RealFX Q-Plus Chews/
			Q-Chew TM (N=6)
C _{max} (µg/l)	354.4±87.6	698.1±189.5	1051.9±393.1
T _{max} (h)	4.7±0.3	2.3±0.5	3.3±0.8
AUC _{total} (µg/l.h)	3845.9±689.8	5314.8±1432.4	4147.1±671.8
L_{z} (1/h)X10 ⁻²	9.9±0.02	9.9±0.01	15.4±0.04
AUMC _{total} (µg/l.h ²)	48141.3±12506.9	66231.8±22169.0	32103.6±5607.5
T _{1/2} (h)	8.3±1.4	8.0±1.3	5.5±0.9
MRT (h)	12.9±2.0	11.4±1.7	7.9±1.3
Cl (l/h)	153.8±28.4	125.4±27.1	143.4±29.7
V _z (l)	2011.0±664.7	1255.2±190.6	987.1±138.4
$V_{ss}(l)$	2131.7±686.1	1279.0±194.8	1140.0±286.7

 Table 10.5: PK parameters of quercetin administered via various oral carrier systems

*Data are shown as means ± SEM (Standard Error of Mean)

	Placebo			Quercetin		
Variable	Pre	Post	Post-Pre	Pre	Post	Post-Pre
W0 ₂ ,	3.00	2.98	-0.01	3.06	3.05	-0.01
l·min ⁻¹	± 0.72	± 0.68	± 0.05	± 0.74	± 0.74	± 0.05
V _T (L)	2.13	2.08	-0.06	2.23	2.07	-0.17
	± 0.49	± 0.48	± 0.18	± 0.63	± 0.54	± 0.20
RR (bpm)	51.37	50.87	-0.50	49.59	52.74	3.15
	± 7.85	± 6.86	± 3.71	± 8.30	± 9.57	± 5.14
V _E (L/min)	107.82	104.42	-3.40	107.89	106.90	-0.99
	± 23.20	± 24.23	± 14.27	± 25.60	± 26.41	± 12.05
RER	1.09	1.09	0.01	1.12	1.08	-0.04
	± 0.13	± 0.15	± 0.15	± 0.09	± 0.11	± 0.11
Blood Lactate,	7.57	7.80	0.24	7.87	7.69	-0.18
mmol·l ⁻¹	± 2.20	± 1.67	± 0.94	± 1.18	± 1.57	± 0.93
HR (bpm)	194	194	0	195	196	1
	± 10	± 10	+ 4	± 8	± 8	± 4
Pain	6±2	7±3	0±1	6±3	6±3	0±1
RPE	19±1	19 ± 1	0±1	18 ± 1	18±2	0±1
Duration (sec)	593	594	1	601	607	6
	± 96	± 83	± 36	± 77	± 71	± 21

Table 11.1: Responses to peak exercise before and after 5 days of placebo or quercetin-supplemented food bar consumption. Values are mean ± SD

 VO_2 , oxygen uptake; V_T , tidal volume; RR, respiratory rate; V_E , expired volume; RER, respiratory exchange ratio (RER). HR, heart rate; RPE, rating of perceived exertion.

Table 11.2: Mean ± S	SD ratings of Dela	ayed Onset Mus	cle Soreness (DO	MS) after a V
O _{2max} test that was co	onducted before a	and after 5 days	of placebo or que	ercetin-
supplemented food b	ar consumption			

	Plac	cebo	Quercetin		
Time of Rating	Pre	Post	Pre	Post	
10 min post	4.8 ± 1.7	4.8 ± 2.1	3.7 ± 2.2	3.8 ±1.8	
24 hr post	2.0 ± 1.7	3.2 ± 1.5	1.3 ± 1.5	2.3 ± 2.5	
48 hr post	1.6 ± 1.9	1.3 ± 1.3	0.6 ± 0.6	1.6 ± 2.8	
72 hr post	1.3 ± 2.2	0.6 ± 0.9	0.6 ± 0.8	0.8 ± 1.3	

Ratings were assessed by using a pencil-paper scale with a 10 cm line anchored with "No Soreness" and "Unbearable Pain" on the left and right, respectively.

	Day 1		Da	ay 3	Day 6	
	Placebo	Quercetin	Placebo	Quercetin	Placebo	Quercetin
Male		#	#	19.31	#	988.18
(n=5)	#			± 43.18		± 356.59
Female	#	#	#	#	#	1196.70
(n=6)						± 696.44
Total	#	#	#	8.78	#	1101.92
(n=11)				± 29.11		$\pm 552.48^{*^{\dagger}}$

Table 11.3: Mean ± SD plasma quercetin concentrations (ng/ml) after 1, 3, or 6 days of supplementation

Quercetin concentration below the detectable limit (3 ng/ml); *Significantly greater than placebo on the same day (P < 0.05); *Significantly greater than day 3 of the same variable (P < 0.05).

Table 11.4: Mean ± SD urine quercetin	concentrations ((ng/ml) a	fter 1, 3, or	6 days
of supplementation				

	Day 1		D	bay 3	Day 6	
	Placebo	Quercetin	Placebo	Quercetin	Placebo	Quercetin
Male (n=5)	127.68± 102.36	76.93±99.59	47.12± 44.74	75.20± 69.01	19.05± 44.74	687.57± 569.46
Female (n=6)	20.24± 21.76	35.32± 42.75	27.32± 16.93	113.55±112.77	11.86± 16.05	640.81± 579.78
Total (n=11)	69.08± 87.04	54.23±73.17	36.32±32.42	96.12± 93.09*	15.13± 18.61	662.07± 546.25* [†]

*Significantly greater than placebo on the same day (P < 0.05); [†]Significantly greater than day of the same variable (P < 0.05)

	Placebo			Quercetin		
Measure	Baseline	Pre-ex.	Post-ex.	Baseline	Pre-ex.	Post-ex.
Glucose, mM	5.30 ±	5.45 ±	6.38 ±	5.49 ±	5.41 ±	6.15 ±
	0.53	1.31	0.79*†	0.35	1.37	1.05
Lactate, mM	1.17 ±	2.47 ±	8.46 ±	1.27 ±	2.44 ±	8.28 ±
	0.43	0.81	2.41*†	0.35	0.47	3.93*†
Glycerol, mM	$0.06 \pm$	$0.04 \pm$	0.19 ±	$0.05 \pm$	$0.06 \pm$	0.17 ±
	0.02	0.02	0.05*†	0.03	0.03	0.05*†
Quercetin, µM	$0.02 \pm$	$0.02 \pm$	$0.02 \pm$	$0.02 \pm$	4.76 ±	7.68 ±
	0.00	0.00	0.00	0.00	2.56*	3.77*†

Table 12.1: Comparison of select blood concentrations among trials

Values are means \pm SD.

Different from baseline (*) or Pre-ex. (†) within trials; (P<0.05)

	Plac	ebo	Querc	etin
Measure	SS	TT	SS	TT
RPE	10.9 ± 2.4	17.4 ± 2.9*	11.4 ± 2.1	17.0 ± 1.8*
RP	1.0 ± 1.2	3.7 ± 3.3*	1.5 ± 1.6	3.6 ± 3.1*
RTC (mm)	57.0 ± 20.0	69.0 ± 22.5	48.7 ± 16.6	66.6 ± 21.6*
RM (mm)	89.9 ± 28.9	93.8 ± 21.2	82.3 ± 24.2	80.2 ± 34.9

Table 12.2: Perceptual performance data during final minute of steady-state (SS) and time trial (TT) exercise

Values are means \pm SD. Different from SS within trials (*) (P<0.05). Abbreviations defined in text.

Subject Number	Placebo	Quercetin
1	123.6	138.2
2	155.8	144.2
3	140.8	114.8
4	113.7	116.0
5	180.4	143.9
6	182.1	195.0
7	196.7	213.3
8	129.4	141.7
9	172.8	163.6
10	139.3	140.7
Mean	153.5	151.1
SD	28.3	31.6

Table 12.3: 15-minute time trial work performance (kJ)

	Visit 1	Informed consent document administered
PHASE 1	Visit 2	Baseline testing of criterion measures* (Blood sample will not be assessed at
		this visit)
	Visit 3	Criterion measure assessment and blood sample (Arm 1 – dominant/non
		dominant chosen in balanced order at random)
		Exercise
		Post-exercise criterion measures
	Visits 4-8	Criterion measures one arm and blood samples
PHASE 2	Visit 8	Begin quercetin supplementation (7 days pre-exercise, through Visit 15),
		wear activity monitor, and continue daily POMS surveys -
		Ingest two First Strike Bars at about 8am and again at about 8pm each day
PHASE 3	Visit 9	Baseline testing of criterion measures (Blood sample will not be assessed at
		this visit)
	Visit 10	Criterion measure assessment and blood sample (Arm 2 contralateral arm
		from day 2)
		Exercise (24 maximal eccentric elbow flexion contractions)
		Post-exercise criterion measures
	Visits 11-15	Criterion measures (Arm 2) and blood samples

Table 13.1: Detailed schematic of the testing schedule

*Criterion measures are POMS survey, soreness, isometric strength, isokinetic strength, range of motion, circumference of the upper arm, and blood sample. (Blood samples were not assessed on Visit 2 or 9)



Figure 2.1: Diagrammatic representation of structure of skin (31).

Figure 2.2: Different mechanisms of action of permeation enhancers (35) (a) Action at intercellular lipids domain of the stratum corneum. (b) Interaction with protein structures leads to splitting of the stratum corneum that is clinically unacceptable. (c) Action within corneocytes leading to swelling, keratin denaturation and vacuolation within individual horny layer cells of stratum corneum.



Figure 2.3: **Bent spoon conformation of laurocapram**, modified from (37) where N1 represents nitrogen atom in laurocapram ring and C13, C16, C22 and C23 represent carbon atoms at 13, 16, 22 and 23 positions respectively. N1, C16, C22 and C23 form an angle of 62° that leads to energy of conformation, E equal to 6.1 kcal/mole.



N1-C16-C22-C23 = 62° E = 6.1 kcal / mole



Figure 4.1: Permeation profile of DEET in presence of laurocapram formulations



Figure 4.2: Permeation profile of DEET in presence of N-0915 formulations



Figure 5.1: DSC of untreated and solvent treated human SC



Figure 5.2: DSC of untreated and laurocapram formulations treated human SC



Figure 5.3: DSC of untreated and N-0915 formulations treated human SC



Figure 5.4: DSC of untreated and DMBIS treated human SC



Figure 5.5: DSC of untreated and DMMCBI treated human SC



Figure 5.6: DSC of untreated and TBDOC treated human SC



Figure 5.7: IR spectrum of SC treated with selected vehicles



Figure 5.8: IR spectra of SC treated with laurocapram in selected vehicles



Figure 5.9: IR spectra of SC treated with N-0915 in selected vehicles



Figure 5.10: IR spectra of SC treated with DMBIS in selected vehicles



Figure 5.11: IR spectra of SC treated with DMMCBI in selected vehicles



Figure 5.12: IR spectra of SC treated with TBDOC in selected vehicles



Figure 6.1: Standard plot of absorbance vs. number of dermal fibroblasts


Figure 6.2: Standard plot of absorbance vs number of epidermal keratinocytes

Figure 6.3: Scanning electron microscopy (SEM) images of skin surface treated with various formulations of penetration modifiers. SC = stratum corneum; PG = propylene glycol; N-0915 = 3-dodecanoyloxazolidin-2-one; DMBIS = *S*,*S*-Dimethyl-*N*-(4-bromobenzoyl) iminosulfurane; DMMCBI = *S*,*S*-Dimethyl-*N*-(2-methoxycarbonylbenzenesulfonyl) iminosulfurane; TBDOC = *tert*-Butyl 1 –dodecyl-2-oxoazepan-3-yl-carbamate.







Figure 6.4: Permeation profile of penetration modifiers across human cadaver epidermis



Figure 6.5: Permeation profile of penetration modifiers across human cadaver dermis



Figure 10.1: Calibration plot of quercetin in methanol matrix

Figure 10.2: Calibration plot of quercetin in plasma matrix



Figure 10.3: Mean total quercetin in plasma following administration of quercetin via various oral carrier systems. Points T0, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, T13 on X-axis represent baseline, 15min, 30min, 45min, 1hr, 2hrs, 3hrs, 4hrs, 6hrs, 8hrs, 12hrs, 24hrs, 28hrs and 32hrs



Figure 10.4: Plasma profile of quercetin in subjects following administration of First StrikeTM Bar. UM 3, 9, 11, 12, 13 and 15 represent subjects that received First StrikeTM Bar. Points T0, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, T13 on X-axis represent baseline, 15min, 30min, 45min, 1hr, 2hrs, 3hrs, 4hrs, 6hrs, 8hrs, 12hrs, 24hrs, 28hrs and 32hrs



Figure 10.5: Plasma profile of quercetin in subjects following administration of RealFXTM **Q-Plus**TM **Chews.** UM2, 4, 8, 14, 17 and 18 represent subjects that received Q-PlusTM Chews. Points T0, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, T13 on Xaxis represent baseline, 15min, 30min, 45min, 1hr, 2hrs, 3hrs, 4hrs, 6hrs, 8hrs, 12hrs, 24hrs, 28hrs and 32hrs.



Figure 10.6: Plasma profile of quercetin in subjects following administration of Quercetin fortified Tang[®] suspension. UM1, 5, 6, 7, 10 and 16 represent subjects that received Q-Plus[™] Chews. Points T0, T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, T13 on X-axis represent baseline, 15min, 30min, 45min, 1hr, 2hrs, 3hrs, 4hrs, 6hrs, 8hrs, 12hrs, 24hrs, 28hrs and 32hrs



Figure 11.1: Mean ± SD effect of placebo and quercetin supplementation

on $\dot{V}O_{2max}$ expressed in ml/kg/min. There was no significant effect across conditions or over time





Figure 11.2: Calibration plot of quercetin in plasma



Figure 11.3: Calibration plot of quercetin in urine







Figures 13.2(A-D) Isokinetic strength of subjects in Placebo (A, C) and Quercetin (B, D) groups



D





Figure 13.3 (A-B): Arm circumference of subjects in Placebo (A) and Quercetin (B) groups

Figure 13.4 (A-B): Arm angle of subjects in Placebo (A) and Quercetin (B) groups







B









Figure 13.7 (A-B): Creatine kinase levels of subjects in Placebo (A) and Quercetin (B) groups

Figure 13.8 (A-B): C- reactive protein (CRP) levels of subjects in Placebo (A) and Quercetin (B) groups









Structure 4.1: Structures of penetration modifiers





S,*S*-dimethyl-*N*-(4-methoxycarbonylbenezenesulfonyl)

iminosulfurane (DMMCBI)



S,S-dimethyl-N-(4-bromobenzoyl)

iminosulfurane (DMBIS)



tert-butyl 1 -dodecyl-2-oxoazepan-3-yl-carbamate (TBDOC)





Structure 8.2: Structure of quercetin, 3, 5, 7, 3', 4'-pentahydroxyflavone



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