ABSTRACT

Faqir Muhammad. Topical absorption and toxicity studies of jet fuel hydrocarbons in skin.
(Under the directions of Dr. Jim E. Riviere)

Kerosene-based fuels have been used for many decades. Over 2 million military and civilian personnel each year are occupationally exposed to various jet fuel mixtures. Dermatitis is one of the major health concerns associated with these exposures. In the past, separate absorption and toxicity studies have been conducted to find the etiology of such skin disorders. There was a need for integrated absorption and toxicity studies to define the causative constituents of jet fuel responsible for skin irritation. The focus of this thesis was to study the percutaneous absorption and to identify the hydrocarbons (HC) causing irritation in jet fuels so that preventive measures could be taken in the future.

The initial study was conducted to understand the possible mechanism for additive interactions on hydrocarbon absorption/disposition in silastic, porcine skin and isolated perfused porcine skin flap (IPPSF) models. The influence of JP-8 (100) additives (MDA, BHT, 8Q405) on the dermal kinetics of $^{14}$C-naphthalene and $^{14}$C/$^3$H-dodecane as markers of HC absorption was evaluated. This study indicated that individual and combination of additives influenced marker disposition in different membranes. MDA was a significant suppressor while BHT was a significant enhancer of naphthalene absorption in IPPSF. The 8Q405 significantly reduced naphthalene content in dosed silastic and skin indicating a direct interaction between additive and marker HC. Similarly, the individual MDA and BHT significantly retained naphthalene in the stratum corneum of porcine skin, but the combination of both of these additives statistically decreased the marker retention in the stratum corneum suggesting a potential biological interaction. This study concluded that all components of a chemical mixture should be assessed since the effects of single components administered alone or as pairs may be confounded when all are present in the complete mixture. However, this study indicated that the marker HC absorption was similar across JP-8 and JP-8 (100) due to the opposite effects of MDA and BHT on HC absorption.
The remaining studies were focused on neat HC absorption and toxicity potential in pig skin. There were no published reports regarding dose-related percutaneous absorption of jet fuel HC that are crucial for risk assessment studies. Three dosing mixtures (1X, 2X, and 5X) comprising 5 aliphatic (C11-C15) and 2 aromatic (naphthalene and dimethyl naphthalene (DMN)) HC were dosed using in vitro porcine skin flow-through diffusion cells with hexadecane as the diluent. Perfusate samples were analyzed with gas chromatography flame ionization detector (GC-FID) using a headspace solid phase micro-extraction (SPME) fiber technique. Absorption parameters were estimated and quantitative structure permeability relationship model was constructed to predict the permeability of unknown jet fuel HC using their known physico-chemical properties. This study suggested a dose related increase in percutaneous absorption of aromatic HC. This dose related increase in absorption of HC focuses attention to occupational settings where workers are exposed to jet fuels on daily basis. In order to assess the effects of jet fuel pre-exposure on subsequent dermal absorption of HC, pigs were exposed to JP-8 jet fuel soaked cotton fabrics for 1-day and with repeated daily application for 4-days to mimic the real occupational scenario. A dosing mixture comprising 8 aliphatic (C9-C16) and 6 aromatic HC (ethyl benzene, o-xylene, trimethyl benzene (TMB), cyclohexyl benzene (CHB), naphthalene and DMN) was dosed to in vitro flow-through diffusion cells containing 1 and 4-day JP-8 pre-exposed and control skin using water + ethanol (50:50) as diluent. Perfusate samples were analyzed with GC-FID and SPME fiber technique. The data revealed that there was 2-3 fold and 3-4 fold increase in percutaneous absorption of short chain aliphatic and most of aromatic HC through 1-day and 4-day JP-8 pre-exposed skin, respectively. Three aromatic HC (naphthalene, CHB and DMN) were found to have persistent skin retention in 4-day JP-8 pre-exposures. Stratum corneum studies with Fourier Transform Infrared (FTIR) spectroscopy suggested that lipid extraction might be the primary mechanism for this increase in hydrocarbon absorption through JP-8 pre-exposed skin. These studies indicated that the single dose absorption data from naive skin for jet fuel HC may not be optimal to predict the toxic potential for repeated exposures.
Finally, in vivo studies with the individual hydrocarbons (HC) of jet fuel were conducted to identify the causative agents in fuel induced skin irritation. In order to mimic occupational scenarios, cotton fabrics soaked with 300µl of JP-8 and 14 different aliphatic and aromatic HC were placed on back of pigs for 1-day and with repeated daily exposures for 4-days. The 1-day in vivo HC exposures revealed no erythema but significant redness was observed in 4-day tridecane, tetradecane, pentadecane and JP-8 exposed sites. The aromatic HC did not produce any macroscopic lesions in 1 or 4-day in vivo exposures. Epidermal thickening and epidermal cellular layers were significantly ($P < 0.05$) different in tridecane, tetradecane, pentadecane and JP-8 treated sites as compared to control sites. No significant differences were observed in aromatic HC exposed sites. Subcorneal micro abscesses filled with inflammatory cells were observed with most of long chain aliphatic HC and JP-8 in 4-day in vivo exposures. Ultrastructural studies depicted that jet fuel HC induced cleft formation in the intercellular lipid lamellar bilayers. The degree of damage to the stratum corneum and epidermis was proportional to the length of in vivo HC exposures. This study indicated that the aliphatic HC are the main source of jet fuel caused irritation. A comparison of absorption and toxicity data for aliphatic HC revealed that toxic point might be in the transition of absorption to retention phases of these HC as tridecane is the last HC that was absorbed through the skin and tetradecane was the first HC to be retained in the skin. Furthermore, the macroscopic and microscopic alterations caused by these two HC in these in vivo studies were similar to the JP-8 exposed animals, suggesting that tridecane and tetradecane might be toxicologically important HC in jet fuel induced skin irritation.
TOPICAL ABSORPTION AND TOXICITY STUDIES OF JET FUEL HYDROCARBONS IN SKIN

by

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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

COMPARATIVE BIOMEDICAL SCIENCES

Raleigh

2004

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DEDICATION

This thesis is dedicated

To my supportive father M. Hussain and to the memory of my extraordinary mother, whose love and sacrifices made everything possible for me,

To my understanding wife Tanzila, for her unconditional love

To my beautiful son Safwan, for bringing happiness in my family

To several of my teachers for their continuous guidance
BIOGRAPHY

Faqir Muhammad was born February 15, 1972 in Punjab, a province of Pakistan. He completed his primary and high school education from T.T. Singh district in Punjab. He graduated with a Doctor of Veterinary Medicine degree from University of Agriculture, Faisalabad, Pakistan in 1995 with distinction. He then received a Master of Science degree in Veterinary Pharmacology from the same university in 1997. Following graduation he was employed by the department of Livestock and Dairy Development, Lahore, Pakistan as veterinary officer in May 1997. He served there for about two years, and then he was selected as lecturer in the Faculty of Veterinary Medicine, University of Agriculture, Faisalabad, Pakistan in August 10, 1999. He married Tanzila Mahmood on August 15, 1999. Because of his excellent academic career, the Government of Pakistan awarded him a merit scholarship in 2001 for PhD studies abroad. He came to United States in August 2001 and selected North Carolina State University to pursue his Doctor of Philosophy degree. He joined the lab of Dr. Jim E Riviere on full time basis in order to complete the requirements of Doctor of Philosophy in Comparative Biomedical Sciences (Pharmacology).
ACKNOWLEDGEMENTS

First, I would like to thank Allah Almighty for His great blessings and granting me with the opportunity, wisdom and friendly environment to complete this piece of work.

My gratitude, appreciation, and respect is extended to Dr. Jim E. Riviere for his acceptance of me as his student, his belief in my abilities, his guidance, and his interest in my future. I have valued his advice and knowledge he has shared with me over these three years, and for his interest in me as an individual. I would like to acknowledge and thank Dr Nancy A. Monteiro-Riviere for her constant drive to make me a better professional and for her tolerance of all of my imperfections. I am grateful to Dr. Ronald E Baynes for useful discussions on mixture interaction studies. I am grateful and appreciative of Dr Muquarrab A. Qureshi for helping me to seek admission to NCSU and providing me with the support that allowed me to pursue my dream. I would like to thank the U.S. Air Force Office of Scientific Research for funding this research (Grant No. F49620-01-1-0080).

I would like to thank post doc and fellow graduate students of my lab especially Dr. Summer and Dr. Deon for their friendship, and for enduring together all the difficult moments we have been through. I am especially grateful to the staff at the Center for Chemical Toxicology Research and Pharmacokinetics for their technical support and trouble shooting assistance, with special thanks to Al Inman, Jim Brooks and Jim Yeatts. Thanks to Beth Barlow and Connie Engel for their assistance in conducting various in vitro and in vivo experiments. Many thanks are extended to Jiming Wang for helping me with computer problems and thanks to Luann Kublin for her administrative assistance.

Words cannot express how much I love and miss my father, and how thankful I am to him for allowing me to come to the United States to pursue PhD studies. My wife has been my support through this entire academic pursuit. Her personal strength and belief in me have kept me going during times when initiative was weak. I thank her for her support, unconditional love, understanding, and ability to accept me for me and nothing more. My son, the unique blessing of God (SWT), has made the last year of my studies wonderful and memorable. I owe a tremendous debt of gratitude to Mr. Rafique, Mrs. Shamim Rafique and their children for taking care of my father in my absence. I
also owe immense gratitude to my father in law, mother in law and brothers in law Ijaz, Shoaib, Zubair and Irfan Anwar for their sincere, devoted and untiring efforts for me and for my family. I extend my hand in gratitude to uncle Ahmad Saeed, Mrs. Misbah Liquat, Mr. Liquat, and so many other relatives for their continual help. I pay special thanks to Dr. Sikandar Hayat, Professor and Dean FVS, for his unending belief in me regardless of the situation.

I am also thankful to Mrs. Rana Qureshi for taking care of my wife and continuously inspiring me towards higher goals. I am grateful to my friends Mr. Sajid, Mr. Manzoor, Mr. Waseem, Mr. Arif and their families for making Raleigh feel like home. I am very thankful to my friends Dr. Azeem, Mian Ashraf, Dr Masood, and Dr Fahim for their sincere help whenever I need. Finally, I want to thank Allah Almighty and all the individuals once again for helping me through out the course of time. You will never know just how much I appreciate what you have done for me, not just for this degree, but for making sure that I see all of life's possibilities, not just the limitations.
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1. INTRODUCTION

In the era of systems toxicology, skin is one of the most important organs that is affected with a variety of toxic insults. This protective barrier serves to keep chemicals and other foreign constituents from entering and interfering with our complicated and finely tuned biochemical and hormonal internal systems. However, the skin is not a total barrier (Dugard and Scott, 1984) and many chemicals have been shown to penetrate into (Schaefer et al., 1982) and through the skin (USEPA, 1992), thus causing local or systemic toxicity. Moreover, knowing the exposure to the skin for a specific chemical does not allow predicting local or systemic toxicity without information about absorption and penetration of that chemical into and across the skin. It is essential to have reliable information about the flux of chemicals across the skin, the magnitude and duration of exposure in the workplace or environment in order to determine potential risks.

Amongst the occupational skin diseases, jet fuel induced skin irritation is believed to be a major health concern in the professional workers. Jet A, JP-8 and JP-8 (100), the most widely used jet fuels by the military, are complex mixtures of hydrocarbons including their thousands of isomers and performance additives (Subcommittee on Jet Propulsion 8 fuel of Committee on Toxicology, 2003). Aircraft fuel maintenance workers may be exposed to liquid fuel for more than 10 min. In cold climates, JP-8 aerosol may be formed when a cold jet engine is started because JP-8 has a low flash point. This plume coming from the jet engine for a short period of time on startup is visible as a white cloud and contains fuel aerosol droplets in addition to ice crystals. A crew chief standing in this plume might get sufficient JP-8 aerosol on the clothing and skin to raise concern about skin penetration (McDougal and Rogers, 2004). Due to the complexity of jet fuels, it is not easy to determine their hazardous effects. The question is: what types of interactions can be experimentally determined or what markers should be selected to see their toxic effects on skin after exposure to a mixture of hundreds of individual constituents? There are some studies available in the literature regarding percutaneous absorption and in vivo toxicity of jet fuels. Such studies have used a maximum of 2-4 marker hydrocarbons to access the toxicity of these complex mixtures. As a result of
this limited data, it was not possible to conclude what is responsible for skin irritation in jet fuel. Our laboratory decided to expand the range of marker hydrocarbons and assessed their in vitro toxicity in different cell culture systems (Chou et al., 2002, 2003). The dermal absorption and in vivo toxicity of these markers HC remained to be investigated. Also, there were concerns in the literature that performance additives in jet fuels might modulate the marker absorption or penetration in skin. In an effort to answer all these issues and to propose the irritation-causing marker constituents in jet fuels, the following studies were conducted in this thesis.

The first set of experiments (chapter 3) describes the effects of JP-8 (100) performance additives (MDA, BHT, 8Q405) on the dermal absorption and disposition of marker HC (naphthalene, dodecane) in different membrane model systems. These studies represent novel data demonstrating mechanisms of various mixture interactions in synthetic (silastic) and biological membranes (porcine skin and IPPSF). Most of the mixture interaction studies in literature were conducted in a single membrane system. These studies emphasized the simultaneous use of simple and complex membranes to assess true mixture interactions and suggest the suitable membrane model for predictive purposes. Furthermore, the absorption and disposition parameters of both markers were demonstrated across JP-8 and JP-8 (100) jet fuels.

In spite of the fact that absorption and toxicity of jet fuels were sometime addressed in the literature, no attention had been paid towards the dose related absorption of fuel constituents. Such studies are crucial to having a true understanding of occupational risk. Most previous studies used one marker radioactive HC at a time to look at its percutaneous absorption and thus were not cost effective. There was a need to establish an efficient and economical analytical system for the simultaneous analysis of a number of neat HC of jet fuels. Chapter 4 of this thesis presents a novel approach for the analysis of neat HC from biological matrices by using solid phase micro extraction (SPME) technique with headspace sampling. This is a solvent free extraction technique that can be easily connected to a gas chromatograph (GC). This chapter defines and compares the dose related percutaneous absorption of selected aliphatic and aromatic hydrocarbons present in jet fuel mixtures.
A quantitative structure permeability relationship model for the prediction of permeability of unknown jet fuel hydrocarbons is also derived using their known physico-chemical properties.

The dose related increase in percutaneous absorption of HC investigated in chapter 4, is critical from a risk assessment point of view as prolonged exposure to jet fuels may lead to enhanced absorption of such HC resulting in local or systemic toxicity in exposed personnel. These studies guided us to consider the effect of repeated/chronic skin exposure to jet fuels on a regular basis. There is scarce information regarding prolonged dermal exposure to jet fuels. Repeated application of petroleum middle distillates to skin causes chronic irritation and inflammation (Freeman et al., 1990; Grasso et al., 1988). Fabric soaked with jet fuels for 4 days produced significant skin damage in pigs (Monteiro-Riviere et al., 2001b). The reduction in integrity in the dermal barrier by repeated exposure to kerosene-based fuels might increase systemic exposure to other occupational toxicants and toxic components of the fuel itself during subsequent exposures (Ritchie et al., 2003). There is no information in the literature regarding percutaneous absorption of jet fuel HC after repeated or pre-exposure to fuels. Chapter 5 of this thesis describes such studies regarding percutaneous absorption of jet fuel HC through the JP-8 pre-exposed porcine skin. These studies address whether pre-exposure of skin to jet fuel would affect the penetration of selected marker HC and if single dose absorption data from naive skin for jet fuel marker HC can be used to predict the toxic potential for repeated skin exposures to jet fuels. These studies for the first time provide evidence regarding the effect of jet fuel exposure on the subsequent dermal absorption of marker HC and emphasize the need to consider the repeated fuel exposure factor in risk assessment studies. This chapter also provides evidence behind the mechanism for the increase in HC absorption through JP-8 pre-exposed skin. A sensitive technique "Fourier Transform Infrared (FTIR) Spectroscopy" is used to gain insight into this mechanism. FTIR spectroscopy provides information on the vibrational modes of stratum corneum lipid layer components and probes the structure on a molecular level (Casel and Mantsch, 1984). The effects of jet fuel HC on the stratum corneum of porcine skin and their FTIR interpretations are detailed in this chapter.
Finally, Chapter 6 presents the studies conducted to identify the specific fuel HCs that may be responsible for jet fuel induced skin irritation. There is no information regarding in vivo irritation with individual jet fuel HC that is necessary to understand the toxicity of jet fuels. This section provides an analysis of in vivo irritation caused by individual aliphatic and aromatic HC as compared to that of JP-8. A combination of macroscopic, morphological and ultra-structural studies are conducted to identify the irritant fuel constituents. This chapter also discusses the comparison of dermal absorption, in vitro and in vivo toxicity of different jet fuel HC and concludes the potential HC that may be responsible for jet fuel induced skin irritation. The experiments conducted in this thesis may be helpful to take preventive measures in order to reduce the hazardous risks of jet fuels in exposed individuals.
2. LITERATURE REVIEW

The skin

As an ectosomatic system, the skin of mammals is a multifunctional organ that covers and protects the body. Since it is in direct contact with the environment, the skin must vary considerably with the mode of life of the species and so it is structurally one of the most diverse systems. Apart from its protective role, the skin has a wide spectrum of other functions including thermoregulation, prevention of dehydration, mechanical support, neurosensory reception, immunological functions, endocrine secretions and metabolism of internal as well as external molecules. Achieving these goals has resulted in the evolution of a complex structure involving several different layers, each with particular properties. The major layers include the epidermis, the dermis, and the hypodermis.

Structure of the epidermis

The major cell type of the epidermis is the keratinocyte. It comprises >90% of the cells of the epidermal layer. Its thickness varies among various mammalian species due to variations in the number of epidermal cell layers (Monteiro-Riviere, 1991, 1998). The epidermis can be divided into several layers based on the state of keratinocyte differentiation (Stenn, 1983).

The basal cells are columnar epithelial cells that are linked to the basal lamina by hemidesmosomes and to each other by desmosomes. These cells are relatively undifferentiated cells that lack the biochemical markers that are typical of the more differentiated cells in the upper layers (Eckert, 1989). The spinous layer is composed of irregular polyhedral shaped cells and situated directly above the basal layer. It appears spine-like (thus spinous) due to the presence of numerous desmosomes joining adjacent cells. The stratum granulosum consists of several layers of flattened cells lying parallel to the epidermal-dermal junction. This layer contains electron-dense keratohyalin granules that contain profilagrin (Dale et al., 1985).

The stratum lucidum is a transition zone between the granular and cornified layers. This is a zone of extensive cellular remodeling. This layer is only present in very thick skin regions and contains a proteinaceous, viscous fluid, eleidin that is analogous to keratin (Monteiro-Riviere, 1991). The
upper most layer of epidermis is called stratum corneum. The terminal stage of keratinocyte
differentiation is its metamorphosis into the corneocyte. The corneocyte is a flattened polyhedron that
is held to adjacent corneocytes by modified desmosomes and an interdigitating system of ridges and
grooves.

**Basement membrane**

The basement membrane also known as epidermal-dermal junction has many functions. It can
act as a selective barrier between the epidermis and dermis, restricting some molecules (larger than
40KD) and permitting the passage of others like Merkel cells, T lymphocytes, monocytes and

**Structure of the dermis**

The dermis comprises the largest fraction of the skin and is responsible for providing its
structural strength. The skin contains 18-40% of total body water, with most of this water found
primarily in the dermis (Monteiro-Riviere, 1991). The dermis consists of two main layers, papillary
and reticular.

The common skin appendages are the hairs, hair follicles, sebaceous glands, apocrine and
eccrine sweat glands and arrector pilli muscles. Numerous species differences exist in regard to the
temporal pattern of hair growth. The importance of hair growth cycle to cutaneous toxicology and
percutaneous absorption is obvious, in that response to specific agents may be dependent upon the
state of hair growth. If possible, hair growth should be synchronized within animals of a study. This
can be accomplished by clipping all animals 24 hours before treatment, a process that induces a new
hair growth cycle in all animals (Monteiro-Riviere, 1991).

Dermal vasculature serves the functions of nutrition and thermal regulation. The average
blood flow to the skin has been reported to vary from 0.5 to 1.0 up to 100 ml/min/100g, depending
upon species, body site, technique of measurement, and temperature. Normal resting blood flow in
humans is approximately 3-10 ml/min/100g. Regional, species (Monteiro-Riviere et al., 1990) and
environmental variations in cutaneous blood flow should be taken into considerations when assessing
the percutaneous absorption of certain compounds and the response of skin to cutaneous toxicants (Johnson et al., 1986; Pence and Song, 1986).

The hypodermis
This is a layer of mesenchymally derived adipose cells. The hypodermis is the innermost layer of skin and it functions to provide a cushion between the external skin layers and the internal structures such as bone and muscle. It also provides an energy reserve, allows for skin mobility, molds body contours, and insulates the body (Mukhtar, 1992).

Formation of skin barrier

Upon leaving the basal layer, the keratinocytes start to differentiate and during migration through the stratum spinusum and stratum granulosum undergo a number of changes in both structure and composition. The keratinocytes synthesize and express numerous different structural proteins and lipids during their maturation. The last sequences of the keratinocyte differentiation are associated with profound changes in their structure, which result in their transformation into chemically and physically resistant cornified squames of the stratum corneum, called corneocytes. Late in the process of differentiation, characteristic organelles (lamellar bodies) appear in the granular cells (Bouwstra et al., 2003).

The lamellar bodies, which play an essential role in stratum corneum formation, are ovoid organelles enriched mainly in polar lipids and catabolic enzymes, which deliver the lipids required for the generation of the stratum corneum. In response to a certain signal (possibly the increase in calcium concentration), the lamellar bodies move to the apical periphery of the uppermost granular cells, fuse with the plasma membrane and secrete their content into the intercellular spaces by exocytosis. The lipids derived from the lamellar bodies are subsequently modified and rearranged into intercellular lamellae orientated approximately parallel to the surface of the cells (Elias and Menon, 1991). In this orientation process, the lipid envelope probably acts as a template (Marekov and Steinert, 1998).
Lamellar bodies serve as a carrier of precursors of stratum corneum barrier lipids, which consist mainly of glycosphingolipids, free sterols and phospholipids. After the extrusion of lamellar bodies at the stratum granulosum/stratum corneum interface, the polar lipid precursors are enzymatically converted into nonpolar products and assembled into lamellar structures surrounding the corneocytes. Hydrolysis of glycolipids generates ceramides, while phospholipids are converted into free fatty acids. The change in lipid composition and cell structure results in the formation of a very densely packed structure in the stratum corneum (SC). Due to the impermeable character of the cornified envelope, the major route of penetration resides in the tortuous pathway between the corneocytes as revealed by confocal laser scanning microscopy and X-ray microanalysis studies (Simonetti et al., 1995; Meuwissen et al., 1998). It is for this reason that the lipids play an irreplaceable role in the skin barrier, which makes their mutual arrangement in the lamellar domains a key process in the formation of the skin barrier.

Lipid composition in stratum corneum

The major lipid classes (Ponec et al., 1998) in stratum corneum are ceramides (CER), cholesterol (CHOL) and free fatty acids (FFA). CER are the most abundant group of lipids in SC, comprising 40% to 50% of the total lipid mass, and cholesterol represents another 20% to 27% of the total. Cholesterol esters represent about 10% of the total lipid in human SC, but only 1% to 2% in pigs. The FFA content is more variable, ranging from 9% to 26%. Cholesterol sulfate is a minor component, whereas phospholipids and glycolipids are trace components (Wertz, 1992). As discussed above, at a late stage of epidermal differentiation, when the lamellar body contents are extruded into the extracellular space, the phospholipids and glycolipids are broken down (Cox and Squier, 1986). As a part of this catabolic process, phosphorylcholine is cleaved from sphingomyelin and the sugars are removed from the glycosylceramides to produce CER. The CER head groups are very small and contain several functional groups that can form lateral hydrogen bonds with adjacent ceramide molecules. The acyl chain length distribution in the CER is bimodal with the most abundant chain lengths being C24-C26. Only a small fraction of CER has an acyl chain length of C16-C18 (Bouwstra
et al., 2003). The chain lengths of C24 and C26 are much longer than those in phospholipids in plasma membranes. In human SC (Stuart and Downing, 1999) eight subclasses of ceramides (HCER) have been identified. These HCER, referred to as HCER 1-8, differ from each other by the head-group architecture (sphingosine, phytosphingosine or 6-hydroxysphingosine base) linked to a fatty acid or an á-hydroxy fatty acid of varying hydrocarbon chain length. In human SC CER1 and CER4 have a very exceptional molecular structure: a linoleic acid is linked to a á-hydroxy fatty acid with a chain length of approximately 30-32 C-atoms. In this respect the HCER are different from ceramides isolated from pig SC (pigCER), in which only pigCER1 has this exceptional molecular structure (Wertz and Downing, 1999) and pigCER5 has an unusual short chain length (acyl chain length of C16-C18). FFA are composed almost entirely of saturated species ranging from 14 to 28 carbons in length, the most abundant being 22:0 and 24:0. FFA and cholesterol sulfate are the only charged lipids in the SC, and may be necessary for the formation of bilayers in the absence of phospholipids (Wertz et al., 1986). Another important lipid in SC is cholesterol sulfate. Its concentration is lower in the outer SC than in the inner portion of this layer. Although cholesterol sulfate is present in small amounts (typically 2-5% w/w), this lipid plays an important role in the desquamation process of SC (Sato et al., 1998).

**Percutaneous transport**

Molecules traverse membranes either by passive diffusion or by active transport. A passive diffusion process implies that the solute flux is linearly dependent on the solute concentration gradient; while, active transport processes typically involve a saturable mechanism (Friedman, 1986). Simple permeation experiments using excised mammalian skin have shown that percutaneous flux is directly proportional to the concentration gradient (Blank and Scheuplein, 1969). Transport across the skin occurs primarily by passive diffusion.

Consider a single membrane with aqueous phases on both sides, one a reservoir of solute, the other a sink. Since a concentration gradient exists between the source and the sink, there is a flux of
the solute molecules through the membrane. At steady state, the flux due to passive diffusion may be described by Fick's 1st law:

\[ J = Kp \cdot a \]

Where \( J \) = flux of the permeant, \( Kp \) = permeability coefficient of the permeant through the membrane, and \( a \) = activity gradient across the membrane. The permeability coefficient \( Kp \) is the inverse of the "resistance", which the membrane offers to solute transport, and is defined by

\[ Kp = KD/h \]

Where \( K \) = membrane-aqueous phase partition coefficient of the solute, \( D \) = diffusion coefficient of the solute in the membrane, and \( h \) = diffusion path length through the membrane. The flux is a rate process that can be described in general terms as

\[ \text{Rate} = \frac{\text{(driving force)}}{\text{(resistance)}} \]

The driving force for diffusion is the activity gradient, which, to a first approximation, can be equated to the concentration gradient across the permeability barrier. It follows that if the driving force remains the same, the molecular flux across the membrane will be primarily determined by the solute's size (which governs the value of \( D \)) and lipophilicity (which will control the value of \( K \)).

Generally, percutaneous absorption involves the following sequence of events

1. Partitioning of the molecule into the stratum corneum (SC) from the applied vehicle phase
2. Diffusion through the SC
3. Partitioning from the SC into the viable epidermis
4. Diffusion through the epidermis and upper dermis
5. Capillary uptake

Hence, the movement of molecules through the skin involves transport through a number of resistances in series. It follows that the overall rate of transport is dictated by the least permeable barrier (Mukhtar, 1992).

\textbf{Evidence that the SC is the rate-limiting barrier}
The results of several elegant experiments have shown that the SC is the rate-limiting barrier to the penetration of many chemicals through the skin. As described earlier that the SC lipids, particularly ceramides, are important components of the epidermal permeability barrier. The topical application of nicotinamide increased ceramide and free fatty acid levels in the SC, and decreased transepidermal water loss in dry skin; thus, nicotinamide improved the permeability barrier by stimulating de novo synthesis of ceramides, with upregulation of palmitoyltransferase and other intercellular lipids (Tanno et al., 2000). The influence of dilution on hydrocortisone permeation through excised human SC was investigated. The permeation profiles of this drug from various cream bases (diluted and undiluted) were found to be very similar with no significant differences. Thus, the permeability of SC, which was not affected by the cream bases, is the rate-limiting step for drug permeation (Refai and Muller-Goymann, 2002). Scheuplein (1976) tape stripped the skin, thereby removing the SC, and compared the resulting permeability with that of unstripped skin. Removal of the SC increased the permeabilities of many solutes by orders of magnitude, strongly implicating the SC as the primary rate-limiting barrier. An analytical approach has also been employed (Flynn, 1981) to identify the rate-limiting barrier within the skin. Assuming that the SC, viable epidermis, and dermis act as membranes in series, the total resistance of the skin is the sum of the individual resistances:

\[ R_{\text{total}} = R_{\text{SC}} + R_{\text{VE}} + R_{\text{D}} \]

Since permeability and resistance are inversely related so

\[ R = h/KD \]

Therefore,

\[ R_{\text{total}} = h_{\text{SC}}/K_{\text{SC}}D_{\text{SC}} + h_{\text{VE}}/K_{\text{VE}}D_{\text{VE}} + h_{\text{D}}/K_{\text{D}}D_{\text{D}} \]

Where the subscripts SC, VE, and D indicate stratum corneum, viable epidermis, and dermis, respectively. For a number of molecules, K and D have been estimated; then, assuming values for h, one can calculate the individual resistances. For example, Scheuplein (1966) determined the skin resistance to the passage of water assuming an \( h_{\text{SC}} \) value of 20µm. Using commonly accepted values
for the other parameters, he found that the SC presented a 1000-fold larger resistance than that offered by the viable epidermis and dermis. This observation can not be applied to lipophilic solutes.

Hi et al (2003) conducted a mechanistic study of alkyl azacycloheptanones as skin permeation enhancers with hairless mouse skin and concluded that these enhancers act, in part but to a significant extent, by inducing a higher partitioning tendency of the permeant into the transport rate-limiting lipoidal domains of the SC. Monteiro-Riviere et al (2001c) demonstrated that extraction of lipids increased the transepidermal water loss to a level similar to repeated tape stripping at all body sites in the pig. This study suggested that strategies that could biochemically alter epidermal lipid composition may increase absorption of simultaneously administered topical compounds and may be useful to enhance drug delivery.

In general, for polar solutes, the diffusional resistance of the SC is large compared to that presented by the viable epidermis and dermis. For more lipophilic molecules, the resistance of the SC is smaller, due to the larger K. However, the SC maintains a rate-controlling role since a lipophilic molecule does not favorably partition out of the SC into the more aqueous viable epidermis. From the description of SC components and morphology, one can envisage that a diffusing molecule can take one or more of the following penetration pathways

1. The transcellular path, indicating that the molecules transfer sequentially and repeatedly through the "bricks" and "mortar" (Elias, 1983)
2. The paracellular path, via the tortuous but continuous intercellular lipids
3. The transappendageal path via hair follicles, etc.

Most molecules follow the first two penetration paths yet the absorption of a compound into skin is affected by various anatomical properties resulting in regional diversity.

**Factors affecting percutaneous absorption**

**Skin age**

It is well known that the pre-term skin is highly permeable than the full term skin (West et al., 1987). It has been reported that transepidermal water loss is insensitive to age. However, the water-
retention capability of old skin (subjects older than 65 years) is different from that of young adults (aged < 45 years) (Potts et al., 1984). Roskos et al (1989) revealed that aged skin was less permeable to relatively "polar" permeants than young skin; however, for lipophilic solutes, no age-related differences were found. Monteiro-Riviere and Stromberg (1985) described pig epidermis and dermis ultrastructurally at one, three, five, nine and fourteen weeks and compared to the human integument. They found that pig integument is morphologically similar to that of man and would serve as a useful animal model when age changes are accounted for. There are also age-related differences in epidermal and dermal thickness, and blood flow of rats and mice, thus age factor should be considered when evaluating cutaneous toxicity and absorption studies in different-aged animals (Monteiro-Riviere et al., 1991).

**Skin condition**

Percutaneous penetration through damaged or diseased skin is expected, and has been shown, to be different than that through intact tissue. An occlusive environment adds another variable for consideration in percutaneous absorption as compared to the non-occlusive state (Qiao et al., 1993; Qiao and Riviere, 1995). Occlusion prevents the evaporation of endogenous water causing the SC to become excessively hydrated, decreasing the diffusional resistance to the compound (Walters, 1989). The results obtained with essential fatty acid-deficient animals showed that increased transepidermal water loss and SC lipid abnormalities are related (Elias et al., 1980). In vivo experiments have indicated that the penetration of drugs, such as hydrocortisone, across normal human skin is different from that through psoriatic lesions (Wester et al., 1983). Similarly, Chapter 5 of this thesis describes the increase in percutaneous absorption of fuel hydrocarbons through the JP-8 pre-exposed skin as compared to un-exposed skin.

**Species selection**

Although the basic structure of skin is similar in all terrestrial mammals, between-and within-species differences exist in the thickness of the epidermis and dermis in various regions of the body (Monteiro-Riviere, 1991). For example, in the pig, epidermal and stratum corneum thickness is
almost twice that in cattle and horses. Stratum corneum thickness in sheep is similar to that in cattle, while the epidermis in sheep is only half as thick. Other investigators have speculated that transappendagial transport of drugs across skin in cattle and sheep may be more important than the transcellular or intercellular pathways as expected in humans and other animal species (Pitman and Rostas, 1982; Chen and Wilkinson, 1989). Skin appendages including hair follicles, sweat glands, arrector pili muscles, and sebaceous glands are more prevalent in certain body regions. These appendages may create aqueous pores that allow the passage of some small polar compounds into the skin (Riviere, 1990). Regional variations in stratum corneum thickness as well as composition have also been noted (e.g. palmar/plantar surfaces vs. abdominal skin) (Greene et al., 1970).

Except for pig skin, skin from other domestic animals previously described may not serve as a suitable model for absorption in humans. Porcine skin was utilized in these jet fuel studies because it is functionally and structurally similar to that of human skin (Monteiro-Riviere, 1991). Swine are sparsely covered with hair like humans. The pigmentation characteristics and the vasculature of pigs are similar to those of humans (Monteiro-Riviere, 1996, Cole, 1990). They also have similar lipid composition and their epidermal turnover kinetics (approximately 30 days) are very similar (Morris et al., 1987; Monteiro-Riviere and Riviere, 1996). Therefore, percutaneous absorption of toxic substances through pig skin should mimic absorption through human skin. Studies have demonstrated that the range of percutaneous absorption of carbaryl, lindane, malathion, and parathion in pig’s skin in vivo (Carver and Riviere, 1989) or in vitro (Chang et al., 1994) was similar to that observed in humans (Feldmann and Maibach, 1974). The permeability of hydrophilic chemicals (mannitol, water, and paraquat) and lipophilic chemicals (carbaryl, aldrin, and fluazifop-butyl) in pig ear skin was compared with human abdominal skin and rat dorsal skin (Dick and Scott, 1992). This study demonstrated that for hydrophilic chemicals, pig ear skin and rat skin over-estimated permeability in human skin. While permeability was generally higher in animal skin than in human skin for the lipophilic chemicals, permeability of carbaryl in human and pig skin was almost identical. Other in vitro studies have demonstrated similar permeability values in pig skin and human skin for
hydrophilic chemicals such as acetylsalicylic acid, urea (Bronaugh et al., 1982), and benzoic acid (Bhatti et al., 1988).

Anatomical site

The permeability coefficient of a penetrant across the stratum corneum is inversely proportional to the diffusion pathlength. Hence, one might expect that permeability coefficient to be smaller at anatomic sites where the stratum corneum is thickest (e.g., plantar surfaces). The absorption and penetration of both hydrophilic and lipophilic chemicals have been compared at various body sites and no differences in absorption rates of paraquat, mannitol, water, and ethanol were observed between different body sites (Scott et al., 1991). In contrast, significant site differences in percutaneous absorption of various pesticides had been reported in humans (Maibach et al., 1971; Wester and Maibach, 1985). Carbaryl and most topical insecticides are usually applied to the dorsum for control or treatment of external parasites in domestic animals. Several studies have demonstrated regional variation in penetration of chemicals in porcine skin (Qiao et al., 1993; Qiao and Riviere, 1995), rat skin (Bronough, 1985) and Rhesus monkey skin (Wester et al., 1980). It has been reported that parathion penetrated through nonoccluded skin in the following order: back > shoulder > buttocks > abdomen. Due to these reasons and because topical formulations are usually applied to the dorsum of domestic animals, skin was dermatomed from the back of pigs for diffusion flow through experiments described in chapters 3, 4 and 5.

Skin metabolism

The skin possesses the capability of metabolizing permeants (Noonan and Wester, 1989). Basal keratinocytes possess microsomal enzymes necessary for metabolism of topically applied drugs. Potts et al (1989) have shown that a lipophilic derivative of salicylic acid is very slowly transported through the epidermis in the absence of enzymatic activity. In contrast, when the epidermis is enzymatically competent, the lipophilic derivative is hydrolyzed and salicylic acid is rapidly transported through and out of the skin. Similar results obtained by Kao et al (1984) suggest that the epidermis converts lipophilic compounds into more polar, water-soluble forms in order to
promote their rapid elimination from the skin. Thus, the epidermis can serve as a metabolic barrier to transdermal drug delivery.

Skin blood flow

In principle, changes in blood flow through the dermis can affect percutaneous absorption. In reality, stratum corneum is the rate-limiting barrier, and only if transport across the stratum corneum is very rapid can blood flow rate control the systemic appearance of the penetrant. Percutaneous absorption of nitroglycerine has been postulated to be blood flow rate controlled (Nakashima et al., 1987). In contrast to increased uptake due to augmented blood flow, vasoconstriction or reduced blood flow can theoretically decrease the percutaneous absorption of molecules. The "deep delivery" of topically applied permeants has been reported and attributed to the successful bypassing of the cutaneous capillary network (Marty et al., 1989). Other physiological effects of altered cutaneous blood flow also could affect drug disposition within skin. When blood flow is decreased to conserve body heat, the biochemical milieu of the skin changes. In addition to the direct effects of the resultant hypoxia, epidermal generation of lactic acid continues, but removal is impaired because of low perfusion. The production of lactic acid results in decreased tissue pH, which could affect pH-partitioning phenomena for weak acids or bases (Riviere and Williams, 1992). In addition, species and regional differences in blood flow exist (Monteiro-Riviere et al., 1990) that would complicate pooling different skin sites into a common tissue.

Physicochemical factors

When skin is hydrated, the tissue softens, swells, and wrinkles. As discussed earlier, that hydration (occlusion) of the stratum corneum promotes the percutaneous absorption of many chemicals through the skin. Similarly, the binding of diffusing molecule to various components of the skin can retard percutaneous absorption. The lipophilicity and hydrophilicity of permeants are important determinants in this context and have been described earlier. The physicochemical properties of jet fuel hydrocarbons and their effects on percutaneous absorption are elaborated in chapter 4 of this thesis. The temperature is another physicochemical factor that affects the dermal
absorption. Temperature of the stratum corneum typically falls in the range 30-37°C. However, temperature above 65°C for protracted times (>1 min) result in severe structural alterations (Flynn et al., 1981). A 10º change in stratum corneum temperature approximately doubles the in vitro permeability (Golden et al., 1987).

**Methods of assessing dermal absorption**

**In vivo methods**

In vivo percutaneous absorption experiments remain the standard to which all other measurements should be compared. Procedures for measuring percutaneous absorption in vivo have been reviewed (Franz et al., 1993). Animal models are used extensively in dermal absorption studies to evaluate dermal toxicity of topically applied compounds since many of these studies cannot be performed using human subjects. The results obtained from animal studies are thus used to predict percutaneous absorption in humans. One should only develop animal models with the highest possible degree of human correlation. There are various methods that have been used in vivo dermal absorption studies.

In the indirect method, the dermal penetration of the test compound is determined from the extent of excretion from urine or feces. The method was originally developed by Feldmann and Maibach (1970) for use in experimental studies with humans. As the compound may be retained in the tissues, a correction must be made by administration of a single parenteral dose and a determination of the extent of excretion of the dose. The use of radiolabeled test compounds allows rapid measurement of excretion of the label. Adequate time must be allowed for the excretion of administered radioactivity, usually 5 days. However, radioactivity must be checked daily until it reaches background level. This method has advantage that it may be used in human subjects if the test compound is not toxic. Bioavailability and area under curve (AUC) ratios are other major techniques used for indirect in vivo transcutaneous drug absorption. The absolute bioavailability of a topically applied chemical/drug is determined with a sensitive assay of blood or urine after topical and intravenous drug administration. Wester and Maibach (1983) compared three methods (plasma
nitroglycerine AUC, plasma total radioactivity AUC, urinary total radioactivity) for determination of absolute bioavailability of topical nitroglycerine. They concluded that the difference in estimate between that of the absolute bioavailability (56.6%) and that of $^{14}$C (72.7-77.2%) is the percentage of compound metabolized in the skin as the compound was being absorbed.

The direct method permits the study of absorption kinetics and provides a better approximation for estimating the absorption of substances with a slow excretion rate from the body. After application of the radioactive test compound to a group of animals, they are killed at certain time intervals, and various tissues are assayed for radioactivity. This method is time consuming and cannot be used in human beings. There are other methods for in vivo absorption assessment like disappearance of test compound from application site, physiological responses, whole body autoradiography (Bloomquist and Thorsell, 1977), histology, fluorescence, and affinity of the test compound for a specific organ or tissue (Cyr et al., 1959). Unfortunately, in vivo methods for dermal absorption studies are not widely used as compared to in vitro methods due to many limitations like small sample size, unknown vehicle, poor analytical method etc. These factors make comparisons and extrapolations very difficult. But in vivo studies are still considered a golden standard to observe the direct irritant effects of toxicants. Such in vivo toxic effects of different jet fuel hydrocarbons are reviewed and presented in chapter 6 of this thesis.

**Ex vivo methods**

Various approaches have been employed to overcome the basic limitations in extrapolations regarding in vivo dermal absorption studies. One such approach involves human-xenograft models, which allow compound absorption through human skin to be assessed in a laboratory animal. When human skin is not available, pigskin has been employed as an acceptable and reliable alternate model. The isolated perfusion of cat (Feldberg and Paton, 1951) and dog (Wheatley et al., 1961) skin has been previously reported but these models were never further developed for percutaneous absorption studies. The isolated perfused porcine skin flap (IPPSF) is a true ex vivo model that has advantages over most in vitro systems in that it has intact vasculature and its predictions have been correlated
well with in vivo absorption data for several drugs and insecticides (Riviere et al., 1986, 1995). The IPPSF is biochemically and physiologically viable thus can be used to assess cutaneous toxicity of topically applied chemicals (Monteiro-Riviere, 1993). This model has a major advantage in that toxicity and dermal absorption can be assessed simultaneously. The major disadvantage of this model is that systemic-mediated immunological and neural feed back responses to topically applied substances cannot be assessed. In spite of this, the IPPSF has been successfully used to study skin distribution, transdermal drug delivery, and systemic targeting of anticancer drugs to tumor-bearing flaps (Williams and Riviere, 1990, Vaden et al., 1994). Such perfused models are best suited to studying mechanisms of absorption, which involve interactions between the various steps involved in percutaneous penetration. The IPPSF has been found to be the best membrane model in studying the mechanism of additive interactions in jet fuel mixtures (Muhammad et al., 2004b). This study is presented in chapter 3 of this thesis.

**In vitro methods**

In vitro measurements on skin samples mounted in a diffusion cell are the most established procedure for predicting percutaneous absorption (Bronaugh and Collier, 1993; Scott et al., 1993). In addition to being more practical than corresponding in vivo investigations, in vitro approaches provide additional information essential for pharmacokinetic analysis or estimations of the level of first-pass metabolism in the skin. It has been shown to be particularly applicable to transdermal drug development, safety assessment of cosmetics (Beck et al., 1991) as well as risk assessment of potentially toxic compounds (Scott, 1989). These in vitro methods have been used extensively in studies that correlate structural features of compounds with their permeability across the skin. In general, it is accepted that standard in vitro protocols provide reliable estimates of percutaneous absorption.

In vitro percutaneous absorption measurements can be made with one or two chamber diffusion cells. One-chamber cells are topologically equivalent to application of a compound to the skin. For two chamber cells, the skin disk is placed between the two chambers and the compound in
question diffuses from a donor phase through the skin into an acceptor phase. This is referred to as infinite dose conditions, where the concentrations of compound in the donor compartment are not significantly diminished during the study. However, this approach fully hydrates the skin which may not be comparable to in vivo conditions. This design is suitable for structure activity analysis. It is recommended that the apparatus is made of inert materials such as glass or Teflon. Permeation may be measured by collecting receptor fluid using static (one time) or continuous (flow-through) collection. The choice of experimental conditions is dictated by the solubility of the compound in the receptor fluid. A static collecting device may be used when permeation does not result in a concentration that exceeds 10% of the maximal solubility in the receptor fluid. For conditions where solubility in the receptor fluid is a concern, a flow-through apparatus is recommended. The other major advantage of the flow-through diffusion system over static one is that the skin is more likely to be viable and the penetrating agent is continuously removed from the skin that is analogous to the continual perfusion of blood in the in vivo situation (Bronaugh and Stewart, 1985; Grummer and Maibach, 1991).

Other benefits of using flow-through diffusion cells are that the hydrophobic substances are more likely to be absorbed even into a saline receptor fluid with a continuous flow than with static flow system (Wester et al., 1985). The receptor volume of the flow-through diffusion cell is usually less than a half-milliliter so that it can be completely flushed out during sampling intervals. Flow rates are usually set at 5-10 times the receptor volume to allow for adequate removal of absorbed molecules. Studies have demonstrated that changes in perfusion rate (Crutcher and Maibach, 1979; Chang and Riviere, 1991), receptor fluid constituents (Bronaugh and Stewart, 1984), environmental conditions (Chang and Riviere, 1991), and skin section thickness (Scott and Ramsey, 1987) will dramatically alter the penetration of the marker constituent. Having considered these findings, the flow-through diffusion cell experiments described in chapters 3, 4, and 5 of this thesis used dermatomed skin sections (400-500 microns thickness). Furthermore, bovine serum albumen was
used as the receptor fluid because it mimics blood and it facilitates partitioning of lipophilic hydrocarbons from the skin sections into the receptor fluid.

The comparisons made between in vitro and in vivo percutaneous absorption experiments on animal skin revealed a good correlation for hydrophilic compounds (Bronaugh and Maibach, 1985; Scott and Clowes, 1992). However, the in vitro/in vivo comparisons of hydrophobic compounds are more problematic (Bronaugh and Stewart, 1984). This is mostly due to an accumulation of compounds in the dermis, which is important for compounds that are poorly soluble in the receptor fluid and where the dermis is not removed. The inclusion of the amount in the viable tissues as part of the cumulative dose absorbed is likely to improve the correlation. It is also important to use split-thickness skin for hydrophobic chemicals. In general, the results from animal studies support the reliability of in vitro measurements as a good prediction of in vivo percutaneous absorption.

**Analytical techniques**

The following analytical techniques have been used in this thesis.

**Radiochemical assay**

Radioisotopes commonly used in biomedical research possess low energy and a short range of air or fluid penetration. Therefore, they require direct contact with the scintillation medium and special technology for efficient indirect detection of radioactivity (Bitelli, 1982). Among these radioisotopes, tritium ($^3$H) and radioactive carbon ($^{14}$C) have many applications in cell biology, pharmacology, toxicology and clinical research. For an ideal liquid scintillation detector, the amount of light generated must be proportional to the energy transferred and the detection should be perfectly linear. However, an energy transfer loss frequently occurs as a result of absorption of light by solid materials, chromogenic interposition, solution turbidity or pH changes in the scintillation fluid (Carvalho, 1999). The process of radioactive detection involves scintillation fluids composed of aromatic solvents that increase the efficiency of energy transfer to the organic fluorine compound, improving the detection of light emission. The primary and most extensively used scintillation fluid
contains 2,5-diphenyloxazole, and is known as PPO. Toluene, xylene and dioxane are also widely used for this purpose (Meechan, 1997).

We mostly use $^{14}$C-labelled chemicals for transdermal absorption and disposition studies in our laboratory because $^{14}$C-labelled drugs are widely used in the pharmaceutical industry to study their absorption, disposition, metabolism and excretion in both animals and humans to establish pharmacokinetic and metabolism parameters (Garner et al., 2000). $^{14}$C is the radiolabel of choice because of its stability once chemically incorporated into a chemical or drug molecule. $^{14}$C has been measured in biomedical research for nearly 50 years by decay counting, primarily liquid scintillation counting by virtue of the fact that this isotope is a low energy $\alpha$-emitter (Horrocks, 1976). However, the success achieved in beta-ray nuclide standardization is not relevant for radionuclides decaying by electron-capture (Malonda and Carles, 2000). We have used this technique to study the mixture interactions in percutaneous absorption and disposition studies of a number of chemicals (Baynes et al., 2001, 2000; Riviere et al., 1999; Chang et al., 1994; Carver and Riviere, 1989). Chapter 3 of this thesis presents the absorption studies conducted with $^{14}$C-labelled jet fuel marker hydrocarbons in various membrane model systems.

The use of $^{14}$C however, has a number of practical limitations due to the long half-life of this isotope (5740 years). In order to have relatively short sample counting times, many $^{14}$C atoms must be present in the sample, typically of the order of 5-10 picomoles $^{14}$C/ml of plasma or urine (Vogel et al., 1995). For chemicals that are administered at microgram doses, analysts often struggle to develop sensitive detection methods to measure the sample radioactivity. Moreover, the radiolabelled chemicals are much more expensive than the neat chemicals and also we could not look at multiple solutes with radiolabelled components. Due to these and aforementioned limitations, we used alternate analytical methods like solid-phase microextraction technique for the detection of neat chemicals in subsequent studies presented in chapters 4 and 5.
Solid-phase microextraction (SPME) with gas chromatography (GC)

In general, most organic pollutants of interest in environmental samples have to be extracted and enriched before their instrumental determination. In the past, sample preparation was dominated by the conventional liquid-liquid extraction, a time-consuming multi-step method for which large amounts of solvents were necessary. Liquid-liquid extraction has been largely replaced in the past few years with solid-phase extraction (SPE) using a variety of different sorbents (Eisert, et al., 1995; Font et al., 1993). These SPE methods are simple, and less time-consuming than classical liquid-liquid extraction as many samples can be enriched in parallel. Moreover, inexpensive and less (toxic) solvents are needed. However, SPE is still a multi-step process that is prone to loss of analytes if it is not fully automated and still needs toxic organic solvents for the elution step. Furthermore, SPE is limited to semi-volatile compounds because the boiling points of the analyst must be substantially above that of the solvents.

By coating the sorbent on a fine rod of fused-silica (a kind of inverted fused-silica capillary column, where the polymeric film is on the outside) the limitations of SPE may be overcome. This new extraction technique, solid-phase microextraction (SPME), was initially introduced by Pawliszyn and Belardi (1989). The SPME method shows several attractive features: it is very simple, fast, and easy to automate by use of a commercially available auto-sampler (Berg, 1993; Arthur et al., 1992). Unlike other extraction processes; no solvent is necessary for the extraction. The fiber is contained within a microsyringe for protection and ease of sampling. The fiber is withdrawn into the needle of the microsyringe and the needle of the syringe is used to pierce the sample vial. The fiber is then exposed to the sample where the analytes partition between the stationary film on the fiber and the aqueous sample phase. The fiber is then withdrawn from the sample and inserted into the injector of a gas chromatograph where the analytes are thermally desorbed. In SPME, an exhaustive extraction does not occur but equilibrium is established as analytes partition between the stationary phase and the sample phase. At equilibrium, a linear relationship exists between the number of moles of an
analyte absorbed by the fiber and the analyte concentration in the sample phase (Pawliszyn and Arthur, 1990).

So far, SPME has been applied to the extraction of organic compounds from different matrices including air (Chai et al., 1993), water (Eisert et al., 1995), and soil (Popp et al., 1994). SPME parameters were examined on water contaminated with hydrocarbons including benzene and alkylbenzenes, n-alkanes, and polycyclic aromatic hydrocarbons (Langenfeld et al., 1996). SPME coupled with GC has been applied to study the binding properties between bovine serum albumen and volatile organic compounds such as benzene, toluene, ethylbenzene, propylbenzene and butylbenzene (Yuan et al., 1999). Theodoridis et al (2000) reviewed the applications of SPME for the analysis of biological samples and illustrated the impact of this technique on various analytical fields (e.g. toxicology, forensics, clinical, biochemical, pharmaceutical, and natural products).

Headspace SPME/GC has been investigated as a possible alternative to conventional gas chromatography for fuel spill identification (Lavine et al., 2000). This technique had been evaluated for use in the quantification of aroma volatile production by Granny Smith apples during cool storage (Matich, et al., 1996), to isolate the volatile compounds formed during peroxidation of fatty acids in vegetable oils (Jelen, et al., 2000), as well as screening of volatile aliphatic and aromatic hydrocarbons in blood (Liu et al., 2000). Headspace SPME/GC has been used to analyze groundwater for benzene and toluene (Arthur et al., 1992) and has been used for analyses involving food and beverage components (Hawthorne, et al., 1992). The headspace technique is advantageous when either the volatility of the analyte permits a headspace determination or undesirable components in the bulk sample make direct SPME sampling undesirable. Headspace sampling prolongs fiber life and is faster than direct sampling (Lavine, 1995). Finally, recovered fuels cannot be analyzed by direct SPME sampling because of irreversible fiber damage caused by excessive swelling of the poly(dimethylsiloxane) coating, which is a direct result of the fiber being in intimate contact with the fuel layer (Stromquist, 1994). Based on aforementioned studies, we used headspace SPME as an analytical technique to analyze perfusate samples for jet fuel hydrocarbons in chapters 4 and 5.
Fourier Transform Infrared (FTIR) spectroscopy

FTIR spectroscopy is an emergent physicochemical technique that is becoming a powerful tool for obtaining structural information from cellular constituents of biological membranes. FTIR spectra reflect the complex chemical composition of the cells, and some of the bands are assigned to distinct functional groups or chemical substructures (Helm et al., 1991). Previous work was conducted on samples from a variety of organs, such as the brain (Levine and Wetzel, 1994), breast (Fabian et al., 1995), and lung (Benedetti et al., 1990). This technique, which requires neither reagent nor sample preparation, is nondestructive, quite sensitive, and highly selective because of its ability to be a spectral fingerprint for molecular components (Frank et al., 1998). Malins et al (1996) applied it to the investigation of pathological conditions such as breast cancers. This technique has been demonstrated to be a convenient tool for pharmacotoxicological studies (Melin et al., 1996).

As discussed earlier, that the main barrier for permeation of most molecules through the skin is the stratum corneum. FTIR has been established as a tool to study such complex biological membranes. The dominant molecular vibrations of the CH$_2$ groups as well as the symmetric and asymmetric bending vibrations of CH$_3$ are sensitive to the conformational state of the aliphatic chains. Peak positions, intensities and half widths of these characteristic vibrations give information about the structure and conformational state of lipids in membranes and have extensively been used to study phase transitions in biomembranes (Chapman, 1984). In the highly ordered, solid-like lamellar gel phase the group absorptions of the stretching vibrations of CH$_2$ are at smaller wave numbers than in the fluid crystalline phase (Mantsch, 1984). The intense ester carbonyl stretching vibration as well as the symmetric and asymmetric stretching vibrations of the phosphate ester is sensitive to the interactions of the lipid head groups with water molecules or ions. Upon hydration, the symmetric stretching vibration of the phosphate group is shifted towards lower wave numbers (Cameron et al., 1980). In addition, it is described that differences in the packing of lipid molecules can influence the absorption maximum of the asymmetric phosphate ester stretching vibration via crystal field effects (Wallach et al., 1979).
Over the last decade, Fourier transform infrared attenuated total reflectance (FTIR-ATR) spectroscopy has been used to investigate penetration kinetics in human stratum corneum and elucidate extent and mechanism of percutaneous penetration enhancement in vivo in human subjects (Pellet et al., 1997). There are some limitations in its application because the typical range of sampling depth with FTIR-ATR is 0.7-2.1 μm, however it still has the advantage of utilizing in vivo samples. We employed FTIR as a biophysical tool to investigate the mechanism of increase in percutaneous absorption of jet fuel HC. We used dried stratum corneum sheets (SC) in our experiments based on previous studies of Panchangnula et al. (2001) and Levang et al. (1999) who used dried SC for evaluation of lipids. These experiments are described in detail in Chapter 5 of this thesis.

Toxicity assessment techniques

Light and transmission electron microscopy

The constituents in chemical mixtures can interact with skin altering the marker absorption. It is therefore, important to determine whether topical exposure to chemical mixtures can induce detectable biological changes in exposed skin. These interactions may correlate with percutaneous absorption of the marker constituents and provide further understanding of the mechanism for increase in transdermal absorption of the marker chemical. Biological effects may not necessarily be macroscopic, but may be subtle and require various microscopic techniques for evaluation.

Light microscopy is a simple technique used to assess whether topical application of chemicals has altered skin morphology. Exposed skin sections can be harvested and fixed in formalin. Later, skin sections can be processed and stained for microscopic evaluation (Monteiro-Riviere, 1991). For routine light microscopy, skin sections can be stained with hematoxylin and eosin (H&E) to determine morphological changes in skin. Although prolonged exposure to solvents and surfactants can induce macroscopic changes such as tissue edema and dry skin, light microscopic evaluation of similarly exposed IPPSFs have revealed minor alterations of the epidermis (King and Monteiro-
Riviere, 1991). Light microscopy has been successfully employed to assess the skin irritation of different jet fuels (jet A, JP-8, JP-8 (100)) (Monteiro-Riviere et al., 2001b).

Chemically induced skin irritation is a major human health problem. There is considerable interest in understanding the effects of various irritant exposures on the epidermal barrier because this provides protection from the penetration of irritants. Irritation of the skin is accompanied by a complex array of epidermal and dermal metabolic responses. The modulating and initiating effect of the keratinocytes is influenced by the reaction of the barrier and the pathway of the irritant into the nucleated (viable) parts of the epidermis. Studies have investigated the different structural changes of the nucleated portions of the epidermis (stratum granulosum, spinosum and basale) as induced by different chemical irritants with the use of transmission electron microscopy (TEM) (Willis et al., 1993, Monteiro-Riviere et al., 2004). However, few studies have investigated the barrier structures of skin after chemical irritation since the intercellular spaces of the stratum corneum appear empty with routine TEM using double fixation with glutaraldehyde and osmium tetroxide. Stratum corneum lipids consists of ceramides, fatty acids, cholesterol, and cholesterol sulfate, with ceramides the dominant component (Long et al., 1985). The aliphatic side chains of the ceramides are largely saturated or monounsaturated (Wertz and Downing, 1983) as are the free fatty acids of the stratum corneum. This indicates that the osmium tetroxide, which reacts with the double bonds of unsaturated lipids, might be less than optimal for the fixation of stratum corneum lipids. Ruthenium tetroxide, a stronger oxidizing agent, reacts well with both saturated and unsaturated molecules and has been shown to react strongly with polar lipids that show no reaction with osmium tetroxide (Gaylarde and Sarkany, 1968). TEM is thus, a powerful tool to investigate the chemical induced alterations in stratum corneum lipids after ruthenium tetroxide staining. We used this technique to study the extraction of lipids from stratum corneum with different jet fuel hydrocarbons in chapter 6 of this thesis.
Jet Fuels

The studies regarding the composition of jet fuels, percutaneous absorption of different jet fuel hydrocarbons, effects of additives on marker HC disposition, effect of in vivo pre-exposures on secondary in vitro dermal penetration and skin toxicity of jet fuels are reviewed in subsequent chapters 3, 4, 5, and 6 of this thesis.
LITERATURE CITED


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3. COMPARATIVE MIXTURE EFFECTS OF JP-8 (100) ADDITIVES ON THE DERMAL ABSORPTION AND DISPOSITION OF JET FUEL HYDROCARBONS IN DIFFERENT MEMBRANE MODEL SYSTEMS

Faqir Muhammad, James D. Brooks and Jim E. Riviere

ABSTRACT

Jet fuel are complex mixtures of hydrocarbon fuel components and performance additives. Three different membrane systems, silastic, porcine skin and the isolated perfused porcine skin flap (IPPSF) were used to gain insight into the possible mechanism for additive interactions on hydrocarbon component absorption. Influence of JP-8 (100) additives on the dermal kinetics of $^{14}$C-naphthalene and $^{14}$C/$^3$H-dodecane as markers of hydrocarbon absorption, were evaluated using analysis of means (ANOM) and analysis of variance (ANOVA). This study indicated that the naphthalene absorption through silastic membrane was significantly different with JP-8 plus individual additives as compared to controls i.e. JP-8 and JP-8(100). The porcine skin data indicated that neither individual nor combinations of additives affected naphthalene absorption. The third membrane system (IPPSF) showed that only MDA and BHT were important additives altering naphthalene absorption. MDA was a significant suppressor while BHT was a significant enhancer of naphthalene absorption. MDA significantly decreased dodecane absorption in skin flaps. All individual and combinations of two additives with JP-8 affected naphthalene and dodecane surface retention in silastic membrane. The IPPSF indicated that only 8Q405 is a significant modulator of surface retention for both marker hydrocarbons. The 8Q405 significantly reduced naphthalene contents in dosed silastic and skin indicating a direct interaction between additive and marker hydrocarbons. The MDA and BHT, which significantly retained naphthalene in the stratum corneum of porcine skin individually, led to a statistical decrease in its retention in the stratum corneum when in combination (MDA + BHT) suggesting a potential biological interaction. These observations demonstrate that the single membrane system may not be suitable for the final prediction of complex additive interactions in jet fuels. Rather a combination of different membrane systems may provide the insight to elucidate the possible mechanism for additive interactions. Finally, it is important to assess all components of a chemical mixture since the effects of single components administered alone or as pairs may be confounded when all are present in the complete mixture.
INTRODUCTION

U.S. Air Force, U.S. Army and NATO armed forces exclusively use the kerosene-based jet fuels such as JP-8 and JP-8 (100). The Navy, which has used (lower volatility) kerosene based JP5 since approximately 1952 to power carrier-based aircraft, is proposing transition by 2008 to exclusive use of JP-8/JP-8 (100) to meet implementation requirements for the Joint Strike Fighter aircraft (Ritchie et al., 2003). The U.S. Department of Defense is planning to completely replace use of unleaded gasoline with JP-8/JP-8(100) by 2010 as the fuel for vehicle and mechanical equipment (Maurice et al., 2000). Direct exposure to such fuels occurs in military and civilian avionics, aircraft maintenance, and in fuel manufacturing/handling personnel through dermal contact with raw fuel and/or aerosol; dermal contact with clothing and gloves saturated with fuel; or to fuel contaminated food or water (Harris et al., 1997; Pleil et al., 2000).

Due to their excessive use, an increasing number of self-reported and medically diagnosed symptoms have been documented from fuel-exposed workers. (Olsen et al., 1998; AFIERA, 2001). Neurological effects and irritant dermatitis have been reported in JP-8 exposed workers (Smith et al., 1997; Zeiger and Smith 1998). Significant effects on the immune, hepatic, respiratory and cardiovascular systems have been observed in several animal exposure studies (Porter, 1990; Grant et al., 2000; Harris et al., 2000; Robeldo et al., 2000).

JP-8(100), a highly thermal stable fuel has identical composition to JP8, except for addition of the thermal stability performance package. The name JP-8(100) was selected because addition of the performance package increases the thermal stability of JP-8 by 100°F (Kalt et al., 2001). This performance package is composed of (1) an antioxidant butylated hydroxytoluene or BHT added at 25ppm; (2) the metal deactivator or MDA (N,N-disalycyldene-1,2-propanediamine, added at 2ppm; (3) the detergent/fuel stabilizer/dispersant (8Q405 or proprietary) added at 100ppm; and (4) a heavy aromatic naphtha solvent (6-10% naphthalene) added at 129ppm (Heneghan et al., 1996; Kalt et al., 2001). Phenolic antioxidants such as BHT, classified as chain-breaking antioxidants in fuel, prevent the formation of soluble gums and insoluble particulate deposits on fuel system components produced.
by oxidation (Papas 1993). Metal deactivator additives suppress the catalytic effect that some metals in fuels induce on the surfaces of fuel systems and tanks. The detergent/dispersant additive is designed to minimize carbon or coke deposits, clean engine deposits, and serve as a high temperature fuel stabilizer (Ritchie et al., 2003).

The skin toxicity and dermal penetration of kerosene based jet fuels have been investigated in numerous in vivo and in vitro animal models. Monteiro-Riviere et al (2001) studied the topical application of Jet-A, JP-8 and JP-8(100) jet fuels on pig skin that resulted in dermal irritation after high doses and occlusion. The application of JP-8(100) increased transepidermal water loss and caused a moderate erythema and moderate to severe edema (Kanikkannan et al., 2001). We have previously assessed the percutaneous absorption and cutaneous deposition of topically applied neat Jet-A, JP-8, JP-8(100) or aged JP-8 by monitoring the absorptive flux of marker components like dodecane, naphthalene and hexadecane (Riviere et al., 1999). The results indicated that naphthalene absorption into the perfusate was similar across all fuel types; however, total penetration of naphthalene into and through skin was higher in JP-8(100). Our laboratory has investigated the influence of military additives (DiEGME, 8Q21 and Stadis450) on the dermal disposition of marker aliphatic and aromatic constituents in Jet-A. The data suggested that various combinations of these three performance additives in at least Jet-A could potentially alter the dermal disposition of aromatic and aliphatic fuel hydrocarbons in skin (Baynes et al., 2001). These studies suggested that the influence of JP-8(100) performance additives on the dermal kinetics of marker fuel constituents must be evaluated in skin. As we have previously shown, the products of two-factor interactions were not predictable from single-factor exposures and, by extension, cannot be extrapolated to three-factor interactions (Baynes et al., 2001). We have demonstrated that results obtained from a single membrane system may not be predictable for multiple/complex membrane systems. This situation raises the need to investigate the effects of mixture interactions on the dermal disposition of marker components simultaneously using simple and complex membrane systems. Therefore, we selected three membrane systems to demonstrate the mixture interactions in the present study. 1) the synthetic
silastic membrane which is suitable to depict chemical-chemical interactions; 2) porcine skin sections which has stratum corneum and viable epidermis and depicts chemical-biological interactions; and 3) the Isolated Perfused Porcine Skin Flap (IPPSF) which has intact vasculature and mimics in vivo exposures for human extrapolations (Wester et al., 1998). The pigskin was selected as our experimental model because of its anatomical and physiological similarities to human skin (Monteiro-Riviere, 1991). Dermal absorption of chemicals in the IPPSF is predictive of in vivo absorption in human (Wester et al., 1998). We used full factorial experiments (2x2x2) to evaluate the influence of JP-8(100) additives, individually and as mixtures, on the dermal kinetics of marker hydrocarbons using the above mentioned three membrane systems.

**MATERIALS AND METHODS**

**Chemicals**

JP-8 jet fuel was kindly supplied by Major T. Miller from Wright Patterson Air Force Base. $^{14}$C-Naphthalene (Specific activity = 8.1 mCi/mmoll) and $^{14}$C-dodecane (Specific activity = 8.8 mCi/mmoll) was obtained from Sigma Chemical (St. Louis, MO). $^3$H-dodecane (Specific activity =10,000 mCi/mmoll) was custom-radiolabeled by the NCI Chemical Carcinogen Reference Standard Repository (Chemsyn Science Laboratories, Lenexa, KS). Naphthalene and dodecane were selected as the representative markers of aromatic and aliphatic hydrocarbons respectively with the greatest percentage in jet fuel. Each hydrocarbon was dissolved in methylene chloride prior to formulations with JP-8 jet fuels. All three chemicals were more than 98% radio chemically pure. JP-8(100) performance additives BHT (25ppm), MDA (2ppm) and 8Q405 (70ppm) were added to JP-8 to prepare mixtures as shown in Table 1. These mixtures were prepared according to the mandatory recommendations for JP-8(100) formulation (Heneghan et al., 1996; Kalt et al., 2001).

**Flow Through Diffusion Cell Experiments**

The flow through diffusion cell system, as previously described by Bronaugh and Stewart (1985), was used to perfuse silastic (Polydimethylsiloxane) membranes (250μm thickness) obtained from Dow Corning (Midland, MI) and porcine skin. Porcine skin was obtained from the dorsum of
female Yorkshire pigs. Skin was dermatomed by a Padgett Dermatome (Padgett Instruments Inc., Kansas City, MO) to a thickness of 450-550μm. Circular sections of silastic and skin were punched to provide a dosing surface area of 0.64 cm² and then placed into two-compartment Teflon flow through diffusion cells. Both silastic and skin discs were perfused with Krebs-Ringer bicarbonate buffer spiked with dextrose (0.12%) and bovine serum albumin (4.5%). The temperature of the perfusate and flow through cell was maintained at 37ºC using a Brinkmann constant temperature circulator (Brinkmann, Westbury, NY). ¹⁴C-Naphthalene or ¹⁴C-Dodecane was added to the specified jet fuel mixtures, and twenty microliters of these jet fuel mixtures were topically applied to each diffusion cell (4-5 silastic or skin sections per treatment) to deliver 340 μg/cm² and 1,100 μg/cm² of naphthalene and dodecane, respectively, reflecting the concentrations seen in fuel. The pH of the perfusate was maintained between 7.4 and 7.5. Perfusate flow rate was 4.0 ml/h and perfusate samples were taken at 0, 10, 20, 30, 45, 60, 75, 90, 105, 120, 150, 180, 240, and 300 min. At the end of the perfusion, the dose area was swabbed with soapy solution to determine surface content, tape-stripped 6 times to determine stratum corneum content, and the dosed area was removed from the silastic or skin disc with a 0.64 cm² punch biopsy to determine dose area silastic or skin deposition. These samples, in addition to the remaining peripheral silastic or skin, were saved for radiochemical analysis.

Isolated perfused porcine skin flaps

Skin flaps were prepared as previously reported by Riviere et al (1986) and perfused with oxygenated (95% O₂, 5% CO₂) Krebs-Ringer bicarbonate buffer spiked with glucose and bovine serum albumin. The perfusion chambers were maintained at a temperature of 37ºC and relative humidity of 50-60% to mimic mildly humid occupational exposures. After perfusing the skin flaps for 1h, a flexible template measuring 1.0x5.0 cm (Stomahesive, ConvaTec-Squibb, Princeton, NJ) was affixed to the skin surface with Skin Bond (Pfizer Hospital Products, Inc., Largo, FL) to provide a surface area of 5.0 cm² for dosing solutions. Fifty microliter dosing mixtures were applied to the dose site of each skin flap (n=4 per treatment) providing an applied surface concentration of 65μg/cm² ¹⁴C-
Naphthalene and 175 ìg/cm² 3H-Dodecane. Perfusate samples (3ml) were collected every 5min for the first 40 min, and every 10min until 1.5 h, and then every 15min until termination at 5h. The skin flap viability was assessed by monitoring vascular resistance (VR) (perfusate pressure/flow) and glucose utilization (GU). Arterial perfusate samples were collected hourly and compared with venous samples to determine GU, while VR was defined as the ratio of arterial pressure to perfusate flow rate. At the end of the experiment, the dose area was swabbed with soapy solution to determine surface contents, tape-stripped twelve times to determine the stratum corneum contents, and skin and fat tissue was obtained from the dose site and surrounding areas and was digested by Soluene (Packard Chemical Co., Downers Grove, IL) for radiochemical analysis.

Radiochemical Analysis

For determination of 14C-dodecane, 3H-dodecane and 14C-naphthalene, perfusate samples, surface swabs, dosed silastic or skin, stratum corneum samples and peripheral silastic or skin were combusted in a Packard Model 307 Tissue Oxidizer (Packard Chemical Co.) and then were analyzed by Liquid Scintillation Counter (Packard Model 1900 TR, Packard Chemical Co.) for total determination of radioactivity.

Calculations and Statistics

The absorption parameters including flux, permeability and diffusivity of the marker hydrocarbons in jet fuel mixtures were determined in silastic and porcine skin sections. Flux (ìg/cm²/h) was determined at steady state from the slope of the cumulative mass per unit area vs time (h) curve. Permeability (cm/h) was determined by dividing the steady state flux (slope of cumulative ìg/cm² versus time curve) over the marker concentration applied to the surface. Diffusivity (cm²/h) was obtained by the following relation: D=L²/6ô, where L=250ìm for silastic or L=500ìm for skin and ô is the lag time obtained by extrapolating the steady state portion of the curve back to the time or X-axis. Absorption was defined as the total amount (ìg) detected in the perfusate for the entire 5-h perfusion period. Tissue retention was defined as the total amount (ìg) detected in a particular
fraction/layer of silastic or skin. Tissue penetration was the cumulative amount (µg) in SC + dosed skin.

The mixture interactions among jet fuel additives BHT, MDA and 8Q405 were assessed using a full factorial analysis of variance (ANOVA). The measurements analyzed were total absorption, flux, permeability, diffusivity, surface, stratum corneum, dosed area and dosed area + peripheral silastic or skin. All analysis was carried out using SAS 8.01 for Windows software (SAS Institute Inc., Cary, NC). As reported previously in the literature, in order to detect the treatment effects, a standard confidence interval based on the significance level of α<0.05, standard deviation, and sample number is not appropriate for multiple-treatment data (Baynes et al., 2001). Budsaba et al (2000) introduced the use of compass plots to define a more appropriate confidence interval, based on the analysis of means (ANOM) around the grand mean of all treatments. If the plotted points fall between the upper and lower decision lines (limits) then it is interpreted as a measure of random variability. On the other hand, if the plotted points fall outside the limits, this suggest nonrandomness of treatment means and a significant (p<0.05) treatment effect. These upper and lower limits represent the 95% confidence interval as we calculated this interval using variance and the exact critical values where α = 0.05, n = 4. The ANOM and ANOVA are not exactly equivalent (Nelson, 1983). The ANOVA has superior ability to detect differences in the k sample means and are more useful for a complex design but ANOVA is usually followed by some additional analysis. We used ANOVA-LSD and ANOVA-TTEST to identify significant differences between treatment groups for each parameter assessed in this study. At the same time, ANOVA lacks the kind of appealing graphical visualization produced by the ANOM (Lapin, 1997). Therefore, in the present study, we plotted mixture interactions in jet fuel with JP-8(100) additives in the form of compass plots by labeling ANOVA results with letters and significant ANOM results with arrows on each plot. We used absolute amount (µg) for absorption and tissue disposition of marker hydrocarbons instead of percentages because the same percent absorbed/dispositioned from a higher dose will have more toxicologic impact than the same percentage from a lower dose (Muhammad et al., 2004).
RESULTS

Naphthalene absorption was two to many fold greater than dodecane, while dodecane tissue retention at the end of an experiment was two to many fold greater than naphthalene in IPPSF and other membrane systems respectively (Figures 1,2,3,4).

Mixture interactions in the marker absorption

Mixture interactions in the absorption of marker hydrocarbons of JP-8 with all three additives of JP-8(100) in the three membrane systems are shown in Figure 1. Although the shapes of compass plots were different in silastic and skin, ANOM did not reveal any significant interaction in naphthalene absorption except in the IPPSF model. The ANOVA, which is more sensitive than ANOM, depicted some significant interactions among various treatments (Figure 1). The silastic membrane was designed to depict chemical-chemical interactions, which indicated that the addition of single additive (MDA, BHT, 8Q405) to JP-8 jet fuel made the naphthalene absorption significantly different from JP-8 alone or with the combination of additives. The second membrane system (porcine skin) used to detect chemical-biological interactions indicated no significant interaction with any additive mixture. The third membrane system (skin flap) that has intact vasculature and more closely mimics the in vivo situations, suggests that addition of MDA to JP-8 significantly decreased naphthalene absorption while addition of BHT to JP-8 significantly enhanced marker absorption (Figure 5). Addition of 8Q405 had no effect on naphthalene absorption.

Dodecane absorption in the three membrane systems is shown in Figure 1 in the form of compass plots. The ANOM failed to detect any significant interactions. The ANOVA indicated that the dodecane absorption in silastic membrane flow through cells was significantly decreased by addition of MDA to JP-8 as compared to JP-8 alone. In porcine skin, the MDA statistically increased the marker absorption as compared to that of JP-8 alone. This conflicting picture was rectified in the skin flap, which revealed that MDA significantly antagonized the dodecane absorption as compared to that of both JP-8 and JP-8(100). There was no other significant additives interaction in any other membrane systems.
Mixture interactions in hydrocarbon surface retention

The amount of naphthalene retention (µg) on the surface of all three membranes is shown in Figure 2. The silastic membrane surface retention data indicated that naphthalene surface retention was similar in JP-8 and JP-8(100). The addition of individual additives to JP-8 made the naphthalene retention significantly greater than that of JP-8 alone, while the combination of two additives (MDA+BHT, MDA+8Q405, BHT+8Q405) with JP-8 statistically decreased the naphthalene retention as compared to single additives, but still higher than that of JP-8 alone. The addition of all three additives to JP-8 further decreased the naphthalene retention on silastic membrane, thus making hydrocarbon retention in JP-8 and JP-8(100) similar. Both ANOM and ANOVA on porcine skin data indicated that MDA and 8Q405 as single additives, and MDA + BHT as combination additives, led to synergistic interaction in naphthalene surface retention. Whereas BHT + 8Q405 antagonized the surface retention. The ANOVA of IPPSF data elaborated that 8Q405 was the only important additive modulating the naphthalene surface retention.

Dodecane surface retention in silastic membrane was significantly higher in JP-8 and JP-8(100) than that of combinations of additives with JP-8. The porcine skin again indicated a different spectrum of additive interactions. Here ANOVA depicted that dodecane surface retention was significantly less in JP-8(100) than JP-8. The 8Q405 and BHT + 8Q405 were important inhibitors of surface retention of dodecane in skin (Figure 2). The skin flap compass plots showed that dodecane surface retention was similar in JP-8 and JP-8(100). In this scenario, 8Q405 was the most important surface retention enhancer.

Mixture interactions in stratum corneum, dose area and tissue penetration

The majority of significant additive interactions were observed in naphthalene retention in the stratum corneum of porcine skin (Figure 3). Both ANOM and ANOVA indicated that two single additives MDA & BHT significantly increased naphthalene stratum corneum contents while 8Q405 and all combinations of two additives statistically lowered naphthalene in stratum corneum as compared to JP-8 and JP-8(100). No significant additive interaction was observed in the stratum
corneum of skin flaps except with MDA + BHT. Here ANOVA depicted a significant decrease in naphthalene contents of stratum corneum in skin flaps as compared to JP-8(100). The shapes of compass plots for dodecane retention in stratum corneum of skin were entirely different from naphthalene (Figure 3), indicating a different spectrum of additive interactions in both marker hydrocarbons. In skin flaps, the effects of additive interactions on dodecane retention in stratum corneum were similar to that of naphthalene.

The amount of naphthalene (µg) in dosed membrane or skin of three membrane systems is represented in Figure 4. In silastic membrane 8Q405 alone and in combination with MDA significantly reduces the naphthalene retention in the dosed area as compared to both JP-8 and JP-8(100). In porcine skin, 8Q405 alone and in combination with BHT, significantly reduced the marker contents in the dosed area as compared to JP8 alone. While in the IPPSF, ANOVA revealed MDA + BHT as significant reducers of naphthalene retention in the dosed area as compared to JP-8(100). For dodecane tissue retention in the dosed area of all three membranes ANOM failed to depict any significant interaction (Figure 4). The ANOVA indicated that only MDA + BHT significantly retained dodecane in silastic dosed area. No significant interaction was encountered in porcine skin. ANOVA of skin flap data demonstrated that JP-8 alone and in combination with MDA + BHT significantly retained less dodecane in the dosed area as compared to JP-8(100). The tissue penetration for both of these marker hydrocarbons in skin flaps is also similar across JP-8 and JP-8(100) with no significant additive interactions (Figure 3).

The flux, permeability and diffusivity of naphthalene and dodecane were similar across silastic and skin membranes in both JP-8 and JP-8(100) (Table 2). ANOVA indicated a few significant differences in some additive interactions in both membranes for the said absorption parameters.

**DISCUSSION**

Naphthalene absorption was higher than dodecane and dodecane tissue retention was higher than naphthalene in all the membranes studied, a finding consistent with previous work (Riviere et al.,
Our focus in the present study was on the effects of mixture additives upon the marker disposition across the three membrane systems.

**Mixture interactions in the marker hydrocarbon absorption**

The IPPSF absorption data indicated that MDA and BHT canceled the effects of each other resulting in naphthalene absorption in JP-8(100) being similar to JP-8. By studying the marker hydrocarbon disposition in three membrane systems, some insight into the potential mechanism behind this observation might be possible. MDA inhibited naphthalene absorption in skin flaps possibly due to its surface adsorptive properties secondary to the presence of phenol and amine functional groups (Striebich et al., 2000). Our data indicated that in JP-8 + MDA treatment, 13 to 17 times more naphthalene was retained on the surface of porcine skin and the IPPSF compared to what was absorbed. Due to this surface adsorptive property, MDA might be assumed to retain fuel hydrocarbon more on the skin surface preventing absorption. This also suggests that slight changes (e.g. 1-2%) in surface retention from MDA might dramatically impact the amount subsequently absorbed.

Both ANOM and ANOVA indicated that BHT significantly increased the absorption of naphthalene in IPPSF but not in porcine skin. BHT is classified as a chain breaking antioxidant; which break the auto-oxidation chain reaction by donating a hydrogen atom to a lipid radical, producing a stable product and an antioxidant free radical (Papas, 1993). BHT is insoluble in water and has an octanol water partition coefficient (log Ko/w) of 5.11-5.20 (Geyer et al., 1986; Lanigan and Yamarik, 2002). This high log Ko/w suggests that the lipophilic BHT would tend to stay in stratum corneum. Our porcine skin data indicated that naphthalene retention on the surface by BHT is similar to JP-8 and JP-8(100) (controls), but significantly greater in stratum corneum as compared with controls. However, the behavior of BHT in the IPPSF was entirely different from that in porcine skin. Here naphthalene surface and stratum corneum retention by BHT is similar to JP-8 and JP-8(100) (Figures 1,2,3) but its absorption was significantly greater than controls. One hypothesis is
that BHT also modulates the dermal uptake, which then enhances the diffusion of hydrophilic naphthalene out of stratum corneum thereby increasing the naphthalene absorption in IPPSF. This would be consistent with BHT's reported pharmacologic action in skin (Lanigan and Yamarik, 2002). This interaction was only present in the biologically intact IPPSF model.

The only significant additive interaction for dodecane was found with JP-8 + MDA in the IPPSF. The decrease in dodecane absorption by MDA can be explained on similar grounds as for naphthalene. Since MDA has surface adsorptive properties, dodecane is retained on the surface of the tissue as seen in our data (1.6 ± 0.14 µg of dodecane was absorbed and 97.34 ± 13.2 µg were retained on the surface of IPPSF).

**Mixture interactions in hydrocarbon surface retention**

Our naphthalene data suggested a chemical interaction in silastic membrane where the combination of two and three additives resulted in antagonistic action on naphthalene surface retention (Figure 2). As discussed earlier, MDA has surface adsorptive properties such that maximum surface retention of naphthalene on skin surface was found with JP-8 + MDA and JP-8 + MDA + BHT treatments. The differences in spectrums of additive effects on dodecane surface retention in various membranes again indicated the influence of different membrane systems upon chemical interactions. By looking solely at dodecane surface retention in silastic membrane, it was difficult to explain the similarity between JP-8 and JP-8(100). In examining porcine skin cells, it was evident in Figure 2 that 8Q405 and BHT + 8Q405 antagonized its surface retention making it significantly less in JP-8(100) than JP-8. In contrast, the skin flap data showed that 8Q405 acted as a potent synergistic additive for dodecane surface retention, making its retention similar in JP-8(100) and JP-8.

8Q405 is a detergent/dispersant added to JP-8(100) for specific fuel performance objectives. Chemically, 8Q405 is polybutenylsuccinimide, an ashless dispersant consisting of a polar polyamine head group conjugated to a succinimide group (Gergel, 1984). Dispersants of the polybutenylsuccinimide type are particularly important as they prevent agglomeration of particles produced by oil degradation and wear of metallic parts by maintaining them in suspension in the oil
(Hui et al., 1997). Based on these studies, it is postulated that 8Q405 could form micelles with marker hydrocarbon and retain them on the surface of membranes. Alternatively, the surface retention of marker hydrocarbon by 8Q405 in synthetic and biological membranes might also be attributed to its viscous nature. The lack of this effect for naphthalene surface retention in the IPPSF may reflect the different surface conditions of the IPPSF compared to the diffusion cell experimental models.

**Mixture interactions in stratum corneum, dose area and tissue penetration**

Important additive interactions in naphthalene retention in the stratum corneum were observed with 8Q405 in porcine skin (Figure 3). As discussed earlier, that 8Q405 could form micelles with marker hydrocarbon thus retaining naphthalene on the surface of the skin rather than being absorbed into the stratum corneum (Figures 2,3). 8Q405 significantly reduced naphthalene retention in dosed silastic and skin indicating a potential direct chemical reaction between additive and marker hydrocarbon (Figure 4). MDA and BHT, which significantly retained the naphthalene in the stratum corneum of porcine skin individually, led to a statistical decrease in naphthalene contents in stratum corneum in combination (MDA + BHT). This indicated that some kind of biological interaction took place in the stratum corneum of porcine skin with these two additives together. This same biological interaction with MDA + BHT was observed in stratum corneum and dosed skin of the IPPSF (Figures 3,4) indicating a significant decrease in naphthalene and dodecane contents in these regions. It is not surprising to observe the synergistic action between MDA and BHT because MDA inhibits the fuel oxidation catalyzed by metals (Striebich et al., 2000) and BHT stabilized biomembranes against lipid peroxidation (Dwight & Hendry, 1996), the rate of which is accelerated by the presence of metal ions (Papas 1993). Furthermore, BHT had also been reported to have a pharmacologic action in the skin (Lanigan and Yamarik, 2002). These additives would be expected to act synergistically in live skin as well as IPPSF membrane models.

The total penetration of naphthalene and dodecane in this study was found to be similar in JP-8 and JP-8(100) (Figure 3). Riviere et al (1999) and Kanikkannan et al (2001) reported that total penetration/permeation of naphthalene was greater in JP-8(100) as compared to JP-8 jet fuel. This
difference might be explained while considering the composition of JP-8(100), which consisted of three performance additives (MDA, BHT, 8Q405) and a heavy aromatic naphtha solvent (6-10% naphthalene) added at 129 ppm (Heneghan et al., 1996; Kalt et al., 2001). In the present study, we added only three additives (MDA, BHT, 8Q405) to JP-8 in order to make JP-8(100). It must be considered that JP-8(100) preformed package itself added 6-10% naphthalene to the fuel formulation. It is possible that the increase in total penetration of naphthalene with full JP-8(100) in the above mentioned studies might be due to a direct enhancer effect of increased naphthalene in the dosed fuel.

Although flux, permeability and diffusivity of marker hydrocarbons were similar across silastic and skin membranes in both JP-8 and JP-8(100), the addition of single or combination of additives to JP-8 indicated significant effects on the above absorption parameters (Table 2). The manufacturer commonly delivers the additive chemical package to the flight line. The additive may be injected into fuel while leaving a fuel truck to enter an aircraft fuel tank, or directly into the aircraft fuel tank itself. There are chances of equipment failures, accidental spills, and unavoidable leaking providing an opportunity for human flight line contact with the undiluted additive. Human contact with the diluted additive package, after addition to JP-8 stock, would appear to occur, as does human contact to the JP-8(100) or its combustion products (Ritchie et al., 2003). The modulation of fuel component absorption due to additives suggests that predicting the toxicity of exposure to different additive factors is problematic. Finally, one must consider what the toxicological implications of the presence of opposing additives (MDA and BHT) are on interpreting equivalent naphthalene fluxes in mixtures containing both additives compared to those without any additives. Do these opposing mechanisms, which cancel out individual modulating effects of each additive, also modify the potential for direct skin toxicity compared to naphthalene alone? This dimension of complex chemical mixture toxicology has not been adequately addressed.

In conclusion, this study indicated that MDA is a significant antagonist of both naphthalene and dodecane absorption while BHT is a potent synergist of naphthalene absorption in IPPSF. Porcine skin did not depict any significant effect of additives on marker absorptions. The 8Q405 has
no effect on marker absorption, but significantly retained it on the surface of membranes. The combination of two additives affected dodecane retention more than naphthalene on surface of silastic membrane while the reverse was true for porcine skin surface retention. 8Q405 alone, and in combination with BHT, significantly reduced naphthalene contents in porcine skin but not in IPPSF. These observations demonstrate that a single membrane system may not be suitable for the final prediction of complex additive interactions in jet fuels. Rather a combination of different membrane systems may provide the insight for possible mechanism for additive interactions. A membrane system such as the IPPSF, which most closely mimics the in vivo situation, should probably be used for predictive purposes.

ACKNOWLEDGMENT

This work was supported by the U.S. Air Force Office of Scientific Research, Grant F49620-01-1-0080.
REFERENCES


<table>
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<tr>
<th>No additive</th>
<th>Single additive</th>
<th>Two additives</th>
<th>Three additives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JP-8 + BHT</td>
<td>JP-8 + MDA + 8Q405</td>
<td></td>
</tr>
<tr>
<td></td>
<td>JP-8 + 8Q405</td>
<td>JP-8 + BHT + 8Q405</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 3.2. Mean (SEM)* Steady State Flux, Permeability and Diffusivity Following Topical Doses of Naphthalene and Dodecane in Jet Fuel Mixtures in Silastic and Porcine Skin

<table>
<thead>
<tr>
<th>Naphthalene (Silastic membrane)</th>
<th>Flux (µg/cm²/hr)</th>
<th>Permeability (cm/hr x 10^-3)</th>
<th>Diffusivity (cm²/hr x 10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JP-8 (n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JP-8+MDA (n=5)</td>
<td>19.29 (0.60) b</td>
<td>1.78 (0.06) d</td>
<td>1.690 (490) b</td>
</tr>
<tr>
<td>JP-8+BHT (n=5)</td>
<td>28.23 (1.62) a</td>
<td>2.30 (0.13) a</td>
<td>16,775 (5,672) a</td>
</tr>
<tr>
<td>JP-8+8Q405 (n=4)</td>
<td>27.17 (0.84) a</td>
<td>2.22 (0.07) ab</td>
<td>3,855 (907) b</td>
</tr>
<tr>
<td>JP-8+MDA+BHT (n=5)</td>
<td>26.86 (0.71) a</td>
<td>2.19 (0.06) ab</td>
<td>2,164 (403) b</td>
</tr>
<tr>
<td>JP-8+MDA+8Q405 (n=4)</td>
<td>21.22 (0.52) b</td>
<td>1.99 (0.05) bcd</td>
<td>1,408 (411) b</td>
</tr>
<tr>
<td>JP-8+BHT+8Q405 (n=4)</td>
<td>21.75 (0.73) b</td>
<td>2.03 (0.06) bc</td>
<td>1,469 (790) b</td>
</tr>
<tr>
<td>JP-8(100) (n=5)</td>
<td>21.01 (0.86) b</td>
<td>2.04 (0.07) bc</td>
<td>251 (23) b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.93 (0.08) cd</td>
<td>6,823 (5,836) b</td>
</tr>
</tbody>
</table>

Dodecane (Silastic membrane)

<table>
<thead>
<tr>
<th>Dodecane (Silastic membrane)</th>
<th>Flux (µg/cm²/hr)</th>
<th>Permeability (cm/hr x 10^-3)</th>
<th>Diffusivity (cm²/hr x 10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JP-8 (n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JP-8+MDA (n=5)</td>
<td>1.46 (0.08) a</td>
<td>0.041 (0.002) a</td>
<td>3,577 (1.956) ab (n=3)</td>
</tr>
<tr>
<td>JP-8+BHT (n=5)</td>
<td>0.70 (0.03) b</td>
<td>0.020 (0.001) b</td>
<td>9,819 (4,607) a</td>
</tr>
<tr>
<td>JP-8+8Q405 (n=4)</td>
<td>0.90 (0.07) b</td>
<td>0.026 (0.002) b</td>
<td>2,187 (628) b</td>
</tr>
<tr>
<td>JP-8+MDA+BHT (n=5)</td>
<td>0.75 (0.04) b</td>
<td>0.021 (0.001) b</td>
<td>1,805 (336) b</td>
</tr>
<tr>
<td>JP-8+MDA+8Q405 (n=4)</td>
<td>0.89 (0.15) b</td>
<td>0.025 (0.004) b</td>
<td>3,741 (1,313) ab (n=4)</td>
</tr>
<tr>
<td>JP-8+BHT+8Q405 (n=4)</td>
<td>0.84 (0.08) b</td>
<td>0.024 (0.002) b</td>
<td>3,601 (1,822) ab</td>
</tr>
<tr>
<td>JP-8(100) (n=5)</td>
<td>0.74 (0.24) b</td>
<td>0.021 (0.007) b</td>
<td>2,972 (1,247) ab</td>
</tr>
<tr>
<td></td>
<td>1.34 (0.05) a</td>
<td>0.038 (0.001) a</td>
<td>2,484 (873) b (n=3)</td>
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</table>

Naphthalene (Pig Skin)

<table>
<thead>
<tr>
<th>Naphthalene (Pig Skin)</th>
<th>Flux (µg/cm²/hr)</th>
<th>Permeability (cm/hr x 10^-3)</th>
<th>Diffusivity (cm²/hr x 10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JP-8 (n=5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JP-8+MDA (n=5)</td>
<td>2.21 (0.27) a</td>
<td>0.21 (0.03) a</td>
<td>455 (49) a</td>
</tr>
<tr>
<td>JP-8+BHT (n=5)</td>
<td>2.63 (0.09) a</td>
<td>0.20 (0.01) a</td>
<td>337 (23) b</td>
</tr>
<tr>
<td>JP-8+8Q405 (n=4)</td>
<td>2.48 (0.28) a</td>
<td>0.19 (0.02) a</td>
<td>324 (39) b</td>
</tr>
<tr>
<td>JP-8+MDA+BHT (n=5)</td>
<td>2.17 (0.16) a</td>
<td>0.17 (0.01) a</td>
<td>330 (42) b</td>
</tr>
<tr>
<td>JP-8+MDA+8Q405 (n=5)</td>
<td>2.88 (0.35) a</td>
<td>0.23 (0.03) a</td>
<td>176 (15) c</td>
</tr>
<tr>
<td>JP-8+BHT+8Q405 (n=4)</td>
<td>2.64 (0.47) a</td>
<td>0.21 (0.04) a</td>
<td>158 (9) c</td>
</tr>
<tr>
<td>JP-8(100) (n=5)</td>
<td>2.70 (0.48) a</td>
<td>0.21 (0.04) a</td>
<td>161 (8) c</td>
</tr>
<tr>
<td></td>
<td>2.24 (0.25) a</td>
<td>0.21 (0.02) a</td>
<td>402 (37) ab</td>
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</tbody>
</table>

Dodecane (Pig Skin)

<table>
<thead>
<tr>
<th>Dodecane (Pig Skin)</th>
<th>Flux (µg/cm²/hr)</th>
<th>Permeability (cm/hr x 10^-3)</th>
<th>Diffusivity (cm²/hr x 10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JP-8 (n=5)</td>
<td></td>
<td></td>
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<tr>
<td>JP-8+MDA (n=5)</td>
<td>0.090 (0.01) b</td>
<td>0.0025 (0.00) b</td>
<td>1,179 (331) bc</td>
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<tr>
<td>JP-8+BHT (n=5)</td>
<td>0.164 (0.03) a</td>
<td>0.0047 (0.00) a</td>
<td>352 (111) c</td>
</tr>
<tr>
<td>JP-8+8Q405 (n=4)</td>
<td>0.123 (0.01) ab</td>
<td>0.0035 (0.00) ab</td>
<td>455 (33) c</td>
</tr>
<tr>
<td>JP-8+MDA+BHT (n=5)</td>
<td>0.171 (0.05) a</td>
<td>0.0049 (0.00) a</td>
<td>271 (47) c</td>
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<td>JP-8+MDA+8Q405 (n=4)</td>
<td>0.077 (0.01) b</td>
<td>0.0022 (0.00) b</td>
<td>2,565 (778) a</td>
</tr>
<tr>
<td>JP-8+BHT+8Q405 (n=4)</td>
<td>0.097 (0.01) b</td>
<td>0.0028 (0.00) b</td>
<td>1,147 (330) bc</td>
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<tr>
<td>JP-8(100) (n=5)</td>
<td>0.079 (0.01) b</td>
<td>0.0022 (0.00) b</td>
<td>1,652 (435) ab</td>
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<td></td>
<td>0.094 (0.02) b</td>
<td>0.0027 (0.00) b</td>
<td>1,174 (384) bc</td>
</tr>
</tbody>
</table>

*Superscripts represent significant differences among treatments within a parameter (p<0.05). Means with the same letter are not significantly different.
Figure 3.1. Compass plots indicating the naphthalene and dodecane absorption in various jet fuel mixtures in the three membrane systems, SMFT (silastic membrane flow through), PSFT (porcine skin flow through), IPPSF (isolated perfused porcine skin flap). Arrows and letters indicate ANOM and ANOVA results respectively.
Figure 3.2. Compass plots indicating the naphthalene and dodecane surface retention in various jet fuel mixtures in the three membrane systems, SMFT (silastic membrane flow through), PSFT (porcine skin flow through), IPPSF (isolated perfused porcine skin flap). Arrows and letters indicate ANOM and ANOVA results respectively.
Figure 3.3. Compass plots indicating the naphthalene and dodecane stratum corneum contents in various jet fuel mixtures in two membrane systems, PSFT (porcine skin flow through), IPPSF (isolated perfused porcine skin flap). The total penetration of both markers is studied in IPPSF only. Arrows and letters indicate ANOM and ANOVA results respectively.
Figure 3.4. Compass plots indicating the naphthalene and dodecane deposition in dose area in various jet fuel mixtures in the three membrane systems, SMFT (silastic membrane flow through), PSFT (porcine skin flow through), IPPSF (isolated perfused porcine skin flap). Arrows and letters indicate ANOM and ANOVA results respectively.
Figure 3.5. Significant perfusate rate of absorption (µg/h) profiles of naphthalene and dodecane after dosing isolated perfusate porcine skin flaps with JP-8, JP-8+MDA and JP-8+BHT.
4. DOSE RELATED ABSORPTION OF JP-8 JET FUEL HYDROCARBONS THROUGH PORCINE SKIN WITH QUANTITATIVE STRUCTURE PERMEABILITY RELATIONSHIP ANALYSIS

F. Muhammad, R.E. Baynes, N.A. Monteiro-Riviere, X.R. Xia and J.E. Riviere

Published in Toxicology Mechanisms and Methods 14 (2004) 159-166.
ABSTRACT

The effects of dosage on the percutaneous absorption of jet fuel hydrocarbons is not clear yet is essential for human risk assessment. The present study is an ongoing approach to assess the dose related percutaneous absorption of a number of aliphatic and aromatic hydrocarbons. The first treatment (1X) was comprised of mixtures containing undecane (4.1%), dodecane (4.7%), tridecane (4.4%), tetradecane (3%), pentadecane (1.6%), naphthalene (1.1%) and dimethyl naphthalene (1.3% of jet fuels) in hexadecane solvent per porcine skin flow through diffusion cell. Other treatments (n=4 cells) were 2X and 5X concentrations. Perfusate samples were analyzed with gas chromatography-flame ionization detector (GC-FID) using head space solid phase micro-extraction fiber technique. We have standardized the assay to have a good linear correlation for all the tested components in media standards. Absorption parameters including diffusivity, permeability, steady state flux and percent dose absorbed were estimated for all the tested hydrocarbons. This approach provides a baseline to access component interactions among themselves and with the diluent (solvents). A quantitative structure permeability relationship (QSPR) model was derived to predict the permeability of unknown jet fuel hydrocarbons in this solvent system by using their physicochemical parameters. Our findings suggested a dose related increase in absorption for naphthalene and dimethyl naphthalene (DMN).

Key words: absorption, GC-SPME, hydrocarbons, jet fuels, porcine skin, QSPR
INTRODUCTION

Millions of occupational workers are exposed to jet fuel mixtures. Dermal absorption of these mixture components has been reported to be toxicologically important. Jet A, JP-8 and JP-8 + 100 are commonly used jet fuels. Commercial aircraft primarily use Jet A whereas military aircraft use JP-8 and JP-8 + 100. Jet fuels contain hundreds of aliphatic and aromatic hydrocarbons. JP-8, the major jet fuel used by US military consists of approximately 81% aliphatic hydrocarbons (9% C_{8-9}, 65% C_{10-14}, and 7% C_{15-17}) with the remainder being aromatic hydrocarbons (Committee on Toxicology, 1996). The US Department of Labor, Bureau of Labor Statistics estimated that in 1992 over 1.3 million workers were exposed to jet fuel (Harris et al. 2000). The use of JP-8 by government and industry worldwide is over 59 billion gallons per year (Armburst Aviation Group 1998).

Jet fuels have been found to be potentially toxic for the professional workers. Neurological effects and irritant dermatitis have been reported in workers exposed to JP-8 jet fuel (Smith et al. 1997; Zeiger and Smith 1998). Significant effects on the immune, hepatic, neurological, and respiratory systems have been observed in several animal exposure studies (Grant et al. 2000; Harris et al., 2000; Robledo et al. 2000).

The National Research Council’s Subcommittee on Jet-Propulsion Fuel 8 (2002) concludes that in addition to inhalation exposures, the potential exists for a substantial contribution by the dermal route, including mucous membranes and the eyes, either with vapors and aerosols or by direct skin contact with JP-8. The subcommittee further mentioned that aircraft fuel-maintenance workers may be exposed to liquid jet fuel for more than 10 minutes which gives ample opportunity for dermal exposure. Prolonged JP-8 skin contact can induce irritation, contact dermatitis, and sensitization (Ullrich 1999; Wolfe et al. 1997). Topical application of jet fuels can cause gross morphological changes in porcine skin (Monteiro-Riviere et al. 2001) and release of tumor necrosis factor-α and interleukin-8 in cultured human epidermal keratinocytes (Allen et al. 2000) and in porcine keratinocytes (Allen et al. 2001a). These findings suggest that toxicologically relevant components in
jet fuels can readily penetrate the skin to elicit these dermatological effects. Several dermal absorption studies in rats (McDougal et al. 2000) and porcine skin flaps and skin sections (Baynes et al. 2001; Riviere et al. 1999) have assessed the dermal disposition of several jet fuel components and their additives. Among them, the aliphatic hydrocarbons could play a major role in jet fuel associated skin irritation because they were more likely to be sequestered in the epidermis than aromatic hydrocarbons (Baynes et al. 2000, 2001) and due to an increase propensity to induce IL-8 release (Allen et al. 2001b). We used porcine skin in this study because of its similarities to human skin (Montagna 1966, 1967; Weinstein 1966). Pig epidermis was found to be similar ultrastructurally to that reported for human (Monteiro-Riviere 1986). Porcine skin is similar to man in respect of its permeability to selected xenobiotics (Bertek et al. 1972; Bronaugh et al. 1982; Wester and Maibach 1977). These studies indicate that pig is a valuable model for toxicologic studies involving the skin.

Our studies have indicated that individual aliphatic hydrocarbons (C11-C16) were toxic to human epidermal keratinocytes (HEK) cells and are capable of inducing the release of proinflammatory cytokines (Chou et al. 2002). Higher cytotoxicity associated with shorter chain aliphatic hydrocarbons did not correlate to an increase in IL-8 stimulation, which peaked at mid-chain lengths, suggesting a different structure-activity relationship for these two toxicological endpoints in keratinocyte cell cultures. We further evaluated the in vitro dose required to cause 50% HEK mortality (LD50) and the highest noncytotoxic (5% HEK mortality) dose of aromatic hydrocarbons in the culture media as well as IL-8 release at selected doses (Chou et al. 2003). There was a dose related differential response in IL-8 release at 24 hrs. IL-8 increased significantly with both doses of methyl naphthalene and naphthalene.

In many in vitro percutaneous absorption studies, individual radioactive hydrocarbons were employed as markers. The potential disadvantage in such studies is that these are not reflecting the absorption of individual hydrocarbons. Also, there are no reports regarding dose related percutaneous absorption of jet fuel hydrocarbons which is necessary for risk assessment studies. In this study, we determined the simultaneous percutaneous absorption of five neat aliphatic (undecane, dodecane,
tridecane, tetradecane, pentadecane) and two aromatic (naphthalene, dimethyl naphthalene) hydrocarbons using hexadecane as solvent. We analyzed our perfusate samples by gas chromatography (GC) equipped with a flame ionization detector (FID) by using the solid phase microextraction (SPME) fiber technique with headspace sampling. This study was conducted to accomplish the following objectives:

1) to define the dose-related percutaneous absorption of selected aliphatic and aromatic hydrocarbons present in jet fuel mixtures.
2) to standardize the assay for neat jet fuel hydrocarbons using SPME and GC-FID.
3) to compare the percutaneous absorption by topical administration of a number of hydrocarbons.
4) to derive the quantitative structure permeability relationship (QSPR) model for prediction of permeability of unknown jet fuel hydrocarbons using their known physicochemical properties.

MATERIALS AND METHODS

Test compounds

Six aliphatic and two aromatic hydrocarbons were selected for this study (Table 1). Undecane (C11), dodecane (C12), tridecane (C13), tetradecane (C14), pentadecane (C15), hexadecane (C16), and 1,2 dimethyl naphthalene (C12), all with greater than 98% purity, were purchased from Sigma Chemical Co. (St. Louis, MO). Naphthalene (C10) was purchased from Supelco (Supelco Inc., Park Bellefonte, PA). Hexadecane was used as solvent/diluent in these absorption studies. Three different treatments of 20 ìl of the dose mixtures were topically applied to each porcine skin flow through diffusion cell. The first treatment (1X) was comprised of mixtures containing undecane 4.1% of JP8 (606.8ìg), dodecane 4.7% (705ìg), tridecane 4.4% (660ìg), tetradecane 3.0% (456ìg), pentadecane 1.6% (246.4ìg), naphthalene 1.1% (255.6ìg) and dimethyl naphthalene 1.3% (263.4ìg) per cell using hexadecane as the diluent. Treatment two was 2X and the third treatment was 5X the applied dose.

Flow through diffusion cell experiments

The flow through diffusion cell system, previously described by Bronaugh and Stewart (1985), was used to perfuse porcine skin that was obtained from the dorsal area of weanling female
Yorkshire pigs. The skin was dermatomed to a thickness of 200-300ìm with a Padgett Dermatome (Padgett Instruments, Kansas City, MO). Each circular skin section was punched to provide a dosing surface area of 0.64 cm² and then placed into a two-compartment Teflon flow through diffusion cell. Skin discs were perfused with Krebs-Ringer bicarbonate buffer spiked with dextrose and bovine serum albumen. The temperature of the perfusate and flow through cell was maintained at 37ºC using a Brinkmann constant temperature circulator (Brinkmann, Westbury, NY). The pH of perfusate was maintained between 7.4 and 7.5. Perfusate flow rate was 4.0 ml/hr. Perfusate samples were taken at 0, 10, 20, 30, 45, 60, 75, 90, 105, 120 min, and then at 2.5, 3, 4 and 5hrs. These samples were saved for chromatographic analysis.

Chromatographic analysis

Chromatographic analysis was performed on a GC 5890 (Hewlett Packard series II) equipped with FID detector. We first confirmed the identity of individual hydrocarbon with GC-MS in our laboratory and then used GC-FID for further analysis because of its availability and good sensitivity for hydrocarbon components. The sensitivity of GC-FID for all the standards was 0.1 ìg/ml. The limit of detectability in samples with SPME fiber was 0.1-1.0 ng/ml for undecane, dodecane, tirdecane and 5ng/ml for naphthalene, DMN. This is the advantage of using SPME fiber because it involves solvent free extraction and very low levels can be detected in the samples. GC-FID conditions were as follows: EC-5 capillary column (30m× 0.25mm I.D., 0.25ìm film thickness; Alltech associates, Inc. Deerfiled, IL, USA); injection port temperature, 275°C; Detector temperature, 300°C; oven temperature, 60-200°C (held at 60°C for 1.50 min, then increased at 5°C/min from 60-200°C); carrier gas (helium) flow rate, 0.8ml/min; carrier + makeup gas (He) flow rate, 22.5ml/min; hydrogen flow rate, 45ml/min; air flow rate, 300-320ml/min. Solid phase microextraction (SPME) devices and 100-ìm polydimethylsiloxane (PDMS) fiber assemblies were purchased from Supelco (Bellefonte, PA, USA).
Standard curves

Stock solutions of each of the tested hydrocarbons were prepared individually in acetone. All stock solutions were mixed together and serially diluted in acetone to have concentrations of 5, 2, 1, 0.5, and 0.1µg/ml. Equal volumes of perfusion media were spiked with each of above concentrations to have standard solutions of 500, 200, 100, 50, and 10ng/ml of media accordingly. Then 500µl from these standard solutions were drawn into 2ml glass vials. A micro stirring bar (7mm L × 2mm D) was placed into these vials. The vials were sealed and SPME fiber was inserted into the head space (HS) of these vials. The fiber was exposed to headspace sampling for 30 mins at 37ºC with continuous stirring. The SPME fiber was then injected manually into GC and held for 5 mins. The peak areas obtained were then plotted against standard concentrations and linear relationships with good correlation coefficients were obtained for all the tested hydrocarbons (Table-2). The same procedure was adopted for the analysis of perfusate samples.

Calculations and Statistics

The perfusate concentrations (ng/ml) were determined for the three dosing (1X, 2X, 5X) protocols. Absorption was defined as the total percentage of initial dose detected in the perfusate for the entire 5-hr perfusion period. Flux (µg/cm²/h) for individual hydrocarbons were determined at steady state from the slope of cumulative mass per unit area versus time (h) curve. Permeability (cm/h) was determined by dividing the steady state flux (slope of cumulative µg/cm² versus time curve) by hydrocarbon concentration applied to surface in µg/ml. Diffusivity (cm²/h) was obtained by the following relation: D=L²/6δ, where L=250µm and δ is the lag time obtained by extrapolating the steady state portion of the curve back to the time or x-axis.

Statistical analysis of steady state flux (Jss), permeability, diffusivity, and percent dose absorbed data were conducted using multiple comparison tests and ANOVA with significance level P<0.05. All analysis were carried out using SAS 8.1 for Windows software (SAS Institute, Cary, NC). A least significance difference (LSD) procedure and Student t test were used for multiple comparisons on all parameters assessed. QSPRs models provide a significant tool for assessing the
percutaneous penetration of chemicals such as jet fuel hydrocarbons for dermal toxicology and risk assessment. Therefore, a multiple regression analysis of our experimentally determined permeability constants and reference physical parameter data was conducted with Microsoft Excel version 97 using least sum of squares method. A QSPR model was suggested to predict the permeability of unknown jet fuel hydrocarbons in this solvent system using their physicochemical properties.

RESULTS

Dose related differences in aromatic hydrocarbons

The concentration vs time profiles in Figure 1 (upper panel) demonstrate a dose dependent increase in absorption of naphthalene in porcine skin. The higher (5X) dose attained a significantly higher concentration of approximately 500ng/ml in perfusate as compared to lower doses (2X and 1X) that gave 185 and 61ng/ml concentration in perfusate samples, respectively. Table 3 indicates that the 5X naphthalene dose also had a significant greater flux (3.63±0.24 ìg/cm²/h) than that of 2X and 1X doses (1.24±0.26 and 0.43±0.12 ìg/cm²/h respectively). There were no statistically significant differences in permeability, diffusivity and percent dose absorbed in all three naphthalene doses (Table 3). Dimethyl naphthalene concentration vs time profiles are shown in Figure 1 (lower panel) indicating a dose dependent absorption in porcine skin for three dosing protocols. With the 5X dose of dimethyl naphthalene, a maximum 88ng/ml concentration was detected in perfusate as compared to 37 and 19ng/ml for 2X and 1X doses respectively. As listed in Table 3, 5X dimethyl naphthalene flux (0.58±0.09ìg/cm²/h) is significantly greater than that of 2X and 1X doses (0.23±0.05and 0.13±0.01ìg/cm²/h respectively), while the permeability, diffusivity and percent dose absorbed are not significantly different in the three doses of dimethyl naphthalene.

Dose related differences in aliphatic hydrocarbons

Undecane concentration vs time profiles are presented in Figure 2. The maximum concentration achieved by undecane in perfusate with the 5X dose is 5ng/ml. All the calculated parameters for undecane are statistically similar for the three doses. The concentration vs time profiles for dodecane are similar to undecane, but with a reduced perfusate concentration of about
3.4 ng/ml with the 5X dose (Figure 3). The maximum concentration achieved by tridecane in perfusate was only 1 ng/ml with the 5X dose. Also, there was no dose dependent trend evident in the profiles (Figure 4). There were no significant differences among 1, 2 and 5X doses with regards to flux, permeability, diffusivity nor the percent dose absorbed of dodecane and tridecane. This can be anticipated since the size of the hydrocarbon is increased, the percutaneous absorption profiles are decreased correspondingly. The high molecular weight tetradecane was not detected in the perfusate samples at the low doses, while high background values for pentadecane confounded the results. The data for these two hydrocarbons were excluded from further analysis.

Absorption parameter differences among tested hydrocarbons

In this study, only one solvent (hexadecane, a normal jet fuel component) was used in all doses with the aim that this would provide an opportunity to depict individual hydrocarbon absorption parameters and to compare them with each other independent of solvent interactions with multiple fuel components. Generally, naphthalene flux, permeability and percent dose absorbed were significantly greater than those for dimethyl naphthalene, undecane, dodecane and tridecane for all the three doses (Table 3). The dimethyl naphthalene comparison to undecane, dodecane and tridecane was similar to that of naphthalene. There were no significant differences in flux, permeability, diffusivity and percent dose absorbed for the three doses of undecane, dodecane and tridecane (Table 3).

QSPR Analysis

In order to study the relationship of permeability of these hydrocarbons to their physicochemical properties such as molecular weight, logarithm of octanol-water partition coefficient (log Ko/w) and water solubility, a multiple regression analysis was performed with level of significance (α = 0.05). The analysis revealed a good correlation coefficient (R^2 = 0.9985). This high correlation coefficient may be due to the fact that for this small set of studied hydrocarbons (naphthalene, DMN, undecane, dodecane and tridecane), the available physicochemical properties in literature varies in the same order of magnitude as permeability. This can be anticipated from
coefficients in equation (1) that permeability (Kp) is inversely related to molecular weight and log Ko/w, but directly related to water solubility.

\[
\log K_p = C + \alpha MW + \beta \log Ko/w + \gamma H_2O solubility
\]  

(1)

Where:  Intercept \ (C = 0.3908)

\[
\text{Molecular weight coefficient (} \alpha = -0.0004) \\
\text{Log Ko/w coefficient (} \beta = -0.565) \\
\text{Water solubility coefficient (} \gamma = 0.0073)
\]

A plot of actual and predicted values of log Kp is shown in Figure 5.

**DISCUSSION**

Due to the chemical complexity of jet fuels and their irritant effects on skin, it is difficult to assess the percutaneous absorption of all fuel components. One must instead evaluate the absorption of individual marker components in a known dosing mixture. There is limited data in the literature on the percutaneous absorption of neat jet fuel hydrocarbons in other experimental models or vehicles. McDougal et al (2000) studied the skin absorption and penetration of neat JP-8 jet fuel components using static diffusion cells with rat skin. A study of neat naphthalene absorption in rats suggested that 50% of the applied dose was excreted in urine by 12 hrs, with the predominant urinary metabolites being 2,7-, and 1,2-dihydroxynaphthlene (Turkall et al. 1994). Naphthalene was also studied in vitro using monkey skin in comparing the percutaneous absorption in acetone using a series of aliphatic compounds (Sartorelli et al. 1998). To our knowledge, there are no dose related percutaneous absorption studies of neat or radiolabeled jet fuel components necessary for understanding human risk assessment. Therefore, this study characterized the dermal absorption of the representative aliphatics (undecane, dodecane, tridecane) and aromatics (naphthalene, dimethyl naphthalene) hydrocarbons and determined the influence of three dosing mixtures on dermal absorption of these marker components. The data obtained illustrated distinct differences between various aliphatic and aromatic components in terms of percutaneous absorption and many of these differences can be explained on the basis of known physicochemical properties.
The flux and absorption profiles depicted a significant dose related increase in absorption of naphthalene and DMN (Table 3, Figure 1). These observations are in accordance with Chou et al (2003) who found a dose related increase in IL-8 release from human epidermal keratinocytes by aromatic hydrocarbons such as naphthalene and DMN. The percent dose absorbed, permeability and diffusivity across the three dosing treatments were statistically insignificant, indicating linear first order pharmacokinetics. The same percentage absorbed from the higher dose will be more toxic than the same percentage absorbed from the lower dose of the same component. Linearity across the dose range supports the use of permeability constants in risk assessment calculations. With the straight chain hydrocarbons such as undecane, dodecane and tridecane, the dose related trend of absorption become non-significant (Figures 2,3,4). The high molecular weight and log octanol-water PC (log \( K_{o/w} \)) for these aliphatic hydrocarbons (Table 1) would result in greater partitioning into the stratum corneum. This interaction may then limit diffusion of these hydrocarbons through skin resulting in the loss of linearity and dose related absorption profiles.

In general, the absorption parameters for aliphatics and lower dose aromatics determined in this study are less as compared to other studies (Baynes et al. 2000; McDougal et al. 2000; Riviere et al. 1999). The most likely reason for this disparity is that McDougal et al (2000) used rat skin dosed with 2ml of whole jet fuel in static diffusion cells, while other authors used radiolabeled hydrocarbons in their studies. Rat skin is generally more permeable than porcine or human skin. Another difference is the use of solvents and type of flow through diffusion cells. In the present study, we used only one solvent (hexadecane) in all dosing mixtures. Chou et al (2002) reported that the mortality of HEKs decreased dramatically when neat aromatic hydrocarbons were dosed in the presence of 50% hexadecane suggesting an antagonistic effect between aliphatic and aromatic hydrocarbons when dosed in combination. Alternatively, the absorption of aromatic hydrocarbons may have been reduced when dosed in an aliphatic vehicle. When a chemical is applied in a vehicle, the relative affinity of the chemical in the skin versus the partitioning of the chemical from the vehicle will determine whether the chemical will have a tendency to stay in the vehicle or to be driven into
the skin by the thermodynamics of the situation (Barry et al. 1985; Jepson and McDougal 1997). In other words, solvents will affect partition coefficients; thus, permeability coefficients and percentage doses absorbed can vary many fold.

Interpreting diffusivity data in skin is complicated by the fact that the stratum corneum is a lipid domain and the viable epidermis is comparably a hydrophilic entity with quasi-like liquid properties (Potts and Francoeur 1991). Diffusivity differences between the SC and viable epidermis have been proposed in the development of various kinetic models in skin (Bunge and Cleek 1995), and the viable epidermis can represent the decisive resistance to drug transport (Wenkers and Lippold 1999). Diffusivities in the SC and viable epidermis can also act differently depending on molecular weight (Bunge and Cleek 1995). However, there were no significant differences between naphthalene and other marker diffusivities in this study suggesting that in jet fuel hydrocarbon mixtures, molecular weight differences may not be sufficient to influence diffusivity in skin.

There are several reports in literature indicating that skin absorption parameters for particular components are dependent upon their physical properties (Baynes et al. 2001; Moss et al. 2002). QSPRs have been derived to model the percutaneous absorption of exogenous chemicals by many researchers. Most of these relationships are based on experimental data from the published literature. We used QSPR analysis as an attempt to statistically relate the experimentally determined percutaneous penetration of a range of jet fuel hydrocarbons to known physicochemical parameters. Abraham et al (1999) suggested that the general linear solvation equations can be applied to the correlation and then to the prediction of some particular solute property such as partitioning. The multiple regression equation (1) in our study suggests that molecular weight, log Ko/w and water solubility are the main determinants of transdermal penetration. Equation (1) indicated the inverse dependence of permeability upon molecular weight (characterized by negative regression coefficient) similar to Potts and Guy (1995). These authors argued that increasing molecular volume increases the hydrophobic surface area and that this will increase partitioning into (and hence, permeability through) a lipid membrane. Conversely, larger molecules diffuse more slowly since they require more
"space" to be created in the medium, and this in turn leads to diminished permeability. The permeability observations in this study (Table 3) are in accordance with that of McDougal et al (2000) who also found that chemicals with lower log Ko/w had larger permeability coefficients than chemicals with larger log Ko/w. An inverse relationship of permeability to log Ko/w was observed with our QSPR model. This is also in compliance to Gobas and Mackey (1987) who elaborated that the initial gill uptake efficiency of rainbow trout is directly proportional to the compounds with log Ko/w of 0.5-3.0 and then become constant for higher log Ko/w compounds (3.0-8.0). Since the hydrocarbons studied have high log Ko/w ranging from 3.37 to 7.57 (Table 1), this was anticipated that more hydrophobic components tend to stay in skin lipids rather than to pass through. Equation (1) indicates that the coefficient for log Ko/w (0.565) is higher than the coefficients for molecular weight (0.0004) and water solubility (0.007), suggesting that log Ko/w is better determinant for prediction of skin permeability of jet fuel hydrocarbons. Our laboratory has recently reported on a simplified and rapid in vitro technique to determine log Ko/w for such hydrocarbons using a membrane coated fiber (Xia et al. 2003). Log Ko/w may be employed to predict the permeability of unknown components.

In conclusion, our studies indicated a dose related increase in percutaneous absorption of naphthalene and dimethyl naphthalene suggesting linear first order pharmacokinetics. This is critical from the risk assessment point of view as greater or prolonged exposure to jet fuels may lead to enhanced absorption of such hydrocarbons resulting in local or systemic toxic effects in exposed individuals. The suggested multiple regression equation can be used to predict the permeability of unknown jet fuel hydrocarbons from this solvent by using their physicochemical properties. In addition, we have demonstrated the use of the SPME fiber as an analytical technique in detecting the percutaneous absorption of neat jet fuel marker components. The dermal absorption of jet fuel components have been studied using one lipophilic vehicle. However, it is anticipated that in a more aqueous vehicle, there will be an increased tendency for jet fuel components to move into a more lipophilic stratum corneum and probably increase absorption. Such screening of jet fuel hydrocarbons
with respect to their percutaneous absorption will be helpful to test the selected hydrocarbons in cell culture or other in vitro systems for toxicity studies. In future, we will study the percutaneous absorption of jet fuel hydrocarbons in different solvent systems using normal and hyperplastic/diseased skin.

ACKNOWLEDGMENTS

This work was supported by the U.S. Air Force Office of Scientific Research F49620-01-1-0080. The authors wish to thank Jim Brooks, Jim Yeatts and Beth Barlow for their technical assistance.
REFERENCES


Committee on Toxicology. 1996. Permissible exposure levels for selected military fuel vapors, National Academy Press, Washington DC.


Table 4.1. Physicochemical characteristics of the JP-8 jet fuel hydrocarbons.

<table>
<thead>
<tr>
<th>Component</th>
<th>Molecular weight&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Log K&lt;sub&gt;o/w&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Water solubility (mg/L)&lt;sup&gt;ab&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>128.2</td>
<td>3.37</td>
<td>31.7</td>
</tr>
<tr>
<td>Dimethyl naphthalene</td>
<td>156.2</td>
<td>4.38</td>
<td>14.6</td>
</tr>
<tr>
<td>Undecane</td>
<td>156.3</td>
<td>6.94</td>
<td>0.0044</td>
</tr>
<tr>
<td>Dodecane</td>
<td>170.3</td>
<td>7.24</td>
<td>0.0037</td>
</tr>
<tr>
<td>Tridecane</td>
<td>184.4</td>
<td>7.57</td>
<td>0.0047</td>
</tr>
<tr>
<td>Tetradecane</td>
<td>198.4</td>
<td>7.20</td>
<td>0.0022</td>
</tr>
<tr>
<td>Pentadecane</td>
<td>212.4</td>
<td>7.71</td>
<td>0.00008</td>
</tr>
<tr>
<td>Hexadecane</td>
<td>226.5</td>
<td>8.25</td>
<td>0.0009</td>
</tr>
</tbody>
</table>

Values obtained from <sup>a</sup>Baynes et al 2000, <sup>b</sup>Howard and Meylan 1997, <sup>c</sup>McDougal et al 2000.
Table 4.2. Regression equations for the calibration of selected jet fuel components with GC-FID using head space SPME fiber.

<table>
<thead>
<tr>
<th>Component</th>
<th>Regression equation</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undecane</td>
<td>$Y= 4546.7x - 10776$</td>
<td>0.999</td>
</tr>
<tr>
<td>Dodecane</td>
<td>$Y= 5611x - 18414$</td>
<td>0.999</td>
</tr>
<tr>
<td>Tridecane</td>
<td>$Y= 5513.1x - 19767$</td>
<td>0.999</td>
</tr>
<tr>
<td>Tetradecane</td>
<td>$Y= 3421.4x - 22274$</td>
<td>0.999</td>
</tr>
<tr>
<td>Pentadecane</td>
<td>$Y= 1756.7x + 46910$</td>
<td>0.997</td>
</tr>
<tr>
<td>Hexadecane</td>
<td>$Y= 771.12x - 6116.1$</td>
<td>0.997</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>$Y= 252.97x + 1196.4$</td>
<td>0.999</td>
</tr>
<tr>
<td>Dimethyl naphthalene</td>
<td>$Y= 537.43x + 2459.7$</td>
<td>0.999</td>
</tr>
</tbody>
</table>
Table 4.3. Mean ± SEM* Flux, permeability, diffusivity and percent dose absorbed of selected hydrocarbons in porcine skin sections exposed to three dosing mixtures for 5 hours.

<table>
<thead>
<tr>
<th>Hydrocarbon</th>
<th>Flux (µg/cm²/h)</th>
<th>Permeability (cm/hr*1,000)</th>
<th>Diffusivity (cm²/h*1,000,000)</th>
<th>Percent Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IX dose (n=4)</td>
<td>0.43±0.12bA</td>
<td>0.0333±0.0090aA</td>
<td>244±40ABC</td>
<td>0.3555±0.120aA</td>
</tr>
<tr>
<td>2X dose (n=4)</td>
<td>1.24±0.26bA</td>
<td>0.0485±0.0101aA</td>
<td>211±17BC</td>
<td>0.4653±0.101aA</td>
</tr>
<tr>
<td>5X dose (n=5)</td>
<td>3.63±0.24bA</td>
<td>0.0569±0.0066aA</td>
<td>213±14AB</td>
<td>0.5424±0.066aA</td>
</tr>
<tr>
<td>Dimethyl-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naphthalene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IX dose (n=4)</td>
<td>0.13±0.01bB</td>
<td>0.0095±0.0007ab</td>
<td>153±9ab</td>
<td>0.0700±0.007ab</td>
</tr>
<tr>
<td>2X dose (n=4)</td>
<td>0.23±0.05bB</td>
<td>0.0088±0.0020ab</td>
<td>151±4ab</td>
<td>0.0650±0.017ab</td>
</tr>
<tr>
<td>5X dose (n=5)</td>
<td>0.58±0.09ab</td>
<td>0.0088±0.0014ab</td>
<td>153±3ab</td>
<td>0.0644±0.011ab</td>
</tr>
<tr>
<td>Undecane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IX dose (n=3)</td>
<td>0.03±0.00bc</td>
<td>0.0002±0.0000bc</td>
<td>349±58bc</td>
<td>0.0066±0.001bc</td>
</tr>
<tr>
<td>2X dose (n=4)</td>
<td>0.03±0.00bc</td>
<td>0.0002±0.0000bc</td>
<td>420±24bc</td>
<td>0.0050±0.001bc</td>
</tr>
<tr>
<td>5X dose (n=5)</td>
<td>0.04±0.01bc</td>
<td>0.0003±0.0000bc</td>
<td>311±53bc</td>
<td>0.0046±0.001bc</td>
</tr>
<tr>
<td>Dodecane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IX dose (n=4)</td>
<td>0.01±0.00bcd</td>
<td>0.0003±0.0001bc</td>
<td>293±37abc</td>
<td>0.0033±0.001bc</td>
</tr>
<tr>
<td>2X dose (n=4)</td>
<td>0.02±0.00bc</td>
<td>0.0002±0.0001bc</td>
<td>244±36bc</td>
<td>0.0020±0.001bc</td>
</tr>
<tr>
<td>5X dose (n=5)</td>
<td>0.03±0.01bc</td>
<td>0.0001±0.0000bc</td>
<td>293±63bc</td>
<td>0.0014±0.000bc</td>
</tr>
<tr>
<td>Tridecane</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IX dose (n=3)</td>
<td>0.004±0.00bd</td>
<td>0.0001±0.0000bc</td>
<td>195±25bcd</td>
<td>0.0015±0.000bc</td>
</tr>
<tr>
<td>2X dose (n=4)</td>
<td>0.006±0.00bc</td>
<td>0.0001±0.0000bc</td>
<td>214±18bcd</td>
<td>0.0015±0.001bc</td>
</tr>
<tr>
<td>5X dose (n=4)</td>
<td>0.008±0.00bc</td>
<td>0.0001±0.0000bc</td>
<td>172±7bcd</td>
<td>0.0014±0.000bc</td>
</tr>
</tbody>
</table>

*Lower case superscripts represent significant differences between treatments within a parameter. Upper case superscripts represent significant differences among various hydrocarbons for a specific treatment (P<0.05). Means with the same letter are not significantly different.
Figure 4.1. Perfusate concentrations (ng/ml) of naphthalene (upper panel) and dimethyl naphthalene (lower panel) after dosing porcine skin sections with 1X dosing mixture (- - , n=4), 2X dosing mixture (- - , n=4) and 5X dosing mixture (- - , n=5). *indicates the first time point at which the 5X dose profile becomes statistically different from 2X and 1X doses.

Figure 4.1. Perfusate concentrations (ng/ml) of naphthalene (upper panel) and dimethyl naphthalene (lower panel) after dosing porcine skin sections with 1X dosing mixture (- - , n=4), 2X dosing mixture (- - , n=4) and 5X dosing mixture (- - , n=5). *indicates the first time point at which the 5X dose profile becomes statistically different from 2X and 1X doses.
Figure 4.2. Perfusate concentrations (ng/ml) of undecane after dosing porcine skin sections with 1X dosing mixture (-Ã- , n=4), 2X dosing mixture (- , n=4) and 5X dosing mixture (- , n=5).
Figure 4.3. Perfusate concentrations (ng/ml) of dodecane after dosing porcine skin sections with 1X dosing mixture (- –, n=4), 2X dosing mixture (- –, n=4) and 5X dosing mixture (- –, n=5).
Figure 4.4. Perfusate concentrations (ng/ml) of tridecane after dosing porcine skin sections with 1X dosing mixture (Å-, n=4), 2X dosing mixture (·-, n=4) and 5X dosing mixture (·-, n=5).
Figure 4.5. A plot illustrating the relationship between actual vs model predicted Kp (---) values.
5. EFFECT OF IN VIVO JET FUEL EXPOSURE ON SUBSEQUENT IN VITRO DERMAL ABSORPTION OF INDIVIDUAL AROMATIC AND ALIPHATIC HYDROCARBON FUEL CONSTITUENTS

F. Muhammad, N.A. Monteiro-Riviere, R. E. Baynes and J.E. Riviere

Submitted for publication to *Toxicological Sciences*. 
ABSTRACT

The percutaneous absorption of topically applied jet fuel hydrocarbons (HC) through skin previously exposed to jet fuel has not been investigated, although this exposure scenario is the occupational norm. Pigs were exposed to JP-8 jet fuel soaked cotton fabrics for 1 and 4 days with repeated daily exposures. Pre-exposed and unexposed skin was then dermatomed and placed in in-vitro flow through diffusion cells. Five cells with exposed skin and four cells with unexposed skin were dosed with a mixture of 14 different HC consisting of nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, ethyl benzene, o-xylene, trimethyl benzene (TMB), cyclohexyl benzene (CHB), naphthalene, and dimethyl naphthalene (DMN) in water + ethanol (50:50) as diluent. Another five cells containing only JP-8 exposed skin were dosed solely with diluent in order to determine the skin retention of jet fuel HC. The absorption parameters of flux, diffusivity and permeability were calculated for the studied HC. The data indicated that there was 2-3-fold and 3-4-fold increase in absorption of nonane, undecane, dodecane and tridecane through 1 and 4-day JP-8 pre-exposed skin, respectively. Similarly, the aromatic HC like ethyl benzene, o-xylene and TMB were absorbed 2-3 times more than controls in 1-day, and greater than 4 times in 4-day JP-8 pre-exposed skin experiments. The absorption of naphthalene and DMN was 1.5 times greater than the controls in both 1 and 4-day pre-exposures. The CHB, naphthalene and DMN had significant persistent skin retention capable of further absorption in 4-day pre-exposures as compared to 1-day exposures. The possible mechanism of an increase in HC absorption in fuel pre-exposed skin may be via lipid extraction from the stratum corneum as indicated by Fourier Transform Infrared (FTIR) spectroscopy. This study suggests that the pre-exposure of skin to jet fuel significantly enhances the subsequent in vitro percutaneous absorption of HC so single dose absorption data for jet fuel HC from naïve skin cannot be used to predict the toxic potential for repeated exposures and that for certain compounds, persistent absorption may occur days after the initial exposure.
INTRODUCTION

Different jet fuels contain different additives based on the performance of the aircraft. The principal difference between fuels is the freezing and flash point specifications. Military aircraft fly under extreme conditions compared with commercial or civilian aircraft, requiring specific additives to the basic hydrocarbon fuel components of Jet A to increase performance (White, 1999). JP-8 jet fuel is the standard military fuel for all types of vehicles, including the U.S. Air Force aircraft inventory. As such, JP-8 presents the most common chemical exposure in the Air Force, particularly for flight and ground crew personnel during preflight operations and for maintenance personnel performing routine tasks. Personal exposure at an Air Force base occurs through occupational exposure for personnel involved with fuel and aircraft handling and/or through incidental exposure, primarily through inhalation of ambient fuel vapors. Because JP-8 is less volatile than its predecessor fuel (JP-4), contact with liquid fuel on skin and clothing may result in prolonged exposure. The slowly evaporating JP-8 fuel tends to linger on exposed personnel during their interaction with previously unexposed colleagues, providing an additional potential route for exposure (Pleil et al., 2000). JP-8 has the potential to induce local and systemic toxicity from cutaneous exposures (McDougal and Rogers 2004).

Various toxic effects have been observed in workers exposed to jet fuel. Neurological effects and irritant dermatitis have been reported in workers exposed to JP-8 jet fuel (Zeiger and Smith, 1998; Smith et al., 1997). Significant effects on the immune, hepatic, neurological, and respiratory systems have been observed in several animal exposure studies (Grant et al., 2000; Harris et al., 2000; Robledo et al., 2000).

Topical application of jet fuels can cause gross morphological changes in porcine skin (Monteiro-Riviere et al., 2001; Rhyne et al., 2002) and the release of tumor necrosis factor-α and interleukin-8 (IL-8) in cultured human epidermal keratinocytes (HEK) and in porcine keratinocytes (Allen et al., 2000, Allen et al., 2001). Different aliphatic and aromatic HC are found to induce IL-8
release from HEK (Chou et al., 2002, Chou et al., 2003). These findings suggest that toxicologically relevant components in jet fuels readily penetrate skin to elicit these dermatological effects. Several dermal absorption studies in rats (McDougal et al., 2000) and porcine skin flaps and skin sections (Riviere et al., 1999; Baynes et al., 2001) have assessed the dermal disposition of several jet fuel components and additives.

Chronic exposure to jet fuel has been shown to cause human liver dysfunction, emotional dysfunction, abnormal electroencephalograms, shortened attention spans, decrease sensorimotor speed and changes in immune functions (Harris et al., 2001). Repeated application of petroleum middle distillates to the skin causes chronic irritation and inflammation (Freeman et al., 1990; Grasso et al., 1988). Fabric soaked with jet fuels for 4 days and evaluated on day 5 produced significant skin damage in pigs (Monteiro-Riviere et al., 2001). The disruption of barrier function of skin, as indicated by an increase in trans-epidermal water loss after exposure to JP-8, might result in increased permeation of its own components and/or other chemicals exposed to skin (Kanikkannan et al., 2001a, Monteiro-Riviere et al., 2001). Pre-exposure of skin to laurocapram, a compound similar to some of the JP-8 performance additives enhanced the penetration of sodium lauryl sulfate (SLS) suggesting that an increase in irritation at the exposed site is possible (Szolar-Platzer et al., 1996). These authors further reported that pre-exposure of skin to SLS alone did not increase the SLS flux value significantly, compared to the laurocapram pre-treated skin.

We have observed the dose related increase in the percutaneous absorption of the jet fuel aromatic HC with hexadecane (a fuel constituent) as a solvent (Muhammad et al., 2004). Repeated daily exposure to jet fuels is the more likely scenario in occupational setting. Polar solvent, in contrast to a non-polar one such as hexadecane, would mimic the post-work environment of occupational worker’s activities including hand washing, showering or exposure to aqueous vehicles. There is no available information on percutaneous absorption of jet fuel components after repeated or pre-exposure to fuel. Understanding this process is necessary to interpret pertinent exposure data and to minimize hazardous health risks in humans. Are skin-retained HC from past fuel exposure susceptible
to mobilization by subsequent aqueous solvent exposure; Do such retained HCs modify subsequent HC absorption? The present study was designed to assess the in vitro percutaneous absorption of fourteen selected jet fuel HC after single and repeated in vivo exposures to JP-8. Specifically, this study was conducted to accomplish the following objectives:

Whether in vivo pre-exposure of skin to jet fuel would affect the subsequent in vitro percutaneous absorption of selected marker components.

Whether single dose application data from naïve (control) skin for jet fuel marker HC can be used to predict the toxic potential for repeated exposures.

**MATERIALS AND METHODS**

**In vivo exposure experiments**

In order to mimic occupational exposure conditions, cotton fabric strips (25 × 8 cm) were used to expose the pigs to JP-8 jet fuel and covered an area of 12× 8 cm. Pigs were sedated with an intramuscular injection of ketamine/xylazine/telazol and hairs were clipped from the back 24 h before dosing. A total of 4 cotton fabric strips were used on each pig. Two of them were dosed with 2 ml of JP-8 in the middle of strip to saturate the marked dosing area in 1-day in vivo exposures, thus the total dose was 20µl/cm². These two dosed strips were redosed with the same amount of JP-8 on the 2nd, 3rd, and 4th day after the first application in the 4 day exposure study. The other two strips were used as controls. All the strips were removed from the body of pig after 24 h and after 4 days of JP-8 application in 1 and 4-day exposures respectively. One dosed and non-dosed area was dermatomed for diffusion skin flow through experiments.

**In vitro flow-through experiments**

The flow-through diffusion cell system, as previously described by Bronaugh and Stewart (1985), was used to perfuse porcine skin. The skin was dermatomed to a thickness of 400-500µm with a Padgett Dermatome (Padgett Instruments, Kansas City, MO). This thickness was employed to
assure that base of hair follicles were not sectioned. The skin was trimmed to provide a dosing surface area of 0.64 cm$^2$ and then placed into a two-compartment Teflon flow-through diffusion cell. For these experiments, 8 aliphatic (nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane) and 6 aromatic (ethyl benzene, o-xylene, trimethyl benzene, cyclohexyl benzene, naphthalene, dimethyl naphthalene) hydrocarbons were used as dosing mixture with water + ethanol (50:50) as the diluent. The dosing mixture was comprised of nonane (288 µg), decane (292 µg), undecane (1236.6 µg), dodecane (1410 µg), tridecane (1320 µg), tetradecane (912 µg), pentadecane (492.8 µg), hexadecane (308µg), naphthalene (510.4 µg), dimethyl naphthalene (526.8 µg), ethyl benzene (346.8µg), o-xylene (348 µg), cyclohexyl benzene (380 µg) and trimethyl benzene (355.6 µg) per diffusion cell in water + ethanol (50:50) as solvent. Aqueous ethanol was selected as a solvent to insure detection of topically applied HCs as well as mimic occupational activities such as washing or showering. A total of 20 µl of this dosing mixture was dosed topically in diffusion flow-through cells. This dosing concentration was selected for better chromatographic detection for most of selected HC in perfusates (Muhammad et al., 2004). The skin flow-through diffusion cells were dosed separately in the following order for both 1 and 4-day exposure experiments: first 5 cells (pre-exposed skin + dosing mixture); next 5 cells (pre-exposed skin + diluent only in order to access the skin retention of jet fuel HC); last 4 cells (control skin "not pre-exposed to JP-8" from the same pig + dosing mixture).

Skin flow-through cells were perfused with Krebs-Ringer bicarbonate buffer with dextrose and bovine serum albumen. The temperature of the perfusate and flow-through cell was maintained at 37°C using a Brinkmann constant temperature circulator (Brinkmann, Westbury, NY). The pH of perfusate was maintained between 7.4 and 7.5. Perfusate flow rate was 4.0 ml/hour. Sampling was accomplished in an open-air system. Perfusate samples were taken at 0, 10, 20, 30, 45, 60, 75, 90, 105, 120, 150, 180, 240, 300 min and saved immediately in a freezer until chromatographic analysis.
Chromatographic analysis

Solid phase microextraction (SPME) fiber with headspace sampling was used to assay the perfusates as described previously (Muhammad et al., 2004). Chromatographic analysis was performed on a GC 5890 (Hewlett Packard series II) equipped with FID detector. GC-FID conditions were as follows: DB-5 capillary column (30m× 0.25mm I.D., 0.25im film thickness; J & W Scientific); injection port temperature, 280ºC; detector temperature, 300ºC; oven temperature, 40-280ºC (held at 40ºC for 3 min, then increased at 8ºC/min up to 160ºC then at 30ºC/min up to the final temperature of 280ºC for 1min); carrier gas (helium) flow rate, 1.24ml/min; carrier + makeup gas (He) flow rate, 22.5ml/min; hydrogen flow rate, 45ml/min; air flow rate, 300-320ml/min. SPME devices and 100-im polydimethylsiloxane (PDMS) fiber assemblies were purchased from Supelco (Bellefonte, PA, USA).

Standard curves

Stock solutions of each of the tested hydrocarbons were prepared individually in acetone. All stock solutions were mixed together and serially diluted in acetone to yield concentrations of 10, 5, 2.5, 1, 0.5, and 0.1ig/ml. Equal volumes of perfusion media were spiked with each of above concentrations to have standard solutions of 1000, 500, 250, 100, 50, and 10ng/ml of media accordingly. 300il from these standard solutions were drawn into GC glass vials. A tiny stir bar was placed into these vials, sealed and exposed to head space (HS) SPME fiber for 25 min at 37ºC along with continuous stirring. The SPME fiber was then injected into the GC and held for 5 min. The peak areas obtained were then plotted against standard concentrations and linear relationships with good correlation coefficients were obtained for all the tested hydrocarbons. The same procedure was adopted for the analysis of perfusate samples.

FTIR experiments with the stratum corneum

Stratum corneum (SC) sheets were prepared from the dissected porcine skin according to the method described by Baynes et al (2000). Briefly, the dissected skin was trimmed to fit between
preheated aluminum blocks and placed in an oven at 60°C for 6-8 minutes. The SC/epidermis was removed using dissection forceps and placed dermis side down into Petri dishes lined with filter paper and containing 0.25% trypsin (Sigma) to dissolve the epidermis. After 24 h in an incubation oven at 35°C, trypsin inhibitor (Sigma) was added to neutralize trypsin and the SC was washed with distilled water. The SC was dried at room temperature for 24 h and then in a desiccator for 48 h. We used dried SC sheets for FTIR analysis based on previous studies of Panchangnula et al (2001); Levang et al (1999); Potts and Francoeur (1992) who used dried SC sheets for evaluation of lipids. Hydrated tissues cannot be evaluated with FTIR since this instrument utilizes transmission beam. If there is water in the tissue, it will absorb all the infrared energy and significantly alter the infrared spectrum. The dried SC discs were scanned by a Fourier Transform Infra Red (FTIR) spectrometer (Spectrum 1000, Perkin Elmer Instruments, CT, USA) and placed in vials. About 2 ml of each of the tested hydrocarbon was added to the SC sample vials (n=4), capped, sealed and allowed to remain undisturbed at room temperature for 24 h. At 24 h, the SC sample was removed and placed under a hood overnight, and then in a desiccator for 48 h to dry them completely. Dried SC were scanned again with FTIR and compared with the respective scans before treatment. The FTIR peak heights and widths were analyzed with Spectrum Software by Perkin Elmer Instruments, CT, USA.

Calculations and Statistics

The perfusate concentrations (ng/ml) were determined for the different dosing protocols. Absorption was defined as the total amount in ng detected in the perfusate for the entire 5 h perfusion period. Absorption parameters like flux, permeability, and diffusivity were calculated as following. Flux (ìg/cm²/h) for individual hydrocarbons were determined at steady state from the slope of cumulative mass per unit area versus time (h) curve. We used all the possible points that were at steady state (linear) in each cumulative absorption curve for determination of slopes. Permeability (cm/h) was determined by dividing the steady state flux (slope of cumulative ìg/cm² versus time curve) by applied surface hydrocarbon concentration. Diffusivity (cm²/h) was obtained by the
following relation: \( D = \frac{L^2}{6 \delta} \), where \( L = 500 \mu m \) and \( \delta \) is the lag time obtained by extrapolating the steady state portion of the curve back to the time or x-axis.

Statistical analysis of steady state flux (\( J_{ss} \)), permeability, diffusivity, and absorption data were conducted using multiple comparison tests and ANOVA with significance level at 0.05. All analysis was carried out using SAS 8.1 for Windows software (SAS Institute, Cary, NC). A least significance difference (LSD) procedure and Student's \( t \) distribution was used for multiple comparisons on all parameters assessed.

RESULTS

Influence of JP8 pre-exposure on the absorption kinetics of aliphatic hydrocarbons

Out of eight topically dosed aliphatic hydrocarbons in flow-through in vitro diffusion cells, only four (nonane, undecane, dodecane and tridecane) were observed in the perfusate samples. Decane and pentadecane were not resolved chromatographically, while tetradecane and hexadecane were not detected in both 1 and 4-day JP-8 pre-exposed skin flow through experiments. The total absorption of nonane (154ng in 1-day and 178ng in 4-day JP-8 pre-exposed skin) was significantly \( (P<0.05) \) greater than that of respective controls (51ng and 80ng) (Tables 1 and 2). The steady state flux and permeability of nonane was significantly greater in both 1 and 4-day pre-exposed skin than control skin. These absorption parameters for dodecane and tridecane were only statistically significant \( (P<0.05) \) in 4-day JP-8 pre-exposure experiments (Tables 1 and 2). The data showed that the total absorption of undecane, dodecane and tridecane was significantly \( (P<0.05) \) greater than their respective controls in 4-day pre-exposed skin unlike the 1-day pre-exposed skin where it was non-significant. The absorption profiles of dodecane shown in figures 1a, b suggests an increased absorption plateau in the 4-day JP-8 pre-exposed skin over the 1-day pre-exposed skin with respect to the control skin. In general, there was a 2-3 fold and 3-4 fold increase in the absorption of nonane, undecane, dodecane and tridecane through 1 and 4-day JP-8 pre-exposed skin respectively, when compared to the control skin (Table 3).
Influence of JP8 pre-exposure on the absorption kinetics of aromatic hydrocarbons

Naphthalene absorption over the entire 5 h perfusion in 1-day JP-8 pre-exposed skin was 17818 ng as compared to 10276 ng in unexposed skin. In 4-day pre-exposed skin, it was 19835 ng with respect to 12030 ng in control skin. This increase was significantly (P<0.05) different in both exposure scenarios (Tables 1 and 2). Similarly, it was evident from these tables that naphthalene flux, permeability and diffusivity were statistically significant from controls in both pre-exposures. The total absorption as well as absorption parameters for dimethyl naphthalene was statistically (P<0.05) different than controls in 4-day pre-exposed skin (Table 2). Both naphthalene and dimethyl naphthalene were approximately 1.5 times more absorbed in JP-8 pre-exposed skin as compared to unexposed skin (Table 3). The benzene containing hydrocarbons such as ethyl benzene, o-xylene, and trimethyl benzene showed a 2 and 4 fold increase in percutaneous absorption through 1 and 4-day JP-8 pre-exposed skin respectively (Table 3). The effect of prolonged JP-8 exposure upon skin was evident by the increased difference from control (naive skin not exposed to JP-8) in absorption profiles of o-xylene in 4-day pre-exposed skin compared to 1-day pre-exposed skin (Figures 2a, b). Tables 1 and 2 summarize the statistically significant (P<0.05) differences in absorption parameters of ethyl benzene, o-xylene, trimethyl benzene, and cyclohexyl benzene in both 1 and 4-day JP-8 pre-exposed skin experiments respectively.

Comparative hydrocarbons absorption kinetics

As seen in Tables 1 and 2, the rank order of marker permeability was naphthalene > o-xylene > ethyl benzene > trimethyl benzene > cyclohexyl benzene > dimethyl naphthalene > nonane > undecane > dodecane > tridecane in both 1 and 4-day JP8 pre-exposed skin. Only cyclohexyl benzene permeated less in 4-day than in 1-day pre-exposed skin. For all components, the rank order of total absorption and steady state flux were similar to that of permeability. There were no statistically significant differences in total absorption and its parameters among aliphatic hydrocarbons in both pre-exposed time points (Tables 1 and 2).
Skin retention of hydrocarbons

The fuel hydrocarbons retained in skin as a result of repeated JP-8 application to pigs were presented in Table 4. Out of all studied hydrocarbons, naphthalene, dimethyl naphthalene, and cyclohexyl benzene were found to have significant ($P<0.05$) skin retention in 4-day JP-8 pre-exposed skin as compared to 1-day pre-exposed skin.

Stratum corneum analysis with FTIR

FTIR analysis of stratum corneum before and after treatment with selected hydrocarbons shed some light on the possible mechanism of increased hydrocarbon absorption in JP-8 pre-exposed skin. Emphasis was placed on the peaks, which appeared at approximately 2850 and 2920 cm\(^{-1}\) and were due to symmetric and asymmetric CH stretching, respectively. The decrease in CH\(_2\) stretching bandwidths, accompanied by a decrease in CH\(_2\) band intensity was observed with most of the aromatic HC (Figure 3) and short chain aliphatic hydrocarbons. The longer chain aliphatic HC like tetradecane and hexadecane showed an increase in CH\(_2\) stretching peak width and peak shift towards higher wave number (Figures 4 & 5). The dimethyl naphthalene skin retention might be due to accumulation of this HC in stratum corneum as indicated in Figure 6 where three peaks (800-650 cm\(^{-1}\)) of dimethyl naphthalene spectrum exactly matched with the SC spectrum after treatment with DMN.

DISCUSSION

Previously, we observed the dose related increase in percutaneous absorption of aromatic hydrocarbons like naphthalene and dimethyl naphthalene suggesting linear first order pharmacokinetics. (Muhammad et al., 2004). This is critical from the risk assessment point of view as greater or prolonged exposure to jet fuels may lead to enhanced absorption of such hydrocarbons resulting in local or systemic toxic effects in exposed individuals. These studies prompted us to consider the effect of chronic/repeated skin exposure to jet fuels on a daily basis. There is limited data in the literature regarding prolonged dermal exposure to jet fuels. Upreti et al (1989) exposed mice to kerosene 15-60 min/d for 7 days by wrapping the hind feet with a muslin cloth wetted with kerosene
and observed systemic consequences of this relatively brief dermal exposure. Ingram et al (1993) applied 3 kerosenes, dermally to mice 3 times/wk for up to 6 wk and found that relative penetration through the skin surface was directly related to the degree of epidermal necrosis observed. There is increasing evidence that severe, long-term fuel-induced dermal irritation, necrosis, and regeneration may be integrally related to possible tumorigenesis (McKee et al., 1994; Walborg et al., 1998). Ritchie et al (2003) suggested that the reduction in the integrity in the dermal barrier by repeated exposure to kerosene-based fuels might increase systemic exposure to other occupational toxicants as well as to toxic components of the fuel itself during subsequent exposures. Therefore, we tested the following hypothesis in this study: "Repeated/pre-exposure of skin to JP8 jet fuel causes disruption in the skin barrier function by extracting/altering the lipids in the stratum corneum layers and thus leads to an increase in the dermal absorption of hydrocarbons on subsequent exposures"

The results obtained in the present study agree with the proposed hypothesis. We observed an increase in the absorption of both aliphatic and aromatic HC upon repeated JP-8 exposures to pig skin. The amount of HC absorption was directly proportional to the length of exposure, as we observed 23 fold increase in absorption after 1-day and 34 fold increase after 4-day JP-8 pre-exposed skin with aliphatic HC. Similarly aromatic HC like ethyl benzene, o-xylene and trimethyl benzene absorbed 2 and 4 times more after 1 and 4-day pre-exposures respectively (Table 3). These observations are in accordance with Kanikkannen et al (2001a, 2001b) who stated that disruption of barrier functions of skin after exposure to JP-8 was hypothesized to increase permeation of its own components, or other chemicals exposed to skin. An alternate explanation for this increase in HC absorption may be that after the previous in vivo exposures, the skin contains levels of aliphatic HC that increase their overall flux/absorption on subsequent in vitro dermal exposure. Since aliphatic HC are highly lipophilic (log Ko/w>5), they may persist in skin for prolonged periods and can be partitioned out later on with polar solvents. This explanation is not appropriate for aromatic HC as the amount "pushed out" of JP-8 pre-exposed skin with diluent (Table 4) is negligible as compared to what is absorbed (Tables 1,2) with dosing mixtures. This suggests a JP-8 alteration of the membrane.
In these studies, polar solvent (ethanol/water) was used to mimic the post-work settings of occupational workers where they come in contact with water at the end of working day as well as provide an experimental scenario where HC absorption is facilitated. The focus of the study was to determine if HC retained in skin from pre-existing fuel exposure modulated subsequent HC exposure. As expected we observed many fold increase in absorption of hydrocarbons through unexposed (control) skin with polar solvent in the present study as compared to our previous study with hexadecane (a fuel constituent) as solvent (Muhammad et al., 2004) with similar topical in vitro doses. One could postulate that water may enhance the absorption of JP-8 constituents retained in the skin of exposed workers.

Out of all the studied HC, aromatics absorbed more efficiently than aliphatic HC (Tables 1,2 and Figures 1,2), a finding consistent with our previous studies (Riviere et al., 1999, Baynes et al., 2001, Muhammad et al., 2004) and with those of McDougal et al (2000). Steady state flux, permeability and diffusivity were statistically different for most studied HC in both 1 and 4-day JP-8 pre-exposures as compared to controls (Tables 1 and 2), suggesting that JP-8 pre-exposure produced changes in the barrier functions of skin thus altering these absorption parameters. This is evident in Table 1 that the in vitro percutaneous absorption of aromatic HC is significantly \( P<0.05 \) different from the control even after single (1-day) in vivo exposure. This finding suggests that dermal absorption studies through naïve skin may not be used to predict percutaneous absorption through fuel pre-exposed skin, which is more likely occupational setting.

In order to gain insight as to the mechanism for the increase in HC absorption through JP-8 pre-exposed skin, we conducted FTIR studies. FTIR spectroscopy provides information on the vibrational modes of stratum corneum lipid layer components and probes the structure on a molecular level (Casel and Mantsch, 1984). Of particular interest in lipid studies are the IR absorbances near 2850 and 2920 cm\(^{-1}\) due to symmetric and asymmetric methylene group (H-C-H) stretching, respectively. Solvent extraction of the SC lipids results in reduction of the methylene group stretching absorbances. Biophysical evidence suggests that stratum corneum lipid domains are the primary
barrier to both water loss and the penetration of compounds into the skin (Van Duzee, 1971). The decrease in CH₂ stretching bandwidths accompanied by a decrease in CH₂ band intensity, suggests an overall extraction of SC lipids (Levang et al., 1999). Similarly, we observed an overall SC lipid extraction with most of aromatic (Figure 3) and short chain aliphatic HC in this study. These observations are in accordance to Monteiro-Riviere et al (2004) who found SC delipidization on in vivo exposures of pigs to jet fuels. These authors used transmission electron microscope (TEM) to study the lipid bilayers of SC after ruthenium tetraoxide staining.

We could not detect long chain aliphatic HC in our perfusate samples. One potential explanation is that these highly hydrophobic HC (log Ko/w > 5) could not partition out of SC lipids to be absorbed through the skin (Riviere et al., 1999, Baynes et al., 2001, Muhammad et al., 2004). We used FTIR as a biophysical tool to address this issue. Figures 4 and 5 present the FTIR spectrum of SC before and after treatment with tetradecane and hexadecane respectively, along with the spectrum of its liquid HC alone as a reference. It was evident that peak widths and peak heights from tetradecane and hexadecane treated SC were higher than that of control SC. A slight peak shift towards greater wave number was also observed in the treated SC. A higher wave number shift in FTIR is an indication of increase in gauche conformers (Potts and Francoeur, 1993). The broadening of peaks at 2850 and 2920 cm⁻¹ is an indication of increased translational movement or mobility of lipid acyl chains (Naik and Guy, 1997). On comparison with the reference HC spectrums; it was revealed that these peak shifts might be due to binding of these long chains HC with the SC lipids. These observations would also support the hypothesis that these long chain aliphatic HC may contribute to jet fuel induced skin irritation.

Another important finding of this study was the formation of dermal depot of certain HC (naphthalene, dimethyl naphthalene and cyclohexyl benzene) as a result of repeated skin exposures to JP-8 (Table 4). The detection of these HC into the perfusates from the JP-8 pre-exposed skin by topically dosing diluent only suggested that such HC might have potential for absorption days post exposure. Naphthalene, being relatively hydrophilic as compared to rest of HC, can partition out of
SC into the viable epidermis/dermis (Baynes et al., 2000). This might be assumed that naphthalene could bind/deposit in the hydrophilic environment of viable epidermis/dermis, thus producing persistent residues. We did not know why cyclohexyl benzene retained in JP-8 pre-exposed skin. Structurally, cyclohexyl benzene is composed of two benzene rings arranged in a perpendicular plane/axes to each other. This structural confirmation might suggest poor absorption through but retention in the skin. FTIR analysis for dimethyl naphthalene revealed that this HC could bind/deposit in SC as was shown in Figure 6. This was evident that three peaks (900-650 cm\(^{-1}\)) of reference dimethyl naphthalene exactly overlapped with the peaks in SC treated with this HC.

In conclusion, our studies indicated a 2-4 fold increase in absorption with most of the aromatic and short chain aliphatic HC through JP-8 pre-exposed porcine skin. Over all lipid extraction from the SC by these HC may be the primary mechanism for this increased absorption as investigated with FTIR. Amount of in vitro percutaneous absorption was proportional to the length of in vivo pre-exposures. Long chain HC like tetradecane, pentadecane and hexadecane may bind in SC as observed with FTIR studies, which may further modulate absorption. Naphthalene, dimethyl naphthalene and cyclohexyl benzene can form depots/residues in JP-8 exposed skin, capable of further absorption days post exposure. Polar solvents can enhance the percutaneous absorption of fuel HC that may be a concern in post-work risk associated with jet fuels. Our results indicate that even single in vivo exposure of skin to JP-8 significantly affect the subsequent in vitro percutaneous absorption of marker HC as compared to absorption through the un-exposed (naïve) skin. Therefore, single dose absorption data from naïve skin for jet fuel marker components may not be optimal to predict the toxic potential for repeated exposures. There is a need to consider the repeated fuel exposure factor in risk assessment studies in occupational settings.

**ACKNOWLEDGEMENT**

This work was supported by US Air Force Office of Scientific Research, Grant F49620-01-1-0080.
REFERENCES


Table 5.1. Mean ± SEM Steady state flux, permeability, diffusivity and absorption of jet fuel hydrocarbons in 1-day JP-8 pre-exposed porcine skin

<table>
<thead>
<tr>
<th>Hydrocarbon</th>
<th>SS Flux (µg/hr/cm²)</th>
<th>Permeability (cm/hr*1,000)</th>
<th>Diffusivity (cm²/hr*1000,000)</th>
<th>Absorption (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aromatic HC</strong></td>
<td></td>
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<tr>
<td>Naphthalene control</td>
<td>4.19 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.16 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>378 ± 22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10276 ± 1023&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Naphthalene exposed</td>
<td>6.60 ± 0.79&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.26 ± 0.03&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>628 ± 73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17818 ± 2255&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMN control</td>
<td>0.62 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.02 ± 0.004&lt;sup&gt;a&lt;/sup&gt;</td>
<td>178 ± 10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1043 ± 202&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMN exposed</td>
<td>0.85 ± 0.21&lt;sup&gt;cD&lt;/sup&gt;</td>
<td>0.03 ± 0.008&lt;sup&gt;d&lt;/sup&gt;</td>
<td>163 ± 16&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1406 ± 447&lt;sup&gt;cD&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethyl Benzene control</td>
<td>1.04 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.06 ± 0.009&lt;sup&gt;b&lt;/sup&gt;</td>
<td>715 ± 75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1586 ± 243&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Ethyl Benzene exposed</td>
<td>3.32 ± 0.52&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.19 ± 0.03&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>1065 ± 97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3137 ± 312&lt;sup&gt;AB&lt;/sup&gt;</td>
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<tr>
<td>TMB control</td>
<td>1.01 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.056 ± 0.008&lt;sup&gt;b&lt;/sup&gt;</td>
<td>391 ± 22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2177 ± 277&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TMB exposed</td>
<td>1.77 ± 0.21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.10 ± 0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>623 ± 74&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4192 ± 497&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHB control</td>
<td>0.35 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.018 ± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
<td>164 ± 9.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>548 ± 103&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHB exposed</td>
<td>0.86 ± 0.11&lt;sup&gt;cD&lt;/sup&gt;</td>
<td>0.045 ± 0.006&lt;sup&gt;d&lt;/sup&gt;</td>
<td>205 ± 16&lt;sup&gt;cD&lt;/sup&gt;</td>
<td>1651 ± 298&lt;sup&gt;cD&lt;/sup&gt;</td>
</tr>
<tr>
<td>O-xylene control</td>
<td>1.47 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.085 ± 0.011&lt;sup&gt;b&lt;/sup&gt;</td>
<td>710 ± 105&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2541 ± 335&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>O-xylene exposed</td>
<td>3.80 ± 0.61&lt;sup&gt;cB&lt;/sup&gt;</td>
<td>0.218 ± 0.04&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>1013 ± 23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4765 ± 490&lt;sup&gt;aB&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Aliphatic HC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonane control</td>
<td>0.03 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.002 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>238 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51 ± 19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nonane exposed</td>
<td>0.08 ± 0.01&lt;sup&gt;cD&lt;/sup&gt;</td>
<td>0.005 ± 0.001&lt;sup&gt;cD&lt;/sup&gt;</td>
<td>342 ± 75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>154 ± 11&lt;sup&gt;cD&lt;/sup&gt;</td>
</tr>
<tr>
<td>Undecane control</td>
<td>0.07 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.001 ± 0.000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>332 ± 101&lt;sup&gt;a&lt;/sup&gt;</td>
<td>151 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Undecane exposed</td>
<td>0.16 ± 0.05&lt;sup&gt;cD&lt;/sup&gt;</td>
<td>0.003 ± 0.000&lt;sup&gt;cD&lt;/sup&gt;</td>
<td>234 ± 14&lt;sup&gt;cD&lt;/sup&gt;</td>
<td>339 ± 98&lt;sup&gt;cD&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dodecane control</td>
<td>0.04 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0005 ± 0.000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>344 ± 76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81 ± 18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dodecane exposed</td>
<td>0.06 ± 0.02&lt;sup&gt;cD&lt;/sup&gt;</td>
<td>0.0009 ± 0.000&lt;sup&gt;cD&lt;/sup&gt;</td>
<td>356 ± 107&lt;sup&gt;a&lt;/sup&gt;</td>
<td>134 ± 37&lt;sup&gt;cD&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tridecane control</td>
<td>0.02 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0003 ± 0.000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>904 ± 212&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46 ± 16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tridecane exposed</td>
<td>0.01 ± 0.00&lt;sup&gt;cD&lt;/sup&gt;</td>
<td>0.0002 ± 0.000&lt;sup&gt;cD&lt;/sup&gt;</td>
<td>343 ± 37&lt;sup&gt;bC&lt;/sup&gt;</td>
<td>34 ± 10&lt;sup&gt;cD&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Lower case superscripts indicate significant ($P$<0.05) differences between treatments (control vs. pre-exposed) within each parameter.
Upper case superscripts indicate significant ($P$<0.05) differences among various hydrocarbons.
Table 5.2. Mean ± SEM Steady state flux, permeability, diffusivity and absorption of jet fuel hydrocarbons in 4 day JP-8 pre-exposed porcine skin

<table>
<thead>
<tr>
<th>Hydrocarbon</th>
<th>SS Flux (µg/hr/cm²)</th>
<th>Permeability (cm/hr*1,000)</th>
<th>Diffusivity (cm²/hr*1000,000)</th>
<th>Absorption (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aromatic HC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naphthalene</td>
<td>4.81 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.19 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>435 ± 17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12030 ± 989&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>7.57 ± 0.47&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.29 ± 0.02&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>514 ± 28&lt;sup&gt;ABC&lt;/sup&gt;</td>
<td>19835 ± 1453&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMN control</td>
<td>0.67 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.03 ± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
<td>185 ± 13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1179 ± 155&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMN exposed</td>
<td>0.99 ± 0.09&lt;sup&gt;DE&lt;/sup&gt;</td>
<td>0.04 ± 0.004&lt;sup&gt;E&lt;/sup&gt;</td>
<td>227 ± 11&lt;sup&gt;aD&lt;/sup&gt;</td>
<td>2056 ± 221&lt;sup&gt;DE&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethyl Benzene</td>
<td>0.61 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.035 ± 0.009&lt;sup&gt;b&lt;/sup&gt;</td>
<td>870 ± 61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>637 ± 136&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethyl Benzene</td>
<td>2.04 ± 0.17&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>0.12 ± 0.009&lt;sup&gt;aC&lt;/sup&gt;</td>
<td>834 ± 54&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>2594 ± 250&lt;sup&gt;BC&lt;/sup&gt;</td>
</tr>
<tr>
<td>TMB control</td>
<td>0.49 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.028 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>456 ± 40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>837 ± 90&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TMB exposed</td>
<td>1.52 ± 0.10&lt;sup&gt;CD&lt;/sup&gt;</td>
<td>0.09 ± 0.005&lt;sup&gt;aD&lt;/sup&gt;</td>
<td>670 ± 124&lt;sup&gt;aAB&lt;/sup&gt;</td>
<td>3821 ± 267&lt;sup&gt;ABC&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHB control</td>
<td>0.29 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.016 ± 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>165 ± 4.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>470 ± 42&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHB exposed</td>
<td>0.49 ± 0.07&lt;sup&gt;EF&lt;/sup&gt;</td>
<td>0.025 ± 0.004&lt;sup&gt;E&lt;/sup&gt;</td>
<td>181 ± 4.6&lt;sup&gt;aD&lt;/sup&gt;</td>
<td>852 ± 136&lt;sup&gt;EF&lt;/sup&gt;</td>
</tr>
<tr>
<td>O-xylene control</td>
<td>1.02 ± 0.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.059 ± 0.016&lt;sup&gt;b&lt;/sup&gt;</td>
<td>816 ± 87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1156 ± 263&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>O-xylene exposed</td>
<td>3.13 ± 0.15&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.180 ± 0.009&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>806 ± 86&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>4865 ± 340&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Aliphatic HC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonane control</td>
<td>0.03 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.002 ± 0.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>409 ± 110&lt;sup&gt;a&lt;/sup&gt;</td>
<td>80 ± 15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nonane exposed</td>
<td>0.07 ± 0.01&lt;sup&gt;EF&lt;/sup&gt;</td>
<td>0.005 ± 0.000&lt;sup&gt;F&lt;/sup&gt;</td>
<td>692 ± 171&lt;sup&gt;aAB&lt;/sup&gt;</td>
<td>178 ± 24&lt;sup&gt;F&lt;/sup&gt;</td>
</tr>
<tr>
<td>Undecane control</td>
<td>0.06 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.001 ± 0.000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>308 ± 52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>135 ± 22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Undecane exposed</td>
<td>0.10 ± 0.02&lt;sup&gt;EF&lt;/sup&gt;</td>
<td>0.002 ± 0.000&lt;sup&gt;F&lt;/sup&gt;</td>
<td>295 ± 78&lt;sup&gt;aCD&lt;/sup&gt;</td>
<td>263 ± 18&lt;sup&gt;F&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dodecane control</td>
<td>0.02 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0002 ± 0.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>245 ± 60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dodecane exposed</td>
<td>0.05 ± 0.01&lt;sup&gt;EF&lt;/sup&gt;</td>
<td>0.0008 ± 0.000&lt;sup&gt;F&lt;/sup&gt;</td>
<td>255 ± 32&lt;sup&gt;aD&lt;/sup&gt;</td>
<td>112 ± 19&lt;sup&gt;F&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tridecane control</td>
<td>0.00 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0001 ± 0.000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>245 ± 28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.4 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tridecane exposed</td>
<td>0.02 ± 0.01&lt;sup&gt;EF&lt;/sup&gt;</td>
<td>0.0003 ± 0.000&lt;sup&gt;F&lt;/sup&gt;</td>
<td>222 ± 18&lt;sup&gt;aD&lt;/sup&gt;</td>
<td>37 ± 9.3&lt;sup&gt;F&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Note:**
- Lower case superscripts indicate significant (P<0.05) differences between treatments (control vs. pre-exposed) within each parameter.
- Upper case superscripts indicate significant (P<0.05) differences among various hydrocarbons.
Table 5.3. Comparative absorption ratios (mean exposed/mean control values) of different hydrocarbons in 1 and 4 day JP-8 pre-exposed porcine skin

<table>
<thead>
<tr>
<th>Hydrocarbons</th>
<th>Absorption ratio 1-day pre-exposure</th>
<th>Absorption ratio 4-day pre-exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>1.73</td>
<td>1.65</td>
</tr>
<tr>
<td>Dimethyl naphthalene</td>
<td>1.35</td>
<td>1.74</td>
</tr>
<tr>
<td>Ethyl Benzene</td>
<td>1.98</td>
<td>4.07</td>
</tr>
<tr>
<td>Trimethyl Benzene</td>
<td>1.93</td>
<td>4.56</td>
</tr>
<tr>
<td>Cyclohexyl Benzene</td>
<td>3.02</td>
<td>1.81</td>
</tr>
<tr>
<td>O-xylene</td>
<td>1.88</td>
<td>4.21</td>
</tr>
<tr>
<td>Nonane</td>
<td>3.03</td>
<td>2.23</td>
</tr>
<tr>
<td>Undecane</td>
<td>2.25</td>
<td>1.95</td>
</tr>
<tr>
<td>Dodecane</td>
<td>1.65</td>
<td>3.50</td>
</tr>
<tr>
<td>Tridecane</td>
<td>0.74</td>
<td>4.90</td>
</tr>
</tbody>
</table>
Table 5.4. Mean ± SEM Comparative skin retention of different hydrocarbons in 1 and 4 day JP-8 pre-exposed porcine skin

<table>
<thead>
<tr>
<th>Hydrocarbons</th>
<th>HC retained (ng) 1-day pre-exposure</th>
<th>HC retained (ng) 4-day pre-exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>50.73 ± 25\textsuperscript{bB}</td>
<td>416.99 ± 106\textsuperscript{abA}</td>
</tr>
<tr>
<td>Dimethyl naphthalene</td>
<td>0 ± 0\textsuperscript{B}</td>
<td>243.91 ± 41\textsuperscript{bA}</td>
</tr>
<tr>
<td>Ethyl Benzene</td>
<td>181.47 ± 92\textsuperscript{abA}</td>
<td>284.89 ± 42\textsuperscript{aA}</td>
</tr>
<tr>
<td>Trimethyl Benzene</td>
<td>347.10 ± 106\textsuperscript{aA}</td>
<td>470.10 ± 112\textsuperscript{aA}</td>
</tr>
<tr>
<td>Cyclohexyl Benzene</td>
<td>10.24 ± 2\textsuperscript{bB}</td>
<td>36.15 ± 7\textsuperscript{cA}</td>
</tr>
<tr>
<td>O-xylene</td>
<td>274.07 ± 97\textsuperscript{aA}</td>
<td>314.89 ± 57\textsuperscript{abA}</td>
</tr>
<tr>
<td>Nonane</td>
<td>11.12 ± 4\textsuperscript{bA}</td>
<td>31.23 ± 8\textsuperscript{bA}</td>
</tr>
<tr>
<td>Undecane</td>
<td>46.16 ± 15\textsuperscript{bA}</td>
<td>26.28 ± 7\textsuperscript{cA}</td>
</tr>
<tr>
<td>Dodecane</td>
<td>24.84 ± 6\textsuperscript{aA}</td>
<td>14.45 ± 3\textsuperscript{cA}</td>
</tr>
<tr>
<td>Tridecane</td>
<td>14.57 ± 1\textsuperscript{aA}</td>
<td>18.71 ± 5\textsuperscript{cA}</td>
</tr>
</tbody>
</table>

Lower case superscripts indicate significant ($P<0.05$) differences among various hydrocarbons within each 1 and 4-day pre-exposure.  
Upper case superscripts indicate significant ($P<0.05$) differences between similar hydrocarbons in 1 and 4-day pre-exposure.
Figure 5.1. Mean perfusate concentrations (ng/ml) of dodecane in 1-day (1a) and 4-day (1b) JP-8 pre-exposed porcine skin flow through experiments. - - Dosing mixture + JP-8 pre-exposed skin, - - Dosing mixture + control skin, - - Diluent + JP-8 pre-exposed skin.
Figure 5.2. Mean perfusate concentrations (ng/ml) of o-xylene in 1-day (2a) and 4-day (2b) JP-8 pre-exposed porcine skin flow through experiments. - - Dosing mixture + JP-8 pre-exposed skin, - - Dosing mixture + control skin, -Ä- Diluent + JP-8 pre-exposed skin.
Figure 5.3. FTIR spectrum of porcine stratum corneum before and after treatment with ethyl benzene.
Figure 5.4. FTIR spectrum of porcine stratum corneum before and after treatment with tetradecane along with the spectrum of liquid tetradecane as a reference.
Figure 5.5. FTIR spectrum of porcine stratum corneum before and after treatment with hexadecane along with the spectrum of liquid hexadecane as a reference.
Figure 5.6. FTIR spectrum of porcine stratum corneum before and after treatment with dimethyl naphthalene along with the spectrum of liquid dimethyl naphthalene as a reference.
6. Comparative In Vivo Toxicity of Topical JP-8 Jet Fuel and Its Individual Hydrocarbon Components: Identification of Tridecane and Tetradecane as Key Constituents Responsible for Dermal Irritation

F. Muhammad, N.A. Monteiro-Riviere, and J.E. Riviere

Submitted for publication to Toxicologic Pathology.
Abstract

Despite widespread exposure to military jet fuels, there remains a knowledge gap concerning the actual toxic entities responsible for irritation observed after topical fuel exposure. The present studies with individual hydrocarbon (HC) constituents of the JP-8 jet fuel shed light on this issue. In order to mimic occupational scenarios, JP-8, 8 aliphatic HC (nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane) and 6 aromatic HC (ethyl benzene, o-xylene, trimethyl benzene, cyclohexyl benzene, naphthalene, dimethyl naphthalene) soaked cotton fabrics were topically exposed to pigs for 1-day and with repeated daily exposures for 4-days. Erythema, epidermal thickness and epidermal cell layers were quantitated. No erythema was noted in 1-day in vivo HC exposures but significant erythema was observed in 4-day tridecane, tetradecane, pentadecane and JP-8 exposed sites. The aromatic HC did not produce any macroscopic lesions in 1 or 4-days of in vivo exposures. Morphological observations revealed slight intercellular and intracellular epidermal edema in 4-day exposures with the aliphatic HC. Epidermal thickness and number of cell layers significantly increased ($P < 0.05$) in tridecane, tetradecane, pentadecane and JP-8 treated sites. No significant differences were observed in the aromatic HC exposed sites. Subcorneal microabscesses containing inflammatory cells were observed with most of the long-chain aliphatic HC and JP-8 in 4-day exposures. Ultrastructural studies depicted that jet fuel HC induced cleft formation within intercellular lipid lamellar bilayers of the stratum corneum. The degree of damage to the skin was proportional to the length of in vivo HC exposures. These data coupled with absorption and toxicity studies of jet fuel HC revealed that specific HCs (tridecane and tetradecane) might be the key constituents responsible for jet fuel induced skin irritation.
Introduction

Aliphatic and aromatic hydrocarbon (HC) mixtures are the primary constituents of JP-8 jet fuel. JP-8 is the major fuel used by U.S. and NATO armed forces, and is also a multipurpose fuel used in ground vehicles, generators, heaters and stoves (Makris, 1994). Although the component mixture can vary from batch to batch, the aliphatic HC tend to dominate the aromatic HC in each fuel batch. On the average, JP-8 jet fuel is composed of 33-61% n-alkanes and isoalkanes, 10-45% naphtenes, 12-22% aromatics, and 0.5-5% olefins (Vere, 2003). Occupational exposures to jet fuel can occur through fuel transport, aircraft fueling and defueling, cold aircraft engine starts, aircraft maintenance, maintenance of equipment and machinery, cleaning or degreasing with fuel, and use of tent heaters (Centers for Disease Control, 1999; Subcommittee on Jet-Propulsion 8 fuel of Committee on Toxicology, 2003).

Skin can be an important route of exposure because of the potential for liquid and aerosol contact with fuel (McDougal and Rogers, 2004). The jet engine maintenance personnel wear fuel permeable cotton coveralls to reduce the possibility of explosion due to the generation of static electricity associated with more protective clothing. Daily exposure to fuels result in saturation of the cotton cloth, resulting in an occluded environment for repeated, long-term exposure to the skin during the typical 8 h workday (Allen et al., 2001). There are several reports that jet fuel can cause local and systemic toxic effects. Significant effects on the immune, hepatic, neurological, and respiratory systems have been observed in several animal exposure studies (Grant et al., 2000; Harris et al., 2000; Robledo et al. 2000). Neurological effects and irritant dermatitis have been reported in workers exposed to JP-8 jet fuel (Smith et al., 1997; Zeiger and Smith 1998).

The different HC of jet fuel have shown the potential for percutaneous absorption or skin retention indicating a potential source for systemic or local toxicity (Baynes et al., 2001; McDougal et al., 2000; Riviere et al., 1999). Previously, we have studied the dose-related HC absorption through skin (Muhammad et al., 2004a) suggesting that prolonged skin exposure to jet fuel may result in enhanced toxic effects. Monterio-Riviere et al (2001a) investigated the cutaneous
toxicity of three jet fuels and concluded that the high-dose fabric-soaked repeated exposure to Jet A, JP-8 and JP-8 + 100 fuels caused the greatest increase in cutaneous erythema, edema, epidermal thickness, cell layers and rete peg depth compared with high-dose non-occluded or low-dose exposure under occluded (HillTop® chambers) and non-occluded conditions. The ultrastructural analysis of skin exposed to three jet fuels revealed low level inflammation accompanied by the formation of lipid droplets in various skin layers, mitochondrial and nucleolar changes, cleft formation in the intercellular lipid lamellar bilayers, as well as disorganization at the stratum granulosum-stratum corneum interface. These changes suggest that the primary effect of jet fuel exposure is damage to the stratum corneum barrier (Monteiro-Riviere et al., 2004). Since all fuels demonstrated a similar toxicologic profile, hydrocarbon constituents and not additives are the primary toxic entities. Studies in our laboratory have indicated that individual and specific aliphatic HC of jet fuel are toxic to human epidermal keratinocytes (HEK) and are capable of inducing release of proinflammatory cytokines such as IL-8 (Chou et al., 2002). We have shown that IL-8 concentration increased significantly by 3 to 10-fold, with the highest increase associated with exposure to hydrocarbons in the C9-C13 chain length. In addition, the cytotoxicity of jet fuel aromatic HC depicted a dose related response in IL-8 release at 24 h from HEK (Chou et al., 2003).

Recently, we have studied the percutaneous absorption of different jet fuel HC through the skin previously exposed to JP-8 jet fuel (Muhammad et al., 2004b). We observed 2 to 4-fold increase in absorption of short chain aliphatic (nonane, undecane, dodecane, tridecane) and aromatic (ethyl benzene, o-xylene, trimethyl benzene, cyclohexyl benzene, naphthalene, dimethyl naphthalene) HC through 1 and 4-day of JP-8 pre-exposed skin. Jet fuels and individual HC may cause lipid extraction from the stratum corneum, as studied with Fourier Transform Infra Red (FTIR) spectrometer (Muhammad et al., 2004b) and transmission electron microscopy (TEM) (Monteiro-Riviere et al., 2004), which could provide a mechanism to explain the increase in HC absorption.

Since our cell culture studies suggested that the aliphatic HC in the range of C9-C13 are more cytotoxic, we hypothesized that specific HC may be responsible for the skin induced irritation from
JP-8 exposure, and secondly that differences observed between jet fuels in vivo are due to additive modulation of cytotoxic hydrocarbon disposition. There are no published reports on the in vivo irritation with individual or specific HC of jet fuels. Therefore, the objective of this study was to access the irritation caused by the individual HC (nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, ethyl benzene, o-xylene, trimethyl benzene, cyclohexyl benzene, naphthalene, and dimethyl naphthalene) at 1 and 4-days of in vivo exposures and to compare these HC toxic effects to that caused by JP-8 mixture.

Materials and Methods

Test compounds

Eight aliphatic HC (n-nonane (C9), n-decane (C10), n-undecane (C11), n-dodecane (C12), n-tridecane (C13), n-tetradecane (C14), n-pentadecane (C15), and n-hexadecane (C16)) all with greater than 98% purity, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Six aromatic HC (ethyl benzene, o-xylene, 1,2,4-trimethyl benzene, cyclohexyl benzene, 1,2-dimethyl naphthalene all with greater than 98% purity, were purchased from Sigma Chemical Co. (St. Louis, MO, USA) except naphthalene, which was purchased from Supelco Inc. (Park Bellefonte, PA, USA). A 1.1% solution of naphthalene was prepared in ethanol. Major T. Miller from Wright Patterson Air Force Base supplied JP-8 jet fuel.

In vivo experimental procedures

These experiments were conducted to mimic complete fuel HC saturation of cotton clothing in workers and to assess their effects after 1 day and with multiple exposures for 4 days with evaluation on day 5. Pigs (n=4 for each of 1 and 4-day in vivo exposures) were sedated with an intramuscular injection of ketamine/xylazine/telazol and hairs were clipped from the back carefully 24 h prior to topical application of the HC. Templates were created from Stomahesive® (Convatec-Squibb, Princeton, NJ) within a dosing area of 1 inch² and a 0.75 cm border for attachment to the skin with Medipore™ tape. The 100% cotton fabric was placed directly inside the template and dosed with 300µl, the volume required to achieve full saturation of the JP-8 and appropriate aliphatic HC
(nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane) and aromatic 
HC (ethyl benzene, o-xylene, trimethyl benzene, cyclohexyl benzene, naphthalene, and dimethyl 
naphthalene) on the cotton fabric. Non-treated fabric served as the controls while fabric soaked with 
ethanol served as the sham control. At 24 h and 96 h post dose, the templates were removed from the 
skin and scored for erythema (Table 1, foot note). After scoring, two 6-mm skin biopsies were taken 
from each site and processed as follows: one was fixed in 10% neutral buffered formalin (NBF) for 
light microscopy (LM) and the other biopsy was embedded in OCT (Sakura Finetek, Torrance CA) 
and frozen in isopentane cooled by liquid nitrogen for stratum corneum lipid staining. After the 
biopsy sampling, all pigs were euthanized with 5ml of Beuthanasia®-D Special solution (Schering-
Plough Animal Health Corp., Kenilworth, NJ). All animal procedures were approved by the NCSU 
IACUC.

Microscopy techniques

For light microscopy (LM), all skin biopsies were fixed in 10% NBF, processed through 
graded ethanols and embedded in paraffin blocks. Thin sections (6µm thickness) were mounted on 
slides and stained with hematoxylin and eosin (H&E). Epidermal thickness excluding the stratum 
corneum was quantitated with an eyepiece reticle on the H&E stained sections. Morphometric 
quantification was conducted using an Olympus PM-10ADS automatic photomicroscope (Olympus 
Optical Co., Tokyo, Japan) with a 40x objective and calibrated ocular micrometer. The epidermal 
thickness and the number of nucleated epidermal cell layers (viable epidermis) were counted at three 
randomly selected locations per slide and averaged.

Transmission electron microscopy (TEM) was used to examine the stratum corneum lipids. 
For staining of stratum corneum lipids, frozen biopsies were sectioned (20 µm) on a cryostat, 
mounted on positively charged slides, air dried, and fixed in Trump's fixative (4% formaldehyde, 1% 
glutaraldehyde in phosphate buffer). The sections were postfixed in 0.25% phosphate-buffered 
ruthenium tetroxide (Polysciences, Inc.) for 45 min at 4 °C to preserve and stain the intercellular 
lipids of stratum corneum. Sections were dehydrated through graded ethanols, cleared in acetone,
infiltrated and embedded in Spurr's resin. Thin sections (800-1000 Å) were mounted on copper grids and examined on a Philips EM208S TEM operating at an accelerating voltage of 80 kV.

**Statistical analysis**

For statistical analysis, the means of erythema, epidermal thickness, and number of epidermal cell layers were calculated and the significant differences ($P < 0.05$) between post-treatment times, 1-day and 4-days of exposure to HC treated sites were determined using the least significant difference in the ANOVA procedure of SAS (version 8.1 for windows; SAS Institute, Cary, NC). Multiple comparisons among different treatments were conducted within each exposure length using the LSD at the $P < 0.05$ level of significance.

**Results**

Macroscopic observations (mean erythema scores) for all the tested HC and JP-8 are summarized in Table 1. After 1-day in vivo exposure, no significant differences in erythema was observed in the HC treatments except for tridecane, tetradecane and pentadecane compared to the other aliphatic HC, aromatic HC, ethanol, JP-8 treated and controls. These three specific HC exhibited moderate erythema. After 4-days of in vivo exposure, significant macroscopic differences in erythema were noted (Table 1, Figure 1). Tridecane, tetradecane and pentadecane erythema scores were similar to JP-8 after 4-days of exposures and significantly different from that of controls, ethanol, aromatic and other aliphatic HC like decane, undecane, dodecane and hexadecane. There were no significant differences among the tested aromatic HC as compared to the ethanol or control (Table 1).

Table 2 represents the epidermal thickness of various studied HC after 1-day and 4-days of in vivo exposures. The mean epidermal thickness was greater in tridecane, tetradecane, pentadecane and JP-8 treated sites in both 1 and 4-day in vivo exposures as compared to the control. There were no significant differences in epidermal thickness for short chain aliphatic HC (nonane, decane) and aromatic HC as compared to the controls. The mean number of epidermal cell layers showed an increase with longer carbon chain lengths of aliphatic HC (Table 3). The HC with a chain length of
C13-C15 (tridecane, tetradecane, pentadecane) showed a significant increase in the number of epidermal cellular layers similar to JP-8 after 4-days of in vivo exposures compared to the controls. The mean number of cell layers in the aromatic HC was similar to controls (Table 3). A comparison of the present in vivo findings with previous in vitro absorption, cytotoxicity and IL-8 release data from our laboratory for the aliphatic HC (C11-C16) was presented in the Table 4. This comparison revealed that the HC with the carbon chain length of C13 (tridecane) and C14 (tetradecane) were more toxic.

Microscopically, different HC exhibited different inflammatory responses in porcine skin. The maximum inflammation was observed with tridecane and tetradecane after 1-day of in vivo exposures (Figure, 2c and d). Intercellular and intracellular epidermal edema and focal areas of dermal inflammatory cells were present. Tridecane produced subcorneal microabscesses containing a mixed population of neutrophils and lymphocytes (Fig. 3a) in all of the 1-day exposures. Tetradecane and pentadecane contained subcorneal microabscesses in 3/4 pigs in the 1-day exposure probably due to uneven contact of the saturated fabric. JP-8 did not produce subcorneal microabscesses after 1-day. Evaluation of all of the aromatic HC were similar to controls except for slight intraepidermal edema. The most significant morphological changes occurred after 4-days. There was an increase in intracellular epidermal edema, intercellular epidermal edema, dermal papillary edema, extensive epidermal rete pegs, and dermal inflammation at the 4-day exposures (Figs. 3c-d) as compared to 1-day HC (Figs. 2b-d and Figs.3a-b). Microscopic observations of all the 4-day treated sites with tridecane, tetradecane, and pentadecane exhibited numerous subcorneal and intracorneal lesions containing parakeratotic cells bordered by a reforming stratum corneum and containing a mix inflammatory population of neutrophils and lymphocytes (Figs. 3c-d). JP-8 treated skin contained focal subcorneal microabscesses with neutrophils and lymphocytes. After 4-days of in vivo exposures to undecane and dodecane, focal areas of parakeratosis were exhibited. The aromatic HC exposed sites for 4-days, only depicted a slight increase in intracellular epidermal edema (Fig. 4b-d).
TEM was used to examine the ultrastructural organization of the intercellular bilipid layers between the stratum corneum layers after fixation with ruthenium tetroxide. Both aliphatic and aromatic HC caused similar damage to the SC intercellular lipids and the intensity of damage was enhanced with prolonged exposure to these HC (Figs. 5b-d and Figs. 6b-d). In control skin, the SC layers appeared normal unaltered with intact desmosomes and normal compact bilipid layers. In 1-day JP-8 treated skin (Fig. 5b) no significant ultrastructural changes were observed except for small lacunae. However, in the 4-day JP-8 (Fig. 6b), individual aromatic HC (e.g. o-xylene, Fig. 6c) and aliphatic HC (e.g. tetradecane, Fig. 6d) exhibited large lacunae (areas with loss of electron-lucent and electron-dense lamellae).

Discussion

Repeated or prolonged exposure to jet fuels is more reflective of the occupational norm. Studies have shown that repeated dermal exposures to jet fuels result in skin irritation (Kinkead et al., 1992; Baker et al., 1999; Monteiro-Riviere et al., 2001a). However, the specific causative fuel components within JP-8 have not been identified. Previously, our laboratory has demonstrated that Jet A, JP-8 and JP-8 (100) all release pro-inflammatory cytokines such as IL-8 from normal human epidermal keratinocytes (Allen et al., 2000). However, these studies also revealed that there were no significant differences among the three different fuel types with respect to IL-8 release. Therefore, one may propose that the components responsible for this response must exist in all three fuels, eliminating performance additives as the causative mechanism (Allen et al., 2001). Additionally, differences between fuels in vivo as well as differences in absorption of components between fuels may be due to modulation of the HC dermal absorption by additives, an effect not seen in culture (Muhammad et al., 2004c; Baynes et al., 2000; Riviere et al., 1999).

Since jet fuels are complex mixtures of aliphatic and aromatic HC, we selected a wide range of aliphatic (C9-C16) and aromatic HC based on our previous cell culture studies (Chou et al., 2002, 2003) and observed their irritant effects in pigs after 1 and 4 days of HC exposures. Pigs were selected as our experimental model because their skin is morphologically, physiologically and
biochemically similar to human skin (Monteiro-Riviere, 2001b; Monteiro-Riviere and Riviere 1996). Literature on the dermal toxicity of jet fuel HC is limited. Our laboratory has demonstrated the dermal absorption and distribution profiles for some of the HC (Muhammad et al., 2004a; Baynes et al., 2000; Riviere et al., 1999). In addition, we have observed the in vitro cytotoxicity and IL-8 release from HEK with different clusters of aliphatic and aromatic HC (Allen et al., 2001, Chou et al., 2002; Chou et al., 2003).

The present in vivo studies suggest that topical application of JP-8 and specific aliphatic HC on pig skin results in dermal irritation after repeated exposure for 4 days. Erythema, and epidermal hyperplasia were pronounced after 4-days of repeated application of the aliphatic HC. A hydrocarbon specific response was demonstrated by epidermal thickness and the number of epidermal cell layers, with tridecane and tetradecane having the greatest proliferative effect followed by JP-8 and pentadecane after 4-days of in vivo exposures. The short chain aliphatic HC like nonane, decane and undecane produced only mild erythema after 4-days of in vivo exposures (Figure 1). These findings are in accordance with Brown and Box (1970) who studied the skin irritancy of alkanes and reported that n-decane was slightly irritant with some epidermal thickening, while n-tetradecane was more irritant with epidermal thickening. Horton et al (1957, 1966) have demonstrated the importance of long-chain aliphatic HC as accelerators of skin carcinogenesis. Results from Sice (1966) and Baxter and Miller (1987) demonstrated that the tumor promoting activity of alkanes is related to their chain length, with maximum activity found in C12-C14 alkanes. The present findings are also in accordance with in vitro studies of Allen et al (2001) who reported that maximum IL-8 was released with C-13 from HEK and also with Chou et al (2002) who found IL-8 peak at mid chain lengths of aliphatic HC.

The macroscopic and microscopic observations for JP-8 in the present study are similar to our earlier studies (Monteiro-Riviere et al., 2001a) indicating the reproducibility of in vivo effects of jet fuels. Subcorneal microabscesses were observed in all of the 4-day JP-8, and most of the tridecane, tetradecane, and pentadecane treated sites. These findings are similar to Monteiro-Riviere et al
(2001a) and other kerosene studies cited in literature. Similar findings were observed in studies of Tagami and Ogino (1973) who found that after 24 h of exposure with kerosene-soaked clothing in volunteers, intraepidermal and subcorneal vesicles containing neutrophils were present. Tridecane, tetradecane, and pentadecane treated sites primarily consisted of multifocal parakeratotic lesions with an inflammatory crust consisting of a few neutrophils and lymphocytes after 4 days. Although tridecane showed this subcorneal microabscesses in all 4 pigs, tetradecane and pentadecane exhibited these microabscesses in 3/4 of the pigs after 1-day of exposure. This could be due to the fact that these lesions were focal and the biopsies did not include the specific area of damage. Serial histologic step sectioning of the paraffin blocks did not reveal any additional subcorneal microabscesses. The typical acute subcorneal microabscesses observed after 1 day of exposure did not resemble the multifocal parakeratotic inflammatory lesions present after 4 days of exposures with the aliphatic HC. These parakeratotic lesions are different and are consistent with the healing phase of the early 1-day subcorneal microabscess. This could signify that during the 4 days of repeated exposure, the individual aliphatic hydrocarbons stimulated a hyperproliferative epidermis that contributes to the progression of the lesion to the stratum corneum, but is resistant to the development of the new subcorneal micropustules. This increase in epidermal hyperplasia seen with the 4-day exposures is typical of a chronic inflammatory process. Although JP-8 consists of a mixture of these hydrocarbons, the relative concentration of each HC is less in the JP-8 mixture, therefore explaining the presence of subcorneal microabscess taking 4 days to develop. The individual aliphatic HC placed on the skin are more concentrated than found in the JP-8 mixture with aromatics and therefore could explain the rapid development of the hyperproliferative state and the progression of the subcorneal lesion into the stratum corneum that was observed in the histology.

Aromatic HC like ethyl benzene, o-xylene, trimethyl benzene, cyclohexyl benzene, naphthalene, and dimethyl naphthalene did not produce any macroscopic or significant microscopic changes in epidermal thickness or lesions after 1 or 4 days of in vivo exposures. Carpenter et al (1975) exposed rats and beagle dogs for 13 wk to mixed xylenes, with no changes in body weight
gain observed relative to controls, and no xylene-related alterations in histopathology. Jenkins et al (1970) exposed rats, guinea pigs, dogs, and monkeys to 3.35 g/m$^3$ o-xylene either for 8h/d, 5d/wk, for 6wk, or for 90 or 127 d continuously, reporting no effects seen on body weight gain, hematological parameters, blood chemistry, or tissue histology.

Biophysical, morphological and biochemical data indicate that the stratum corneum forms a continuous sheath of protein-enriched corneocytes embedded in an intercellular matrix enriched in nonpolar lipids and organized as lamellar lipid layers (Elias, 1983; Elias and Menon, 1991; Landmann, 1986). Lipid layers in the stratum corneum interstices were first visualized by freeze-fracture technology (Elias et al., 1977; Landmann, 1986). However, this method bypasses the more polar domains because of the preferential deviation of the fracture plane to the most hydrophobic surfaces (Hou et al., 1991). The use of ruthenium tetroxide fixation allows for detailed studies of the spatial organization of the intercellular lipids of the stratum corneum (Fartasch and Ponec, 1994; Fartasch et al., 1993; Madison et al., 1987). In the present study, we observed intercellular disruption, loss of cohesion between the layers, and extraction of lipids from some layers. Specific individual HC caused the same magnitude of damage to stratum corneum, as did the JP-8. Similar disorganization of bilayers have been reported in jet fuels treated porcine stratum corneum (Monteiro-Riviere et al., 2004), in humans after application of sodium dodecyl sulfate or acetone irritants (Fartasch, 1997), in humans after kerosene exposure which induced large lacunae in the horny layers (Lupulescu et al., 1973), in murine stratum corneum after topical application of silicone (Menon and Ghadially, 1997), in porcine selective lipid extraction studies in different body regions (Monteiro-Riviere et al., 2001c) and in petrolatum-treated murine skin (Ghadially et al., 1992). It is well known that lipid extraction severely affects the biophysical properties of the horny layer (Wolfram et al., 1972). The present findings clearly indicate that JP-8 and specific individual HC can extract the lipids from the stratum corneum even after 24 h exposure. These findings support the proposed mechanism of lipid extraction for the increase in HC absorption through the JP-8 pre-exposed skin (Muhammad et al., 2004b). Although aromatic HC like o-xylene did not produce macroscopic or microscopic skin lesions, they
had the potential to extract lipids out of stratum corneum of skin (Figs. 4c, 5c). Thus, aromatic HC can lead to an increase in percutaneous absorption on subsequent dermal exposures to themselves or to other chemicals (Muhammad et al., 2004b). The ability of aromatic HC to extract lipids out of stratum corneum of skin as investigated with TEM in the present study confirms our previous finding of stratum corneum delipidization with these aromatic HC investigated with FTIR (Muhammad et al., 2004b).

Macroscopic and microscopic findings in the present study indicate that individual aliphatic HC (tridecane, tetradecane, pentadecane) are the principal source of jet fuel induced irritation. A comparison of absorption and toxicity data for aliphatic HC is shown in Table 4. By comparing all these data, it is evident that tridecane is the last HC that is absorbed through the skin and tetradecane is the first HC retained in the skin. It appears that this transition between absorption and retention of aliphatic HC contributes to a HC's propensity to induce skin irritation. Furthermore, the macroscopic and microscopic lesions produced by tridecane and tetradecane after 1 and 4 days of in vivo exposures duplicate the type of irritation produced with JP-8 mixture. Based on these studies it is postulated that tridecane and tetradecane may be the two most important hydrocarbons responsible for jet fuel induced skin irritation.

Acknowledgements

US Air Force Office of Scientific Research, Grant F49620-01-1-0080, supported this work. Authors greatly appreciate the technical assistance of CCTRP staff.
References


Centers for Disease Control (CDC) (1999). Background document on gulf war-related research for the health impact of chemical exposures during the gulf war: a research planning document.


Table 6.1. Erythema scores* (mean ± SEM) after 1 and 4-days of in vivo jet fuel hydrocarbon exposures

<table>
<thead>
<tr>
<th>Aliphatic HC</th>
<th>1-day</th>
<th>4-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonane (C9)</td>
<td>0.00 ± 0.00(^b)</td>
<td>0.50 ± 0.29(^{cf})</td>
</tr>
<tr>
<td>Decane (C10)</td>
<td>0.00 ± 0.00(^b)</td>
<td>1.25 ± 0.48(^{de})</td>
</tr>
<tr>
<td>Undecane (C11)</td>
<td>0.00 ± 0.00(^b)</td>
<td>2.00 ± 0.41(^{cd})</td>
</tr>
<tr>
<td>Dodecane (C12)</td>
<td>0.75 ± 0.48(^b)</td>
<td>2.00 ± 0.41(^{cd})</td>
</tr>
<tr>
<td>Tridecane (C13)</td>
<td>2.25 ± 0.48(^a)</td>
<td>3.25 ± 0.25(^{ab})</td>
</tr>
<tr>
<td>Tetradecane (C14)</td>
<td>2.25 ± 0.48(^a)</td>
<td>3.50 ± 0.50(^a)</td>
</tr>
<tr>
<td>Pentadecane (C15)</td>
<td>2.00 ± 0.58(^a)</td>
<td>2.50 ± 0.29(^{bc})</td>
</tr>
<tr>
<td>Hexadecane (C16)</td>
<td>0.25 ± 0.25(^b)</td>
<td>1.25 ± 0.25(^{de})</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aromatic HC</th>
<th>1-day</th>
<th>4-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl benzene</td>
<td>0.00 ± 0.00(^b)</td>
<td>0.00 ± 0.00(^f)</td>
</tr>
<tr>
<td>O-xylene</td>
<td>0.00 ± 0.00(^b)</td>
<td>0.00 ± 0.00(^f)</td>
</tr>
<tr>
<td>Trimethyl benzene</td>
<td>0.00 ± 0.00(^b)</td>
<td>0.25 ± 0.25(^f)</td>
</tr>
<tr>
<td>Cyclohexyl benzene</td>
<td>0.00 ± 0.00(^b)</td>
<td>0.25 ± 0.25(^f)</td>
</tr>
<tr>
<td>Dimethyl naphthalene</td>
<td>0.00 ± 0.00(^b)</td>
<td>0.25 ± 0.25(^f)</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>0.00 ± 0.00(^b)</td>
<td>0.00 ± 0.00(^f)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.00 ± 0.00(^b)</td>
<td>0.00 ± 0.00(^f)</td>
</tr>
<tr>
<td>JP-8</td>
<td>0.00 ± 0.00(^b)</td>
<td>3.00 ± 0.41(^{ab})</td>
</tr>
<tr>
<td>Control</td>
<td>0.00 ± 0.00(^b)</td>
<td>0.00 ± 0.00(^f)</td>
</tr>
</tbody>
</table>

Superscripts indicate significant differences among different hydrocarbons within the same treatment

* 0 No significant change, 1 Very slight erythema (barely perceptible), 2 Slight; pale red in defined area, 3 Moderate to severe; red in well-defined area, 4 Severe; beet-red in defined area.
Table 6.2. Mean ± SEM Epidermal thickness (µm) after 1 and 4-days of in vivo exposures

<table>
<thead>
<tr>
<th>Aliphatic HC</th>
<th>1-day</th>
<th>4-day</th>
</tr>
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<tbody>
<tr>
<td>Nonane (C9)</td>
<td>51.89 ± 3.00&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>49.35 ± 1.91&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>Decane (C10)</td>
<td>49.35 ± 1.03&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>51.89 ± 2.73&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Undecane (C11)</td>
<td>56.30 ± 1.75&lt;sup&gt;de&lt;/sup&gt;</td>
<td>64.17 ± 3.66&lt;sup&gt;bcd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dodecane (C12)</td>
<td>55.83 ± 2.09&lt;sup&gt;de&lt;/sup&gt;</td>
<td>62.78 ± 5.40&lt;sup&gt;ed&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tridecane (C13)</td>
<td>85.02 ± 4.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.23 ± 4.05&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Tetradecane (C14)</td>
<td>74.83 ± 3.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82.01 ± 0.46&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pentadecane (C15)</td>
<td>67.42 ± 6.83&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>70.20 ± 2.31&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hexadecane (C16)</td>
<td>60.93 ± 3.62&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>60.70 ± 3.27&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aromatic HC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl benzene</td>
<td>50.74 ± 1.09&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>47.96 ± 1.58&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>O-xylene</td>
<td>51.20 ± 2.22&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>46.33 ± 0.54&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Trimethyl benzene</td>
<td>50.97 ± 2.54&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>48.42 ± 0.69&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyclohexyl benzene</td>
<td>50.04 ± 1.36&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>49.11 ± 1.13&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dimethyl naphthalene</td>
<td>51.66 ± 1.22&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>49.11 ± 1.97&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>48.42 ± 2.15&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>47.49 ± 1.67&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>Ethanol</td>
<td>45.18 ± 1.58&lt;sup&gt;f&lt;/sup&gt;</td>
<td>47.03 ± 2.12&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>JP-8</td>
<td>65.79 ± 3.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>71.12 ± 2.74&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Control</td>
<td>46.57 ± 1.83&lt;sup&gt;f&lt;/sup&gt;</td>
<td>47.26 ± 2.62&lt;sup&gt;d&lt;/sup&gt;</td>
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</table>

Superscripts indicate significant differences among different hydrocarbons within the same treatment
Table 6.3. Number of epidermal cell layers (mean ± SEM) after 1 and 4 days of in vivo exposures

<table>
<thead>
<tr>
<th>Hydrocarbon</th>
<th>1-day Mean ± SEM</th>
<th>4-day Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aliphatic HC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonane (C9)</td>
<td>4.58 ± 0.16&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>3.75 ± 0.21&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Decane (C10)</td>
<td>4.08 ± 0.16&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3.50 ± 0.10&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Undecane (C11)</td>
<td>4.67 ± 0.24&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>5.17 ± 0.40&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dodecane (C12)</td>
<td>4.50 ± 0.17&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>4.50 ± 0.17&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tridecane (C13)</td>
<td>7.00 ± 0.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.42 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tetradecone (C14)</td>
<td>6.58 ± 0.16&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.08 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pentadecane (C15)</td>
<td>6.00 ± 0.56&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>6.25 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hexadecane (C16)</td>
<td>5.17 ± 0.32&lt;sup&gt;de&lt;/sup&gt;</td>
<td>4.75 ± 0.21&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Aromatic HC</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl benzene</td>
<td>4.33 ± 0.24&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>3.92 ± 0.16&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>O-xylene</td>
<td>4.42 ± 0.16&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>3.92 ± 0.16&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>Trimethyl benzene</td>
<td>4.08 ± 0.25&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3.92 ± 0.28&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyclohexyl benzene</td>
<td>4.25 ± 0.08&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3.83 ± 0.22&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dimethyl naphthalene</td>
<td>4.33 ± 0.14&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>3.50 ± 0.10&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>4.00 ± 0.14&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4.08 ± 0.16&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3.92 ± 0.28&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3.75 ± 0.16&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>JP-8</td>
<td>5.67 ± 0.27&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>6.58 ± 0.25&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>4.17 ± 0.10&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4.08 ± 0.16&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Superscripts indicate significant differences among different hydrocarbons within the same treatment.
Table 6.4. Absorption and toxicity comparison of jet fuel aliphatic hydrocarbons in skin and epidermal keratinocytes

<table>
<thead>
<tr>
<th>HC</th>
<th>1\textsuperscript{st} Absorption</th>
<th>1\textsuperscript{st} SC/FTIR</th>
<th>1d micro-abscess</th>
<th>4d Macroscopic</th>
<th>Epidermis Thickness (µ) 1day</th>
<th>Epidermis Thickness (µ) 4day</th>
<th>\textsuperscript{2}In vitro Cytotoxicity 24 h</th>
<th>\textsuperscript{2}In vitro IL-8 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undecane (C11)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>56.3</td>
<td>64.17</td>
<td>75%</td>
<td>+++</td>
</tr>
<tr>
<td>Dodecane (C12)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>55.83</td>
<td>62.78</td>
<td>65%</td>
<td>++</td>
</tr>
<tr>
<td>Tridecane (C13)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>85.02</td>
<td>79.23</td>
<td>70%</td>
<td>+++</td>
</tr>
<tr>
<td>Tetradecane (C14)</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>+++</td>
<td>74.82</td>
<td>82.01</td>
<td>50%</td>
<td>+++</td>
</tr>
<tr>
<td>Pentadecane (C15)</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>+++/++++</td>
<td>67.42</td>
<td>70.19</td>
<td>35%</td>
<td>+</td>
</tr>
<tr>
<td>Hexadecane (C16)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>60.93</td>
<td>60.69</td>
<td>30%</td>
<td>+</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Data obtained from (Muhammad et al., 2004b)
\textsuperscript{2}Data obtained from (Chou et al., 2002)
Figure 6.1. Macroscopic lesions after 4 days of application of JP-8 and aliphatic HC to pig. Cont. (control), DEC (C10), UND (C11), DOD (C12), TRI (C13), TET (C14), PEN (C15), HEX (C16) and JP-8. Note the differential response with varying carbon chain length of HC.
Figure 6.2. Light micrographs of porcine skin following 1-day dermal exposures to (a) control fabric; (b) fabric soaked in JP-8; (c) fabric soaked in tridecane; (d) fabric soaked in tetradecane. Note increase in dermal inflammation in c and d. Epidermis (E). x145.
Figure 6.3. Light micrographs indicating subcorneal microabscesses (arrows) filled with inflammatory cells following 1-day dermal exposure to (a) tridecane; (b) tetradecane; and 4-day dermal exposures to (c) tridecane; (d) JP-8. Note extensive epidermal rete pegs in c and d. Epidermis (E). x76.
Figure 6.4. Light micrographs following 4-day dermal exposures to (a) control fabric; (b) fabric soaked in ethyl benzene; (c) fabric soaked in o-xylene; (d) fabric soaked in dimethyl naphthalene. Epidermis (E). x145.
Figure 6.5. TEM micrographs after ruthenium tetroxide staining of the lipid bilayers between the stratum corneum cell layers following 1-day exposures to (a) control fabric; (b) fabric soaked in JP-8; (c) fabric soaked in o-xylene; (d) fabric soaked in tetradecane. Note the intact lipid bilayers (arrow), and expanded intercellular spaces (*) where the intercellular lipid lamellae appeared extracted. x69,700.
Figure 6.6. TEM micrographs after ruthenium tetroxide staining of the lipid bilayers between the stratum corneum cell layers following 4-day exposures to (a) control fabric; (b) fabric soaked in JP-8; (c) fabric soaked in o-xylene; (d) fabric soaked in tetradecane. Note the intact lipid bilayers (arrow), and expanded intercellular spaces (*) where the intercellular lipid lamellae appeared extracted. x69,700.
7. CONCLUSIONS

These studies have been conducted in an attempt to identify specific constituents in jet fuel mixtures involved in skin irritation. Many unrelated studies had been conducted in the literature to find the possible agents in jet fuel that induced skin irritation. The complex nature of these kerosene based fuels requires one to conduct an array of integrated absorption and toxicity studies in suitable in vitro and in vivo models. The selection of appropriate markers, their percutaneous absorption, effects of performance additives and occupational norms on the transdermal disposition, use of sensitive analytical, biophysical and ultrastructural techniques, mechanistically driven hypothesis, and in vivo toxicity studies of marker constituents in these studies have generated the unique data necessary for risk assessment considerations.

The initial chapter of this thesis (chapter 3) focused on the effects of mixture interactions in the dermal disposition of jet fuel marker hydrocarbons (HC) simultaneously using simple and complex membrane systems. The aim of the study was to compare the marker HC disposition across JP-8 and JP-8 (100) jet fuels in different synthetic and biological membranes. These are the two most commonly used fuel types, which are complex mixtures of hundreds of different aliphatic and aromatic HC and performance additives. It has been recognized that components within a chemical mixture may interact with the toxicant of interest and result in health effects that are not simply additive. It has been reported in the literature that additives in JP-8 (100) might modulate the dermal penetration of fuel constituents and hence has considerable toxicity concerns. Such studies have used single membrane model systems. The present research has been extended in an attempt to demonstrate that single membrane systems are not sufficient to address the effects of complex additive interactions on the transmembrane kinetics of fuel constituents.

The results presented in this chapter indicated that the in vivo mimicking membrane system such as the IPPSF exposed the mechanism for different additive effects on the marker HC (naphthalene and dodecane) absorption. MDA was found to antagonize the absorption of both markers while BHT was a potent synergist of naphthalene absorption. 8Q405 had no effect on marker
absorption in IPPSF suggesting the reason of similar marker disposition across complete JP-8 and JP-8 (100) fuel mixtures, a common finding observed with all the three membrane systems used in this study. In contrast, silastic membrane data indicated that all three individual additives were significant enhancers of naphthalene absorption while porcine skin did not depict any significant effect of additives on marker absorption. These findings are important because silastic and porcine skin alone could not provide the explanation for similar disposition of markers in both jet fuels, emphasizing the need to conduct such mixture interaction studies in a combination of membrane systems. Another advantage of using a combination of membranes is to identify chemical and biological interactions among the additives in these complex mixtures. The experiments conducted in this regard revealed that 8Q405 significantly reduced naphthalene contents in dosed silastic and skin indicating a direct interaction between additive and marker HC. The MDA and BHT individually, retained naphthalene in the stratum corneum of porcine skin but statistically decreased this retention when in combination (MDA + BHT) suggesting a biological interaction. Furthermore, these studies provide evidence that the combination of two additives affected dodecane retention more than naphthalene on the surface of silastic membrane while the reverse was true for porcine skin surface retention.

Finally, the modulation of fuel HC absorption due to additives suggests that predicting the toxicity of exposure to different additive factors is complicated. This is important to consider the toxicological implications of the presence of opposing additives (MDA and BHT) on the interpretation of equivalent naphthalene fluxes in mixtures containing both additives compared to those without any additives. Would these opposing mechanisms, which cancel out individual modulating effects of each additive, also modify the risk for direct skin toxicity compared to naphthalene alone? The additional research must be focused on this dimension of complex chemical mixture toxicology.

These studies revealed that there were no significant differences in HC disposition across JP-8 and JP-8 (100) jet fuels. Similarly the previous studies conducted in our laboratory concluded no significant differences among three different fuel types (Jet A, JP-8 and JP-8 (100)) with respect to
IL-8 release. Therefore, one can propose that components responsible for jet fuel induced skin irritation must exist in all fuel types, eliminating the possibility of performance additives as the causative constituents. The remainder of this thesis was focused on the identification of such HC constituents. Since all fuel types are mixtures of different aliphatic and aromatic hydrocarbons, we proposed that these HC themselves could be responsible for fuel induced skin irritation. Three major aims were sought to be accomplished with these studies. First, to standardize a new sensitive and reliable analytical method for quantification of neat jet fuel marker HC and determine the influence of various dosages on their percutaneous absorption. Second, to expand the range of marker HC and determine the effect of in vivo fuel pre-exposure on the subsequent in vitro dermal absorption of these marker constituents in order to mimic occupational scenarios. And third, to access the in vivo toxicity of marker HC and to correlate the absorption data to that of toxicity data.

In initial studies, we chose two aromatic HC (naphthalene and dimethyl naphthalene) and five aliphatic HC (undecane, dodecane, tridecane, tetradecane, pentadecane) markers because of their occurrence in the literature with respect to percutaneous absorption. Hexadecane, a fuel constituent was used as a non-polar solvent. The percutaneous absorption of these neat markers HC was determined with a novel solid phase microextraction fiber technique with headspace sampling. These studies indicated a dose-related increase in percutaneous absorption of aromatic HC suggesting linear first order pharmacokinetics. The long chain HC (tetradecane and pentadecane) could not get through the skin. These studies have provided evidence to use the absolute amount absorbed instead of percent absorption, as the same percent absorbed from the higher dose may be more toxic than the same percent absorbed from the lower dose. These findings are significant and raise concern for the potential adverse effects of the chronic exposure expected to be encountered by aircraft personnel, most notably fuel handlers and engine mechanics. Due to the complexity of jet fuels, it is not possible to assess the percutaneous absorption of all fuel components. A quantitative structure permeability relationship model was suggested to determine the permeability of unknown fuel HC with the use of their known physicochemical parameters. These results provide useful data that can be applied in risk.
assessment calculations in occupational settings for such long chain aliphatic HC. Most notably, these results justify the continuation of experimentation to include simultaneous in vivo and in vitro studies.

With this in mind, we attempted to expose pigs to JP-8 jet fuel for 1 and 4-days in order to mimic occupational settings where professional workers come in contact with fuel during their routine work. Our laboratory uses pigs as an in vivo model for skin research due to the well-documented similarities of porcine and human skin. Therefore, an in vitro model from the same species would serve as a useful tool for correlating in vivo and in vitro results. We further broadened the range of hydrocarbons and determined the secondary in vitro percutaneous absorption of 8 aliphatic HC (nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane) and 6 aromatic HC (ethyl benzene, o-xylene, trimethyl benzene, cyclohexyl benzene, naphthalene, dimethyl naphthalene) through the JP-8 pre-exposed porcine skin. In contrast to the previous study, a polar solvent (ethanol/water) was used in these studies to mimic the post work environment of occupational workers where they come in contact with water at the end of day, and secondly to investigate the effect of polar vehicle on the percutaneous absorption of fuel HC. These studies indicated a 2-4 fold increase in percutaneous absorption of most of aromatic and short chain aliphatic HC (nonane, undecane, dodecane) through the JP-8 pre-exposed skin. To study the mechanism of this increase in HC absorption, we employed a biophysical tool (Fourier Transform Infrared spectroscopy) instead of molecular techniques. These investigations revealed that overall lipid extraction out of the stratum corneum (SC) with these HC might be the primary mechanism for this many fold increase in secondary percutaneous absorption. Interestingly, the amount of in vitro percutaneous absorption was proportional to the length of in vivo pre-exposure. FTIR studies further indicated that long chain HC like tetradecane, pentadecane and hexadecane might bind in SC that may further modulate absorption. In addition, naphthalene, dimethyl naphthalene, and cyclohexyl benzene could have the potential to form skin depots in JP-8 exposed skin, another significant finding regarding risks associated with occupational scenarios. Our data indicated that polar solvents enhanced the percutaneous absorption
of fuel HC many fold compared to non-polar solvents. Our results suggests that even single in vivo exposure of skin to JP-8 statistically affects the subsequent in vitro percutaneous absorption of marker HC as compared to absorption through the naive skin, therefore, it is postulated that single dose absorption data from naive skin for jet fuel HC may not be optimal for prediction of risk associated with repeated exposures.

The findings of these studies regarding differential absorption and retention of fuel HC in skin indicate the necessity to conduct the toxicity studies in vivo with these markers HC. These individual HC are selected as markers because these are present in the highest concentrations in typical jet fuel lots. To address this, we exposed pigs to these 14 individual HC for 1 and 4 days and compared their macroscopic and microscopic effects to that caused by complete JP-8 mixture exposure. Interestingly, all HC were not toxic in this exposure scenario. Only mid chain aliphatic HC like tridecane and tetradecane produced macro and microscopic lesions similar to that of JP-8. These in vivo observations are similar to our previous in vitro findings where these mid chain aliphatic HC were found to cause maximum release of IL-8 in human epidermal keratinocyte cell cultures. As an objective of this thesis to perform integrated absorption and toxicity studies, the comparison of absorption and toxicity data for aliphatic HC revealed that tridecane is the longest carbon chain (C13) HC that is absorbed through the skin and tetradecane is the shortest carbon chain (C14) HC retained in the skin. This transition of carbon chain length between absorption and retention of these aliphatic HC seems related to fuel induced skin irritation. Additionally, the SC delipidization by jet fuel HC in vivo was confirmed with transmission electron microscope (TEM). The TEM results were correlated to that of FTIR results suggesting that biophysical tools can be successfully used in mechanistic studies.

In conclusion, these studies have suggested that performance additives in jet fuels are not the cause of skin irritation but rather that the fuel HCs are themselves toxic. We have developed a sensitive analytical method for the chromatographic analysis of neat fuel HC. We have provided evidence regarding the dose-related increase in percutaneous absorption of jet fuel HC along with
quantitative structure permeability relationship model. We are the first to demonstrate the significant effects of in vivo fuel pre-exposure on the subsequent in vitro dermal absorption of individual aromatic and aliphatic fuel constituents. Finally, we have shown that specific aliphatic HC components (tridecane and tetradecane) induce the same in vivo toxicity as seen with the complete JP-8 jet fuel, and thus may warrant the consideration as the inciting agents.

Future studies could be directed to investigate the irritant effects of jet fuel mixtures without these specific toxic aliphatic HC, alternatively these specific HC could be modified structurally/chemically, and their absorption and irritation assessed both in in vitro and in vivo models. These in vitro percutaneous absorption and in vivo irritation studies should be repeated in other animal models or preferably in bioengineered skin model systems to minimize the use of animals in such studies.

Future studies can also be targeted towards the correlations of this absorption and toxicity data to that of other pro-inflammatory cytokines such as IL-1 or IL-6. We have demonstrated in vivo exposure effects with full saturation of cotton fabric, further studies could be conducted with sub-saturated to full saturated fuel exposure and correlate their toxic effects to that of certain immune parameters. Finally, since the pre-exposure of skin to fuels resulted in significant dermal absorption of marker HC and in vivo irritation, measures should be refined to reduce the risk to occupational workers.

These studies add to the justification for continuing studies on the potential toxicity mechanisms of two specific aliphatic HC (tridecane and tetradecane). These studies have highlighted the importance of repeated fuel exposure in occupational settings thus this factor must be taken into account in risk assessment considerations in jet fuel literature.