

ABSTRACT

VAN DER MERWE, DEON. The dermal absorption of selected agricultural and industrial chemicals through porcine skin with emphasis on chemical mixture effects. (Under the direction of Jim E. Riviere.)

The rate and extent of dermal absorption are important in the analysis of risk from dermal exposure to toxic chemicals and for the development of topically applied drugs, barriers, insect repellants and cosmetics. The stratum corneum is the primary barrier to dermal absorption and the processes of absorption include partitioning into, and diffusion through, the lipid matrix of the stratum corneum. These studies were aimed at furthering our understanding of these processes and modeling dermal absorption. We used 11 industrial and agricultural compounds, including ^{14}C labeled phenol, 4-nitrophenol, pentachlorophenol, dimethyl parathion, parathion, chlorpyrifos, fenthion, triazine, atrazine, simazine and propazine, and 24 solvent mixtures consisting of combinations of water, ethanol, propylene glycol, sodium lauryl sulphate and methyl nicotinate. Study methods included *in vitro* partitioning using isolated stratum corneum and permeability studies using dermatomed porcine skin in Bronaugh-type flow-through diffusion cells. Fourier transform infrared spectroscopy was used to investigate lipid changes in the stratum corneum. K-means and hierarchical clustering techniques were used to identify patterns in the partitioning and permeability data sets. A model of dermal absorption was developed using a physiological-based (PBPK) approach. Parameters used in the model were generated from the partitioning data as well as transmission electron microscopy and light microscopy. Ethanol and ethanol/water mixtures altered the stratum corneum through lipid extraction, rather than through disruption of lipid order. Partitioning was primarily determined by relative compound solubility between the stratum corneum lipids and the donor solvent. Permeability, however, reflected the result of successive, complex processes and was not consistently correlated with stratum corneum partitioning. These results demonstrated the potential of using large datasets to identify consistent solvent and chemical mixture effects. Diffusion cell studies were conducted to validate the PBPK model under a variety of conditions including different dose ranges ($6.3\text{-}106.9\text{ }\mu\text{g}/\text{cm}^2$ for parathion; $0.8\text{-}23.6\text{ }\mu\text{g}/\text{cm}^2$ for fenthion; $1.6\text{-}39.3\text{ }\mu\text{g}/\text{cm}^2$ for methyl parathion), different solvents (ethanol, 2-propanol and acetone), different solvent volumes ($5\text{-}120\text{ }\mu\text{l}$ for ethanol; $20\text{-}80\text{ }\mu\text{l}$ for 2-propanol and acetone),

occlusion versus open to atmosphere dosing, and corneocyte removal by tape-stripping. The study demonstrated the utility of PBPK models for studying dermal absorption, which can be useful as explanatory and predictive tools; and may be used for *in silico* hypotheses generation and limited hypotheses testing. These data have direct relevance to topical chemical exposure risk assessments.

**THE DERMAL ABSORPTION OF SELECTED AGRICULTURAL AND
INDUSTRIAL CHEMICALS THROUGH PORCINE SKIN WITH EMPHASIS ON
CHEMICAL MIXTURE EFFECTS**

by

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DEDICATION

I dedicate this dissertation my daughter, Liza.

BIOGRAPHY

Deon van der Merwe was born on May 29, 1970 in Carletonville, South Africa, where he completed his primary school education at Laerskool Dagbreek in 1983. He moved to Potchefstroom for his secondary education at Hoërskool Gimnasium where he matriculated in 1988. He received a BVSc degree from the University of Pretoria in 1994. After a period of employment in private veterinary practice, he returned to the University of Pretoria and received a BSc(Hons) in 1998 and a MSc(Vet Sc) (with distinction) in 2000. He then joined the Onderstepoort Research Institute in Pretoria as a Senior Researcher, where he managed projects related to ethnoveterinary medicine and developing farmers. He also worked as a wildlife management consultant. He started a PhD in Comparative Biomedical Sciences (Pharmacology) in 2003 under the supervision of Dr. Jim E. Riviere at North Carolina State University. He is married to Dr. Ronette Gehring and has a daughter, Liza, born on August 26, 2004 in Raleigh, North Carolina.

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If there is any merit to be found in this report of my doctoral research it is, in large part, due to my teachers. They encouraged my sense of wonder and restrained the worst of my wayward tendencies. Teachers who had left deep impressions include my father, who first introduced me to the world of anatomy and physiology on hunting trips in South Africa. Our discussions on the structure and functions of various organs planted a seed that is still growing. Anton Ankiewicz, one of my high-school physics teachers, taught me that originality in science lies at the root of progress and that understanding could be pursued for the love of it. Drs. Fanie Kellerman and Theuns Naudé, whom I had the privilege of having as toxicology professors at the University of Pretoria, introduced me to the world of biologically active plants and their fascinating relationships with the animals that live among them. My interest in this topic, which was initiated by the enthusiasm of these two wonderful teachers, was the catalyst that led to my involvement in research after completing my veterinary training. Dr. Gerry Swan, my MSc supervisor, saw the potential in my desire to study ethnoveterinary plant use and his leadership was critical to the success of my early projects. He shaped my development as a researcher and taught me a range of indispensable skills.

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1. INTRODUCTION

Skin is in constant, direct contact with the environment and is often a site of exposure to environmental contaminants. Skin is also easily accessible for the topical application of therapeutic drugs. An ability to predict the rate and extent of chemical absorption after skin exposure is of great interest to risk assessors and drug developers.

Our understanding of dermal absorption from single solvents has, through the decades-long efforts of many groups and individuals, progressed to a point where models are available to roughly predict absorption rates. These models, and predictions derived from them, are usually specific to certain types of molecules and solvents. Such models are useful for relatively simple, comparative assessment of dermal absorption; but are often not successful when applied to the immensely variable conditions of real-world dermal exposure to chemicals. The development of widely applicable principles that would allow more general predictions is needed and is the focus of a large proportion of current dermal absorption research.

This dissertation reports studies conducted to:

- Understand the principles that govern absorption from chemical mixtures; and
- To develop a model of dermal absorption that is adaptable to a wide range of exposure conditions.

A set of industrial and agricultural compounds, including industrial pollutants, herbicides and pesticides with differing physical-chemical properties, were used to develop a database of *in vitro* dermal absorption and solvent/stratum corneum partitioning from 24 solvent mixtures through porcine skin. Targeted analysis of the database and a variety of additional experiments were used to test hypotheses related to dermal absorption from chemical mixtures and to develop a physiologically based pharmacokinetic model of dermal absorption. Porcine and human skin are similar in structure and composition (Monteiro-Riviere 2001) and principles derived from porcine skin studies are generally applicable to human skin. It is, therefore, hoped that these studies will contribute to the accurate assessment of risk following dermal exposure to potentially toxic substances and to the development of safe and effective drugs.

2. LITERATURE REVIEW

The Skin

Mammalian skin consists of layered tissues at the body's interface with the outside environment. The various skin layers have specialized cells and structures that support the functions of the skin. Skin plays important roles in thermoregulation, control of water loss, resistance to solar radiation and chemical penetration, neurosensory reception, immune responses, mechanical support, apocrine, eccrine and sebaceous secretion, endocrine functions and metabolism (Monteiro-Riviere 1991). The epidermis (30 – 56 μm thick in the pig depending on site and processing method) is the outer layer of the skin (Monteiro-Riviere et al 1990). It is ordered into successive layers including the stratum corneum (outermost layer; 9 – 18 μm in depth) (Monteiro-Riviere et al 1990), stratum granulosum, stratum spinosum and stratum basale. The epidermis is separated from the dermis (500 – 3000 μm in depth) by the basement membrane. The epidermis, and the stratum corneum in particular, forms the primary barrier to transdermal water loss and absorption of xenobiotics (Scheuplein & Blank 1971) and will be the focus of this review. A detailed review of the morphology of the deeper skin layers can be found in (Marks et al 1988).

Stratum corneum

The stratum corneum consists of several layers of terminally differentiated, tightly packed, flattened, keratin-enriched, anucleate, cornified keratinocytes (also known as corneocytes), which are constantly self-renewing through desquamation from the surface, balanced by cell divisions in the lower epidermis (Holbrook & Odland 1975). Corneocyte differentiation involves the formation of a covalently cross-linked protein layer that is deposited at the cytoplasmic membrane of developing corneocytes to form a cornified envelope (Chidgey 2002). Keratin is the main constituent of the corneocyte envelope. It is a water insoluble, thermodynamically stable, fibrous structural protein stabilized by disulfide bridges between cystine residues (Monteiro-Riviere 1991).

The keratinocytes are embedded in an intercellular lipid matrix that fills the intercellular space. The lipid content, as a fraction of the mass of the stratum corneum, is variable with a range in human skin of 1 – 11 % (Raykar et al 1988). The major lipid classes in porcine stratum corneum (% weight of solvent extracted lipids) are ceramides (27.4 - 31.4 %), cholesterol (30.6 – 34.2 %), cholesterol esters (4.3 – 9.4 %), free fatty acids (26.0 – 27.3 %) and triglycerides (5.0 – 6.7 %) (Monteiro-Riviere et al 2001). The relative proportions of the major lipid classes remain similar at different body sites, but the amount of lipids tend to be higher in back skin (Monteiro-Riviere et al 2001) and closer to the surface of the stratum corneum (Cox & Squier 1986). Cholesterol sulphate makes up a small proportion of stratum corneum lipids (2 – 5 %) and the level decreases abruptly close to the stratum corneum surface in both pigs and humans (Cox & Squier 1986; Weerheim & Ponc 2001). Phospholipids are found in the stratum corneum at low concentrations that decrease towards the surface (Cox & Squier 1986).

A typical ceramide consists of a polar head-group and two acyl chains of variable length. Ceramides with relatively long acyl chains consisting of 24 - 26 carbons, which are longer than those of typical plasma membrane phospholipids, predominate in both human and porcine stratum corneum. Examples of shorter chain length ceramides with 16 - 18 carbons and unusual ceramides consisting of linoleic acid linked to ω -hydroxy fatty acid are also found in both species. The free fatty acids are mostly saturated with chain lengths of 22 - 24 carbons (Bouwstra et al 2003).

The stratum corneum lipids are mostly derived from lamellar bodies that are secreted from keratinocytes in the upper stratum granulosum. The lamellar bodies are then modified and oriented parallel to the surfaces of surrounding keratinocytes (Mauro et al 1998; Bouwstra et al 2003). The lamellar bodies also contain hydrolytic enzymes necessary for postsecretory processing, which results in products that are less polar in nature (Mauro et al 1998). The formation of stratum corneum lipids is controlled by calcium, potassium and phosphate ions. It is thought that lower concentrations of these ions are due to increased trans epidermal water loss associated with low stratum corneum lipid content or stratum corneum damage. It initiates increased lipid production and secretion from the keratinocytes – providing a homeostatic and repair control mechanism (Lee et al 1992).

The lipids are arranged into orthorhombic crystalline ceramide bilayers with a 13 nm periodicity separated by bands of fluid lipids with relatively short acyl chains mainly consisting of fatty acids and cholesterol. The ceramide head groups are arranged into dense hydrogen-bonded lattices, which form the framework of orthorhombic crystals. Acyl chains are mutually attracted through Van der Waals forces. The degree of order in the lipid crystalline structure is variable and becomes less ordered at higher temperatures and lower pH (Bouwstra et al 2003). The degree of order in the lipid matrix lessens closer to the skin surface and it has been suggested that this is related to the incorporation of sebaceous lipids into the matrix and a decrease in pH, which is slightly acidic at the skin surface (Pilgram et al 2001; Bouwstra et al 2003). Cholesterol sulphate is required for the stable formation of crystalline lipid bilayers *in vitro* and the abrupt decrease of cholesterol sulphate close to the stratum corneum surface is likely to play a role in desquamation (Cox & Squier 1986; Kitson et al 1992).

Keratinocytes are attached to each other by specialized morphological structures called desmosomes (Burdett 1998; Cozzani et al 2000; Chidgey 2002). Desmosomes are local modifications of the plasma membrane that attaches cells to each other and/or to a matrix or substratum. They are common in epithelial tissues and are particularly plentiful in stratified epithelia where cells experience mechanical stress, such as skin. They are most common in the spinous and granular layers of the epidermis and lower in number in the basal cells and stratum corneum (Chidgey 2002). Desmosomes are formed by two opposing, symmetrically shaped proteinaceous disks of 0.1 – 0.5 µm in diameter and 15 – 20 nm in thickness on the cytoplasmic faces of plasma membranes of adjacent cells. The disks are referred to as plaques. Two layers are recognized in the plaques, a dense submembranous layer and a less dense layer facing the cytoplasm (Burdett 1998; Kowalczyk et al 1999; Chidgey 2002). The extracellular space between the plaques, also referred to as the desmoglea, is approximately 30 nm wide and filled with ordered arrays of protein filaments, spaced at 8 – 10 nm intervals. The filaments are attached to each other in the center of the desmoglea in a cross-bridge structure. Inter-filament attachments are thought to be due to a calcium dependent, zipper-like, homophilic adhesive mechanism between the amino-terminal domains of opposing N-cadherin proteins. The midline of the desmoglea is relatively electron dense due to the area of overlap and attachment between filaments originating from opposing plaques (Shapiro et al 1995; Kowalczyk et al 1999). Keratin filaments are present on the cytoplasmic face of plaques. They are attached to the plaque in a series of loops, which enter the plaque tangentially and curve back into the

cytoplasm. Bundles of filaments from adjacent desmosomes in the same cell are linked to form tonofilaments, which may be important for structural continuity and mechanical support (Kelly 1966). Desmosomes become modified during the differentiation of keratinocytes into corneocytes as the stratum corneum is formed. The desmosome plaques are incorporated into the cornified envelope, while the desmoglea are transformed into electron dense plugs. The resulting structures are referred to as corneodesmosomes and are present in the stratum corneum until they are broken down by proteolytic degradation just below the stratum corneum surface during the process of desquamation (Chidgey 2002).

Viable epidermis

The cells of the epidermal cell layers below the stratum corneum, also called the viable epidermis, contain functional genetic material and the intact biochemical machinery to perform the various specialized functions associated with the viable epidermis. Apart from keratinocytes, which are the most abundant cell type in the viable epidermis, specialized cell types include melanocytes, which produce melanin to increase resistance to UV irradiation, Langerhans cells, which mediate immune responses, and Merkel cells, which have neuroendocrine functions (Kirkel & Herzog 2004). The basal layer contains epidermal stem cells and transit amplifying cells. The transit amplifying cells are destined to lose contact with the basement membrane, move towards the skin surface and undergo differentiation (Lavker & Sun 1983; Adams & Watt 1990). The stratum spinosum, which is the next layer towards the skin surface, is characterized by an abundance of desmosomes. This gives the layer a spinous appearance. Cells of the next layer, the stratum granulosum, are characterized by the presence of multiple keratohyalin granules. The stratum lucidum is present in thick skin regions, such as the soles of the feet and the palms. The cells of the stratum lucidum contain eleiden, which is a viscous fluid protein similar to keratin (Monteiro-Riviere 1991).

Basement membrane

The basement membrane separates the epidermis from the dermis and consists of an extracellular matrix rich in laminin and collagen IV (Briggaman & Wheeler 1975). It is attached to the basal cell layer via hemidesmosomes. Apart from a structural role, hemidesmosomes and their associated anchoring filaments also have functions related to cell adhesion, cell migration and signal transduction (Burgeson & Christiano 1997).

Dermis

The dermis consists of a matrix of collagen, ground substance, muscle and elastic fibers interspersed with blood vessels, nerves, apocrine and eccrine sweat glands, hair follicles and sebaceous glands. It has important thermoregulatory functions mediated through sweat production, changes in blood flow and control of arrector pili muscles; it supports and nourishes the epidermis; and it has sensory functions including touch, pain, itch and temperature. Specialized cell types found in the dermis include fibroblasts, melanocytes, mast cells, eosinophils, neutrophils, lymphocytes, histocytes, and plasma cells (Monteiro-Riviere 1991; MVM 2003).

Dermal absorption

Conceptual models of the stratum corneum barrier

The complexity of the stratum corneum barrier, combined with imperfect knowledge, make the use of physically complete and exact descriptions impossible. Various models have been proposed that aim to capture the fundamental nature of the stratum corneum in relatively simple terms. Most models are designed with a specific use in mind, which influence model assumptions and complexity. Another important source of variation is the level of information and understanding available to the model designer at the time of model design. Early models tend to be less comprehensive and the assumptions more sweeping than later models. Lastly, but as importantly, models contain elements of creativity and intuition expressed as hypotheses. Such hypotheses are often the bases of study design and play a crucial role in the advancement of our understanding.

The “brick and mortar” model

Michaels and coworkers (Michaels et al 1975) described the stratum corneum as two isotropic, homogeneous compartments – a continuous lipid compartment and a discontinuous, multileveled protein compartment. This model was further developed by Elias and coworkers (Elias et al 1981) and became widely known as the “brick and mortar” model with the protein compartment visualized as the bricks of a wall, representing the corneocytes, and the lipid compartment as the mortar, representing the intercellular lipid matrix. Two routes of absorption were proposed, either by alternate transit through the lipid and protein compartments or by

continuous transit through the lipid compartment only. Exclusive transit through the lipid compartment required a considerably increased pathway length to move around the protein “bricks”. This model remains popular in spite of its erroneous assumption of isotropic and homogeneous compartments. The model resembles the light-microscopically-observed stratum corneum structure and is easy to explain to almost any audience. It also offers a convenient way to visualize the lipid pathway of absorption, which is the primary absorption pathway for small, relatively lipophilic molecules (Albery & Hadgraft 1979), such as the compounds used in studies for this thesis. Most subsequent models may be regarded as extensions of the “brick and mortar” model that retain the continuous nature of the lipid compartment, but move away from the assumption of an isotropic, homogeneous lipid compartment.

Stacked monolayer model

The intercellular lipid matrix has a layered molecular arrangement with the alkyl chains of ceramides splayed in opposite directions and interdigitating with neighbouring layers and cholesterol distributed nonrandomly between layers (Swartzendruber et al 1989). This model offered an explanation for the successive lucent and dense bands observed in transmission electron microscopy of the stratum corneum.

Domain mosaic model

The lipids are segregated into crystalline/gel domains bordered by lipids in a fluid state. The crystalline areas are effectively impermeable and the fluid zones offer channels through which molecules can diffuse resulting in tortuous diffusion pathways through the lipids. This model offered an explanation for the maintenance of barrier function in spite of mechanical stress on the skin. It proposed that structural transformations of the lipid organization due to permeation promoters could take place without structural changes in the bulk organization of lipids. It also offered an explanation for the maintenance of corneocyte hydration by water diffusion towards the stratum corneum surface from deeper skin layers while controlling water loss (Forslind 1994).

Laminglex model

Successive crystalline and liquid crystalline layers form a laminglex-like arrangement. The crystalline layers are monolayers formed by ceramides in the splayed chain formation, similar to the stacked monolayer model,

with sphingosine (a product of ceramide metabolism by ceramidase) and fatty acids forming orthorhombic hydrocarbon chain matrices. The remaining cholesterol-rich lipids form liquid layers in direct contact with the crystalline monolayers. This model offers high permeability and resistance to mechanical stress (Norlen 2003).

Sandwich model

Ceramides are arranged into orthorhombic crystalline bilayers. The ceramide head groups form dense hydrogen-bonded lattices, which form the framework of orthorhombic crystals. The two acyl chains of the individual ceramides are pointed in the same direction and are oriented in the opposite direction of the ceramides in the other layer of the bilayer. Neighboring acyl chains are mutually attracted through Van der Waals forces. Bands of fluid lipids with relatively short acyl chains mainly consisting of fatty acids and cholesterol separate the ceramide bilayers and are in contact with the non-polar ceramide acyl chains. The liquid phase lipid layer is assumed to be non-continuous (Bouwstra et al 2003). Norlen (Norlen 2003) pointed out that the model requires that polar groups found in the liquid layer lipids must be in contact with non-polar acyl chains, which he deemed to be an unlikely arrangement. However, the model offers an explanation for structural data obtained by X-ray diffraction, FT-IR and transmission electron microscopy.

Single gel-phase model

The lipid matrix is a lamellar, single gel phase without true first-order phase transitions. The cholesterol concentration is not uniform throughout the gel and lipids in areas with low cholesterol content are tightly packed, generating a gel that is crystal-like in nature, while the gel in areas with high cholesterol content is more liquid in nature. The lamellar gel phase loses cohesion and becomes progressively more perturbed towards the stratum corneum surface. Crystalline segregation and phase separation occurs close to the surface as part of the desquamation process (Norlen 2001).

Mechanisms of absorption

Dermal absorption is variable and depends on numerous factors such as:

- skin factors including site of exposure, age, skin condition, hydration and blood flow;
- environmental factors including temperature, humidity and atmospheric turnover; and

- solute factors including structure, polarity, volatility and concentration (EPA 1992).

The fate of compounds applied to the skin can include evaporation from the skin surface, partitioning into the stratum corneum followed by reversible or irreversible binding, penetration to the viable epidermis followed by metabolism or penetration to the dermis and absorption into the systemic circulation or binding to tissues such as fat. Resistance to solute movement is relatively low in the viable skin compared to the stratum corneum (EPA 1992). Active transport and facilitated transport processes are absent from the stratum corneum because the corneocytes are anucleate and keratinized and cannot produce the specialized protein structures needed for active or facilitated transport (Bouwstra et al 2003).

Solute polarity is a major determinant of the partitioning pattern observed in the stratum corneum. Highly polar compounds tend to partition almost exclusively into the protein portion of the stratum corneum, while the reverse is true for relatively non-polar solutes (Raykar et al 1988). Most compounds of interest for dermal absorption, including those included in this thesis, are relatively non-polar. Dermal absorption, for such compounds, occurs principally through the intercellular lipid matrix (Albery & Hadgraft 1979) in the absence of active or facilitated transport.

It is commonly assumed that the mechanism of transport through the stratum corneum is by a process of mass diffusion as described by Fick's First Law of diffusion (Scheuplein & Blank 1971). Adapted to dermal absorption, Fick's First Law takes the form of:

$$J = (P \cdot D \cdot C) / h$$

where P is the partitioning coefficient between the skin and the solvent, D is the diffusivity within the skin, C is the concentration gradient across the skin and h is the skin thickness.

According to Fick's Law the transfer of solutes through the stratum corneum is a first order process driven by the solute concentration gradient. P and D are assumed to be constant for a particular solute and solvent – resulting in a constant fraction of any dose absorbed per unit time irrespective of the dose concentration.

However, empirical evidence contradicts this view because the fraction of solute absorbed through skin is not

consistently constant across a range of solute concentrations in the donor solvent (Blank 1964; Billich et al 2005). Smaller fractions of the solute are absorbed over time when relatively high solute concentrations are applied to the surface of the skin. This is inconsistent with the predictions of Fick's Law. Fick's Law has been explained in molecular terms by the molecular-kinetic mechanism of Brownian motion in fluid systems (Einstein 1905; Von Smoluchowski 1906), but the assumptions needed include the presence of a dilute, homogeneous suspension; rigid, elastically colliding particles; no solvent-solute interaction and a system that tends towards equilibrium. These assumptions are not compatible with the stratum corneum (Agutter et al 2000).

It is useful to consider the solvent/stratum corneum system from the perspective of the molecular environments created by the physical-chemical properties of the various molecules in the system. The Second Law of Thermodynamics requires that systems tend towards energy equilibrium, which is achieved through energy flow from areas of higher energy to areas of lower energy. For mobile molecules in a constant-temperature system, this energy flow is achieved through molecular movement towards lower energy areas (Atkins 1994). Movements of molecules are dependent on the energy gradients in their immediate location. Gradients are due to the net force resulting from the sum of all the inter-molecular repulsive and attractive forces. These forces can be understood in terms of enthalpy and entropy.

$\text{Enthalpy} = \text{Internal energy} + (\text{Pressure} \times \text{Volume})$

Enthalpy is, therefore, directly proportional to internal energy at constant pressure and volume. Internal energy consists of the potential and kinetic energy of all the individual molecules in the system. At constant temperature (kinetic energy), enthalpy is directly proportional to the net repulsive intermolecular forces experienced by a molecule (potential energy). The intermolecular forces that contribute most significantly to molecular potential energy include charge-charge interactions, London dispersion forces and hydrogen bonds. When a molecule is at a transitional zone, such as the interface between the solvent and the skin surface, it will tend to move towards the side where it experiences relatively less repulsive forces. Enthalpy difference is thus the main driver of partitioning. Entropy, which is a measure of energy dispersal, is another important factor that determine the directional movement of molecules. Due to the spontaneous Brownian movement of mobile molecules and the increased frequency of collisions when molecular density is high, energy density is higher

when molecules are focally distributed. The flow of kinetic energy from high to low density via the mass movement of molecules causes diffusion along concentration gradients from high to low density. It should be remembered that immobile molecules, such as those bound to tissues or experimental equipment, do not participate in mass diffusion. In closed systems, equilibrium is reached when the energy is balanced between the different motive forces acting on molecules. In open systems, such as typical dermal absorption experiments using flow-through cells, equilibrium is never reached because energy gradients are maintained by the removal of solute from the dermal side of the skin.

The conceptual model used to describe the stratum corneum has important implications for the permeation rate of solute molecules via the mechanisms described above. If continuous lamellar structures are assumed, the rate of solute permeation is limited by the rates of successive partitioning from layer to layer, while molecular mobility in the individual domains is less important (Norlen 2003). If the sandwich model, for example, is assumed to be correct, the successive polar and non-polar layers will favor the permeation of molecules that are both water and lipid soluble, while highly polar or highly non-polar molecules will permeate slowly due to the partitioning resistance of opposite-polarity layers. Solutes could be expected to behave differently in other models. In the domain mosaic model, for example, solutes may be expected to move relatively rapidly by mass diffusion in the fluid areas, while movement will be limited through the crystalline areas due to the requirements of successive partitioning. The number of continuous channels through the domain mosaic then becomes the limiting factor – similar to percolation models (Stauffer 1985). Evidence of a direct link between lipid disorder and permeability have been shown, which indicates a link between stratum corneum lipid structure and permeability (Golden et al 1987). These findings support the conceptual models that present the stratum corneum lipids as ordered structures. The important role of lipid structure in the barrier function of the stratum corneum is also supported by the finding that enhancers, such as oleic acid, causes the formation of distinct areas of lipid fluidity (Ongpipattanakul et al 1991).

Evidence obtained from tape-stripping experiments indicate that the barrier properties of the stratum corneum is not uniform throughout its depth, but that the deeper layers of the stratum corneum offer more resistance to chemical penetration (Guy et al 1990).

The rate of partitioning between the stratum corneum and the viable skin can be a rate-limiting factor for highly non-polar molecules due to the more polar nature of the viable skin (Guy & Hadgraft 1988).

Although the stratum corneum lipid matrix is the most important route of dermal absorption for most chemicals, other routes such as the hair follicles, may be important for certain specialized formulations. Adapalene-loaded microspheres, for example, were shown to penetrate through the hair follicles and not the stratum corneum lipid matrix (Rolland et al 1993).

Mathematical models of dermal absorption

Mathematical constructs can be used to represent the processes of dermal absorption quantitatively, describe experimental data and predict the kinetics of dermal absorption (Roberts et al 2001). Models can be classed into two general types: quantitative structure-activity relationship (QSAR) models; and mathematical models that simulate the effects of partition and transport processes involved in absorption (Fitzpatrick et al 2004).

QSAR models are obtained by regression analyses of physical-chemical properties of permeants, solvents and chemical mixtures with steady-state permeability constants (Potts & Guy 1995; Sartorelli et al 1998; Sartorelli et al 1999; Geinoz et al 2004; Ghafourian et al 2004; Riviere & Brooks 2005). The model proposed by (Potts & Guy 1992) established a permeability (K_p) relationship with octanol/water partitioning K_o/w and molecular weight (MW): $\log K_p = -2.7 + 0.7 \log K_o/w - 0.0061 \text{ MW}$. This model has been widely recognized as a useful indicator of dermal absorption potential in spite of its modest correlation with permeability ($R^2 = 0.67$). This can partly be attributed to the convenience of using simple, easily obtainable physical-chemical parameters in the regression (Howes et al 1996). The QSAR approach is hampered by the scarcity of high quality, comparable absorption data (Fitzpatrick et al 2004). Steady-state permeability also does not predict absorption over time frames outside the steady-state portion of the absorption/time curve. Another drawback is that regressions obtained from experimental data generated using a single solvent do not predict permeability from different solvents or solvent mixtures. However, recent work that included solvent mixture factors in the regression may overcome this problem (Riviere & Brooks 2005).

Models that simulate the effects of chemical partitioning into skin and the transport across skin over time vary in their degree of correlation with skin physiology and anatomy (McCarley & Bunge 2001; Roberts et al 2001). At the one extreme are data-based models that describe the aggregate result of all the processes involved in determining the flux/time curve of dermal absorption. It uses single or multiple compartments and first order rate equations chosen to optimally describe experimental data, with no physiological relevance or implied fidelity to anatomical structure or physiology. Such models cannot be used to extrapolate beyond the experimental range of doses, conditions and species used in generating the experimental data (Krishnan & Andersen 1994; McCarley & Bunge 2001).

At the other extreme are detailed mechanistic physiological-based pharmacokinetic (PBPK) models that are derived from attempts at complete mathematical descriptions of body compartments, tissues and processes of partitioning, solute transfer and metabolism that influence the dermal distribution, absorption and elimination of drugs (Krishnan & Andersen 1994). The effects of changes in skin physiology and structure, environmental change, altered solvent systems, rates of metabolism and skin pathology may be investigated using such models. Parameters can also be scaled to reflect different dose ranges, pathological changes and species, breed, polymorphic and life-stage differences. The advantages of PBPK models are, however, difficult to realize because the necessary anatomical and physiological parameters are often not available and the processes are not well understood. The inclusion of uncertain parameters restricts degrees of freedom. Ideal, complete PBPK models are not possible as long as complete knowledge of the modeled system is not available. Most PBPK models are, therefore, simplified representations of reality, based on assumptions regarding the most important processes and structures that determine solute kinetics. In its simplest form PBPK models represent the skin as two well-stirred compartments representing polar and non-polar skin layers (McCarley & Bunge 2001). However, highly detailed, multiple compartment models have also been developed (Williams et al 1990; Williams & Riviere 1995), as well as other models of varying complexity (McCarley & Bunge 2001; Roberts et al 2001). Assuming well-stirred compartments is a common practice. It allows the use of first-order, ordinary differential equations describing average concentration over time, instead of the second-order, partial

differential equations required for describing concentration change in both space and time (McCarley & Bunge 2001).

Mathematical models can also be classified according to their association with various conditions of dermal absorption. Each set of conditions requires its own set of assumptions to construct practical mathematical models – leading to a large number of mathematically distinct models. Choosing a specific model depends on the experimental conditions that the modeler intends to simulate. An example is a classification by Roberts and coworkers (Roberts et al 2001) of *in vitro* skin permeability models based on assuming different numbers of compartments, the presence or absence of metabolism, evaporation, adsorption and shunt transport, solute-vehicle, vehicle-skin and solute-skin interactions, and combinations of infinite or finite donor concentration conditions, infinite or finite receptor sink conditions and removal of the donor phase after reaching steady state.

Assessment of dermal absorption

Data showing the amount of chemicals/drugs absorbed through the skin are required by regulatory agencies for risk assessments and therapeutic assessments. It is also crucial to the development of topically applied drugs. The specific requirements of different regulatory agencies vary between precise requirements, such as the requirement of *in vivo* rat studies for pesticides by the USEPA, to relatively vague statements regarding toxicokinetic data. Irrespective of the specific requirements set by regulatory agencies, pressure on industry to reduce the number of animals used in testing is increasing and there is a growing need for alternative *in vitro* dermal absorption assessment methods (Howes et al 1996).

Dermal absorption can be characterized best if studied under conditions similar to those under which exposure is expected - in the target species, *in vivo* and using highly sensitive and accurate analytical methods. Reaching such a high standard is, however, rarely possible due to the ethical concerns associated with *in vivo* testing and the limitations of resources and analytical methods. Practical alternatives are used to indicate what could be expected under ideal testing conditions. Alternative methods can be classified according to the level of confidence that can typically be achieved as a predictor for *in vivo* exposure scenarios. Methods ordered according to their levels of confidence, from low to high, are as follows: mathematical models, model

membranes, stratum corneum, dermatome slices, non-viable whole skin, viable whole skin, perfused skin and *in vivo* (Howes et al 1996). The species used to predict dermal absorption in the target species, which is most commonly the human, can also be classified according to the level of confidence typically expected. Commonly used species, ordered from low to high confidence, are: mouse, rabbit, rat, guinea pig, pig, primate and human (Howes et al 1996).

Experiments that allow the calculation of permeability constants (K_p) are often favored because K_p values are generally the most important basis of assessing dermal absorption for regulatory purposes (EPA 1992; Howes et al 1996; OECD 2000). The variety of possible exposure scenarios are limitless and the use of any single, standard method of assessing dermal absorption is inadequate for predicting absorption under very dissimilar conditions. One of the key challenges facing regulators is to judge the appropriateness of K_p values obtained from a variety of experimental sources for a particular exposure scenario of interest (EPA 1992).

In vivo methods

The preservation of complete physiological processes makes *in vivo* methods physiologically more relevant than *in vitro* methods. Direct estimates of absorption are, however, not possible and it can be more challenging to establish K_p values (EPA 1992).

Common techniques for quantifying *in vivo* absorption include (EPA 1992):

- Detection of radioactivity, parent compound or metabolite levels in excreta, blood, plasma and other tissues
- Quantification of compound disappearance from the skin surface or the donor solution
- Biological response estimates
- Tape-stripping

In vitro methods

Extrapolation of dermal absorption results obtained by *in vitro* methods to *in vivo* exposure scenarios can be challenging. *In vitro* methods are, however, the mainstay of dermal absorption assessment. It is more suited to

the estimation of K_p values and lends itself better to relatively simple, high throughput, cost-effective testing. It also offers better control of experimental conditions, direct sampling from under the skin and the opportunity to test the dermal absorption of highly toxic compounds in human skin (EPA 1992).

In vitro methods include (EPA 1992):

- Diffusion cells
- Perfused skin
- Powdered stratum corneum binding

Diffusion cells will be considered in more detail. It is the most common method used for the estimation of K_p values and was utilized in studies for this thesis. Diffusion cells have some distinct advantages. It is relatively simple and cheap, while still producing results that are relevant to *in vivo* absorption. Sample collection can be automated, the receptor fluid can be manipulated to improve solute partitioning and skin nutrients can be replenished via the receptor fluid to maintain skin viability (Bronaugh & Stewart 1985).

The validity of results obtained from diffusion cell studies to *in vivo* exposure scenarios are dependent on three assumptions: *in vitro* and *in vivo* skin surface conditions are similar; the dermis does not affect absorption; and *in vivo* physiological processes does not affect absorption (EPA 1992). These assumptions should be considered when specific diffusion cell techniques are selected.

Early studies commonly used side-by-side diffusion chambers. A major disadvantage of side-by-side diffusion cells is the occlusion of the skin between two cells containing donor and receptor fluid. This leads to skin hydration and altered skin surface conditions (EPA 1992).

Non-occluded, single-chambered cells open to the atmosphere reduce the problem of stratum corneum hydration and allows for the evaporation of volatile compounds, which more closely resembles most *in vivo* scenarios (Franz 1975). This type of diffusion cell has become the most commonly used system in dermal

absorption studies. The receptor fluid may be static, sampled at intervals and replaced with equal volumes of fresh receptor fluid (Franz 1975) or constantly flowing and collected into vials (Bronaugh et al 1982).

Steady-state flux is desirable for the optimal estimation of K_p values. K_p values can also be derived from pseudo-steady-state conditions, but requires gradient estimates to be made based on fewer data points and it is therefore associated with more uncertainty. Unless a large excess dose is applied to simulate infinite dose conditions, steady-state flux cannot be achieved. Solvent evaporation can cause super saturation of the solute, which affects the transdermal concentration gradient. This can be relevant to *in vivo* exposure scenarios, but should be differentiated from absorption under unsaturated conditions. The problem can be avoided by using relatively large donor solvent volumes. However, using large volumes of an aqueous solution can cause skin hydration (Bronaugh & Stewart 1985).

Using a saline solution as the receptor fluid may be appropriate for hydrophilic compounds, but lipophilic compounds require the use of a receptor fluid that more closely resembles the lipophilic properties of blood. Blood-resembling receptor fluids also help to maintain skin viability (EPA 1992). Low volumes of receptor fluid can also be a limiting factor when compounds with low rates of partitioning into the receptor fluid are tested. This problem can be reduced by using continuous flow diffusion cells (Wester et al 1985).

Compounds penetrating the *in vivo* skin does not traverse the whole thickness of the dermis to be absorbed systemically, but are taken up by capillaries at a depth of c. 200 μm . Using full-thickness skin in diffusion cells, however, requires penetration through the full thickness of the dermis to reach the receptor fluid. This problem can be lessened by using dermatomed, split-thickness skin resembling the effective thickness of *in vivo* skin (EPA 1992).

Maintaining the viability of the skin may be important if the metabolic capacity of the skin is a significant factor in the absorption of a compound. Although the use of fresh skin is assumed to be associated with better predictability for *in vivo* exposure (EPA 1992), freezing of the skin, which affects skin viability, did not affect permeation of a variety of compounds (Bronaugh et al 1986; Delterzo et al 1986).

Analytical techniques

Fourier transformed infrared spectroscopy (FT-IR)

FT-IR is a spectroscopy technique that can be used to detect information on molecular identity and structure relatively rapidly and economically. It can be used to study translucent solids, liquids and films by IR transmission, or opaque substances by IR reflection. Poly-atomic molecules with N molecules generally have $3N-6$ distinct vibrations. Each vibration is associated with a set of quantum states related to the kinetic energy of the vibration. Electro-magnetic waves in the infrared (IR) range interact with molecular vibrations that involve changes in dipole moments during vibration (dipole moment = charge $\times$ distance). It causes the energy of the IR wave to be absorbed and the vibration to assume a higher quantum state. Energy absorption from a spectrum of IR waves traveling through or reflecting off a substance results in a pattern of energy absorption that is related to the functional groups and the spatial arrangement of molecules in the substance. By applying Fourier-transformations to the resulting wave patterns the frequencies and amplitudes of energy absorption can be visually inspected (Parker 1983; Franck et al 1998). The stratum corneum lends itself to FT-IR techniques because of low water content and the ordered character of the stratum corneum lipids (Ongpipattanakul et al 1994).

The IR wavelengths of most relevance to FT-IR studies of lipid bilayers are found between the $3000\text{--}2800\text{ cm}^{-1}$ region, related to C-H₂ stretching (Parker 1983; Casal & Mantsch 1984). Lipid bilayers are associated with typical IR absorption patterns and detectable changes when bilayers are disrupted (Moore et al 1997). Increased lipid acyl chain mobility causes shifts to a higher wave number, also called “blue shift”, of peaks around 2854 cm^{-1} (CH₂ symmetric vibration) and 2925 cm^{-1} (CH₂ asymmetric vibration) (Vaddi et al 2002). These effects have been used to study temperature dependent stratum corneum lipid phase behavior (Krill et al 1992) and dermal absorption enhancer mechanisms (Yokomizo & Sagitani 1996; Jaiswal et al 1999). The absorption spectra of C-H bonds in certain solvents, such as ethanol, may overlap the absorption spectra of stratum corneum lipids and should be considered when absorption patterns are analyzed. Lipid-induced IR absorption in other regions of the IR spectrum, such as IR absorption due to COOH in the 1740 cm^{-1} region, can aid in the

differentiation between lipid and solvent spectra (Van der Merwe & Riviere 2005). Absorption spectra associated with organic solutes, such as jet fuel components, may also overlap and mask lipid absorption spectra (Muhammad et al 2005).

Liquid scintillation counting

Liquid scintillation counting is a method for quantifying the concentration of β -particle emitting nuclides in a liquid medium. β -Particle emitting nuclides include H^3 , C^{14} , S^{35} and P^{32} . Target molecules are labeled with β -particle emitting nuclides. Samples are placed in clear glass vials and suspended or dissolved in a scintillation liquid that contains solvents and fluors. Solvent molecules absorb energy from β -particles and transfer the energy to fluor molecules, which release the energy in the form of light. Scintillation counters detect light emissions through highly sensitive, photomultiplier-based detectors. Counting efficiency depends on the type of nuclides used and on the composition of the scintillation liquid. Efficiency of less than 100 % is known as “quenching” and requires the use of correction factors (Polach 1992).

C^{14} is the most commonly used nuclide in organic molecules. It has a physical half-life of 5730 years, a biological half-life of 12 days and an effective half-life of 12 days (bound) and 40 days (unbound). The specific activity is 4.46 Ci/g. It has relatively low radiation energy levels of 156 keV maximum and 49 keV average. Its penetration potential is 24 cm in air, 0.28 mm in water/tissue and 0.25 mm in plastic. The radiation hazards posed by C^{14} can be controlled relatively easily. About 1 % of C^{14} derived β -particles is typically transmitted through intact stratum corneum. Fat is considered to be the tissue most vulnerable to adverse effects due to possible accumulation of labeled compounds. C^{14} does not normally present an external radiation hazard, but can be hazardous when internalized. Safe handling requirements therefore include control of exposure via breathing, eating, drinking, open wounds or skin contact (USNRC 1996; NCHPS 2005).

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**3. COMPARATIVE STUDIES ON THE EFFECTS OF WATER, ETHANOL AND
WATER/ETHANOL MIXTURES ON CHEMICAL PARTITIONING INTO PORCINE STRATUM
CORNEUM AND SILASTIC MEMBRANE**

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ABSTRACT

The effects of water and ethanol vehicles on stratum corneum and silastic membrane partitioning of 11 industrial and agricultural compounds were studied to aid in characterizing and assessing risk from skin exposure. Zero percent, 50% and 100% aqueous ethanol solutions were used as solvents for ^{14}C labeled phenol, 4-nitrophenol, pentachlorophenol, dimethyl parathion, parathion, chloropyrifos, fenthion, triazine, atrazine, simazine and propazine. Compound partitioning between the solvents and porcine stratum corneum/silastic membrane were estimated. Stratum corneum was exposed to aqueous ethanol ranging from 0% to 100% v/v ethanol in 20% increments and Fourier transform infrared spectroscopy (FT-IR) was used to obtain an index of lipid disorder. Gravimetry and FT-IR were used to demonstrate lipid extraction in aqueous ethanol solutions. Partitioning patterns in silastic membranes resembled those in stratum corneum and were correlated with octanol/water partitioning. Partitioning was highest in water and was higher from 50% ethanol than from 100% ethanol, except for parathion, 4-nitrophenol, atrazine and propazine. Correlation existed between molecular weight and partitioning in water, but not in ethanol and ethanol/water mixtures. Lipid order, as reflected in FT-IR spectra, was not altered. These studies suggest that stratum corneum partitioning of the compounds tested is primarily determined by relative compound solubility between the stratum corneum lipids and the donor solvent. Linear relationships existed between octanol/water partitioning and stratum corneum partitioning. Partitioning was also correlated with molecular weight in water solvent systems, but not in ethanol and ethanol/water mixtures. Ethanol and ethanol/water mixtures altered the stratum corneum through lipid extraction, rather than through disruption of lipid order.

Keywords: Dermal absorption; Partitioning; Chemical mixtures

INTRODUCTION

Skin exposure to potentially toxic industrial and agricultural chemicals is a common occurrence. Predicting the rate at which chemicals move across skin is important for the assessment of risk associated with skin exposure to these compounds. Most toxicological risk assessment studies assess dermal absorption after neat chemical exposure, yet environmental and occupational exposures are to chemicals in solvents.

Estimating skin permeability depends on adequate description and understanding of the processes that influence the barrier properties of skin. The stratum corneum is the primary barrier to exogenous chemical absorption and water loss through mammalian skin. It consists of layers of tightly packed, flattened, keratin-enriched, anucleate corneocytes, embedded in an intercellular lipid matrix (Bouwstra et al 2003). The main constituents of the lipid matrix are long chain ceramides, fatty acids, cholesterol and triglycerides (Monteiro-Riviere et al 2001). These lipids form long lamellae parallel to the corneocyte surfaces. Inside the lamellae, at physiological temperatures, the lipids are arranged in bilayers consisting of ordered, crystalline phases on both sides of a narrow, central band of fluid lipids (Monteiro-Riviere 1986; Bouwstra et al 2003). Partitioning into corneocytes is dependent on the lipophilicity of the compound. More hydrophilic compounds tend to partition into the corneocytes proteins, while more lipophilic compounds tend to partition into the stratum corneum lipids (Raykar et al 1988). The intercellular lipid matrix is therefore the main route for the passage of lipophilic exogenous chemicals through intact stratum corneum (Albery & Hadgraft 1979).

The rate of chemical absorption through the skin is primarily determined by two factors: partitioning into the stratum corneum and its resistance to diffusion (Scheuplein & Blank 1971). If Fickian diffusion is assumed, permeability should be proportional to diffusivity and partitioning into the stratum corneum lipids. Also, if diffusivity and partitioning can be predicted, it follows that permeability should be predictable. The prediction of partitioning and diffusivity is, however, problematic. The complexity of skin structure, variability in skin lipid composition and thickness, changes in skin induced by solvents and permeants and the effects of metabolizing enzymes on permeants with the possible occurrence of finite capacity processes, makes it unlikely that skin permeability can be accurately predicted based only on permeant and solvent physical-chemical data. Added complexity due to the practically limitless solvent systems that can be encountered causes prediction of

skin permeability and its use in risk assessment to be laden with uncertainty. That said, the use of large sets of empirical data does offer the potential for identifying characteristics of permeants and solvent systems that show consistent effects across a wide range of experimental conditions. This approach has been used with some success to predict skin permeability in relatively simple systems based on quantifiable molecular characteristics, such as molecular size (weight and volume), octanol/water partition coefficients, H-bonding capacity and electric charge (Potts & Guy 1992). These physico-chemical parameters are predictive of absorption due to their influence on partitioning and diffusivity. However, solvents may change the partitioning and diffusion behavior of compounds in stratum corneum depending on the physico-chemical properties of the solvent and solvent effects on the stratum corneum (Raykar et al 1988; Kai et al 1990; Rosado et al 2003). Solvent induced changes in the stratum corneum could also change its resistance to diffusion. Empirical characterization of the effects of commonly encountered solvents on the stratum corneum could improve the prediction of skin absorption in many real-world scenarios.

Water and ethanol are commonly used solvents in the chemical and pharmaceutical industries. Increased permeant flux has been demonstrated using a variety of permeants, methods and skin models using aqueous ethanol solvents in the 40–70% (v/v) range, compared to higher and lower ethanol concentrations (Berner et al 1989; Kurihara-Bergstrom et al 1990; Megrab et al 1995; Kim et al 1996; Levang et al 1999; Panchagnula et al 2001). The suggested mechanisms by which ethanol affects stratum corneum permeability include lipid extraction, increased lipid fluidity, effects on the putative pore pathway, enhanced drug solubility in stratum corneum lipids, changes in stratum corneum hydration, altered keratinized protein and permeant-ethanol copermeation. Pretreatment of human stratum corneum to extract lipids have been demonstrated to influence stratum corneum partitioning of lipophilic compounds from an aqueous donor solution (Raykar et al 1988). The effects of ethanol pretreatment on the barrier properties of hairless mouse skin to nicotinamide in an aqueous solution indicated that lipid extraction compromised the stratum corneum barrier (Kai et al 1990). This study builds on previous work investigating chemical partitioning into the stratum corneum from water, ethanol and water/ethanol mixtures by extending the range of model compounds to those with toxicological significance and by determining the effects on stratum corneum/solvent partitioning using ethanol, water and ethanol/water

mixtures as donor solvents, not as pretreatments. Partitioning patterns and solvent effects on stratum corneum lipid order and lipid extraction were used to illuminate the processes that determine the rate of skin absorption.

MATERIALS AND METHODS

Chemicals

CCl₄ and C-14 radio labeled phenol, 4-nitrophenol, pentachlorophenol, dimethyl parathion, parathion, atrazine, and simazine were obtained from Sigma (St. Louis, MO). Chlorpyrifos, fenthion, triazine (1,3,5-triethylhexahydro-1,3,5-triazine; also called 1,3,5-triethylhexahydro-s-triazine) and propazine were obtained from American Radiolabeled Chemicals (St. Louis, MO). The purity ranged from 95% to 99.5% and the radioactivity ranged from 9 to 76.6 mCi/mmol. All C-14 radio labels were situated in the ring structure of the molecules. Pure ethanol was obtained from Aaper Alcohol and Chemical Co. (Shelbville, KY).

Stratum corneum/vehicle and silastic membrane/vehicle partition coefficient determination

Stratum corneum/vehicle and silastic membrane/vehicle partition coefficients were estimated according to methods previously described (Baynes 2000). In short, stratum corneum and epidermis layers were removed from abdominal skin of female weanling Yorkshire pigs after heat treatment and then immersed in 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) for 24 h. The stratum corneum was then dried in a Fisherbrand Dessicator Cabinet (Fisher Scientific, Pittsburgh, PA) with DrieriteTM anhydrous calcium sulfate (WA Hammond Drierite Company, Xenia, Ohio), weighed (5–8 mg per sample) using a Mettler AE 200 scale (Mettler Toledo, Columbus, OH) and placed in vials. Three ml of the solvents with 100 μ g radio labeled compound was added to the stratum corneum sample vial ($n=5$), and capped. After 24 h, the stratum corneum sample was removed and gently blotted on KimwipeTM to remove excess solution. Two hundred and fifty μ l of the vehicle was removed, by pipetting from the center of the fluid mass, for direct counts using Ecolume (ICN Costa Mesa, CA). For determination of radio labeled compound in the stratum corneum, stratum corneum samples were combusted in a Packard Model 306 Tissue Oxidizer (Packard Chemical Co., Downers Grove, IL) and then analyzed using a Packard Model 1900TR Liquid Scintillation Counter (Packard Chemical Co., Downers Grove, IL). The same method was used to estimate silastic membrane/vehicle partitioning. Biomedical

grade silastic membrane was obtained from Dow Corning Corporation (Hemlock, MI). The research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

FT-IR

FT-IR transmission spectra were obtained using a Perkin–Elmer Spectrum 1000 FT-IR spectrometer (Perkin–Elmer Inc., Wellesley, MA). Dried stratum corneum was prepared as for stratum corneum/vehicle partition coefficient determination. Circular stratum corneum samples (c. 32 mm diameter) were cut from a single sheet of stratum corneum for each experiment ($n=3$). The stratum corneum samples were immersed in 10 ml of solvent for 12 h in closed 20 ml vials. The solvents used were pure water, aqueous ethanol in 10% increments of ethanol v/v and pure ethanol. Treated samples were gently blotted on Kimwipe™ to remove excess solution. Samples were then suspended in a demountable FT-IR liquid cell (Pike Technologies, Madison, WI) for determination of transmission spectra. Samples were then exposed to a desiccating atmosphere at room temperature for 24 h and the transmission spectra of the dried samples were determined.

Lipid extraction

Dried stratum corneum was prepared as for stratum corneum/vehicle partition coefficient determination. Stratum corneum samples ($n=4$), within a weight range of 40–60 mg, were weighed at the start of the procedure using a Mettler AE 200 scale (Mettler Toledo, Columbus, OH) and placed in closed vials with 10 ml water, 50% aqueous ethanol (v/v) or 100% ethanol. The stratum corneum samples were removed after 24 h, gently blotted on Kimwipe™. The samples were then placed in a desiccating atmosphere for 24 h and weighed again. The solvents were evaporated at 50 °C using nitrogen gas in a Zymark TurboVap evaporator (Zymark Corporation, Hopkinton, MA) and the precipitate was redissolved in 0.5 ml CCl₄. A thin film of precipitate was prepared by spreading a drop of the redissolved precipitate solution on the surface of a KBr crystal (Pike Technologies, Madison, WI) and allowing the CCl₄ to evaporate at room temperature. FT-IR transmission spectra were obtained using a Perkin–Elmer Spectrum 1000 FT-IR spectrometer (Perkin–Elmer Inc., Wellesley, MA).

Data processing and statistical analysis

For partition coefficient determinations, radioactivity content in the vehicle mixture and stratum corneum were normalized to 1000 mg vehicle (C_{vehicle}) and 1000 mg stratum corneum ($C_{\text{stratum corneum}}$), respectively. The log stratum corneum/vehicle partition coefficient was determined from the equation: $\log PC = \log C_{\text{stratum corneum}} / C_{\text{vehicle}}$. Standard errors were determined for all data sets. Log K octanol/water values were obtained from the literature (Howard & Meylan 1997), except for triazine. No published log K octanol/water values for triazine were available. The SPARC On-Line Calculator (2003 version; available at: <http://ibmcl2.chem.uga.edu/sparc/>) was used to calculate an estimated log K octanol/water for triazine.

FT-IR peaks at 2955 cm^{-1} were used to indicate absorbance due to asymmetric vibration associated with CH_3 functional groups. Peaks around 2854 cm^{-1} indicated CH_2 symmetric vibration and 2925 cm^{-1} indicated CH_2 asymmetric vibration (Parker 1983). "Blue-shift" of these peaks to higher wave numbers were used to indicate decreased lipid order. Peaks around 1740 cm^{-1} indicated absorbance due to $\text{C}=\text{O}$ bonds associated with COOH functional groups (Raykar et al 1988).

RESULTS

Partitioning

Partitioning into the stratum corneum was highest across all compounds when water was used as a solvent (Figure 3.1; Table 3.1). Partitioning from 50% ethanol was higher than from 100% ethanol, except for 4-nitrophenol, parathion, atrazine and propazine, which did not show any significant difference between 50% and 100% ethanol. Stratum corneum partitioning was correlated with octanol/water partitioning (Figure 3.2). Divergence between octanol/water partitioning and stratum corneum/solvent partitioning became wider in 50% and 100% ethanol as the octanol/water partitioning coefficient increased. The decreased slope of the regression line in 50% and 100% ethanol illustrates this effect. Significant correlation was observed between molecular weight and stratum corneum partitioning when water was used as a solvent (Figure 3.3), but correlation was poor when 50% or 100% ethanol was used.

Lipid order and extraction

FT-IR did not reveal consistent, significant V_aCH_2 or V_sCH_2 peak shifting (Figure 3.4). Gravimetry revealed increasing loss of stratum corneum mass after treatment with 50% and 100% aqueous ethanol solutions and no significant change in mass after treatment with water (Figure 3.5). The FT-IR spectra, obtained from the precipitates of the extracts, indicated that the extracted material predominantly consisted of the lipid fraction of the stratum corneum (Figure 3.6). Cellular debris were observed in the extracts using light microscopy, indicating that some of the loss in mass was caused by loss of material other than lipids. Infrared absorption in the 1740 cm^{-1} region, associated with COOH groups, was lowered in a concentration dependent manner (Figure 3.7). It is consistent with loss of lipid-associated COOH groups. Dried aqueous ethanol treated samples also revealed concentration dependent lowering of the V_aCH_2 and V_sCH_2 peaks, which also is consistent with loss of lipids from the stratum corneum (Figure 3.8). Water did not extract observable amounts of lipid.

The presence of ethanol in stratum corneum was detected through its influence on infrared absorption in the 2955 cm^{-1} region associated with CH_3 groups (Figure 3.8). The enhanced CH_3 associated absorption was absent after drying the samples for 24 h, indicating that ethanol was not present in the samples in significant quantities after 24 h.

DISCUSSION

Dermal absorption and stratum corneum partitioning studies from water are traditionally used to predict risk from skin exposure. Many exposures, however, are from chemical mixtures rather than from pure water. Investigations of the effects of mixed solvents on processes that determine absorption are therefore relevant to the appropriate use of data from studies using aqueous solvents analyzing risk from mixture exposure.

Triazine (1,3,5-triethylhexahydro-1,3,5-triazine; CAS# 7779-27-3) was used as an industrial biocide to control bacteria and fungi in adhesives, fuels, oil storage tanks, cutting fluids, paints, slurries, rubber products and industrial processing chemicals. Although the product had been registered as a biocide since 1967, Data-Call-Inn notices were issued in 1987 and 1992. Reregistration is dependent on additional toxicity and ecological effects data (United States Environmental Protection Agency 1997). Information on triazine's physical

properties is largely absent from the available scientific literature. The $\log K$ octanol/water value used in this study should be interpreted with caution, since the accuracy of the calculation was not be validated experimentally. Studies on the physical properties of this compound are needed to assist in the interpretation of data for risk analysis procedures.

Partitioning between a donor solvent and a membrane is a function of the relative solubility of a compound in the donor solution and the membrane. The observed partitioning pattern therefore reflects changes in the stratum corneum that alter its solvation properties relative to the donor solvent. The higher partition coefficients obtained in water, compared to pure ethanol and aqueous ethanol, can be explained by the fact that most of the tested compounds are relatively non-polar with $\log$ octanol/water partition coefficient values ranging from 1.46 to 5.12, with the exception of triazine, which is hydrophilic. Ethanol consists of a short two-carbon chain, which confers on it the ability to interact with and dissolve relatively non-polar molecules; and a hydroxyl group, which gives it the ability to interact with relatively polar molecules and to form hydrogen bonds. It therefore has the ability to act as a solvent for organic molecules with a wide range of octanol/water partition coefficients. In contrast, water is not an effective solvent for relatively non-polar organic molecules.

The range of stratum corneum/ethanol partition coefficients was much narrower than the range seen with $\log$ stratum corneum/water. The influence of partitioning on absorption rate suggests that the expected range of absorption rates of compounds from ethanol will be narrower than the absorption rates from water. The increasing divergence observed between $\log$ octanol/water partitioning coefficient and $\log$ stratum corneum/solvent partitioning coefficient in 50% and 100% ethanol as the $\log$ octanol/water partitioning coefficient increased is therefore consistent with the hypothesis that $\log$ stratum corneum/solvent partitioning coefficient is determined by the relative solubility of the solute in the solvent and in the stratum corneum. Divergence also occurred between pure ethanol and aqueous ethanol, as expected. However, the effect was inconsistent exceptions were *p*-nitrophenol, atrazine, propazine and parathion. The $\log$ octanol/water partitioning coefficient range of these compounds are between 1.9 and 4. Compounds outside this range exhibited the expected divergence. It is not clear that the effect is dependent on $\log$ octanol/water partitioning coefficient. It may be related to specific interactions between functional groups on the compounds and stratum corneum components. More compounds across a wide range of partitioning behavior should be studied to

determine the consistency of the effect. Pentachlorophenol exhibited a lower partitioning into stratum corneum and silastic from water than predicted from its $\log K_{o/w}$ value. The pK_a of pentachlorophenol is 4.7, which causes ionization of a significant proportion of the molecules when in a solvent with a pH close to neutral. Polarization increases water solubility thereby lowering stratum corneum/water partitioning.

The stratum corneum has low water content and stratum corneum lipids are ordered into bilayers. These characteristics contribute to the suitability of FT-IR as a useful technique for studying stratum corneum lipid structure (Ongpipattanakul et al 1994). FTIR spectra of lipid bilayers show typical absorption patterns in the 3000–2800 cm^{-1} region that are related to C–H₂ stretching (Parker 1983). Disruption of lipid bilayer structures leads to identifiable changes in the absorption spectra of infrared light passed through, or reflected off, the stratum corneum (Moore et al 1997). This has been used successfully to study the temperature dependent phase behavior of skin lipids (Krill et al 1992) and the effect of chemical absorption promoters on the lipid bilayer structure (Yokomizo & Sagitani 1996; Jaiswal et al 1999). Shifts to a higher wave number of peaks around 2854 cm^{-1} (CH₂ symmetric vibration) and 2925 cm^{-1} (CH₂ asymmetric vibration) indicates decreased order in a lipid membrane due to changes in the mobility of lipid acyl chains (Vaddi et al 2002). In the present study, high variability was observed between stratum corneum samples. This is due to variability in lipid composition and stratum corneum thickness. However, the pattern of solvent induced change was consistent when individual samples were compared before and after treatment. Ethanol, due to its C–H groups, also absorbs IR energy in the 3000–2800 cm^{-1} region. This may confound estimates of peak change due to changes in lipid order. However, ethanol adds to the FT-IR absorbance in the 2955 cm^{-1} region due to the presence of CH₃ groups (Parker 1983). This effect is easily detectable in samples tested directly after removal from ethanol, while it is absent from samples exposed to the atmosphere for 24 h (Figure 3.8). It suggests that ethanol did not contribute significantly to FT-IR spectra obtained after 24 h. The expected "blue-shift" of FT-IR absorbance peaks in the 3000–2800 cm^{-1} region due to reduced lipid order after ethanol treatment was not found (Figure 3.4). This may be explained by lipid extraction or loss of lipid disorder with ethanol evaporation. Additionally, the extracted portion of the stratum corneum, which would show most evidence of disorder, does not contribute to the FT-IR absorption obtained after treatment. This finding agrees with results previously reported in the literature (Kai et al 1990).

The ability of ethanol to extract lipids from the stratum corneum has been described previously (Sugibayashi et al 1992; Levang et al 1999). We demonstrated lipid extraction under the same conditions as those used to determine stratum corneum/solvent partitioning using gravimetry in combination with FT-IR (Figure 3.5, Figure 3.6 and Figure 3.7). Lipid extraction results in lowered IR absorbance due to C-H₂ groups in the 3000–2800 cm⁻¹ region as well as lowered absorbance due to COOH in the 1740 cm⁻¹ region. Although the conditions under which lipid extraction was demonstrated is unlikely to be encountered in topical drug formulations, it indicates a substantial potential for lipid extraction under conditions of excessive skin exposure to ethanol, which may occur in an occupational exposure scenario. Lipid extraction from the stratum corneum results in a lowered mass of lipids within the membrane and a smaller capacity for accepting lipophilic molecules from the donor solution, thereby contributing to lowered partitioning into the stratum corneum. Lipid extraction could also change the lipid composition and solvation properties of the stratum corneum lipids. Stratum corneum samples were dehydrated through storage in a desiccating atmosphere before estimation of control sample weights. However, the hygroscopic nature of pure ethanol could cause further dehydration of the stratum corneum. Lipid extraction, loss of cellular debris and dehydration therefore contributed to the loss of stratum corneum mass after ethanol treatment. It should also be noted that an exposure time of 24 h is likely to show extreme solvent effects, which may differ from solvent effects associated with shorter exposure times. However, initial investigation did not show significant difference between 12 and 24 h changes in FT-IR spectra. More work on lipid extraction, as previously done with other solvents (Monteiro-Riviere et al 2001), is needed to determine the rate and extent of extraction of different types of lipids.

Significant correlation between molecular weight and partitioning into stratum corneum when water was used as a solvent was observed. This may be explained by the relative lipophilic nature of the compounds tested. It has been theorized that increased molecular volume, associated with increased molecular weight, increases the hydrophobic surface area associated with larger molecules and therefore partitioning into stratum corneum (Potts & Guy 1995). Larger molecules are associated with stronger London dispersion forces than smaller molecules due to larger molecular surfaces available for interaction with neighboring molecules, which accounts for the generally higher lipophilicity of larger molecules (Streitwieser 1992). In water, which is strongly lipophobic, increased molecular weight would therefore explain the increased partitioning into the

relatively lipophilic environment within the stratum corneum lipids as molecular weight increases. The correlation of partitioning with molecular weight is, therefore, a reflection of the influence of molecular weight on lipophilicity and not due to a direct causal relationship between partitioning and molecular weight. This hypothesis is in agreement with previous studies showing that lipophilicity drives partitioning behavior in lipid bilayers (Xiang & Anderson 1994). It is also in agreement with the theorized influence of molecular size on partitioning behavior based on scaled-particle theory (Mitragotri et al 1999), which predicts that more energy is required for large molecules to partition into lipid bilayers. Since the size of large molecules tends to reduce partitioning into lipid bilayers, correlation between molecular weight and partitioning may not be present for compounds outside the molecular weight range of the compounds used in this study. Lower correlation between molecular weight and stratum corneum partitioning when ethanol is used as a solvent supports the previously discussed hypothesis that ethanol is a more universal solvent for organic compounds than water. The influence of molecular weight on lipophilicity therefore does not predict the solubility of organic compounds in ethanol to the extent that it does in water. Correlations of molecular size and partitioning should be interpreted with caution when used in attempts to predict permeability due to the effect of molecular size on diffusivity. Molecular size and shape influence diffusivity independent of compound lipophilicity (Xiang & Anderson 1994; Mitragotri et al 1999).

This study demonstrated the determining influence of solvents on processes that control the rate of cutaneous absorption. The predictive value of octanol/water partitioning for partitioning into stratum corneum from water is dependent on similarities between the relative solvation properties of octanol and water to stratum corneum and water. This has important implications for the use of data obtained from compound behavior in one solvent system, typically water, to predict its behavior in another solvent system. The study also suggested that the use of large data sets to identify consistent solvent influences over the behavior of a wide range of chemical permeants offers a workable approach to reduce uncertainty in the risk assessment of real-world dermal exposure to chemicals in commonly used solvents and solvent mixtures. Additional work to elucidate the mechanisms by which broadly repeatable solvent effects function will increase the utility of this approach in chemical and pharmaceutical development.

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Table 3.1. Log Koctanol/water, molecular weight and log of partitioning between the stratum corneum (sc)/silastic (si) of triazine (TRI), phenol (PHE), p-nitrophenol (PNP), simazine (SIM), atrazine (ATR), methyl parathion (MPA), propazine (PRO), ethyl parathion (EPA), fenthion (FEN), chlorpyrifos (CPY) and pentachlorophenol (PCP). Partitioning values are followed by the standard error of the mean (SEM) (n = 5).

	TRI	PHE	PNP	SIM	ATR	MPA	PRO	EPA	FEN	CPY	PCP
Log Ko/w	-0.11	1.46	1.91	2.18	2.61	2.86	2.93	3.83	4.09	4.96	5.12
Mol. Weight	171.29	94.113	139.11	201.66	215.69	263.21	229.71	291.26	278.33	350.59	266.34
Log Psc/water	0.892	1.081	1.246	1.570	1.722	1.922	1.969	2.952	3.006	3.784	2.534
SEM	0.034	0.037	0.021	0.025	0.113	0.078	0.087	0.065	0.036	0.031	0.037
Log											
Psc/ethanol+water	0.549	0.784	0.717	1.276	0.695	1.016	0.801	0.987	1.042	1.644	1.629
SEM	0.049	0.046	0.008	0.023	0.045	0.052	0.100	0.056	0.047	0.031	0.036
Log Psc/ethanol	0.337	0.663	0.741	0.880	0.883	0.840	0.905	1.090	0.800	0.960	1.004
SEM	0.077	0.048	0.059	0.008	0.045	0.054	0.064	0.037	0.058	0.066	0.094
Log Psi/water	-1.746	-0.099	-0.945	0.398	1.281	2.058	1.582	2.027	2.442	2.718	0.091
SEM	0.220	0.043	0.211	0.116	0.028	0.009	0.017	0.035	0.036	0.035	0.069
Log											
Psi/ethanol+water	-2.383	-0.876	-1.649	-0.993	-0.878	0.035	-0.665	0.524	0.470	1.221	-0.941
SEM	0.241	0.028	0.034	0.089	0.025	0.015	0.020	0.047	0.016	0.008	0.071
Log Psi/ethanol	-1.984	-1.963	-2.110	-1.783	-1.753	-1.865	-1.850	-0.906	-1.681	-1.508	-2.360
SEM	0.028	0.013	0.027	0.036	0.045	0.020	0.021	0.121	0.016	0.016	0.038

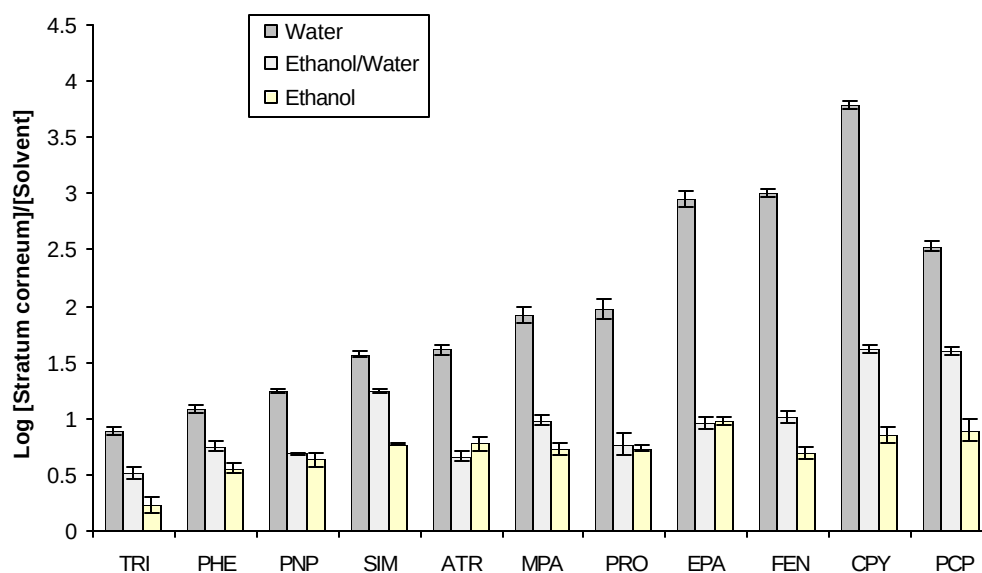


Figure 3.1. The partitioning between the stratum corneum and the solvent expressed as the log of stratum corneum/solvent concentrations of triazine (TRI), phenol (PHE), p-nitrophenol (PNP), simazine (SIM), atrazine (ATR), methyl parathion (MPA), propazine (PRO), ethyl parathion (EPA), fenthion (FEN), chlorpyrifos (CPY) and pentachlorophenol (PCP) (n = 5).

Plate 1

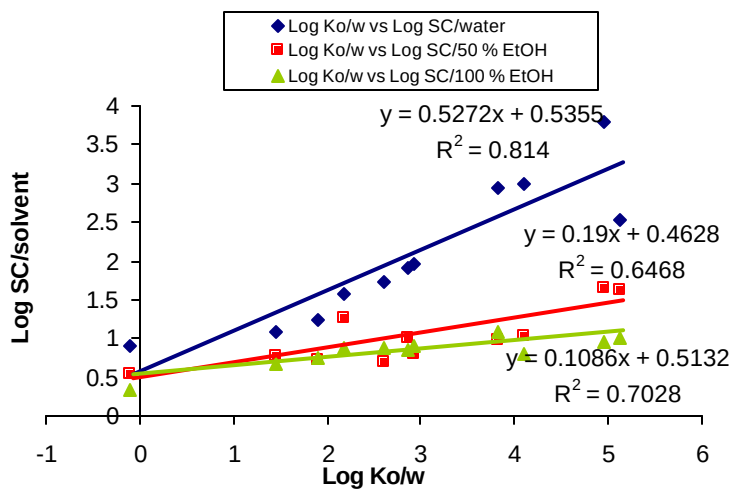


Plate 2

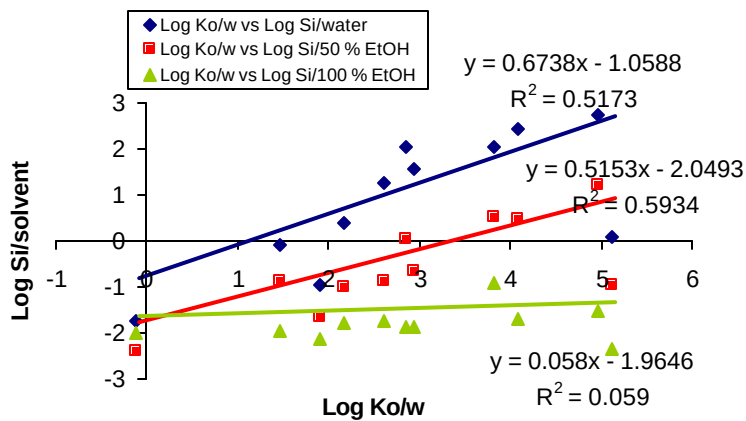


Figure 3.2 Log Ko/w plotted against the mean log membrane/solvent using stratum corneum (Plate 1) and silastic (Plate 2) as membranes and water, 50 % ethanol and 100 % ethanol as solvents. The linear regression line, regression equation and R^2 value of the plot for each solvent is displayed ($n = 5$).

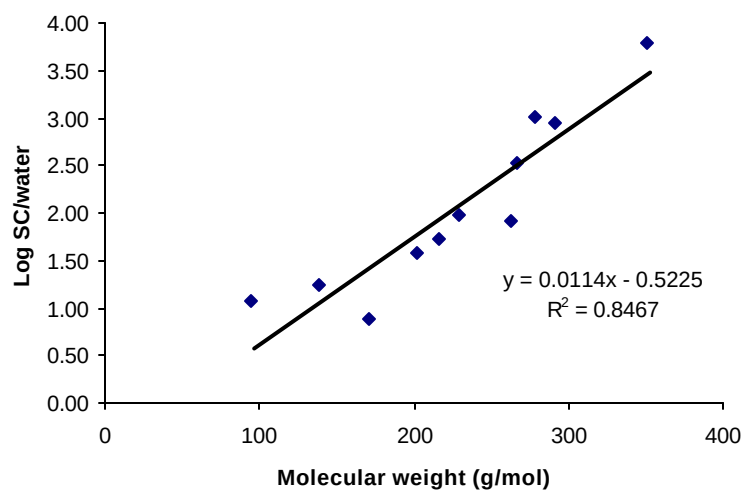


Figure 3.3. Molecular weight plotted against the mean log SC/water. The linear regression line, regression equation and R^2 value is displayed ($n = 5$).

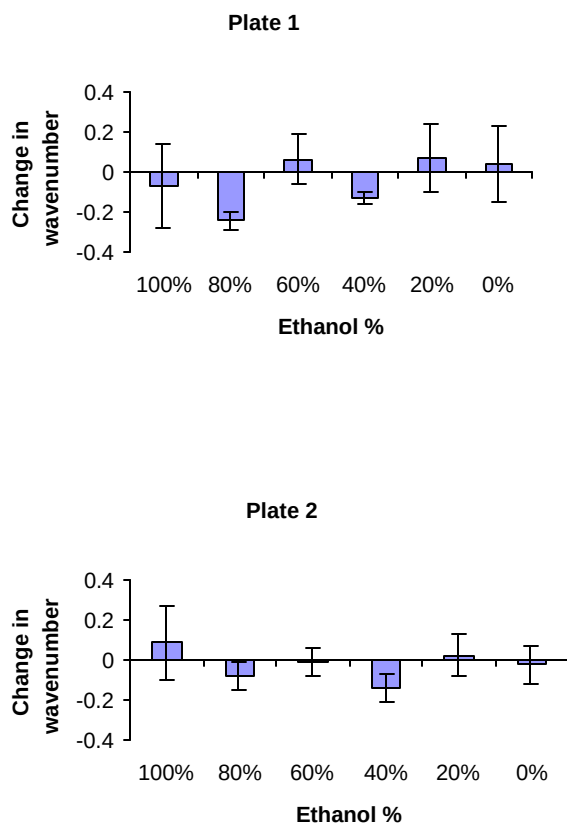


Figure 3.4. Change in wave number after aqueous ethanol treatment of VaCH₂ absorbance in the 2917 cm⁻¹ region (Plate 1) and VsCH₂ absorbance in the 2849 cm⁻¹ region (Plate 2) (n=4).

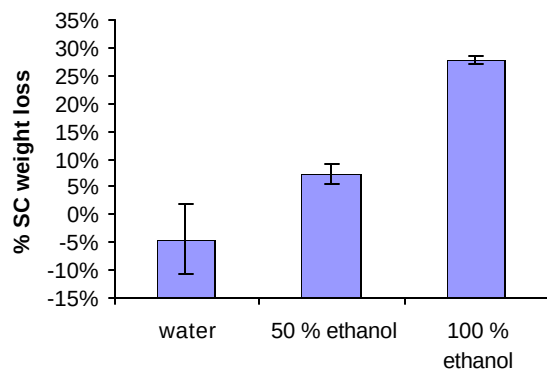


Figure 3.5. Percentage SC weight loss after 24 hr extraction using water, 50 % ethanol and 100 % ethanol (n = 4).

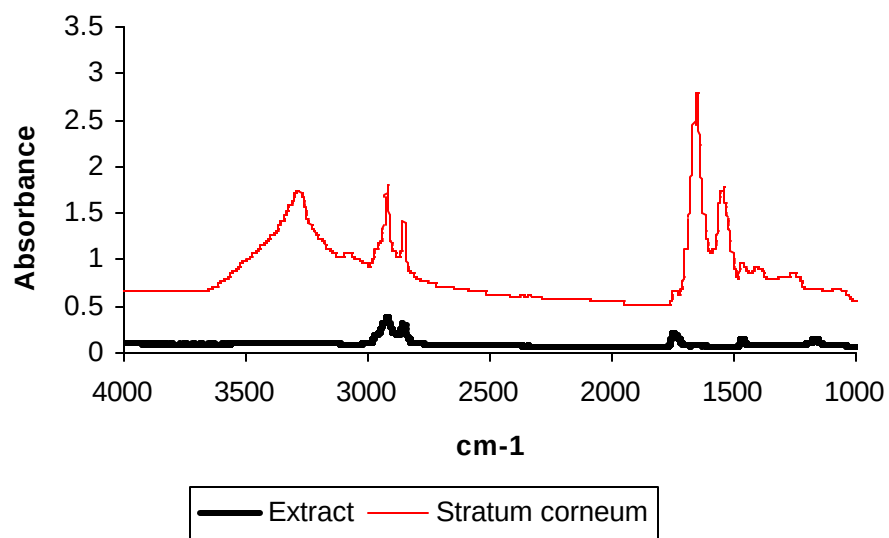


Figure 3.6. Representative FT-IR absorbance spectra of typical stratum corneum and the precipitate from an ethanol extract of stratum corneum. The peaks between 2800 cm⁻¹ and 3000 cm⁻¹ are due to IR absorbance at C-H bonds, while the peak at 1743 cm⁻¹ is attributed to C=O bonds in carboxyl groups (Parker 1983).

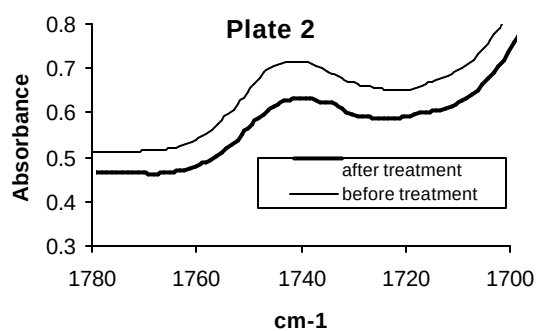
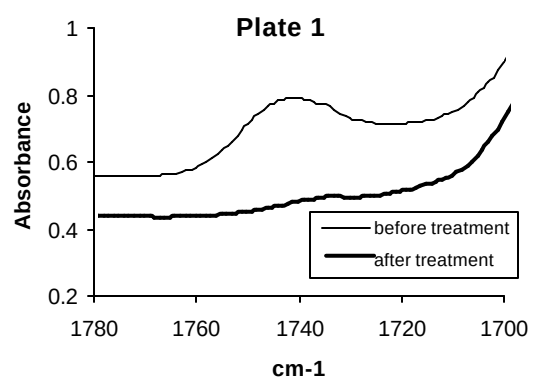


Figure 3.7. Representative FTIR absorbance spectra of stratum corneum before and after 12 hr exposure to 100 % ethanol (Plate 1) and 10 % ethanol (Plate 2) showing change in absorbance due to COOH in the 1740 cm⁻¹ region.

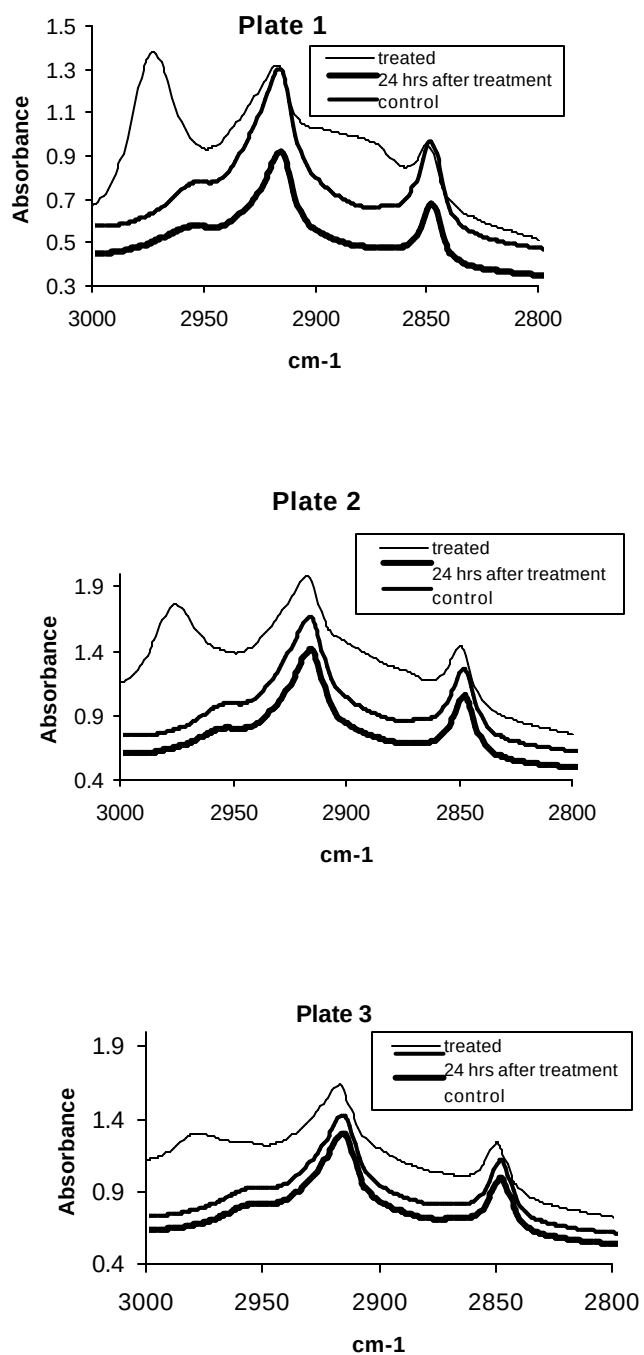


Figure 3.8. Representative FTIR absorbance spectra of stratum corneum before and after 12 hr exposure to 90 % ethanol (Plate 1), 60 % ethanol (Plate 2) and 30 % ethanol (Plate 3) and again after 24 hours in a dessicating atmosphere, showing change in $\text{Va}(\text{CH}_3)$ absorbance in the 2955 cm^{-1} region.

**4. EFFECT OF VEHICLES AND SODIUM LAURYL SULPHATE ON XENOBIOTIC
PERMEABILITY AND STRATUM CORNEUM PARTITIONING IN PORCINE SKIN**

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ABSTRACT

Dermal contact with potentially toxic agricultural and industrial chemicals is a common hazard encountered in occupational, accidental spill and environmental contamination scenarios. Different solvents and chemical mixtures may influence dermal absorption. The effects of sodium lauryl sulphate (SLS) on the stratum corneum partitioning and permeability in porcine skin of 10 agricultural and industrial chemicals in water, ethanol and propylene glycol were investigated. The chemicals were phenol, *p*-nitrophenol, pentachlorophenol, methyl parathion, ethyl parathion, chlorpyrifos, fenthion, simazine, atrazine and propazine. SLS decreased partitioning into stratum corneum from water for lipophilic compounds, decreased partitioning from propylene glycol and did not alter partitioning from ethanol. SLS effects on permeability were less consistent, but generally decreased permeability from water, increased permeability from ethanol and had an inconsistent effect on permeability from propylene glycol. It was concluded that, for the compounds tested, partitioning into the stratum corneum was determined by the relative solubility of the solute in the donor solvent and the stratum corneum lipids. Permeability, however, reflected the result of successive, complex processes and was not predictable from stratum corneum partitioning alone. Addition of SLS to solvents altered partitioning and absorption characteristics across a range of compounds, which indicates that partition coefficients or skin permeability from neat chemical exposure should be used with caution in risk assessment procedures for chemical mixtures.

Keywords: Skin permeability; Dermal absorption; Stratum corneum partitioning; Water; Ethanol; Propylene glycol; Phenol; *p*-Nitrophenol; Pentachlorophenol; Methyl parathion; Ethyl parathion; Chlorpyrifos; Fenthion; Simazine; Atrazine; Propazine

Abbreviations: SLS, Sodium Lauryl Sulphate; CMC, Critical Micelle Concentration; log K_o/W , log of the octanol/water partitioning ratio

INTRODUCTION

Dermal contact with potentially toxic agricultural and industrial chemicals is a common hazard encountered in occupational, accidental spill and environmental contamination scenarios. The risk of toxicity is related to the ability of these compounds to reach the site of toxic effect. This usually involves dermal penetration and absorption, unless the skin surface is the site of toxicity. Since chemicals may be encountered in a variety of solvents, solvent mixtures and chemical mixtures; the effects of solvents and chemicals in the mixture on the dermal kinetics of the chemical of interest is relevant. Most current dermal risk assessment guidelines only deal with single chemical exposure. The large variety of solvents and chemical mixtures that may be encountered prohibits the empirical characterization of the dermal kinetics of all possible mixtures. However, certain mixture-effects may be reasonably predicted from studies on the effects of similar mixtures on similar compounds.

The outer layer of the skin, the stratum corneum, is generally accepted to be the primary barrier to skin absorption for most compounds (Bouwstra et al 2003) and the intercellular lipid matrix of the stratum corneum is the main route for the dermal absorption of chemicals (Albery & Hadgraft 1979). Absorption through intact stratum corneum usually involves two processes—partitioning into the stratum corneum and movement through the lipid matrix (Scheuplein & Blank 1971). Mixture effects on the rate and extent of either of these processes will conceivably influence the rate and extent of dermal absorption.

Effects on dermal absorption rates have been demonstrated using a variety of surfactants, including sodium lauryl sulphate (SLS) (Frankild et al 1995; Effendy et al 1996; Baynes & Riviere 1998; Lopez et al 2000; Nielsen 2000; Riviere et al 2001; Shokri et al 2001; Baynes et al 2002; Nokhodchi et al 2003). Surfactants may change skin barrier properties by causing skin irritation (Lee & Maibach 2004). If present above the critical micelle concentration (CMC), reversible, altered structural organization of stratum corneum lipids may occur. Surfactants can also reduce the amount of chemical available for absorption through the formation of micelles in the donor solvent. Different, and occasionally apparently contradictory, surfactant influences on dermal absorption may be encountered depending on the interplay between the various surfactant effects (Riviere et al 2001; Shokri et al 2001; Baynes et al 2002).

Water, ethanol and propylene glycol are common solvents and their effects on dermal absorption across a range of compounds have been described (Berner et al 1989; Kurihara-Bergstrom et al 1990; Megrab et al 1995; Kim et al 1996; Levang et al 1999; Panchagnula et al 2001). Comparing the dermal absorption of a range of compounds from these solvents with and without the addition of SLS offers an opportunity to study SLS effects on absorption when in combination with solvents relevant to a significant number of real-world dermal exposure hazards. SLS is common in mixtures associated with skin exposure. It is included in chemical formulations as emulsifiers, stabilizers and wetting agents (Shokri et al 2001) in products such as pharmaceutical vehicles, cosmetics, foaming dentifrices and foods (Nikitakis et al 1991). In this study, 10 representative agricultural and industrial compounds, chosen to characterize a range of physical properties, were used to investigate the effects of SLS on partitioning into isolated porcine stratum corneum and permeability in dermatomed porcine skin from water, ethanol and propylene glycol.

MATERIALS AND METHODS

Chemicals

C-14 radio labeled phenol, *p*-nitrophenol, pentachlorophenol, methyl parathion, ethyl parathion, atrazine, and simazine were obtained from Sigma (St. Louis, MO). Chlorpyrifos, fenthion, and propazine were obtained from American Radiolabeled Chemicals (St. Louis, MO). Purity ranged from 95 to 99.5% and radioactivity ranged from 9 to 76.6 mCi/mmol. All C-14 labels were situated in the ring structure of the labeled molecules. Pure ethanol was obtained from Aaper Alcohol and Chemical Co. (Shelbville, KY). Sodium lauryl sulphate (99%; GC Grade), Bovine serum albumin (Fract V; cold alcohol precipitated), NaCl (Certified A.C.S.), KCl (Certified A.C.S.), CaCl (Certified A.C.S.; anhydrous), KH₂PO₄ (Certified A.C.S.), MgSO₄·7H₂O (Certified A.C.S.), NaHCO₃ (Certified A.C.S.) and dextrose (Certified A.C.S.; anhydrous) was obtained from Fisher Scientific (Pittsburgh, PA). Pure propylene glycol was obtained from Sigma (St. Louis, MO). Amikacin (250 µg/ml) was obtained from Abbott Labs (Chicago, IL). Heparin (1000 units/ml) was obtained from Elkins Sinn (Cherry Hill, NY). Penicillin G Sodium (250,000 units/ml) was obtained from Pfizer Inc. (New York, NY).

Stratum corneum/solvent partitioning

Stratum corneum/solvent partition coefficients were estimated using methods described previously (Baynes 2000). Briefly, abdominal skin of female weanling Yorkshire pigs was immersed in 0.25% trypsin (Sigma, St. Louis, MO) for 24 h. The stratum corneum was then removed after heat treatment, dried in a Fisherbrand Dessicator Cabinet (Fisher Scientific, Pittsburgh, PA) with Drierite™ anhydrous calcium sulfate (WA Hammond Drierite Company, Xenia, OH), weighed (5–8 mg per sample) using a Mettler AE 200 scale (Mettler Toledo, Columbus, OH) and placed in vials. The solvent (3 ml), and 100 µg radio labeled compound was added to the stratum corneum sample vial ($n = 5$) and capped for 24 h. Then 250 µl of the solvent was removed for direct radiolabel counts using Ecolume (ICN Costa Mesa, CA). Excess solvent was removed from the stratum corneum by gentle blotting on Kimwipe™. The stratum corneum samples were combusted in a Packard Model 306 Tissue Oxidizer (Packard Chemical Co., Downers Grove, IL). Samples were analyzed using a Packard Model 1900TR Liquid Scintillation Counter (Packard Chemical Co., Downers Grove, IL).

It should be noted that SLS could not be dissolved directly into ethanol and propylene glycol. SLS, as a 40% mass/mass aqueous solution, was added to the solvents at a ratio of 25% (v/v). This resulted in 10% (m/v) SLS in the SLS-containing mixtures.

Permeability

Porcine skin disks, dermatomed from fresh skin to a thickness of 500 µm and presenting an exposed surface of 0.32 cm², were used as barrier membranes in a flow-through diffusion cell system according to the methodology of (Bronaugh & Stewart 1985), as adapted by (Chang & Riviere 1991). The dose volume was 20 µl. The target dose was 10 µg/cm². Actual doses, as estimated from the dosing stock solutions, were influenced by solvent interactions and non-specific binding with glassware. Doses used, followed by their standard errors in brackets were: 10.34 µg/cm² (0.24) for methyl parathion, 15.15 µg/cm² (0.33) for ethyl parathion, 5.69 µg/cm² (0.13) for chlorpyrifos, 5.93 µg/cm² (0.06) for fenthion, 7.89 µg/cm² (0.06) for phenol, 13.71 µg/cm² (0.05) for *p*-nitrophenol, 13.43 µg/cm² (0.16) for pentachlorophenol, 8.61 µg/cm² (0.03) for atrazine, 6.87 µg/cm² (0.08) for simazine and 10.69 µg/cm² (0.14) for propazine. The receptor solution, designed to mimic a blood plasma environment, consisted of 13.78 g NaCl, 0.71 g KCl, 0.56 g CaCl, 0.32 g KH₂PO₄, 0.58 g MgSO₄·7H₂O,

5.50 g NaHCO₃, 2.40 g dextrose, 90.0 g bovine serum albumin, 0.25 ml amikacin, 10 ml heparin and 0.1 ml penicillin G sodium made up to 2 l with glass distilled water. An 8 h experimental period was used, which allowed the estimation of adequate flux/time curves while avoiding complications due to skin degradation. Constant perfusate flow provided infinite sink conditions, maintaining a concentration gradient across the membrane. Perfusate was collected at 15 min intervals for the first 2 h, and 1 h intervals thereafter ($n = 5$). Radiolabel in the perfusate was determined by liquid scintillation as described above for solvents.

Data processing and statistical analysis

For partition coefficient determinations, radioactivity content in the vehicle mixture and stratum corneum were normalized to 1000 mg vehicle (C_{vehicle}) and 1000 mg stratum corneum ($C_{\text{stratum corneum}}$), respectively. The log stratum corneum/vehicle partition coefficient was determined from the equation: $\log PC = \log C_{\text{stratum corneum}}/C_{\text{vehicle}}$. The log K octanol/water values were obtained from the literature (Howard & Meylan 1997).

For permeability estimations the receptor fluid was assumed to be an infinite sink because of the constant flow of receptor fluid out of the diffusion cell. Permeability (cm/hr) was estimated by dividing the slope of the steady-state portion of the cumulative mass absorbed/time curve with the concentration in the donor solvent.

Differences between means were assumed to be statistically significant at or above the 95% confidence level as determined by t distribution tests assuming normal population variable distributions. The research adhered to the 'Principles of Laboratory Animal Care' (NIH publication #85-23, revised 1985).

RESULTS

Stratum corneum/solvent partitioning

Partitioning estimations were summarized in Table 4.1. The partitioning into stratum corneum from water of phenol and *p*-nitrophenol, which have relatively low log Ko/w values of 1.46 and 1.91, respectively, was not altered by the addition of SLS. For compounds with higher log Ko/w values, except simazine, partitioning was reduced when SLS was added (Figure 4.1). Partitioning from water increased as compound log Ko/w values

increased and log K_o/w was correlated with log $P_{\text{stratum corneum/solvent}}$ ($R^2 = 0.7915$). The addition of SLS to water removed the influence of compound log K_o/w values on stratum corneum partitioning (Figure 4.1).

Addition of SLS to ethanol did not alter stratum corneum/solvent partitioning except for simazine, which showed an increased partitioning and ethyl parathion, which showed decreased partitioning. Partitioning was weakly correlated with log K_o/w in both ethanol and ethanol-SLS mixtures (Figure 4.2).

Addition of SLS to propylene glycol reduced stratum corneum/solvent partitioning for atrazine, methyl parathion and pentachlorophenol. Ethyl parathion, fenthion and chlorpyrifos partitioning appeared to be reduced, but it was not statistically significant. It may, however, be reasonable to assume that *p*-nitrophenol partitioning was reduced, because it narrowly fell outside the chosen statistical significance level of 95% (P -value = 0.061). Simazine showed an increased partitioning. Phenol partitioning appeared to be unchanged. Partitioning was weakly correlated with log K_o/w in both propylene glycol and propylene glycol-SLS mixtures (Figure 4.3).

Permeability

Permeability estimations were summarized in Table 4.2. Permeability from propylene glycol was generally lower than permeability from water or ethanol (Table 4.2). The effects of SLS on permeability from water, ethanol and propylene glycol was summarized in terms of the ratio of permeability from solvents with SLS to solvents without SLS (Figure 4.4). The flux/time estimations of phenol were presented in (Figure 4.8) as a representative plot to illustrate typical flux/time curves obtained. Permeability estimation values were tabulated in Table 4.2. SLS generally reduced permeability from water except for phenol, which did not show significant change when SLS was present. SLS generally increased permeability from ethanol, except for ethyl parathion. SLS increased permeability from propylene glycol for phenol, *p*-nitrophenol, simazine, ethyl parathion, fenthion, chlorpyrifos and pentachlorophenol, but permeability was decreased for atrazine and methyl parathion. SLS did not change permeability for propazine.

The relationships between partitioning and permeability was represented in Figure 4.5, Figure 4.6 and Figure 4.7. The flux/time curves estimated for phenol was displayed as a representative curve to demonstrate typical flux/time curves (Figure 4.8).

DISCUSSION

These results are of interest in the field of risk assessment, because such mixture-studies have not been conducted for this wide variety of chemicals. It offered the opportunity to observe mixture -effects that are relevant across a range of compounds, which adds confidence to predictions of vehicle effects on the dermal absorption of similar compounds. Although these studies were conducted in porcine skin, the results are relevant to human dermal absorption due to the histological and biochemical similarity of human and porcine skin (Reifenrath et al 1984; Monteiro-Riviere 1986).

The stratum corneum was processed using trypsin, heat and desiccation. Some alterations in the stratum corneum environment can be expected due to the processing procedures, such as removal of free water and changes in keratinocyte protein structure. Stratum corneum partitioning of lipophilic compounds in trypsinized, dried human stratum corneum was shown by (Raykar et al 1988) to be correlated with partitioning into extracted stratum corneum lipids. If the stratum corneum lipids are assumed to be the primary route of absorption through the stratum corneum, then partitioning into stratum corneum lipids is of interest. Differences in the effects of solvents in isolated stratum corneum compared to hydrated, intact stratum corneum may be due to variations in the extent of solvent penetration. Assuming that solvent effects on partitioning in isolated stratum corneum are quantitatively the same as in intact stratum corneum may not be valid. However, it is reasonable to assume that the differences would be largely in extent, while the overall pattern remains similar. This hypothesis is supported by the data of the present study.

Assuming that a solvent and non-electrolyte solute do not react chemically with each other, the solubility of a solute in a solvent or chemical mixture is a function of the change in enthalpy and entropy when the solute dissolves. The total energy required for overcoming the solvent intermolecular bonds and the energy released upon association of the solute molecules with the solvent determines the change in enthalpy whereas change in

entropy is determined by the amount of disorder introduced to the system (Atkins 1994). Since systems tend towards lower energy (Atkins 1994), change in enthalpy and entropy will determine the solute partitioning between the solvents at equilibrium. According to this hypothesis, the changes in partitioning patterns observed when SLS is added to the solvent should be influenced by the effects of SLS on intermolecular forces. Water exhibits relatively strong intermolecular attraction due to charge interactions and H-bonding. Non-polar compounds are not attracted strongly to water molecules and therefore do not overcome the intermolecular bonds between water molecules readily. Lipids in the stratum corneum, however, contain long carbon chains that can attract non-polar molecules through the action of London dispersion forces. The energy of the system will therefore be balanced, at equilibrium, when non-polar solutes are present at higher concentrations in the stratum corneum lipids than in water. The difference in concentration should be correlated with lipophilicity, expressed as $\log K_{o/w}$. This hypothesis is supported by the data presented in Figure 4.1, which shows an increased partitioning into the stratum corneum from water as the $\log K_{o/w}$ of the solute increases. SLS, when present at concentrations above the CMC, causes the formation of micelles around the non-polar solute molecules. The concentration of SLS used in this study was well above the CMC, which is ca. 0.23% (m/v) (Mukerjee & Mysels 1971). The polar region of the surfactant is strongly attracted to water due to charge interactions, which maintains a favorable energy balance in the system, at equilibrium, when solute is present in the solvent at relatively high concentrations. The lipophilicity of the solute is therefore expected to have a weaker influence on its partitioning when SLS is added to the system due to increased lipophilicity in the solvent as shown in Figure 4.1.

The hypothesis presented above is also supported by the partitioning behavior of the tested compounds in ethanol. Ethanol contains a short carbon chain and a polar group, which allows ethanol to attract polar and non-polar molecules. The addition of SLS, therefore, did not substantially change the partitioning behavior of solutes across a range of lipophilicity (Figure 4.2). There was, however, still a weak correlation between partitioning into stratum corneum and lipophilicity, which indicates that the stratum corneum has a higher lipophilicity than ethanol.

Propylene glycol mixed with SLS slowly solidifies into a paste-like consistency at room temperature. Trapping of solute in the solidified solvent before partitioning reached equilibrium explains the lowered partitioning from propylene glycol when SLS was added (Figure 4.3). Similar to partitioning from ethanol, weak correlation between lipophilicity and stratum corneum partitioning was found, indicating that the stratum corneum has a higher lipophilicity than propylene glycol.

In the present study, permeability showed large inter-compound variability compared to partitioning; and permeability was not related to compound lipophilicity and stratum corneum partitioning across all solvent systems (Figure 4.5, Figure 4.6 and Figure 4.7). Although partitioning is essential for dermal absorption, permeability is influenced by the diffusivity of a compound in the stratum corneum lipids and the pathway length. The relationship between partitioning, diffusivity and pathway length allows qualitative deductions to be made from comparisons between partitioning and permeability data. Since the pathway length was unchanged between permeability estimations in the same solvent system and partitioning was not correlated with permeability within solvents systems, it can be concluded that the physical and chemical factors that predict partitioning do not predict diffusivity.

The addition of SLS to ethanol generally increased permeability, while partitioning from ethanol was largely unaffected by SLS. It suggests that the effect of SLS on permeability from ethanol is largely due to an effect on diffusivity. This effect may be related to the addition of water with the SLS rather than an effect of SLS itself. Binary mixtures of water and ethanol have been shown to increase dermal absorption compared to pure ethanol. Pure ethanol dehydrates the stratum corneum, which normally decreases permeability. With the addition of water, however, ethanol may have a disruptive effect on stratum corneum lipid structure causing increased permeability. Other suggested mechanisms include effects on the putative pore pathway, increased solubility in stratum corneum lipids and ethanol copermeation (Berner et al 1989; Kurihara-Bergstrom et al 1990; Megrab et al 1995; Kim et al 1996; Levang et al 1999; Panchagnula et al 2001).

SLS generally decreased permeability and partitioning from water when compared to permeability from pure water, with the effect on partitioning increasing with an increase in compound lipophilicity. Phenol, which did

not show a significant change in permeability, is the compound in the group with the lowest lipophilicity. Phenol partitioning was also not significantly changed by the addition of SLS (Table 4.1). Although this suggests that the lack of effect of SLS on phenol permeability and partitioning from water may be related, there is no consistent correlation between permeability and partitioning in the other compounds and it is possible that a small SLS effect could be masked by experimental variability. If only the direction of effect is considered, however, the similarity of SLS effects on partitioning and permeability from water suggests that the effect on partitioning is related to the effect on permeability and that the relationship is strong enough not to be completely masked by differences in diffusivity. From a practical perspective the data suggests that washing contaminated skin using water and a surfactant should, apart from removing some of the contaminating chemical, reduce absorption when compared to washing in water without a surfactant. However, should the surfactant induce skin irritation, as might be expected in an in vivo situation, this physicochemical advantage may be reduced.

Pure propylene glycol dehydrates the stratum corneum, which decreases permeability. A 66% aqueous solution of propylene glycol, however, enhances permeability (Panchagnula et al 2001). Proposed mechanisms of absorption enhancement include a cosolvency effect (Barry 1983) and a carrier mechanism (Hoelgaard & Mollgaard 1985). In the present study, the influence of SLS on permeability from propylene glycol was not consistent, although partitioning was generally reduced (Figure 4.3 and Figure 4.4; Table 4.2). Since SLS was added to propylene glycol in the form of an aqueous solution, the influence of water was confounded with the influence of SLS. SLS is not soluble in pure propylene glycol. An aqueous mixture of propylene glycol and SLS therefore resembles more probable real-world exposures to this mixture. An example is when an aqueous solution of SLS is used as a cleaning agent after dermal exposure to a chemical in propylene glycol. The addition of SLS diminished the dehydrating effect of propylene glycol, weakening the decrease in permeability associated with pure propylene glycol and opening up the possibility of absorption enhancement through one or both mechanisms mentioned above.

Models of dermal absorption usually assume permeability to be a first order process. However, changes in the stratum corneum lipid environment induced by solutes and solvent components may be non-linear over time

and concentration (Berner et al 1989; Kurihara-Bergstrom et al 1990; Megrab et al 1995; Kim et al 1996; Levang et al 1999; Panchagnula et al 2001). The stratum corneum lipids include long chain ceramides, fatty acids, cholesterol and triglycerides (Monteiro-Riviere et al 2001), which form parallel lamellae on corneocyte surfaces. Inside the lamellae, the lipids are arranged in bilayers of ordered, crystalline phases bordering a central band of fluid lipids. The bilayer arrangement of the lipids results in continuous bands of polar layers and non-polar layers (Bouwstra et al 2003). Differences in lipid composition and structure occur between deep and superficial layers and on different body areas (Monteiro-Riviere 1986; Bouwstra et al 2003). The complexity of this system allows for the possibility of a variety of interactions and solvent effects on partitioning and diffusivity, as demonstrated by the present study. Caution should therefore be used when attempting to use data obtained from a particular solvent system to predict parameters in a different solvent system.

This study related the partitioning of parent compound into isolated stratum corneum to the absorption of total C-14 through dermatomed skin. The absorption of C-14 reflected the driving concentration of parent compound for diffusion across the stratum corneum. It allowed the estimation of total absorption of the C-14 labeled compound in an experimental system where metabolism may or may not be significant. The absorption/time curve is, according to Fick's law of diffusion, a reflection of the permeant concentration gradient, permeant partitioning, permeant diffusivity and pathway length, which is related to the membrane thickness in a flow-through cell. Metabolism of parent compound in the viable epidermis may alter subsequent partitioning between the viable epidermis and the receptor fluid and could influence the shape of the absorption/time curve. Although the assumption cannot be made that the initial partitioning step in absorption is the overriding determinant of absorption, this first step in the disposition of parent drug across the stratum corneum is generally assumed to be rate-limiting and must be understood before any subsequent metabolism of penetrated parent drug can be taken into account. The approach used in this study revealed generally repeatable solvent influences on absorption across a range of compounds (Figure 4.4) that could be related to independently estimated solvent/stratum corneum partitioning. However, it provides a limited description of the nature of parent compound absorption into the systemic circulation due to the possible influences of permeant metabolism in the epidermis and dermis. This limitation must be considered when conclusions are drawn from the absorption data relative to the risk of systemic toxicity. These data apply to assessing vehicle and surfactant effects on the first step in dermal

absorption–partitioning into the stratum corneum. The use of chromatographic techniques and, ideally, perfused skin models (Riviere et al 1986) are needed to fully elucidate the effects of metabolism on a dermal absorption profile.

It was concluded that, for the compounds tested, partitioning into the stratum corneum was determined by the relative solubility of the solute in the donor solvent and the stratum corneum lipids. Permeability, however, reflected the result of successive, complex processes and is not always predictable from stratum corneum partitioning. This finding has considerable significance on the use of partition coefficients alone, determined from neat chemical exposure, to estimate chemical absorption for mixture exposures. Physical–chemical interactions present within the exposure mixture should be defined before partition coefficients are employed in risk assessment models.

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Table 4.1. Estimated log P stratum corneum/solvent values in water, water plus sodium lauryl sulphate (SLS), ethanol (EtOH), ethanol plus SLS, propylene glycol (PG) and PG plus SLS with standard errors (SE) (n=5) and log P octanol/water (log P o/w) values (Howard and Meylan 1997) for phenol (PHE), p-nitrophenol (PNP), simazine (SIM), atrazine (ATR), methyl parathion (MPA), propazine (PRO), ethyl parathion (EPA), fenthion

	Log		Water			EtOH				PG +			
	Po/w	Water	SE	+ SLS	SE	EtOH	SE	+ SLS	SE	PG	SE	SLS	<u>SE</u>
PHE	1.46	1.081	0.037	1.166	0.025	0.556	0.048	0.500	0.038	0.689	0.097	0.695	0.071
PNP	1.91	1.246	0.021	1.215	0.024	0.633	0.110	0.658	0.073	0.878	0.240	0.455	0.050
SIM	2.18	0.737	0.025	1.313	0.029	0.773	0.008	0.932	0.027	0.784	0.052	0.992	0.062
ATR	2.61	1.722	0.113	1.206	0.022	0.775	0.045	0.604	0.104	1.256	0.094	0.623	0.046
MPA	2.86	1.922	0.078	1.370	0.037	0.733	0.054	0.770	0.037	1.369	0.067	0.856	0.049
PRO	2.93	1.969	0.381	1.210	0.070	0.797	0.064	0.738	0.050	0.992	0.160	0.652	0.036
EPA	3.83	2.952	0.065	1.114	0.074	0.983	0.037	0.760	0.088	1.100	0.226	0.979	0.023
FEN	4.09	3.006	0.036	1.279	0.209	0.693	0.058	0.763	0.033	0.892	0.052	0.767	0.115
CPY	4.96	3.784	0.710	1.417	0.031	0.853	0.066	0.787	0.033	1.241	0.034	0.974	0.044
PCP	5.12	2.534	0.189	1.305	0.094	0.897	0.094	1.189	0.144	1.402	0.056	1.120	0.037

(FEN), chlorpyrifos (CPY) and pentachlorophenol (PCP).

Table 4.2. Estimated permeability values (cm/h) from water, water plus sodium lauryl sulphate (SLS), ethanol (EtOH), ethanol plus SLS, propylene glycol (PG) and PG plus SLS with standard errors (SE) (n=5) for phenol (PHE), p-nitrophenol (PNP), simazine (SIM), atrazine (ATR), methyl parathion (MPA), propazine (PRO), ethyl

	Water +				EtOH +				PG +			
	Water	SE	SLS	SE	EtOH	SE	SLS	SE	PG	SE	SLS	<u>SE</u>
PHE	4.377	0.194	4.380	0.234	4.210	0.291	4.444	0.238	0.127	0.012	0.179	0.015
PNP	2.222	0.344	1.755	0.089	0.350	0.018	1.604	0.074	0.013	0.001	0.030	0.009
SIM	0.484	0.061	0.199	0.039	0.095	0.014	0.355	0.030	0.023	0.002	0.062	0.041
ATR	1.126	0.221	0.703	0.120	0.068	0.002	0.606	0.031	0.035	0.019	0.027	0.005
MPA	4.918	0.634	0.634	0.068	0.178	0.021	0.712	0.052	0.049	0.005	0.036	0.010
PRO	0.233	0.013	0.144	0.028	0.032	0.007	0.093	0.010	0.008	0.002	0.008	0.002
EPA	0.410	0.049	0.236	0.043	0.157	0.032	0.122	0.009	0.035	0.004	0.042	0.023
FEN	0.463	0.013	0.195	0.033	0.102	0.007	0.256	0.042	0.015	0.002	0.027	0.002
CPY	0.061	0.018	0.052	0.013	0.013	0.002	0.036	0.003	0.007	0.001	0.008	0.002
PCP	1.648	0.217	0.393	0.040	0.078	0.020	0.297	0.077	0.013	0.002	0.016	0.003

parathion (EPA), fenthion (FEN), chlorpyrifos (CPY) and pentachlorophenol (PCP).

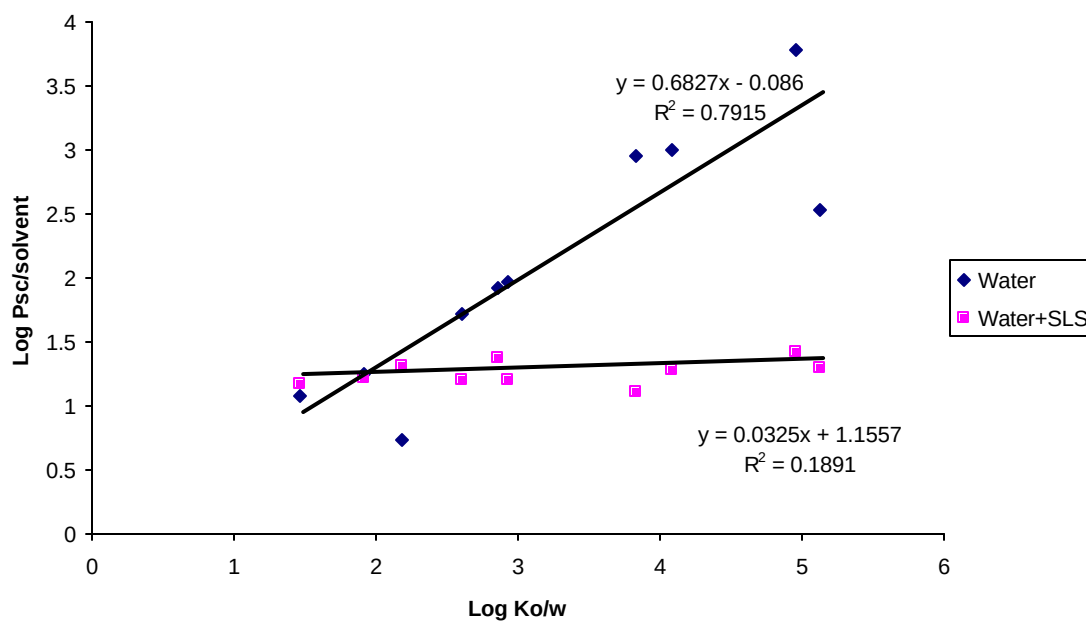


Figure 4.1. $\text{Log } K_{o/w}$ plotted against the mean log partitioning of stratum corneum/solvent from water and water with sodium lauryl sulphate. Compounds represented are, from left to right, phenol, p-nitrophenol, simazine, atrazine, methyl parathion, propazine, ethyl parathion, fenthion, chlorpyrifos and pentachlorophenol. The linear regression lines, regression equations and R^2 values of the plot for each solvent is displayed ($n = 5$).

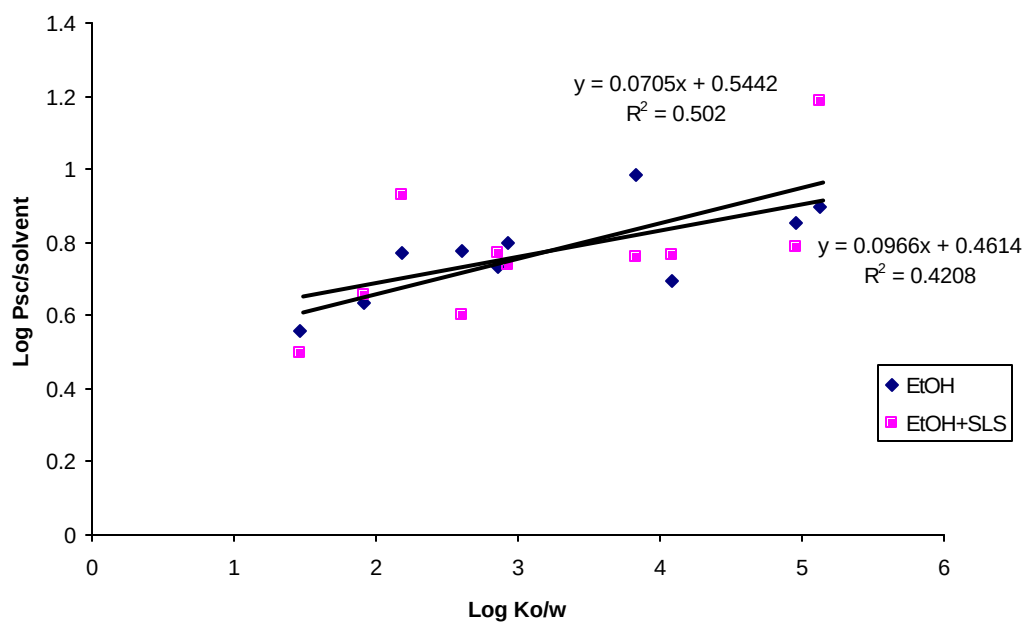


Figure 4.2. Log Ko/w plotted against the mean log partitioning of stratum corneum/solvent from ethanol and ethanol with sodium lauryl sulphate. Compounds represented are, from left to right, phenol, p-nitrophenol, simazine, atrazine, methyl parathion, propazine, ethyl parathion, fenthion, chlorpyrifos and pentachlorophenol. The linear regression lines, regression equations and R^2 values of the plot for each solvent is displayed ($n = 5$).

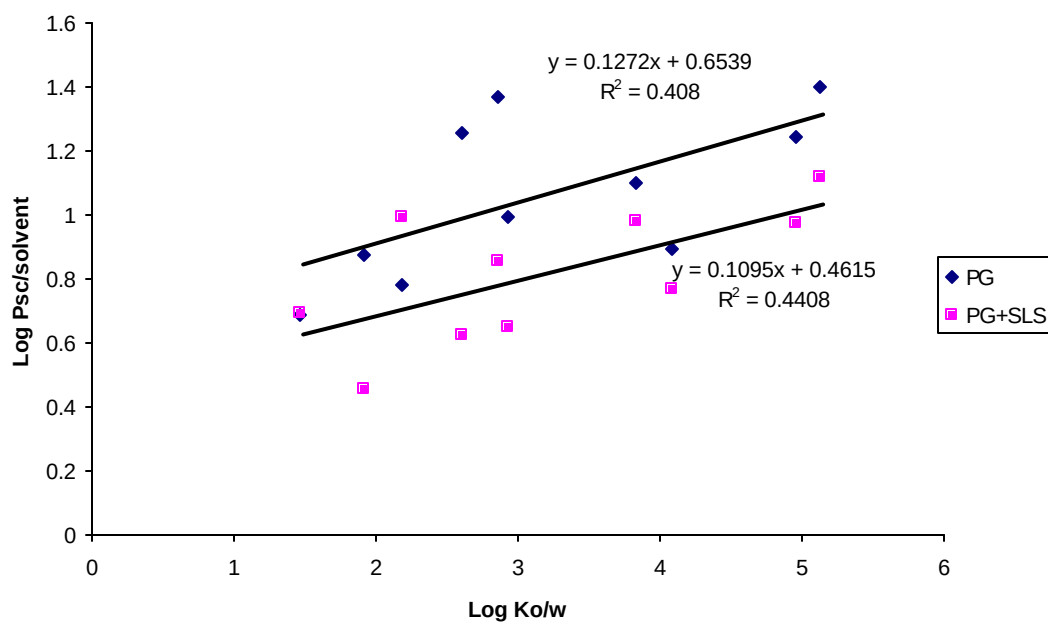


Figure 4.3. Log Ko/w plotted against the mean log partitioning of stratum corneum/solvent from propylene glycol and propylene glycol with sodium lauryl sulphate. Compounds represented are, from left to right, phenol, p-nitrophenol, simazine, atrazine, methyl parathion, propazine, ethyl parathion, fenthion, chlorpyrifos and pentachlorophenol. The linear regression lines, regression equations and R^2 values of the plot for each solvent is displayed ($n = 5$).

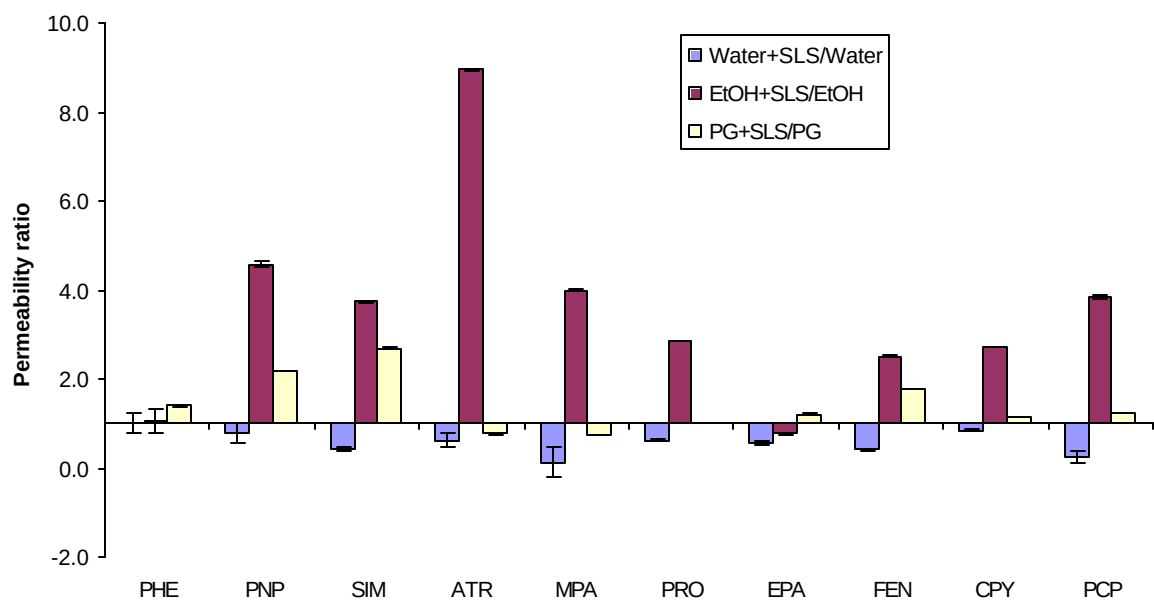


Figure 4.4. The permeability ratios of mean permeability from water with sodium lauryl sulphate (SLS)/water, ethanol with SLS/ethanol and propylene glycol with SLS/propylene glycol of phenol (PHE), p-nitrophenol (PNP), simazine (SIM), atrazine (ATR), methyl parathion (MPA), propazine (PRO), ethyl parathion (EPA), fenthion (FEN), chlorpyrifos (CPY) and pentachlorophenol (PCP). Error bars denote the average standard error of the means comprising the ratio (n = 5).

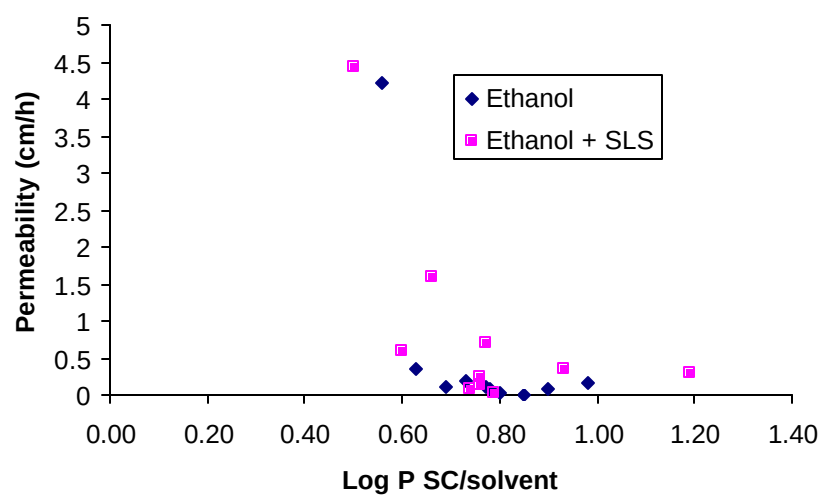


Figure 4.5. Scatterplot to compare mean values of permeability (n=4) and log stratum corneum (SC)/solvent partitioning (n=5) from ethanol and ethanol plus sodium lauryl sulphate for phenol, p-nitrophenol, simazine, atrazine, methyl parathion, propazine, ethyl parathion, fenthion, chlorpyrifos and pentachlorophenol.

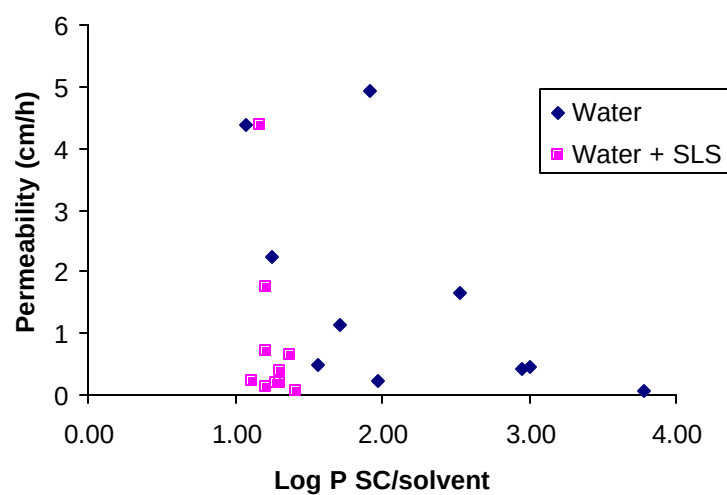


Figure 4.6. Scatterplot to compare mean values of permeability (n=4) and log stratum corneum (SC)/solvent partitioning (n=5) from water and water plus sodium lauryl sulphate for phenol, p-nitrophenol, simazine, atrazine, methyl parathion, propazine, ethyl parathion, fenthion, chlorpyrifos and pentachlorophenol.

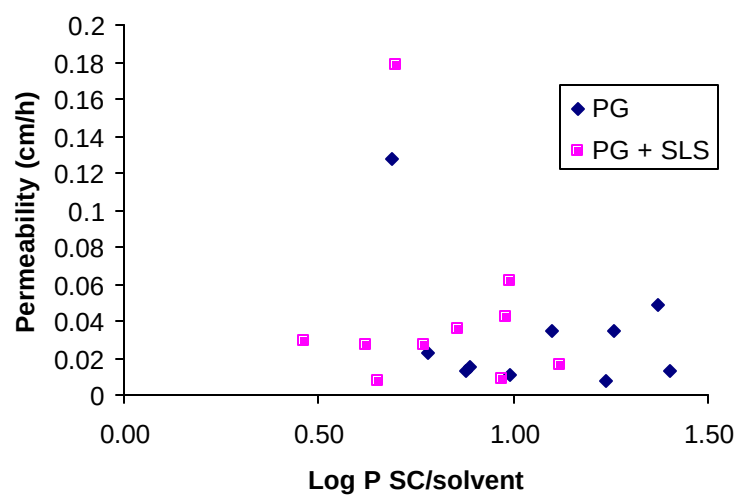


Figure 4.7. Scatterplot to compare mean values of permeability (n=4) and log stratum corneum (SC)/solvent partitioning (n=5) from propylene glycol (PG) and PG plus sodium lauryl sulphate for phenol, p-nitrophenol, simazine, atrazine, methyl parathion, propazine, ethyl parathion, fenthion, chlorpyrifos and pentachlorophenol.

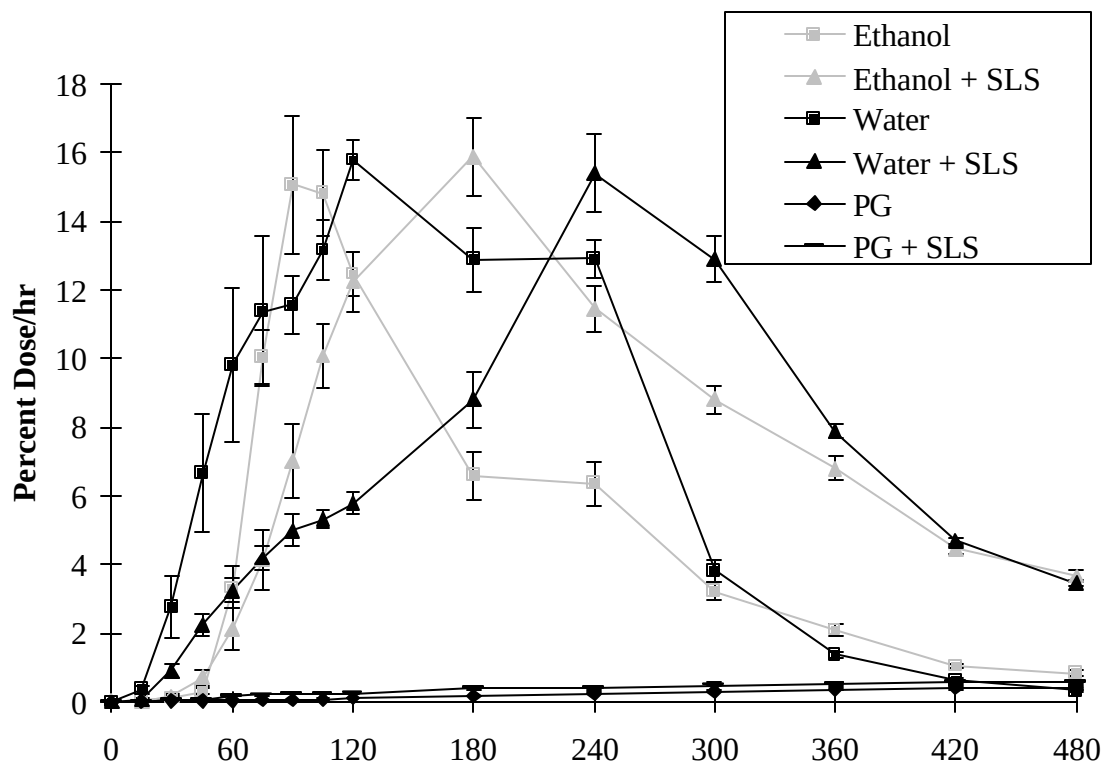


Figure 4.8. Flow-through cell estimated flux/time curves for phenol from ethanol, ethanol plus sodium lauryl sulphate (SLS), water, water plus SLS, propylene glycol (PG) and PG plus SLS. Error bars denote standard errors (n=5).

**5. CLUSTER ANALYSIS OF THE DERMAL PERMEABILITY AND STRATUM CORNEUM/SOLVENT
PARTITIONING OF TEN CHEMICALS IN TWENTY-FOUR CHEMICAL MIXTURES IN PORCINE
SKIN**

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ABSTRACT

Assumptions based on absorption from single solvent systems may be inappropriate for risk assessment when chemical mixtures are involved. We used K-means and hierarchical cluster analyses to identify clusters in stratum corneum partitioning and porcine skin permeability datasets that are distinct from each other based on mathematical indices of similarity and dissimilarity. Twenty four solvent systems consisting of combinations of water, ethanol, propylene glycol, methyl nicotinate and sodium lauryl sulphate were used with 10 solutes, including phenol, p-nitrophenol, pentachlorophenol, methyl parathion, ethyl parathion, chlorpyrifos, fenthion, simazine, atrazine and propazine. Identifying the relationships between solvent systems that have similar effects on dermal absorption formed the bases for hypotheses generation. The determining influence of solvent polarity on the partitioning data structure supported the hypothesis that solvent polarity drives the partitioning of non-polar solutes. Solvent polarity could not be used to predict permeability because solvent effects on diffusivity masked the effects of partitioning on permeability. The consistent influence of the inclusion of propylene glycol in the solvent system supports the hypothesis that over saturation due to solvent evaporation has a marked effect on permeability. These results demonstrated the potential of using cluster analysis of large datasets to identify consistent solvent and chemical mixture effects.

Key words: Cluster analysis; hierarchical clustering; K-means clustering; chemical mixtures; skin permeability; dermal absorption; stratum corneum partitioning; water; ethanol; propylene glycol; phenol; p-nitrophenol; pentachlorophenol; methyl parathion; ethyl parathion; chlorpyrifos; fenthion; simazine; atrazine; propazine; sodium lauryl sulphate; methyl nicotinate

INTRODUCTION

The absorption of small, relatively non-polar solutes through mammalian skin occurs predominantly by partitioning into the stratum corneum lipids, moving through the stratum corneum lipid matrix, the viable epidermis and partitioning into blood in the dermal blood vessels. The stratum corneum forms the primary barrier to dermal absorption (Scheuplein & Blank 1971). The rate and extent of solute flux across skin is related to exposure area, the extent of solute partitioning into the skin, the ease of solute movement through the skin, the solute concentration gradient and the pathway length. The initial concentration in the solvent, the rate of solute movement into the skin and the effects of solvent and solute evaporation into the atmosphere determine the concentration gradient. Evaporation, rate of movement in the skin and solvent/skin partitioning are dependent on the solute concentration gradient and the net influence of all the intermolecular forces acting in the system. The forces include H-bonding, covalent bonding, London dispersion forces and charge-charge interactions. They are dependent on the chemical-physical properties of the solute, the solvent system and the skin. If the physical chemical properties of the skin remain relatively constant, although it should be noted that it could change due to the effects of solutes, it is reasonable to hypothesize that dermal absorption is correlated with quantifiable molecular descriptors of the solute. This approach has been used with some success to develop models of dermal absorption of single solutes from single solvents (Potts & Guy 1995). Such models are, however, inadequate when multiple solvent systems and chemical mixtures are considered due to the vast number of different chemical environments that can be encountered and the altered influence of particular descriptors when the physical-chemical environment of the solvent system is changed. This was demonstrated recently using a hybrid quantitative structure permeation relationship model that incorporated a mixture factor, based on the physical-chemical properties of the solvent, to improve permeability predictions from chemical mixtures (Riviere & Brooks). Complex chemical mixtures are the norm in situations of dermal exposure to potentially harmful chemicals. Assumptions based on absorption from single solvent systems may be inappropriate for risk assessment when chemical mixtures are involved. Large datasets of dermal absorption from a range of solvent systems could potentially indicate consistent solvent effects associated with types of solvent systems, which could reduce uncertainty when predicting dermal absorption.

We used dermatomed porcine skin in flow-through cells to study solvent and chemical mixture effects on dermal absorption. It is a convenient and relatively high throughput in vitro model for the most significant dermal barrier,

the stratum corneum, but does not include the effects of dermal blood flow. Surface area, skin thickness and environmental conditions can be controlled to allow solvent and chemical mixture effects to be isolated from random experimental variability. Hierarchical and K-means clustering were used to identify data structure in a quantifiable manner. Cluster analysis is useful where large numbers of treatments and apparently similar numerical data points prevent intuitive identification of data structure. Cluster analyses identify clusters in a dataset that are distinct from each other based on mathematical indices of similarity and dissimilarity. Identifying the relationships between treatments that have similar effects on dermal absorption could form the bases for hypotheses generation.

METHODS

Chemicals

Atrazine-ring-UL-¹⁴C (specific activity = 15.1mCi/mmol, purity = 98.1%), methyl parathion-ring-UL-¹⁴C; (specific activity = 13.8mCi/mmol, purity = 99.5%), 4-nitrophenol-UL-¹⁴C (specific activity = 6.4mCi/mmol, purity = 99.6%), parathion-ring-UL-¹⁴C (specific activity = 9.2mCi/mmol, purity = 97.1%), pentachlorophenol-ring-UL-¹⁴C (PCP) (specific activity = 11.9mCi/mmol, purity = 98.0%), phenol-UL-¹⁴C (specific activity = 9.0mCi/mmol, purity = 98.5%) and simazine-ring-UL-¹⁴C (specific activity = 15.5mCi/mmol, purity = 99.0%) were obtained from Sigma Chemical Co. (St. Louis, MO). Chlorpyrifos[pyridine-2,6-¹⁴C] (specific activity = 32mCi/mmol, purity = 99.0%), fenthion-ring-UL-¹⁴C (specific activity = 55mCi/mmol, purity = 98.5%) and propazine-ring-UL-¹⁴C (specific activity = 15mCi/mmol, purity = 96.6%) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Absolute (200 proof) ethanol was obtained from Aaper Alcohol and Chemical Co. (Shelbville, KY). Propylene glycol (PG) (purity = 99 %) was obtained from Sigma Chemical Co. (St. Louis, MO). Sodium lauryl sulphate (SLS) (99 %, GC Grade) was obtained from Fisher Scientific (Pittsburgh, PA). Methyl nicotinic acid (MNA) (purity = 99%) were obtained from Sigma Chemical Co. (St. Louis, MO). Double distilled water was obtained from our in-house still. Bovine serum albumin (Fract V; cold alcohol precipitated), NaCL (Certified A.C.S.), KCl (Certified A.C.S.), CaCl (Certified A.C.S.; anhydrous), KH₂PO₄ (Certified A.C.S.), MgSO₄·7H₂O (Certified A.C.S.), NaHCO₃ (Certified A.C.S.) and dextrose (Certified A.C.S.; anhydrous) was obtained from Fisher Scientific (Pittsburgh, PA).). Amikacin (250 µg/ml) was obtained from Abbott Labs (Chicago, IL). Heparin (1,000 units/ml) was obtained from Elkins Sinn (Cherry Hill, NY). Penicillin G Sodium (250,000 units/ml) was obtained from Pfizer Inc. (New York, NY).

The receptor solution consisted of 13.78 g NaCL, 0.71 g KCl, 0.56 g CaCl, 0.32 g KH₂PO₄, 0.58 g MgSO₄·7H₂O, 5.50 g Na HCO₃, 2.40 g dextrose, 90.0 g bovine serum albumin, 0.25 ml amikacin, 10 ml heparin and 0.1 ml penicillin G sodium made up to 2 l with glass distilled water. Water, ethanol and PG were mixed at 1:1 ratios (by volume) with each other. SLS could not be dissolved directly into ethanol and propylene glycol. A 40 % mass/mass aqueous solution was added to the solvents/solvent mixtures at a ratio of 25 % v/v. The proportional compositions of solvent systems (by mass) were summarized in Table 5.5.

Stratum Corneum/Solvent partitioning

Stratum corneum/solvent partition coefficients were estimated according to published methods (Baynes *et al.* 2000). In short: The stratum corneum of female weanling Yorkshire pigs was removed after heat treatment and immersed in 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) for 24 hours, dried in a Fisherbrand Dessicator Cabinet (Fisher Scientific, Pittsburgh, PA) with Drierite™ anhydrous calcium sulfate (WA Hammond Drierite Company, Xenia, Ohio). Stratum corneum samples were weighed (5-8 mg per sample) using a Mettler AE 200 scale (Mettler Toledo, Columbus, OH), placed in vials with three ml solvent and 100 µg radio labeled compound (n = 5) and capped. Solvent samples (250 µl) were removed after 24 hours. Compound concentrations were estimated by direct radiolabel counts using Ecolume (ICN Costa Mesa, CA) and a Packard Model 1900TR Liquid Scintillation Counter (Packard Chemical Co., Downers Grove, IL). The stratum corneum was dried by gentle blotting on Kimwipe™ and combusted in a Packard Model 306 Tissue Oxidizer (Packard Chemical Co., Downers Grove, IL) for scintillation counting. For partition coefficient determinations, radioactivity content in the vehicle mixture and stratum corneum were normalized to 1000 mg vehicle (C_{vehicle}) and 1000 mg stratum corneum ($C_{\text{stratum corneum}}$), respectively. The log stratum corneum/vehicle partition coefficient was determined from the equation: $\log P = \log C_{\text{stratum corneum}}/C_{\text{vehicle}}$.

Permeability

A flow through diffusion cell system, incorporating 500 µm dermatomed porcine skin disks from the backs of female weanling Yorkshire pigs as diffusion barriers, was used according to the methodology of Chang and Riviere (Chang & Riviere 1991) as adapted from Bronaugh and Stewart (Bronaugh & Stewart 1985). The solvent volume was 20 µl. Doses (followed standard errors in brackets) were: 10.34 µg/cm² (0.24) for methyl parathion, 15.15

$\mu\text{g}/\text{cm}^2$ (0.33) for ethyl parathion, $5.69 \mu\text{g}/\text{cm}^2$ (0.13) for chlorpyrifos, $5.93 \mu\text{g}/\text{cm}^2$ (0.06) for fenthion, $7.89 \mu\text{g}/\text{cm}^2$ (0.06) for phenol, $13.71 \mu\text{g}/\text{cm}^2$ (0.05) for p-nitrophenol, $13.43 \mu\text{g}/\text{cm}^2$ (0.16) for pentachlorophenol, $8.61 \mu\text{g}/\text{cm}^2$ (0.03) for atrazine, $6.87 \mu\text{g}/\text{cm}^2$ (0.08) for simazine and $10.69 \mu\text{g}/\text{cm}^2$ (0.14) for propazine. Perfusate ($n = 4$ or 5) was collected at 15 min intervals for the first two hours, and at 1-hour intervals thereafter up to 8 hours. Radiolabel in the perfusate was determined by liquid scintillation as described above. The receptor fluid was assumed to be an infinite sink due to constant receptor fluid flow out of the diffusion cell. Permeability (cm/hr) was estimated by dividing the slope of the steady-state portion of the cumulative mass absorbed/time curve with the concentration in the donor solvent.

Polarity index

A polarity index was used to quantify the relative polarity of the various solvent systems. This allowed relative solvent system polarity to be used in quantitative comparisons. A solvent system polarity index was created by summing the products of the LogP values of each component and their proportional contributions to the total mass of the solvent system (mass of component/mass of total solvent system). The summed products were then normalized by adding the absolute value of the lowest number to each summed product. This resulted in an index where the lowest index value was zero and increased index value represented increased non-polarity. LogP values were obtained from the Syracuse Research Corporation online database and are: -0.31 for ethanol, -1.38 for water, -0.92 for propylene glycol, 0.83 for MNA and 1.60 for SLS. LogP values of the solutes are: 1.46 for phenol, 1.91 for p-nitrophenol, 2.18 for simazine, 2.61 for atrazine, 2.86 for methyl parathion, 2.93 for propazine, 3.83 for parathion, 4.09 for fenthion, 4.96 for chlorpyrifos and 5.12 for pentachlorophenol (Syracuse Research Corporation 2005).

Cluster analysis

Cluster analysis was conducted using the statistics toolbox of Matlab™ version 6.5.0.180913a release13 (The Mathworks Inc, Natick, MA). Two methods were used: K-means clustering and hierarchical clustering (Jain & Dubes 1988; Duda et al 2000).

To perform K-means clustering, variables were assigned to a predetermined number of clusters based on the relatedness of the observations associated with each member of the cluster. Relatedness was determined through an

iterative algorithm to minimize the sum of distances from each object to its cluster centroid. Mean silhouette values were used to determine the optimum number of clusters.

Hierarchical clustering created a multi-level binary cluster tree, which linked clusters formed at a lower level to form higher-level clusters. The Euclidian distances between pairs of variables in the data matrix were computed. Various indices of similarity and dissimilarity were used to link variables into a cluster tree including shortest distance, largest distance, average distance, centroid distance and incremental sum of squares. The relative efficiencies of the clustering solutions based on the similarity and dissimilarity indices were determined by calculating cophenetic correlation coefficients, which compared the cluster tree links with the distance vectors between pairs of data variables.

RESULTS

Partitioning data structure

Stratum corneum/solvent partitioning results were summarized in Table 5.1. The solvent systems were clustered based on their influence on the log stratum corneum/solvent partitioning (logP) values of 10 solutes (Table 5.1) into hierarchical clusters (Figure 5.1) and K-means clusters (Table 5.2). The optimum number of K-means clusters was four with a mean silhouette value of 0.6860 (Table 5.2). Average distance provided the most efficient hierarchical clustering solution (Figure 5.1). The cophenetic correlation coefficients of the cluster trees were: 0.9311 for largest distance, 0.9620 for shortest distance, 0.9663 for average distance, 0.9658 for centroid distance and 0.9137 for incremental sum of squares. Hierarchical clustering and K-means clustering resulted in the same 4-cluster solution – except for solvent systems 5 (ethanol and water) and 6 (ethanol, water and MNA). They were grouped with the K-means cluster 1 (Table 5.2), but were grouped with the hierarchical cluster 2. The hierarchical cluster 2 was similar to the K-means cluster 2.

Permeability data structure

Permeability results were summarized in Table 5.3. The solvent systems were clustered based on their influence on solute permeability values of 10 solutes (Table 5.3) into hierarchical clusters (Figure 5.2) and K-means clusters (Table 5.4). The optimum number of K-means clusters was three with a mean silhouette value of 0.7109 (Table 5.4).

Average distance provided the most efficient hierarchical clustering solution (Figure 5.2). The cophenetic correlation coefficients of the cluster trees were: 0.8889 for largest distance, 0.9265 for shortest distance, 0.9359 for average distance, 0.9356 for centroid distance and 0.8310 for incremental sum of squares. Water and water with MNA formed a separate cluster in hierarchical clustering, but formed part of the first K-means cluster (Table 5.4; Figure 5.2). The second and third K-means clusters were preserved during hierarchical clustering.

Polarity index

The polarity indexes for the solvent systems were summarized in Table 5.5. The average polarity indexes for the hierarchical clusters and K-means clusters based on stratum corneum/solvent partitioning were summarized in Table 5.6.

DISCUSSION

A clustering structure based on solvent polarity emerged from the partitioning data (Table 5.2; Table 5.6; Figure 5.1). Hierarchical cluster one may be described as mildly non-polar (average polarity index: 0.369), cluster two as substantially non-polar (average polarity index: 0.727), cluster three as mildly polar (average polarity index: 0.235) and cluster four as substantially polar (average polarity index: 0.001). K-means clusters showed similar polarity index values (Table 5.6). The inter-molecular attraction between molecules of similar polarity is relatively higher when compared to the inter-molecular attraction between molecules of dissimilar polarity. When other system conditions are equal, solute molecules in a solvent system of dissimilar polarity therefore exist in a state of higher potential energy compared to solute molecules in a solvent system of similar polarity. Due to these effects of inter-molecular forces on enthalpy, energy equilibrium was reached when partitioning into the non-polar environment of the stratum corneum lipids of the relatively non-polar compounds were higher from polar solvents than from non-polar solvents.

The addition of MNA to solvents did not alter partitioning – indicating that MNA had little effect on enthalpy. This was largely due to the low proportions (0.12 – 0.16 % by mass) of the solvent systems consisting of MNA.

The addition of SLS caused solvent systems with relatively high proportions of water and no ethanol, which had low polarity index values indicating high polarity (Table 5.5) to cluster with more non-polar solvents in the partitioning data (Figure 5.1), while the clustering behavior of non-polar solvents was unchanged. Partitioning from water of more polar solutes, such as phenol and p-nitrophenol, was not increased by the addition of SLS, while partitioning from water of more non-polar solutes, such as chlorpyrifos and pentachlorophenol, was increased (Table 5.2). The detection of substantially polar solvent systems as a separate cluster from similar solvent systems with the addition of SLS indicated that the group of solutes partitioned, on average, similarly. However, correlation between the degree of solute non-polarity and the degree of change in partitioning supported the hypothesis that SLS form micelles around non-polar molecules in water and reduces their potential energy in polar solvents (Shokri et al 2001; Baynes et al 2002; van der Merwe & Riviere 2005). These results also agree with previously published analysis, which indicated that partitioning into porcine stratum corneum from polar solvents is correlated with compound lipophilicity when using octanol/water partitioning as a predictor of compound lipophilicity (van der Merwe & Riviere 2005; Van der Merwe & Riviere 2005).

The addition of MNA did not alter the permeability data structure. These results may not be relevant to the effects of MNA *in vivo* because MNA is a vaso-constrictor, but it indicates that MNA does not alter solute diffusivity in the skin.

Hierarchical clustering of the permeability data separated water from ethanol, water from water with SLS and water from ethanol with SLS (cluster 4 in Figure 5.2), but the difference was not large enough to form a separate cluster using K-means clustering (Table 5.4). The difference in clustering reflects differences in the efficiency of the two clustering methods. The separation detected in the hierarchical clusters based on permeability was, however, consistent with the partitioning data structure and may be due to the influence of partitioning on permeability. Ethanol/water mixtures, however, consistently formed a separate cluster due to higher permeability, which was not consistent with the clusters based on partitioning. Since the separation of ethanol/water mixtures cannot be explained based on partitioning, but consistently formed a separate cluster based on permeability (cluster 3 in Figure 5.2), it suggests that this mixture significantly alters diffusivity. This result was consistent with findings from other studies, which reported enhanced dermal absorption when a mixture of ethanol and water was used as a solvent

compared to water or ethanol alone (Berner et al 1989; Kurihara-Bergstrom et al 1990; Megrab et al 1995; Kim et al 1996; Levang et al 1999; Panchagnula et al 2001).

Solvents containing propylene glycol formed a consistent cluster in the permeability data (cluster 2 in Figure 5.2). This was due to consistently low solute permeability when compared to solvents without propylene glycol. Propylene glycol, which has a vapor pressure of 0.129 mmHg at 25 °C, has a low rate of evaporation compared to water and ethanol, which has vapor pressures of 23.8 mmHg and 59.3 mm Hg, respectively, at 25 °C (Syracuse Research Corporation 2005). This supports the hypothesis that water and ethanol evaporation, when the skin surface is open to the atmosphere, causes solute super saturation if all or most of the solvent evaporates within the experimental period. Super saturation provides a high solute concentration gradient across the skin that accelerates solute absorption – an effect that would be absent from experiments using propylene glycol as the solvent due to the low volatility of propylene glycol. Super saturation could also explain the significance of Henry's Law constant in hybrid quantitative structure permeation relationship models of permeability, which take solvent mixture effects into account. This is due to the influence of volatility on the value of Henry's Law, which is defined as vapor pressure divided by water solubility (Riviere & Brooks).

Inconsistencies between the partitioning data structure and the permeability data structure suggested that the degree of solute partitioning into the skin does not predict the rate of solute movement through the skin. Since permeability incorporates both partitioning and rate of movement, permeability cannot be derived from partitioning data alone. It follows that predictive models of dermal absorption over limited periods of time that include partitioning and rate of movement terms should perform better than models that are based solely on partitioning. Solutes partitioned into the stratum corneum may be absorbed over long periods of time in spite of low diffusivity, while low diffusivity can have a more limiting influence on total absorption over the time-span of an 8-hour experiment. Models that predict partitioning could, therefore, be more successful for predicting total absorption over infinite time, but such models may not be clinically relevant.

We concluded that the determining influence of solvent polarity on the partitioning data structure supported the hypothesis that solvent polarity drives the partitioning of non-polar solutes. Solvent polarity did not consistently

predict permeability because solvent effects on diffusivity masked the effects of partitioning on permeability. The consistent reduction of permeability associated with the inclusion of propylene glycol, which has a low volatility compared to water and ethanol, in the solvent system supports the hypothesis that super saturation due to solvent evaporation has a marked effect on permeability. These results demonstrated the potential of using cluster analysis of large datasets to identify consistent solvent and chemical mixture effects on permeability related to identifiable solvent characteristics. Expansion of such datasets should be balanced by including compounds and chemical mixtures representing a range of physical-chemical characteristics. This approach does not allow exact predictions of permeability, but it could be valuable when novel chemical mixtures are assessed. In a practical sense, it could enable improved permeability estimation for the purposes of risk assessment of chemical exposure and the potential for pharmaceutical drug absorption from complex chemical mixtures.

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Table 5.1. The log of stratum corneum/solvent partitioning (LogP) followed by standard errors in brackets. PNP is p-Nitrophenol, PCP is pentachlorophenol, Mparathion is Methyl parathion, MNA is methyl nicotinate, PG is propylene glycol and SLS is sodium lauryl sulphate.

	Phenol (LogP)	PNP (LogP)	PCP (LogP)	Mparathion (LogP)	Parathion (LogP)	Chlorpyrifos (LogP)	Fenthion (LogP)	Simazine (LogP)	Atrazine (LogP)	Propazine (LogP)
Ethanol	0.56 (0.05)	0.63 (0.06)	0.90 (0.09)	0.73 (0.05)	0.98 (0.04)	0.85 (0.07)	0.69 (0.06)	0.77 (0.01)	0.78 (0.05)	0.80 (0.06)
Ethanol+MNA	0.55 (0.10)	0.59 (0.09)	1.06 (0.03)	0.95 (0.09)	1.11 (0.08)	0.83 (0.06)	0.81 (0.04)	0.63 (0.06)	0.73 (0.08)	0.58 (0.06)
Ethanol+SLS	0.50 (0.04)	0.66 (0.07)	1.19 (0.14)	0.77 (0.04)	0.76 (0.09)	0.79 (0.03)	0.76 (0.03)	0.93 (0.03)	0.60 (0.10)	0.74 (0.05)
Ethanol+MNA+SLS	0.51 (0.03)	0.54 (0.08)	0.97 (0.02)	0.95 (0.05)	0.81 (0.06)	0.92 (0.07)	0.65 (0.09)	0.92 (0.03)	0.79 (0.03)	0.75 (0.10)
Ethanol+Water	0.75 (0.05)	0.69 (0.01)	1.60 (0.04)	0.99 (0.05)	0.96 (0.06)	1.61 (0.03)	1.01 (0.05)	1.25 (0.02)	0.67 (0.05)	0.77 (0.10)
Ethanol+Water+MNA	0.77 (0.02)	0.72 (0.02)	1.48 (0.04)	1.10 (0.08)	0.91 (0.03)	1.34 (0.06)	1.06 (0.06)	1.17 (0.03)	0.61 (0.03)	0.76 (0.04)
Ethanol+Water+SLS	0.59 (0.04)	0.48 (0.04)	0.80 (0.03)	0.89 (0.08)	0.81 (0.11)	0.69 (0.03)	0.55 (0.07)	0.95 (0.02)	0.62 (0.06)	0.49 (0.04)
Ethanol+Water+MNA+SLS	0.53 (0.03)	0.65 (0.05)	0.87 (0.03)	0.87 (0.05)	0.68 (0.04)	0.70 (0.04)	0.69 (0.02)	0.77 (0.09)	0.56 (0.06)	0.57 (0.08)
Water	1.08 (0.04)	1.25 (0.02)	2.53 (0.04)	1.92 (0.08)	2.95 (0.06)	3.78 (0.03)	3.01 (0.04)	0.74 (0.02)	1.72 (0.11)	1.97 (0.09)
Water+MNA	1.08 (0.04)	1.22 (0.04)	1.70 (0.13)	1.97 (0.04)	2.99 (0.21)	3.25 (0.06)	2.51 (0.05)	1.57 (0.06)	1.50 (0.04)	2.18 (0.15)
Water+SLS	1.17 (0.03)	1.21 (0.02)	1.30 (0.09)	1.37 (0.04)	1.11 (0.07)	1.42 (0.03)	1.28 (0.02)	1.31 (0.03)	1.21 (0.02)	1.21 (0.07)
Water+MNA+SLS	1.02 (0.02)	1.11 (0.01)	1.34 (0.03)	1.24 (0.04)	1.22 (0.03)	1.29 (0.02)	1.08 (0.02)	1.15 (0.05)	1.18 (0.03)	1.17 (0.04)
Ethanol+PG	1.08 (0.08)	0.88 (0.05)	1.08 (0.02)	0.92 (0.07)	0.78 (0.03)	0.96 (0.05)	0.78 (0.05)	1.09 (0.04)	0.75 (0.09)	0.91 (0.12)
Ethanol+PG+MNA	0.92 (0.07)	0.79 (0.05)	0.96 (0.04)	1.39 (0.04)	1.03 (0.16)	0.85 (0.09)	0.88 (0.01)	1.01 (0.03)	0.95 (0.07)	0.85 (0.07)
Ethanol+PG+SLS	0.56 (0.06)	0.42 (0.04)	0.86 (0.02)	0.91 (0.09)	0.89 (0.04)	0.82 (0.05)	0.73 (0.06)	1.02 (0.09)	0.79 (0.04)	0.64 (0.04)
Ethanol+PG+MNA+SLS	0.58 (0.02)	0.53 (0.04)	0.80 (0.03)	0.86 (0.07)	0.87 (0.06)	0.89 (0.04)	0.66 (0.08)	0.92 (0.07)	0.85 (0.11)	0.75 (0.06)
PG	0.69 (0.10)	0.88 (0.03)	1.40 (0.06)	1.37 (0.07)	1.10 (0.05)	1.24 (0.03)	0.89 (0.05)	0.78 (0.05)	1.26 (0.09)	0.99 (0.16)
PG+MNA	0.94 (0.06)	0.74 (0.11)	1.28 (0.03)	1.24 (0.05)	1.40 (0.03)	1.31 (0.06)	0.85 (0.03)	1.31 (0.04)	0.99 (0.12)	1.20 (0.14)
PG+SLS	0.70 (0.07)	0.46 (0.05)	1.12 (0.04)	0.86 (0.05)	0.98 (0.02)	0.97 (0.04)	0.77 (0.11)	0.99 (0.06)	0.62 (0.05)	0.65 (0.04)
PG+MNA+SLS	0.67 (0.10)	0.54 (0.03)	1.06 (0.04)	0.80 (0.07)	1.03 (0.06)	0.86 (0.05)	0.74 (0.04)	0.91 (0.04)	0.75 (0.06)	0.80 (0.03)
Water+PG	0.80 (0.03)	0.78 (0.01)	1.75 (0.03)	1.29 (0.05)	1.42 (0.11)	2.09 (0.03)	1.58 (0.06)	1.00 (0.02)	0.75 (0.03)	0.95 (0.07)
Water+PG+MNA	0.73 (0.03)	0.63 (0.03)	1.84 (0.01)	1.17 (0.05)	2.02 (0.05)	2.17 (0.02)	1.49 (0.04)	0.94 (0.06)	0.77 (0.04)	0.97 (0.04)
Water+PG+SLS	0.58 (0.03)	0.50 (0.03)	0.92 (0.02)	0.93 (0.05)	0.81 (0.04)	0.72 (0.03)	0.72 (0.02)	1.04 (0.03)	0.66 (0.06)	0.61 (0.04)
Water+PG+MNA+SLS	0.65 (0.04)	0.66 (0.02)	1.12 (0.20)	0.80 (0.07)	0.89 (0.05)	1.10 (0.02)	0.76 (0.02)	0.90 (0.06)	0.67 (0.03)	0.71 (0.03)

Table 5.2. K-means clustering based on stratum corneum/solvent partitioning. MNA is methyl nicotinate, SLS is sodium lauryl sulphate and PG is propylene glycol.

Solvent	Cluster
Ethanol + Water	1
Ethanol +Water + MNA	1
Water + SLS	1
Water + MNA + SLS	1
PG	1
PG + MNA	1
Ethanol	2
Ethanol + MNA	2
Ethanol + SLS	2
Ethanol + MNA + SLS	2
Ethanol + Water + SLS	2
Ethanol + Water + MNA + SLS	2
Ethanol + PG	2
Ethanol + PG + MNA	2
Ethanol + PG + SLS	2
Ethanol + PG + MNA + SLS	2
PG + SLS	2
PG + MNA + SLS	2
Water + PG + SLS	2
Water + PG + MNA + SLS	2
Water + PG	3
Water + PG + MNA	3
Water	4
Water + MNA	4

Table 5.3. Solute permeability (cm/hr x 10⁻³) followed by standard errors in brackets. PNP is p-Nitrophenol, PCP is pentachlorophenol, Mparathion is Methyl parathion, MNA is methyl nicotinate, PG is propylene glycol and SLS is sodium lauryl sulphate.

	Phenol	PNP	PCP	Mparathion	Parathion	Chlorpyrifos	Fenthion	Simazine	Atrazine	Propazine
Ethanol	421 (0.29)	0.35 (0.02)	0.08 (0.02)	0.18 (0.02)	0.16 (0.03)	0.01 (0.00)	0.10 (0.01)	0.095 (0.01)	0.07 (0.00)	0.03 (0.01)
Ethanol+MNA	288 (0.32)	0.36 (0.03)	0.10 (0.02)	1.43 (0.47)	0.14 (0.01)	0.01 (0.00)	0.05 (0.00)	0.037 (0.00)	0.19 (0.05)	0.02 (0.00)
Ethanol+SLS	444 (0.24)	1.60 (0.07)	0.30 (0.08)	0.71 (0.05)	0.12 (0.01)	0.04 (0.00)	0.26 (0.04)	0.355 (0.03)	0.61 (0.03)	0.09 (0.01)
Ethanol+MNA+SLS	271 (0.42)	1.22 (0.13)	0.18 (0.03)	0.71 (0.11)	0.20 (0.02)	0.02 (0.00)	0.10 (0.01)	0.135 (0.00)	0.39 (0.04)	0.04 (0.01)
Ethanol+Water	10.50 (0.41)	4.99 (0.22)	1.43 (0.27)	4.15 (0.32)	0.29 (0.02)	0.15 (0.03)	0.40 (0.04)	0.259 (0.03)	1.75 (0.12)	0.42 (0.04)
Ethanol+Water+MNA	8.00 (0.54)	6.26 (0.49)	0.32 (0.04)	3.08 (0.18)	0.37 (0.05)	0.11 (0.01)	0.18 (0.04)	0.081 (0.01)	0.74 (0.07)	0.06 (0.01)
Ethanol+Water+SLS	5.50 (0.06)	2.21 (0.06)	0.12 (0.04)	0.73 (0.04)	0.18 (0.01)	0.04 (0.01)	0.17 (0.02)	0.340 (0.05)	0.75 (0.07)	0.17 (0.01)
Ethanol+Water+MNA+SLS	3.36 (0.54)	1.17 (0.10)	0.06 (0.01)	0.45 (0.03)	0.28 (0.02)	0.04 (0.01)	0.02 (0.00)	0.293 (0.04)	0.38 (0.02)	0.09 (0.01)
Water	4.38 (0.19)	2.22 (0.34)	1.65 (0.22)	4.92 (0.63)	0.41 (0.05)	0.06 (0.02)	0.46 (0.01)	0.484 (0.06)	1.13 (0.22)	0.23 (0.01)
Water+MNA	3.56 (0.35)	3.12 (0.46)	0.65 (0.11)	3.81 (1.44)	1.12 (0.08)	0.02 (0.01)	0.36 (0.01)	0.218 (0.03)	0.45 (0.18)	0.05 (0.01)
Water+SLS	4.38 (0.23)	1.75 (0.09)	0.39 (0.04)	0.63 (0.07)	0.24 (0.04)	0.05 (0.01)	0.19 (0.03)	0.199 (0.04)	0.70 (0.12)	0.14 (0.03)
Water+MNA+SLS	2.75 (0.23)	1.21 (0.14)	0.27 (0.05)	0.94 (0.12)	0.46 (0.11)	0.04 (0.00)	0.06 (0.00)	0.172 (0.03)	0.37 (0.08)	0.03 (0.00)
Ethanol+PG	1.04 (0.12)	0.128 (0.01)	0.024 (0.00)	0.17 (0.02)	0.02 (0.00)	0.02 (0.00)	0.06 (0.01)	0.034 (0.01)	0.07 (0.00)	0.03 (0.00)
Ethanol+PG+MNA	0.73 (0.10)	0.146 (0.03)	0.007 (0.00)	0.27 (0.05)	0.03 (0.00)	0.02 (0.01)	0.02 (0.00)	0.049 (0.02)	0.08 (0.00)	0.03 (0.00)
Ethanol+PG+SLS	0.85 (0.05)	0.074 (0.01)	0.013 (0.00)	0.08 (0.01)	0.05 (0.01)	0.01 (0.00)	0.06 (0.01)	0.141 (0.03)	0.15 (0.02)	0.07 (0.01)
Ethanol+PG+MNA+SLS	0.74 (0.11)	0.067 (0.00)	0.010 (0.00)	0.15 (0.04)	0.06 (0.00)	0.01 (0.00)	0.01 (0.00)	0.231 (0.03)	0.15 (0.02)	0.07 (0.00)
PG	0.13 (0.01)	0.013 (0.00)	0.013 (0.00)	0.05 (0.01)	0.03 (0.00)	0.01 (0.00)	0.02 (0.00)	0.023 (0.00)	0.03 (0.02)	0.01 (0.00)
PG+MNA	0.22 (0.03)	0.039 (0.01)	0.015 (0.00)	0.17 (0.08)	0.06 (0.00)	0.01 (0.01)	0.01 (0.00)	0.034 (0.01)	0.06 (0.04)	0.01 (0.00)
PG+SLS	0.18 (0.02)	0.030 (0.01)	0.016 (0.00)	0.04 (0.01)	0.04 (0.02)	0.01 (0.00)	0.03 (0.00)	0.062 (0.04)	0.03 (0.00)	0.01 (0.00)
PG+MNA+SLS	0.50 (0.14)	0.026 (0.01)	0.010 (0.00)	0.06 (0.00)	0.06 (0.02)	0.02 (0.01)	0.02 (0.00)	0.165 (0.04)	0.13 (0.07)	0.01 (0.00)
Water+PG	1.32 (0.40)	0.152 (0.01)	0.091 (0.01)	0.23 (0.04)	0.03 (0.02)	0.02 (0.00)	0.09 (0.01)	0.022 (0.01)	0.12 (0.05)	0.02 (0.00)
Water+PG+MNA	0.90 (0.18)	0.324 (0.06)	0.020 (0.00)	0.20 (0.03)	0.03 (0.01)	0.04 (0.02)	0.07 (0.04)	0.044 (0.02)	0.22 (0.11)	0.01 (0.00)
Water+PG+SLS	0.61 (0.05)	0.049 (0.00)	0.020 (0.00)	0.08 (0.01)	0.04 (0.00)	0.04 (0.01)	0.04 (0.01)	0.048 (0.00)	0.10 (0.01)	0.05 (0.00)
Water+PG+MNA+SLS	0.56 (0.03)	0.060 (0.01)	0.010 (0.00)	0.12 (0.03)	0.11 (0.03)	0.01 (0.00)	0.01 (0.00)	0.157 (0.02)	0.16 (0.01)	0.03 (0.01)

Table 5.4. K-means clustering based on permeability. MNA is methyl nicotinate, SLS is sodium lauryl sulphate and PG is propylene glycol.

Solvent	Cluster
Ethanol	1
Ethanol + MNA	1
Ethanol + SLS	1
Ethanol + MNA + SLS	1
Ethanol + Water + SLS	1
Ethanol + Water + MNA + SLS	1
Water	1
Water + MNA	1
Water + SLS	1
Water + MNA + SLS	1
Ethanol + PG	2
Ethanol + PG + MNA	2
Ethanol + PG + SLS	2
Ethanol+ PG c+ MNA + SLS	2
PG	2
PG + MNA	2
PG + SLS	2
PG + MNA + SLS	2
Water + PG	2
Water + PG + MNA	2
Water + PG + SLS	2
Water + PG + MNA + SLS	2
Ethanol + Water	3
Ethanol + Water + MNA	3

Table 5.5. Solvent system numbers, names, proportional mass compositions and polarity indexes. PG is propylene glycol, MNA is methyl nicotinate and SLS is sodium lauryl sulphate.

Number	Solvent system	%Ethanol	%Water	%PG	%MNA	%SLS	Polarity index
1	Ethanol	100	0	0	0	0	1.070
2	Ethanol+MNA	99.84	0	0	0.16	0	1.072
3	Ethanol+SLS	62.76	26.60	0	0	10.64	0.989
4	Ethanol+MNA+SLS	62.67	26.56	0	0.14	10.63	0.990
5	Ethanol+Water	43.30	56.70	0	0	0	0.463
6	Ethanol+Water+MNA	43.92	55.94	0	0.14	0	0.473
7	Ethanol+Water+SLS	39.55	50.38	0	0	10.08	0.723
8	Ethanol+Water+MNA+SLS	39.50	50.31	0	0.13	10.06	0.725
9	Water	0	100	0	0	0	0
10	Water+MNA	0	99.87	0	0.13	0	0.003
11	Water+SLS	0	90.91	0	0	9.09	0.271
12	Water+MNA+SLS	0	90.47	0	0.12	9.41	0.283
13	Ethanol+PropGlyc	43.11	0	56.89	0	0	0.723
14	Ethanol+PropGlyc+MNA	43.05	0	56.81	0.14	0	0.725
15	Ethanol+PropGlyc+SLS	28.46	24.22	37.63	0	9.69	0.766
16	Ethanol+PropGlyc+MNA+SLS	28.43	24.19	37.59	0.12	9.67	0.768
17	PropGlyc	0	0	100	0	0	0.460
18	PropGlyc+MNA	0	0	99.87	0.13	0	0.462
19	PropGlyc+SLS	0	22.18	68.95	0	8.87	0.582
20	PropGlyc+MNA+SLS	0	22.96	67.74	0.12	9.18	0.588
21	Water+PropGlyc	0	49.11	50.89	0	0	0.234
22	Water+PropGlyc+MNA	0	49.05	50.82	0.13	0	0.237
23	Water+PropGlyc+SLS	0	44.72	46.34	0	8.94	0.480
24	Water+PropGlyc+MNA+SLS	0	44.51	46.12	0.12	9.26	0.491

Table 5.6. The average polarity indexes and ranges of the hierarchical clusters and K-means clusters based on stratum corneum/solvent partitioning.

	Average polarity index	Range
Hierarchical clusters		
1	0.369	0.271 - 0.462
2	0.727	0.463 - 1.072
3	0.235	0.234 - 0.237
4	0.001	0.000 - 0.003
K-means clusters		
1	0.325	0.271 - 0.473
2	0.764	0.480 - 1.070
3	0.235	0.234 - 0.237
4	0.001	0.000 - 0.003

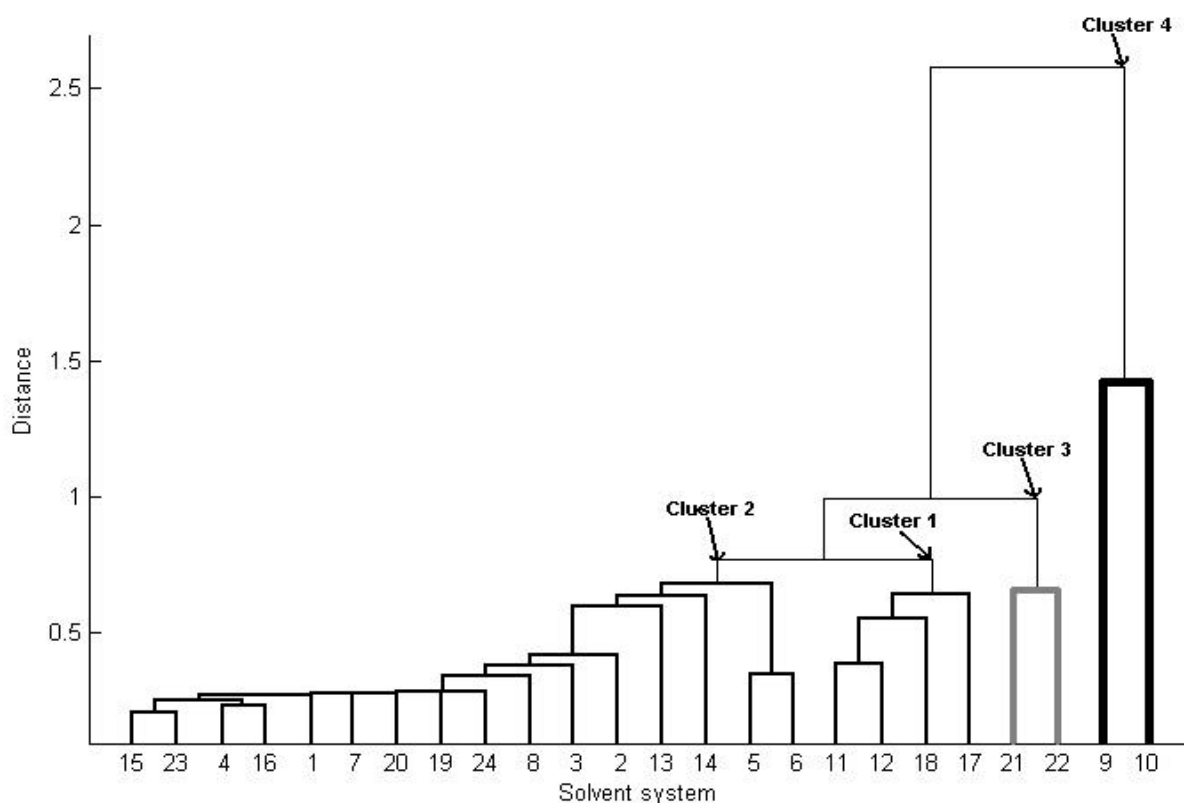


Figure 5.1. A hierarchical average distance cluster tree of 24 solvent systems based on the log of stratum corneum/solvent partitioning values of methyl parathion, ethyl parathion, atrazine, phenol, p-nitrophenol, pentachlorophenol, simazine, propazine, chlorpyrifos and fenthion. The solvent systems are: 1 - ethanol; 2 - ethanol and MNA; 3 - ethanol and SLS; 4 - ethanol, MNA and SLS; 5 - ethanol and water; 6 - ethanol, water and MNA; 7 - ethanol, water and SLS; 8 - ethanol, water, MNA and SLS; 9 - water; 10 - water and MNA; 11 - water and SLS; 12 - water, MNA and SLS; 13 - ethanol and propylene glycol; 14 - ethanol, propylene glycol and MNA; 15 - ethanol, propylene glycol and SLS; 16 - ethanol, propylene glycol, MNA and SLS; 17 - propylene glycol; 18 - propylene glycol and MNA; 19 - propylene glycol and SLS; 20 - propylene glycol, MNA and SLS; 21 - water and propylene glycol; 22 - water, propylene glycol and MNA; 23 - water, propylene glycol and SLS; 24 - water, propylene glycol, MNA and SLS.

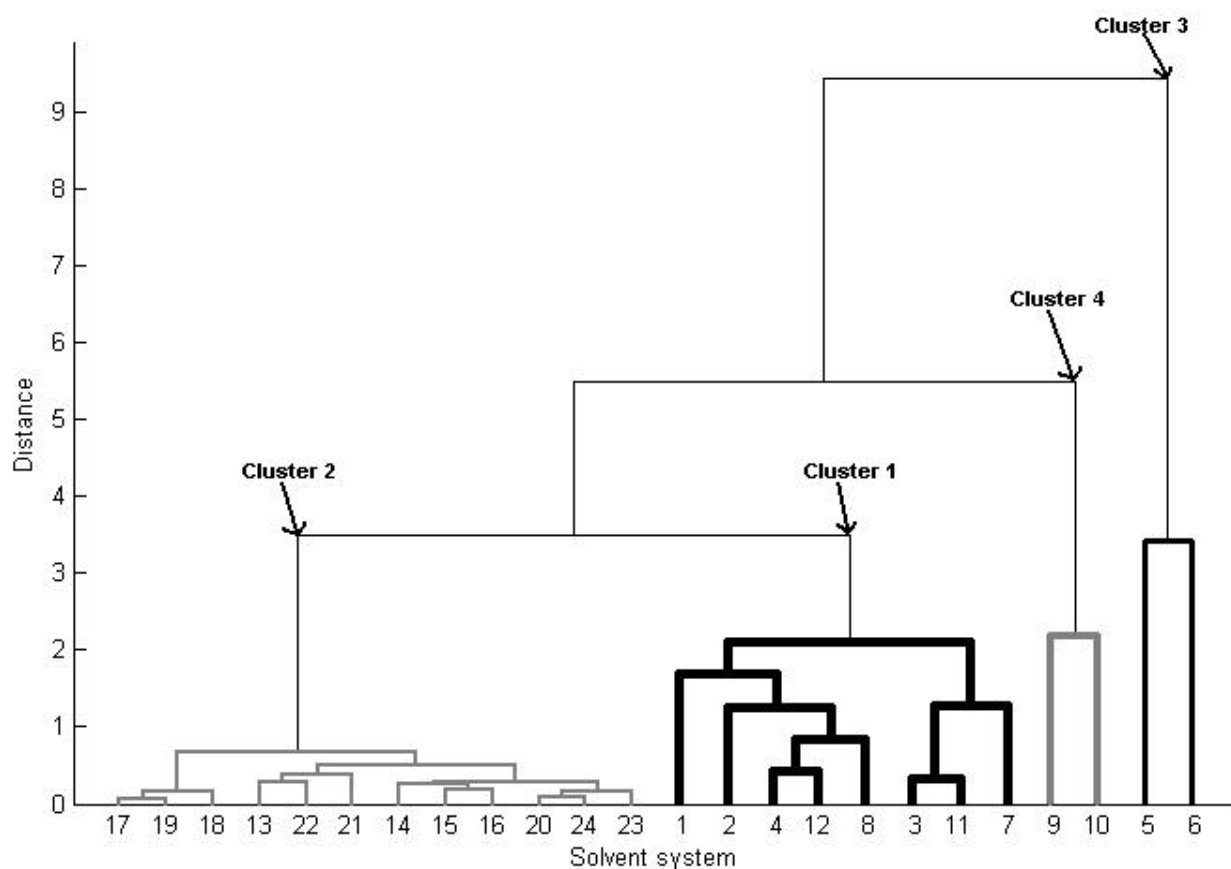


Figure 5.2. A hierarchical average distance cluster tree of 24 solvent systems based on the skin permeability of methyl parathion, ethyl parathion, atrazine, phenol, p-nitrophenol, pentachlorophenol, simazine, propazine, chlorpyrifos and fenthion. The solvent systems are: 1 - ethanol; 2 – ethanol and MNA; 3 – ethanol and SLS; 4 – ethanol, MNA and SLS; 5 – ethanol and water; 6 – ethanol, water and MNA; 7 – ethanol, water and SLS; 8 – ethanol, water, MNA and SLS; 9 – water; 10 – water and MNA; 11 – water and SLS; 12 – water, MNA and SLS; 13 – ethanol and propylene glycol; 14 – ethanol, propylene glycol and MNA; 15 – ethanol, propylene glycol and SLS; 16 – ethanol, propylene glycol, MNA and SLS; 17 - propylene glycol; 18 - propylene glycol and MNA; 19 - propylene glycol and SLS; 20 - propylene glycol, MNA and SLS; 21 – water and propylene glycol; 22 – water, propylene glycol and MNA; 23 – water, propylene glycol and SLS; 24 – water, propylene glycol, MNA and SLS.

**6. A PHYSIOLOGICAL-BASED PHARMACOKINETIC MODEL OF ORGANOPHOSPHATE
DERMAL ABSORPTION**

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ABSTRACT

The rate and extent of dermal absorption are important in the analysis of risk from dermal exposure to toxic chemicals and for the development of topically applied drugs, barriers, insect repellants and cosmetics. *In vitro* flow-through cells offer a convenient method for the study of dermal absorption that is relevant to the initial processes of dermal absorption. This study describes a physiological-based pharmacokinetic (PBPK) model developed to simulate the absorption of organophosphate pesticides, such as parathion, fenthion and methyl parathion through porcine skin using flow-through cells. Parameters related to the structure of the stratum corneum and solvent evaporation rates were independently estimated. Three parameters were optimized based on experimental dermal absorption data, including solvent evaporation rate, diffusivity and a mass transfer factor. Diffusion cell studies were conducted to validate the model under a variety of conditions including different dose ranges (6.3-106.9 $\mu\text{g}/\text{cm}^2$ for parathion; 0.8-23.6 $\mu\text{g}/\text{cm}^2$ for fenthion; 1.6-39.3 $\mu\text{g}/\text{cm}^2$ for methyl parathion), different solvents (ethanol, 2-propanol and acetone), different solvent volumes (5-120 μl for ethanol; 20-80 μl for 2-propanol and acetone), occlusion versus open to atmosphere dosing, and corneocyte removal by tape-stripping. The study demonstrated the utility of PBPK models for studying dermal absorption, which can be useful as explanatory and predictive tools; and may be used for *in silico* hypotheses generation and limited hypotheses testing. The similarity between the overall shapes of the experimental and model-predicted flux/time curves and the successful simulation of altered system conditions for this series of small, lipophilic compounds indicated that the absorption processes that were described in the model successfully simulated important aspects of dermal absorption in flow-through cells. These data have direct relevance to topical organophosphate pesticide risk assessments.

Key words: Dermal absorption; PBPK model; parathion; fenthion; methyl parathion

INTRODUCTION

Knowledge of the rate and extent of dermal absorption is important in the analysis of risk from dermal exposure to toxic chemicals and for the development of topically applied drugs, barriers, insect repellants and cosmetics. Dermal absorption parameters can be estimated from *in vitro* and *in vivo* experimental data (Riviere 2005), but the substantial investment of resources required and the need for reducing the numbers of animals used in research limits the use of the *in vivo* approach. Estimates of absorption parameters under defined experimental conditions also do not necessarily reflect the values of such parameters under different exposure conditions. The limitations of the experimental approach to absorption parameter estimation have generated much interest in the development of mathematical or so-called *in silico* models of skin permeability.

Published models of dermal absorption may be divided into two types: quantitative structure-activity relationship (QSAR) models, and mathematical models that simulate the effects of partition and transport processes involved in absorption (Fitzpatrick et al 2004). QSAR techniques are widely used to predict the behavior of molecules. When applied to dermal absorption, QSAR models are usually based on statistical correlations of physical-chemical properties of permeants, solvents and chemical mixtures with steady-state permeability constants (Potts & Guy 1995; Sartorelli et al 1998; Sartorelli et al 1999; Geinoz et al 2004; Ghafourian et al 2004; Riviere & Brooks 2005). QSAR and steady-state permeability is widely used as an indicator of absorption potential, but its accuracy is hampered by the scarcity of high quality, comparable absorption data (Fitzpatrick et al 2004). Steady-state permeability also does not predict absorption over time frames outside the steady-state portion of the absorption/time curve. A large number of mathematical models that simulate the effects of chemical partitioning into skin and the transport across skin over time have been developed (Williams et al 1990; McCarley & Bunge 2001; Roberts et al 2001). These models vary in their degree of correlation with skin physiology and anatomy. One extreme are models that are similar to traditional compartmental pharmacokinetic models. They are mathematical constructs that describe the aggregate result of all the processes involved in determining the flux/time curve of dermal absorption. The compartments used are not physiologically or anatomically relevant. Such models are not suited to hypotheses generation and testing involving specific anatomical or physiological changes. Physiological-based pharmacokinetic (PBPK) models

of dermal absorption are constructed from mathematical descriptions of body compartments, tissues and processes of partitioning, movement and metabolism that influence the dermal distribution, absorption and elimination of drugs. Model compartments and processes can be linked to skin physiology and anatomy, which makes such models suitable for hypotheses generation and testing involving anatomical, physiological and environmental change. PBPK model parameters can also be scaled to reflect species, breed, life-stage or pathological differences and changes. The advantages of PBPK models are, however, difficult to realize because the necessary anatomical and physiological parameters are often not available and the processes are not well understood. This can result in oversimplification of the physiological processes involved, which limits the advantage of PBPK models over traditional compartmental models. The inclusion of uncertain parameters may also restrict degrees of freedom to the extent that model-based predictions are irrelevant.

The stratum corneum is the most significant barrier to dermal absorption (Monteiro-Riviere 1986; Bouwstra et al 2003). Flow-through diffusion cells offer a convenient method for the study of dermal absorption that is relevant to the initial processes of dermal absorption – including solvent and chemical mixture effects on the skin surface, as well as partitioning into and transport through the stratum corneum. Parameters such as temperature, absorption surface area, skin thickness, and receptor fluid flow rate can be controlled. Results obtained from flow-through cells are generally relevant to *in vivo* dermal absorption (Bronaugh et al 1982; Howes et al 1996). However, the effects of metabolism in the viable epidermis and dermis, differences between the composition of blood and receptor fluid and the resistance to permeant transfer in these layers may cause the results from flow-through cells to deviate from perfused skin or *in vivo* systems and should be considered when results are used to predict *in vivo* absorption parameters.

Small, lipophilic compounds tend to partition into the stratum corneum lipid matrix after contact with the stratum corneum surface (Raykar et al 1988). The stratum corneum lipid matrix is also the principle route of absorption for such compounds (Albery & Hadgraft 1979). It is possible to describe the micro-structure of the stratum corneum lipid pathway in terms of the average corneocyte dimensions and their relative positioning to characterize the pathway length, lateral bilayer diffusional resistance and volume (Johnson et al 1997; Frisch & Barbero 2003). This offers an opportunity to move away from the use of simple, uniform compartments to

represent the stratum corneum, to the use of anatomically more correct descriptions of the absorption pathway. Solvent evaporation, which has an effect on the permeant concentration gradient across the skin, and permeant partitioning into the stratum corneum, can be estimated independently.

The availability of detailed data on skin structure, dermal absorption in flow-through cells and chemical-specific parameters reduce the limitations of PBPK models of dermal absorption. This study describes a PBPK model developed to simulate the absorption of organophosphate pesticides, such as parathion, through normal porcine skin in flow-through diffusion cells. Pigs are important food animals in many cultures around the world. Dermal absorption models of porcine skin could be used in the estimation of chemical residues in food after accidental or therapeutic topical exposure of pigs to pesticides and other potentially hazardous chemicals. It could also be of value in the development of topical medications. Porcine skin is widely accepted as an appropriate surrogate model for human skin due to its physiological and structural similarity to human skin (Monteiro-Riviere 2001; Schmook et al 2001; Singh et al 2002; Chilcott et al 2005). The model was primarily developed and validated for parathion absorption, but it was also applied to the dermal absorption of two other organophosphate compounds: fenthion and methyl parathion. Secondary aims were to demonstrate the utility of the model for hypotheses generation and as an *in silico* experimental system; to identify critical model parameters; to determine the effects of critical model parameters on the shape of the absorption/time curve; to model the effects of solvent evaporation on dermal absorption; and to model the effects of the removal of stratum corneum layers on dermal absorption.

MATERIALS AND METHODS

Software

PBPK models were constructed using acslXstreme® continuous simulation software (Aegis Technologies (Huntsville, AL)).

Chemicals

Methyl parathion-ring-UL-¹⁴C; (specific activity = 13.8mCi/mmol, purity = 99.5%) and parathion-ring-UL-¹⁴C (specific activity = 9.2mCi/mmol, purity = 97.1%) and propylene glycol (PG) (purity = 99 %) were obtained

from Sigma Chemical Co. (St. Louis, MO). Fenthion-ring-UL-¹⁴C (specific activity = 55mCi/mmol, purity = 98.5%) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Absolute (200 proof) ethanol was obtained from Aaper Alcohol and Chemical Co. (Shelbville, KY). Double distilled water was obtained from our in-house still. Bovine serum albumin (Fract V; cold alcohol precipitated), NaCl (Certified A.C.S.), KCl (Certified A.C.S.), CaCl (Certified A.C.S.; anhydrous), KH₂PO₄ (Certified A.C.S.), MgSO₄-7H₂O (Certified A.C.S.), NaHCO₃ (Certified A.C.S.), acetone (GC grade; Certified A.C.S.), 2-propanol (Certified A.C.S.) and dextrose (Certified A.C.S.; anhydrous) were obtained from Fisher Scientific (Pittsburgh, PA). Amikacin (250 µg/ml) was obtained from Abbott Labs (Chicago, IL). Heparin (1,000 units/ml) was obtained from Elkins Sinn (Cherry Hill, NY). Penicillin G sodium (250,000 units/ml) was obtained from Pfizer Inc. (New York, NY). The receptor solution was prepared according to published methods (Riviere et al 1986) and consisted of 13.78 g NaCl, 5.50 g NaHCO₃, 0.58 g MgSO₄-7H₂O, 0.32 g KH₂PO₄, 0.56 g CaCl, 0.71 g KCl, 2.40 g dextrose, 90.0 g bovine serum albumin, 0.1 ml penicillin G sodium, 10 ml heparin and 0.25 ml amikacin made up to 2l with water.

Stratum corneum/solvent partitioning

Parathion, fenthion and methyl parathion partitioning coefficients between isolated porcine stratum corneum and ethanol at equilibrium were obtained from the literature (Van der Merwe & Riviere 2005).

Flow-through diffusion cell system

Porcine skin disks were prepared from the back skin of female weanling Yorkshire pigs, dermatomed to 0.5 mm, and were used as barrier membranes in a flow-through diffusion cell system according to the methodology of (Bronaugh & Stewart 1985), as adapted by (Chang & Riviere 1991). An 8 hr experimental period was used. Perfusate was collected at 15 min intervals for the first two hrs, and 1 hr intervals thereafter. Radioactivity in the perfusate was determined by liquid scintillation counts (Packard Model 2500TR liquid scintillation counter, Packard Chemical Co.). For occlusion experiments (Figure 6.6), diffusion cells were occluded with Parafilm M (SPI Supplies, West Chester, PA) directly after depositing the dose to the skin surface. Perfect occlusion was not achieved, but ethanol evaporation was reduced.

The permeant concentrations used were selected based on the amount of radiolabel needed for efficient detection using scintillation counting. Concentrations were then multiplied to investigate the range of inference of the model and the validity of assumptions related to the use of Fick's First Law.

Solvent evaporation

Solvent evaporation was estimated gravimetrically by estimating weight loss of solvent in flow-through cell in a temperature and humidity controlled chamber using a calibrated Mettler AE 200 scale (Mettler Toledo, Columbus, OH) at 32°C and 30 % relative humidity. Evaporation rates were estimated from the slope of the linear regression of the linear portion of the weight/time curve.

Physical dimension estimates

The physical dimensions of major stratum corneum structures were estimated from TEM micrographs and phase contrast microscopy of dermatomed back skin from female weanling Yorkshire pigs. Skin sections (three sections per pig from three pigs) were fixed in Trump's fixative, processed and then embedded in Spurr's resin. Thin sections (800–1000 Å) were examined using a Philips EM208S transmission electron microscope. Three pictures per sample were examined. Corneocyte layers were counted, corneocyte overlaps were estimated and corneocyte thickness and inter-corneocyte gap widths were estimated. Corneocyte diameters were estimated using an Olympus CK40 inverted phase contrast microscope (Opelco, Sterling, VA).

Stratum corneum removal by tape stripping

The clipped back skin of a female weanling Yorkshire pig was divided into three areas. Adhesive tape (Crystal Clear HP260, Henkel Consumer Adhesives Inc., Avon, OH) was briefly attached to the skin and then pulled away. One area was not tape stripped, one area was tape stripped five times and one area was tape stripped 80 times. The skin was dermatomed to a depth of 0.5 mm after tape stripping for use as barrier membranes in flow-through cells.

The research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

THE MODEL

Model organization

The model was built from a set of differential and algebraic equations using a block diagram format, which enabled conceptual divisions within the model to be visually represented (Figure 6.1). The blocks also correlated with compartments, which enabled the simulation of solute spatial distribution over time. The first block consisted of a dermal exposure simulator and a tortuosity calculator. It simulated a single dose in the dosing chamber of the flow-through cell, which is altered by solvent evaporation, solute evaporation and solute diffusion into the skin. The tortuosity calculation was added to the first block for convenience, but the calculation is independent of time and is, therefore, independent of the flux/time curve. The second block simulated permeant partitioning into the stratum corneum, diffusion through the stratum corneum lipids and partitioning into the extracellular fluid of the viable epidermis. The third block simulated the viable epidermis and the dermis to the dermatomed depth. The viable skin volume was the combined volume of the viable epidermis and the dermis. The fourth block represented the receptor fluid flowing through the receptor fluid chamber of the flow-through cell.

Assumptions

It would be incorrect to assume that the model fully captures all the processes involved in dermal absorption. This is especially true for processes occurring in the deeper layers of the skin where oversimplification is implicit in the assumptions that the viable skin is an insignificant barrier and that metabolism is negligible. The major metabolites of parathion, p-nitrophenol and paraoxon, found in proportion to the parent compound in a perfused porcine skin flap model were 14.6 % and 3.4 % respectively, while 78.5 % of the parent compound remained intact (Chang et al 1994). Since the bulk of the parathion remained intact in a perfused porcine skin model it was assumed that, although the extent of absorption could be altered to some degree by metabolism, the overall shape of the flux/time curve derived from radiolabeled parathion absorption would be similar in the absence of metabolism. The shape of the flux/time curve is mostly determined by the initial processes of dermal absorption, including solvent evaporation effects on solute concentration on the skin surface, partitioning into the stratum corneum and rate of movement across the stratum corneum because the stratum corneum is the

primary barrier membrane. The model is, therefore, mostly relevant to the initial processes of dermal absorption and its use as an *in silico* experimental system should be limited to these processes.

It was assumed that solutes partitioned exclusively into the lipid phase of the stratum corneum and absorption occurred exclusively through the lipid phase. The stratum corneum was assumed to be uniform in character from its surface to its base, which avoided the need for second-order, partial differential equations. The viable skin was assumed to be a well-mixed environment and an insignificant barrier to diffusion. Minimum solvent volume was 0.1 µl. It was assumed that system conditions remained constant throughout the experimental period. The number of corneocyte layers was assumed to be equal to the average number of layers observed in back skin processed for TEM except where changed to test hypotheses Solvent and permeant loss to the experimental apparatus was assumed to be negligible. Permeant evaporation was assumed to be negligible due to the low volatility of organophosphate pesticides.

Tortuosity calculation

The effective tortuosity (t) of the stratum corneum was calculated according to the method of Johnson *et al.* (Johnson et al 1997).

$$t = 1 + (2g/ah)\ln(kd/2s) + (N*kd*kt/s*ah) + (kd/(1+\omega))^2(\omega/ah*g)(N-1) \quad (1)$$

where t is the effective tortuosity (ratio of diffusivity in stratum corneum with impermeable corneocytes to diffusivity in stratum corneum without impediments), kd is corneocyte diameter (equivalent to “d” of Johnson *et al.* 1997), kt is corneocyte thickness (equivalent to “t” of Johnson *et al.* 1997), N is the number of corneocyte layers, ah is the stratum corneum thickness (equivalent to “h” of Johnson *et al.* 1997), g is the vertical gap between corneocytes, s is the lateral gap between corneocytes and ω is the ratio between the long overlap and short overlap of successive corneocytes (Figure 6.2)

The minimum pathway length (minpath) was predicted from:

$$\text{minpath} = ((kt+g+kd2)*(N-1))+kt \quad (2)$$

where d2 is the short leg of corneocyte overlap. The minimum pathway length is equivalent to the geometric pathway length (hg) proposed by Talreja *et al.* in 2001 (Talreja et al 2001), which is given by:

$$hg = ((kd2/((N/(N-1))*kt+g))+1)*ah \quad (3)$$

Where ah is the actual stratum corneum thickness predicted from:

$$ah = ((Kt+g)*(N-1))+Kt \quad (4)$$

The effective skin thickness (h) was predicted from:

$$h = ((ah*t)/10000) + (\text{dermisdepth} - (ah/10000))$$

where dermisdepth is the thickness of the dermatomed skin in cm. (5)

Permeant flux prediction

The fractional rate of permeant entry into the stratum corneum (Jf1) was predicted from:

$$Jf1 = (P*D*area)/(h/2) \quad (6)$$

Where P is the solvent/stratum corneum partitioning coefficient, D is permeant diffusivity in the stratum corneum lipid and area is the surface area of the skin disk.

The fractional rate of permeant returning to the skin surface (Jf2) was predicted from:

$$Jf2 = (1/P*D*area)/(h/2) \quad (7)$$

The solute concentration on the skin surface (Cs) was predicted from:

$$Cs = A_{\text{surface}}/\text{solventvol} \quad (8)$$

where A_{surface} is the amount of permeant on the skin surface and solventvol is the solvent volume on the skin surface. The solvent volume was predicted from the initial solvent volume, altered by the rate of evaporation to a minimum of 0.1 µl.

The rate of change of permeant on the skin surface (achangesurface) was predicted from:

$$\text{achangesurface} = Cs*(-Jf1)+Csc*Jf2-\text{doseevap} \quad (9)$$

where C_{sc} is the solute concentration in the stratum corneum and $dose_{evap}$ is the rate of solute evaporation into the atmosphere

The stratum corneum lipid volume ($S_{clipidvol}$) was predicted from:

$$S_{clipidvol} = (g/kt) * area * ah * 1000 \quad (10)$$

The fractional rate of the permeant in the stratum corneum moving into the viable skin ($Jf3$) was predicted from:

$$Jf3 = S_{Cepipart} \quad (11)$$

where $S_{Cepipart}$ is the water/stratum corneum partition coefficient.

The fractional rate of the permeant in the viable skin moving into the stratum corneum was predicted from:

$$Jf4 = 1/S_{Cepipart} \quad (12)$$

The rate of change of permeant in the stratum corneum ($achangesc$) was predicted from:

$$achangesc = C_s * Jf1 - C_{sc} * Jf2 - C_{sc} * Jf3 + C_{vs} * Jf4 \quad (13)$$

where C_{vs} is the permeant concentration in the viable skin and C_{sc} is the permeant concentration in the stratum corneum.

The rate of change of permeant in the viable skin and receptor fluid ($achangevs$) was predicted from:

$$achangevs = C_{sc} * Jf3 - C_{vs} * Jf4 - R_m - (Q_b * C_{vs})$$

where R_m is the rate of metabolism and Q_b is the receptor fluid flow rate.

The lag time of permeant flux (lag) was predicted from:

$$lag = (minpath^2) / (6 * D * 10000000) \quad (14)$$

It should be noted that this is the theoretical minimum lag time based on the minimum pathway length and the rate of permeant movement in the stratum corneum as predicted from diffusivity. It is unlikely that this lag time will be experimentally observable. The apparent lag time observed during experiments is the result of the

absorption of detectable quantities of permeant, which results in the apparent lag time being longer than the theoretical minimum lag time.

The percent flux (% dose/hour) of permeant exiting from the stratum corneum (starting at time 0 + lag) was predicted from:

$$\text{Percent flux} = (\text{achangevslag} * 100 * 60) / \text{indose} \quad (15)$$

Where indose is the marker dose applied to the skin surface at time 0 and achangevslag is the rate of change in the permeant amount in the viable skin and receptor fluid incorporating the time lag due to permeant diffusion through the stratum corneum.

RESULTS

Solvent evaporation

Evaporation rates from a flow-through cell at 32 °C and 30 % relative humidity were 3.79 µl/minute ($R^2 = 0.9985$) for acetone, 1.93 µl/minute ($R^2 = 0.9960$) for ethanol and 0.54 µl/minute ($R^2 = 0.9835$) for 2-propanol (Figure 6.3).

The apparent lag time observed for parathion in seven different volumes of ethanol (Figure 6.5) was correlated ($R^2 = 0.9082$) with the time to solvent depletion, predicted based on the estimated evaporation rate for ethanol, using an exponential function (Figure 6.4). Apparent lag time could be predicted from: $y = 17.716e^{0.0437x}$ where y is the predicted apparent lag time and x is the predicted time to solvent depletion. The error in the predicted apparent lag time, as a fraction of the apparent lag time, was more significant at lower solvent volumes (Table 6.1).

Physical dimension estimates

Porcine back skin corneocyte diameter was 32.09 micron (standard error: 1.02), corneocyte thickness was 0.19 micron (standard error: 0.006) and number of corneocyte layers was 21.9 (standard error: 1.04; maximum: 28; minimum 14). Vertical and lateral gaps between corneocytes were 0.019 micron (standard error: 0.0006).

Flow-through experiments and simulations

The flux/time curves of parathion dosed in various volumes of ethanol, 2-propanol and acetone were summarized in Figure 6.5. Different volumes could be differentiated based on apparent lag time with increased volumes associated with increased apparent lag times, except for acetone. Acetone evaporated too rapidly for different lag times to be differentiated at the 15-minute observation intervals used during the first two hours of the experiments.

The effects of occluding the top of the flow-through cells on the flux/time curves of parathion dosed in 20 and 40 μl of ethanol were summarized in Figure 6.6. Occlusion increased the apparent lag time of absorption from 45 to 75 minutes (20 μl ethanol) and from 90 to 240 minutes (40 μl ethanol). Peak flux was significantly increased by occlusion ($P = 0.03$).

Observed flux/time curves of parathion dosed in 20 μl and 40 μl ethanol at water bath temperatures of 25 °C and 37 °C and simulated flux/time curves of parathion at 25 °C and 37 °C were summarized in figure 6.7. The apparent lag time was increased at the lower water bath temperature. To achieve optimal simulation of 20 μl ethanol volume at 25 °C diffusivity was 0.0005, evaporation rate was 0.25 $\mu\text{l}/\text{minute}$ and the mass transfer factor was 15. For 20 μl ethanol volume at 37 °C diffusivity was 0.0002, evaporation rate was 0.35 $\mu\text{l}/\text{minute}$ and the mass transfer factor was 20. For 40 μl ethanol volume at 25 °C diffusivity was 0.0008, evaporation rate was 0.18 $\mu\text{l}/\text{minute}$ and the mass transfer factor was 21. For 40 μl ethanol volume at 37 °C diffusivity was 0.0006, evaporation rate was 0.4 $\mu\text{l}/\text{minute}$ and the mass transfer factor was 12.

The influence of the range of observed numbers of corneocyte layers was simulated and summarized in Figure 6.8. All model parameters were kept constant, except for the number of corneocyte layers, which were varied from 14 to 28. To approximate the observed data diffusivity was 0.0004, evaporation rate was 0.4 $\mu\text{l}/\text{minute}$ and the mass transfer factor was 5. The simulation (Figure 6.8, Plate A) predicted that the number of corneocyte layers had a substantial influence on absorption. This hypothesis was supported by the observed effect of removal of corneocyte layers through tape stripping (Figure 6.8, Plate B).

The flux/time curves of six different masses of parathion ($6.3 \mu\text{g}/\text{cm}^2$, $11.1 \mu\text{g}/\text{cm}^2$, $22.5 \mu\text{g}/\text{cm}^2$, $43.3 \mu\text{g}/\text{cm}^2$, $106.9 \mu\text{g}/\text{cm}^2$ and $209.1 \mu\text{g}/\text{cm}^2$) dosed in $20 \mu\text{l}$ ethanol were summarized in terms of percent dose/hour in Figure 6.9. The fractions/time of parathion absorbed decreased with increased concentrations, while the absolute mass/time of parathion absorbed increased with increased concentrations. The fraction of the dose remaining in the skin at the conclusion of the experiments and the disintegrations per minute counted in the skin were summarized in Figure 6.10. The dose fractions remaining in the skin ranged from 71.9 % to 88.7 % and were not correlated with the doses used ($R^2 = 0.5236$). Simulated and observed flux/time curves of $22.5 \mu\text{g}/\text{cm}^2$ and $209.1 \mu\text{g}/\text{cm}^2$ parathion dosed in $20 \mu\text{l}$ ethanol expressed in terms of percent dose/hour were summarized in Figure 6.11. The diffusivity, mass transfer factor and solvent evaporation rate values used to achieve optimal simulations were summarized in Table 6.5. The fractions of the dose absorbed after 8 hours decreased with increased concentrations. The relationship between the concentrations used and the fractions absorbed could be described with a power function ($R^2 = 0.9941$): $y = 6.3945x^{0.4628}$, where y is the dose in $\mu\text{g}/\text{cm}^2$ and x is the fraction absorbed (Figure 6.12).

The flux/time curves of six different masses of fenthion ($0.8 \mu\text{g}/\text{cm}^2$, $1.5 \mu\text{g}/\text{cm}^2$, $3.0 \mu\text{g}/\text{cm}^2$, $5.8 \mu\text{g}/\text{cm}^2$, $11.7 \mu\text{g}/\text{cm}^2$ and $23.6 \mu\text{g}/\text{cm}^2$) dosed in $20 \mu\text{l}$ ethanol were summarized in terms of percent dose/hour in Figure 6.9. The fractions/time of fenthion absorbed decreased with increased concentrations, while the absolute mass/time of fenthion absorbed increased with increased concentrations. The fraction of the dose remaining in the skin at the conclusion of the experiments and the disintegrations per minute counted in the skin were summarized in Figure 6.10. The dose fractions remaining in the skin ranged from 62.2 % to 83.7 % and were not correlated with the doses used ($R^2 = 0.5007$). Simulated and observed flux/time curves of $3.0 \mu\text{g}/\text{cm}^2$ and $23.6 \mu\text{g}/\text{cm}^2$ fenthion dosed in $20 \mu\text{l}$ ethanol expressed in terms of percent dose/hour were summarized in Figure 6.11. The diffusivity, mass transfer factor and solvent evaporation rate values used to achieve optimal simulations were summarized in Table 6.6. The fractions of the dose absorbed after 8 hours decreased with increased concentrations. The relationship between the concentrations used and the fractions absorbed could be described

with a power function ($R^2 = 0.8739$): $y = 1.6268x^{-0.3739}$, where y is the dose in $\mu\text{g}/\text{cm}^2$ and x is the fraction absorbed (Figure 6.12).

The flux/time curves of six different masses of methyl parathion ($1.6 \mu\text{g}/\text{cm}^2$, $2.8 \mu\text{g}/\text{cm}^2$, $5.1 \mu\text{g}/\text{cm}^2$, $10.2 \mu\text{g}/\text{cm}^2$, $19.0 \mu\text{g}/\text{cm}^2$ and $39.3 \mu\text{g}/\text{cm}^2$) dosed in $20 \mu\text{l}$ ethanol were summarized in terms of percent dose/hour in Figure 6.9. The fractions/time of methyl parathion absorbed decreased with increased concentrations, while the absolute mass/time of fenthion absorbed increased with increased concentrations. The fraction of the dose remaining in the skin at the conclusion of the experiments and the disintegrations per minute counted in the skin were summarized in Figure 6.10. The dose fractions remaining in the skin ranged from 59.2 % to 91.6 % and were not correlated with the doses used ($R^2 = 0.527$). Simulated and observed flux/time curves of $5.1 \mu\text{g}/\text{cm}^2$ and $39.3 \mu\text{g}/\text{cm}^2$ fenthion dosed in $20 \mu\text{l}$ ethanol expressed in terms of percent dose/hour were summarized in Figure 6.11. The diffusivity, mass transfer factor and solvent evaporation rate values used to achieve optimal simulations were summarized in Table 6.7. The fractions of the dose absorbed after 8 hours decreased with increased concentrations. The relationship between the concentrations used and the fractions absorbed could be described with a power function ($R^2 = 0.9164$): $y = 11.017x^{-0.4784}$, where y is the dose in $\mu\text{g}/\text{cm}^2$ and x is the fraction absorbed (Figure 6.12).

A sensitivity analysis of the parameters used to optimize the simulation of $22.5 \mu\text{g}/\text{cm}^2$ parathion absorption from $20 \mu\text{l}$ ethanol, including diffusivity, mass transfer factor and solvent evaporation rate was represented in Figure 6.13. As expected, all parameters showed low sensitivity before the apparent lag time. Diffusivity and solvent evaporation rate sensitivity were high at the time period directly following the apparent lag time, but was low at later times. This indicated that these parameters had a high influence during the period from apparent lag time to peak flux, and that these parameters did not have a large influence on the extent of total absorption over the experimental period. Mass transfer factor sensitivity also increased after the apparent lag time, but remained high – indicating that it had a significant influence on the total absorption over the experimental period. Sensitivity analyses using higher volumes of ethanol revealed the same pattern, but delayed the onset of high sensitivity for all parameters.

Sensitivity analysis of the parameters used to describe the stratum corneum and determine effective tortuosity associated with 22.5 $\mu\text{g}/\text{cm}^2$ parathion absorption from 20 μl ethanol was represented in Figure 6.14.

Corneocyte diameter had a significant influence during the period immediately following the apparent lag time. The number of corneocyte layers and the width of the vertical gap between corneocytes was significant from the apparent lag time to the end of the experimental period. The sensitivity associated with the number of corneocyte layers also had a peak during the period immediately following the apparent lag time, which is likely to be associated with its influence on the length of the lipid matrix pathway.

DISCUSSION

Mathematical models of biological structures and processes are becoming common tools in the analysis of complex problems related to risk assessment, where one of the goals is the reduction of variance associated with predictions and another is the explanation of observations (Spear 2002). For a mechanistic model to be plausible, the model must be phenomenologically consistent with observed data. It emphasizes *a priori* model structure and hypotheses relating to our current understanding of processes and variables and its causal relationship to observations (Spear 2002). PBPK models, such as the model presented here, are mechanistic in nature and useful for qualitative, explanatory analysis.

We could adopt one of two approaches to decisions on the inclusion or exclusion of physiological and anatomical detail. A “lumping” approach involves using the smallest amount of biological description required to demonstrate observed data. A “splitting” approach involves the inclusion of as much system biology as can be conceptualized and supported with data (Clark et al 2004). We used the latter approach because, in complex models, the influence of model parameters and variables are not always intuitively apparent and the importance of parameters may be changeable depending on system conditions. Many of the model’s parameters were independently estimated, which made it possible to be detailed without loss of degrees of freedom. Also, the retention of code that account for processes that have little influence under the system conditions primarily modeled, such as metabolism, may be of value when hypothetical questions regarding the probable influence of alternative system conditions are raised. The availability of adequate computing power permits simulations to

be run on highly complex models without any significant time penalty. This diminishes the historical practical advantage of collapsing model sections that have little impact on the model output into single parameters. The possibility of losing degrees of freedom when including more parameters in more complex models should, however, be emphasized. Including more unknown parameters decreases confidence in the validity of simulations. By estimating parameters independently, the dependent parameters in the current model were reduced to three, which each had a uniquely identifiable effect on the flux/time curve.

Under equivalent system conditions, dermal absorption with different solvents, solvent volumes and solutes could be simulated by calibrating solvent evaporation rate, solute diffusivity and mass transfer factor. The values of these parameters were dependent on the simulated system conditions and the assumptions used. They were, therefore, conditional in nature and relevant when interpreted comparatively. The simulated apparent lag time was determined by solvent evaporation rate, which causes a rapid increase in the transdermal concentration gradient at low solvent volumes due to permeant super saturation on the skin surface. This model-derived hypothesis was supported by observed correlations between solvent volume and apparent lag time (Figure 6.5), the increased apparent lag time associated with occlusion (Figure 6.6) and the increased apparent lag time at lower water bath temperatures (Figure 6.7). The evaporation rates required by the model were, however, significantly slower than the evaporation rates observed in a temperature and humidity controlled chamber (Figure 6.2). This may be due to the relatively high rate of atmosphere turnover in the temperature and humidity controlled chamber, a higher temperature in the chamber than that of the skin surface in the flow-through cell due to inefficient heat transfer between the water bath and the skin surface. An uneven skin surface may reduce surface area as the solvent reaches low volumes. The remnants of hair and the hygroscopic nature of solvents such as ethanol can absorb atmospheric water and possibly extract water from the skin surface and the presence of solutes could also alter solvent evaporation rates. Differences between the surface texture, hair density and atmospheric conditions could explain the variation in apparent ethanol evaporation rates at a water bath temperature of 37 °C (varied from 0.3 $\mu\text{l}/\text{cm}^2/\text{min}$ to 0.4 $\mu\text{l}/\text{cm}^2/\text{min}$) needed for optimal simulations.

The apparent lag times observed for single studies should be interpreted with caution due to the possible effects of environmental conditions and inter-individual skin variability on apparent lag time. It should be noted that

the apparent lag time is influenced by the time it takes for the permeant to diffuse through the skin and the sensitivity of the method used for permeant detection. These factors and the limitations on the apparent lag time accuracy due to sampling intervals made the use of predicted apparent lag times based on estimated time to solvent depletion impractical at the relatively low solvent volumes used in the simulations. Predicting the apparent lag time for higher solvent volumes was, however, more successful (Table 6.1; Figure 6.3). More data for higher solvent volumes would be needed to firmly establish a quantitative relationship between apparent lag time and solvent volume, but the existence of a relationship between apparent lag time and the time to solvent depletion due to evaporation was supported in these studies.

The hypothesis that super saturation due to solvent evaporation increases absorption rates is also supported by studies comparing dermal absorption from ethanol with a relatively non-volatile solvent, such as propylene glycol. Permeability from propylene glycol, which never reaches super saturation conditions during the experimental period, is markedly lower than permeability from ethanol, which reaches super saturation conditions early in the experimental period (van der Merwe & Riviere 2005).

The time from apparent lag to peak flux could be simulated by optimizing diffusivity and this correlation was used to determine diffusivity when simulating observed data. The diffusivity used in the model refers to the rate of solute movement in the stratum corneum lipids and should not be confused with the apparent diffusivity used in traditional models, which is based on the observed lag time and membrane thickness. Simulating conditions where the skin surface is open to the atmosphere is relevant to the most common conditions of exposure to environmental toxins, but changing the solvent evaporation rate can simulate different degrees of occlusion or total occlusion.

The mass of solute transferred across the stratum corneum over time, as reflected by the area under the curve, was related to the mass transfer factor. This factor was needed because solute partitioning determined from *in vitro* partitioning between isolated stratum corneum and solvent (Van der Merwe & Riviere 2005) did not fully account for the apparent solute penetration into the stratum corneum and partitioning from the stratum corneum to viable skin under the simulated conditions. This may be due to solvent-solute co-absorption, different

physical conditions between the partitioning experiments and the flow-through diffusion experiments, such as temperature and exposure to the atmosphere vs. total occlusion and immersion in the solvent, and to differences between isolated stratum corneum and the stratum corneum of intact, fresh skin. The lipophilicity of the viable skin is different from that of water and the mass transfer factor adjusted the stratum corneum/water partition coefficient used to describe stratum corneum/viable skin partitioning in the model. Assuming the minimum solvent volume on the skin surface to be 0.1 μl was also a possible source for error in permeant partitioning based on independently estimated partitioning values. The value of the mass transfer factor therefore adjusts for a number of possible sources of error and more work, such as detailed partitioning studies in intact skin, are needed to differentiate between different contributors to the mass transfer factor. Since the mass transfer factor was a significant contributor to the total extent of absorption over the experimental period the model should be used with caution for predictions of the total extent of absorption in the absence of experimental data. However, the model can be a useful tool for predicting dermal absorption under different exposure scenarios comparatively and for developing hypotheses on the effects of specific structural changes on dermal absorption.

Our assumption of constant barrier membrane properties throughout the experimental period was not consistently supported by the observed data. Longer exposure of the skin to ethanol when using higher volumes resulted in higher peak flux (Figure 6.5). Increased peak flux was also observed when flow-through cells were occluded (Figure 6.6). This may be attributed to disruption of the lipid matrix over time due to the penetration of ethanol into the stratum corneum (Kim & Chien 1996; Kim et al 1996), which could enhance solute partitioning into the stratum corneum, increase the rate of solute movement in the lipid matrix and open additional lipid channels to solute transfer.

The number of corneocyte layers had a marked influence on the effective tortuosity, which is correlated with the effectiveness of the stratum corneum as a barrier (Figure 6.8). Sensitivity analysis of the number of corneocyte layers (Figure 6.14) revealed that this parameter had a highly significant influence on the total absorption over the experimental period and that there was a peak in its influence in the period immediately following the apparent lag time. The peak in sensitivity was probably due to its effect on the length of the lipid matrix pathway through the stratum corneum and its consequent influence on the time of diffusion through the stratum

corneum. The sustained significance of the number of corneocyte layers was probably related to its influence on the stratum corneum lipid volume and, therefore, on permeant concentration in the stratum corneum. Variability of the number of corneocyte layers in the stratum corneum is a potential source of inter-experimental variability and predictive uncertainty. This potential variability is illustrated by the inter-experimental variability observed between the absorption of similar concentrations of parathion in different experiments using skin from different pigs (Figures 6.8, 6.9 and 6.11). It also has implications for dermal absorption through skin from different body sites with different stratum corneum thickness and skin with altered corneocyte layers, such as damaged or diseased skin. Finally, it has a significant effect on interspecies comparisons based on skin thickness alone. The model-derived hypothesis of the importance of the effect of the number of corneocyte layers was supported by observed differences in dermal absorption between tape-stripped and control skin. The assumption that the number of corneocyte layers were equal to the average number of layers observed in back skin, may have been an underestimation because of the possibility of losing corneocyte layers during skin preparation.

The simulation of methyl parathion absorption under predicted the flux during the latter part of the simulation. The effect was more pronounced in the simulation of the lower concentration (Figure 6.11). The same phenomenon was observed for the simulation of the lower concentration of parathion. A possible explanation could be that a significant portion of the permeant is bound to structures in the stratum corneum and is therefore unavailable for diffusion. The results of such an effect would be more pronounced at lower concentrations. It is, however, a speculative explanation and further studies are needed to establish the cause of this effect.

It is often assumed that dermal absorption is a first order process of mass diffusion as predicted by Fick's First Law, at least for low solute concentrations at steady state. Our observations across a wide range of parathion and fenthion doses, however, did not show absorption to be a first order process (Figure 6.9). The relationship between dose concentration and absorption of parathion, fenthion and methyl parathion could be described using power functions (Figure 6.12). It is a well established, long standing assumption that Fick's Law offers a reasonable approximation of the processes of dermal absorption (Treherne 1956; Blank 1964a; Wahlberg 1968a; Scheuplein & Blank 1971). It is also the basis for the assumption that permeability can be characterized by a permeability constant (K_p), which is defined as: $K_p = J_{ss}/\Delta C$, where J_{ss} is the steady-state flux and ΔC is

the concentration gradient across the skin. An assumption of Fickian diffusion is inherent in predictions of dermal absorption by QSAR models based on correlations between molecular descriptors and observed K_p values (Potts & Guy 1995; Sartorelli et al 1998; Sartorelli et al 1999; Geinoz et al 2004; Ghafourian et al 2004). K_p is used by regulatory agencies to characterize the dermal absorption of compounds for comparative and risk assessment purposes using standardized methods. However, lack of confidence in the validity of K_p values determined outside the dose range of typical exposures and the concentrations used in chemical products compelled regulatory agencies to recommend that K_p values should be determined using typical product formulations or expected exposure concentrations (EPA 1992; OECD 2000). It is clear that an assumption of the validity of Fick's Law, at least as an adequate approximation, plays a central role in our current understanding of dermal absorption and that there is a need to identify the conditions under which it is applicable.

Fick's Law can be explained in molecular terms by the molecular-kinetic mechanism of Brownian motion in fluid systems (Einstein 1905; Von Smoluchowski 1906), but the assumptions required include the presence of a dilute, homogeneous suspension; rigid, elastically colliding particles; no solvent-solute interaction and a system that tends towards equilibrium. These assumptions are not compatible with biological barriers - including the stratum corneum (Agutter et al 2000). It is also evident from the results of published studies that Fick's Law does not invariably offer a good approximation of dermal absorption (Blank 1964b; Billich et al 2005). Examples of studies where an assumption of Fickian diffusion appears to be reasonable across some spectrum of concentrations can be found (Wahlberg 1968b; Payan et al 2003), but it should be emphasized that a continuation of Fickian diffusion across wider spectra of concentrations cannot be assumed from such studies. Permeability constants can also be misleading when the permeability associated with neat compounds are used to infer the permeability of compounds in solvents (Korinth et al 2005) or mixtures (Riviere & Brooks 2005). Although our model made use of the same expressions as is used in describing Fickian diffusion (equation 6), accurate simulation of observed data required non-constant diffusivity and mass transfer. Since the absorbed fraction of the solute was not correlated with the fraction partitioned into the skin across a range of doses, diffusivity and mass transfer would be constant if Fickian diffusion occurred in the stratum corneum. However,

for the compounds tested, diffusivity and mass transfer appeared to be altered depending on the concentration and solvent used - resulting in non-Fickian absorption patterns.

Diffusivity is related to the kinetic energy of the diffusing molecules and is temperature dependent, although the extent of the effect at the moderate temperature differences in these experiments (Figure 6.7) is expected to be relatively small. Higher temperature may also affect the fluidity of stratum corneum lipids, which could increase diffusivity. However, phase changes indicating altered lipid crystal structure in the stratum corneum are typically detected at significantly higher temperatures than those used in these experiments (Bouwstra et al 2003). Slightly increased diffusivity was, therefore, expected to be associated with higher water bath temperatures, but diffusivity appeared to be increased at lower water bath temperatures (Tables 6.3 and 6.4). This was likely due to the effects of longer contact between ethanol and skin on diffusivity overriding the temperature effect on diffusivity. This hypothesis is supported by the fact that the diffusivity was highest when the contact between ethanol and skin was longest (40 μ l ethanol at 25 °C). The mass transfer factor was also increased at longer ethanol to skin contact times, which may indicate disruptive effects leading to altered permeant partitioning and effective tortuosity. Altered diffusivity due to solvents, such as ethanol, may be explained by the disruptive effects that solvents may have on stratum corneum lipid bilayers (Kim & Chien 1996; Kim et al 1996) and the effects of lipid extraction from the superficial layers of the stratum corneum lipid matrix (Van der Merwe & Riviere 2005). However, the influence of concentration on diffusivity in the stratum corneum has not been adequately explained. Simulations of absorption from higher concentrations of parathion and methyl parathion required increased diffusivity (Tables 6.5 and 6.7). Although no increase in diffusivity was observed for fenthion (Table 6.6), small increases may have been masked by experimental variability. Alternative models of diffusion, such as hop-diffusion (Suzuki et al 2005), have been proposed for cell membranes but viable alternative diffusion models have not been widely used in the stratum corneum. This could be a fruitful area of investigation in future research if methods can be found to track the movement of single molecules through the stratum corneum.

The rate and extent of dermal absorption was not directly correlated with partitioning into the skin and only a small fraction of the compounds that partitioned into the skin were absorbed within the 8-hour experimental

period (Figure 6.12). The extent of absorption/time was, therefore, limited by the rate of solute movement through the skin and not by partitioning into the skin. Similar results were obtained with cyclosporin derivatives in rat and human skin (Billich et al 2005) and with DDT in rat, guinea pig, porcine and human skin (Moody et al 1994). It indicates that small, lipid soluble compounds are likely to be sequestered in the skin after topical exposure. The skin acts as a reservoir from which compounds may be absorbed into the rest of the body over long periods of time.

The study demonstrated the utility of PBPK models of dermal absorption, which can be used for *in silico* hypotheses generation and limited hypotheses testing. The similarity between the overall shapes of the experimental and model-predicted flux/time curves indicate that the absorption processes that were described in the model successfully simulates important aspects of dermal absorption in flow-through cells. The presented model reflects current understanding of the processes of dermal absorption, which is likely to evolve over time. An advantage of PBPK models is the ability to adapt to newer insights and increased knowledge of the modeled processes and structures may be expected to increase the model's predictive and explanatory value.

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Table 6.1. The estimated time to depletion (TD) of different volumes of ethanol compared to the observed apparent lag times (OL) of parathion absorption and the predicted apparent lag times (PL) based on the exponential function: $PL = 17.716e^{0.0437*TD}$.

Volume	TD	OL	PL
5	3	15	20
10	5	15	22
20	10	45	28
30	16	45	35
50	26	60	55
80	41	105	108
120	62	240	268

Table 6.2. Independently estimated and fixed parameters used for all experimental scenarios.

Symbol	Description	Unit	Value
<i>Independently estimated parameters</i>			
kt	corneocyte thickness	μm	0.19
kd	corneocyte diameter	μm	32.09
g	vertical gap between corneocytes	μm	0.019
s	lateral gap between corneocytes	μm	0.019
d1	long leg of corneocyte overlap	μm	27.69
d2	short leg of corneocyte overlap	μm	4.4
dermisdepth	dermatome depth setting	cm	0.5
area	skin surface area	cm^2	0.64
Rdepth	receptor chamber depth	cm	0.48
Qb	receptor fluid flow rate	$\mu\text{l/minute}$	67
<i>Fixed parameters</i>			
Vmax	maximum rate of metabolism	$\mu\text{g/minute}$	0.001
Km	[permeant] at 50 % of Vmax	$\mu\text{g}/\mu\text{l}$	1000
bb	minimum solvent volume on skin surface	μl	0.1
doseevap	rate of permeant evaporation from skin surface	$\mu\text{g/minute}$	0.0001

Table 6.3. Independently estimated and optimized parameters used to simulate observed flux/time curves of 22.5 $\mu\text{g}/\text{cm}^2$ parathion dosed in 20 μl ethanol at a water bath temperature of 25 °C (A) and 22.4 $\mu\text{g}/\text{cm}^2$ parathion dosed in 20 μl ethanol at a water bath temperature of 37 °C (B).

A			
Symbol	Description	Unit	Value
<i>Independently estimated parameters</i>			
dose	amount of permeant deposited onto skin surface	ug	22.5
insolventvol	solvent volume deposited onto skin surface	ul	20
N	number of corneocyte layers		21.9
logP	log [stratum corneum]/[solvent]		1.090
logPsw	log [stratum corneum]/[water]		2.952
<u>Optimized parameters</u>			
solventevaprate	rate of solvent evaporation from skin surface	ul/min	0.25
D	diffusivity in stratum corneum lipid matrix	cm ² /minute	0.0005
MTf	Mass transfer factor		15
B			
Symbol	Description	Unit	Value
<u>Independently estimated parameters</u>			
dose	amount of permeant deposited onto skin surface	ug	22.4
insolventvol	solvent volume deposited onto skin surface	ul	20
N	number of corneocyte layers		21.9
logP	log [stratum corneum]/[solvent]		1.090
logPsw	log [stratum corneum]/[water]		2.952
<u>Optimized parameters</u>			
solventevaprate	rate of solvent evaporation from skin surface	ul/min	0.35
D	diffusivity in stratum corneum lipid matrix	cm ² /minute	0.0002
MTf	Mass transfer factor		20

Table 6.4. Independently estimated and optimized parameters used to simulate observed flux/time curves of 22.5 $\mu\text{g}/\text{cm}^2$ parathion dosed in 40 μl ethanol at a water bath temperature of 25 °C (A) and 22.4 $\mu\text{g}/\text{cm}^2$ parathion dosed in 40 μl ethanol at a water bath temperature of 37 °C (B).

A			
Symbol	Description	Unit	Value
<i>Independently estimated parameters</i>			
dose	amount of permeant deposited onto skin surface	ug	22.5
insolventvol	solvent volume deposited onto skin surface	ul	40
N	number of corneocyte layers		21.9
logP	log [stratum corneum]/[solvent]		1.090
logPsw	log [stratum corneum]/[water]		2.952
<u>Optimized parameters</u>			
solventevaprate	rate of solvent evaporation from skin surface	ul/min	0.18
D	diffusivity in stratum corneum lipid matrix	cm ² /minute	0.0008
MTf	Mass transfer factor		21
B			
Symbol	Description	Unit	Value
<u>Independently estimated parameters</u>			
dose	amount of permeant deposited onto skin surface	ug	22.4
insolventvol	solvent volume deposited onto skin surface	ul	40
N	number of corneocyte layers		21.9
logP	log [stratum corneum]/[solvent]		1.090
logPsw	log [stratum corneum]/[water]		2.952
<u>Optimized parameters</u>			
solventevaprate	rate of solvent evaporation from skin surface	ul/min	0.4
D	diffusivity in stratum corneum lipid matrix	cm ² /minute	0.0006
MTf	Mass transfer factor		12

Table 6.5. Independently estimated and optimized parameters used to simulate observed flux/time curves of 22.5 $\mu\text{g}/\text{cm}^2$ (A) and 209.1 $\mu\text{g}/\text{cm}^2$ (B) parathion dosed in 20 μl ethanol.

A			
Symbol	Description	Unit	Value
<i>Independently estimated parameters</i>			
dose	amount of permeant deposited onto skin surface	ug	22.5
insolventvol	solvent volume deposited onto skin surface	ul	20
N	number of corneocyte layers		21.9
logP	log [stratum corneum]/[solvent]		1.090
logPsw	log [stratum corneum]/[water]		2.952
<u>Optimized parameters</u>			
solventevaprate	rate of solvent evaporation from skin surface	ul/min	0.4
D	diffusivity in stratum corneum lipid matrix	cm ² /minute	0.002
MTf	Mass transfer factor		20
B			
Symbol	Description	Unit	Value
<u>Independently estimated parameters</u>			
dose	amount of permeant deposited onto skin surface	ug	209.1
insolventvol	solvent volume deposited onto skin surface	ul	20
N	number of corneocyte layers		21.9
logP	log [stratum corneum]/[solvent]		1.090
logPsw	log [stratum corneum]/[water]		2.952
<u>Optimized parameters</u>			
solventevaprate	rate of solvent evaporation from skin surface	ul/min	0.4
D	diffusivity in stratum corneum lipid matrix	cm ² /minute	0.005
MTf	Mass transfer factor		6

Table 6.6. Independently estimated and optimized parameters used to simulate observed flux/time curves of 3.0 $\mu\text{g}/\text{cm}^2$ (A) and 23.6 $\mu\text{g}/\text{cm}^2$ (B) fenthion dosed in 20 μl ethanol.

A			
Symbol	Description	Unit	Value
<u>Independently estimated parameters</u>			
dose	amount of permeant deposited onto skin surface	ug	3.0
insolventvol	solvent volume deposited onto skin surface	ul	20
N	number of corneocyte layers		21.9
logP	log [stratum corneum]/[solvent]		0.800
logPsw	log [stratum corneum]/[water]		3.006
<i>Optimized parameters</i>			
solventevaprate	rate of solvent evaporation from skin surface	ul/min	0.3
D	diffusivity in stratum corneum lipid matrix	cm ² /minute	0.001
MTf	Mass transfer factor		16
B			
Symbol	Description	Unit	Value
<u>Independently estimated parameters</u>			
dose	amount of permeant deposited onto skin surface	ug	23.6
insolventvol	solvent volume deposited onto skin surface	ul	20
N	number of corneocyte layers		21.9
logP	log [stratum corneum]/[solvent]		0.800
logPsw	log [stratum corneum]/[water]		3.006
<i>Optimized parameters</i>			
solventevaprate	rate of solvent evaporation from skin surface	ul/min	0.3
D	diffusivity in stratum corneum lipid matrix	cm ² /minute	0.001
MTf	Mass transfer factor		8.5

Table 6.7. Independently estimated and optimized parameters used to simulate observed flux/time curves of 5.1 $\mu\text{g}/\text{cm}^2$ (A) and 39.3 $\mu\text{g}/\text{cm}^2$ (B) methyl parathion dosed in 20 μl ethanol.

A			
Symbol	Description	Unit	Value
<u>Independently estimated parameters</u>			
dose	amount of permeant deposited onto skin surface	ug	5.1
insolventvol	solvent volume deposited onto skin surface	ul	20
N	number of corneocyte layers		21.9
logP	log [stratum corneum]/[solvent]		0.840
logPsw	log [stratum corneum]/[water]		1.922
<u>Optimized parameters</u>			
solventevaprate	rate of solvent evaporation from skin surface	ul/min	0.3
D	diffusivity in stratum corneum lipid matrix	$\text{cm}^2/\text{minute}$	0.002
MTf	Mass transfer factor		5
B			
Symbol	Description	Unit	Value
<u>Independently estimated parameters</u>			
dose	amount of permeant deposited onto skin surface	ug	39.3
insolventvol	solvent volume deposited onto skin surface	ul	20
N	number of corneocyte layers		21.9
logP	log [stratum corneum]/[solvent]		0.840
logPsw	log [stratum corneum]/[water]		1.922
<u>Optimized parameters</u>			
solventevaprate	rate of solvent evaporation from skin surface	ul/min	0.35
D	diffusivity in stratum corneum lipid matrix	$\text{cm}^2/\text{minute}$	0.006
MTf	Mass transfer factor		2.3

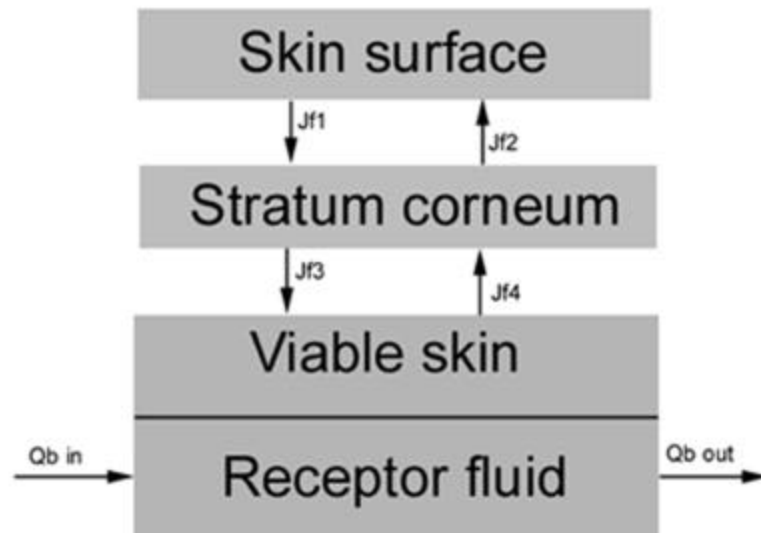


Figure 6.1. Model block diagram showing conceptual model compartments, rate constants for permeant transfer between compartments and receptor fluid flow.

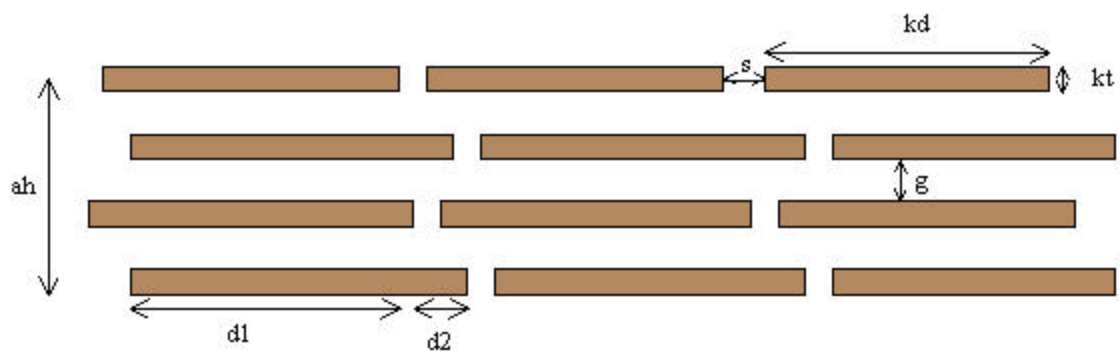


Figure 6.2. Schematic representation of the stratum corneum depicting the parameters used to calculate effective tortuosity and minimum pathway length.

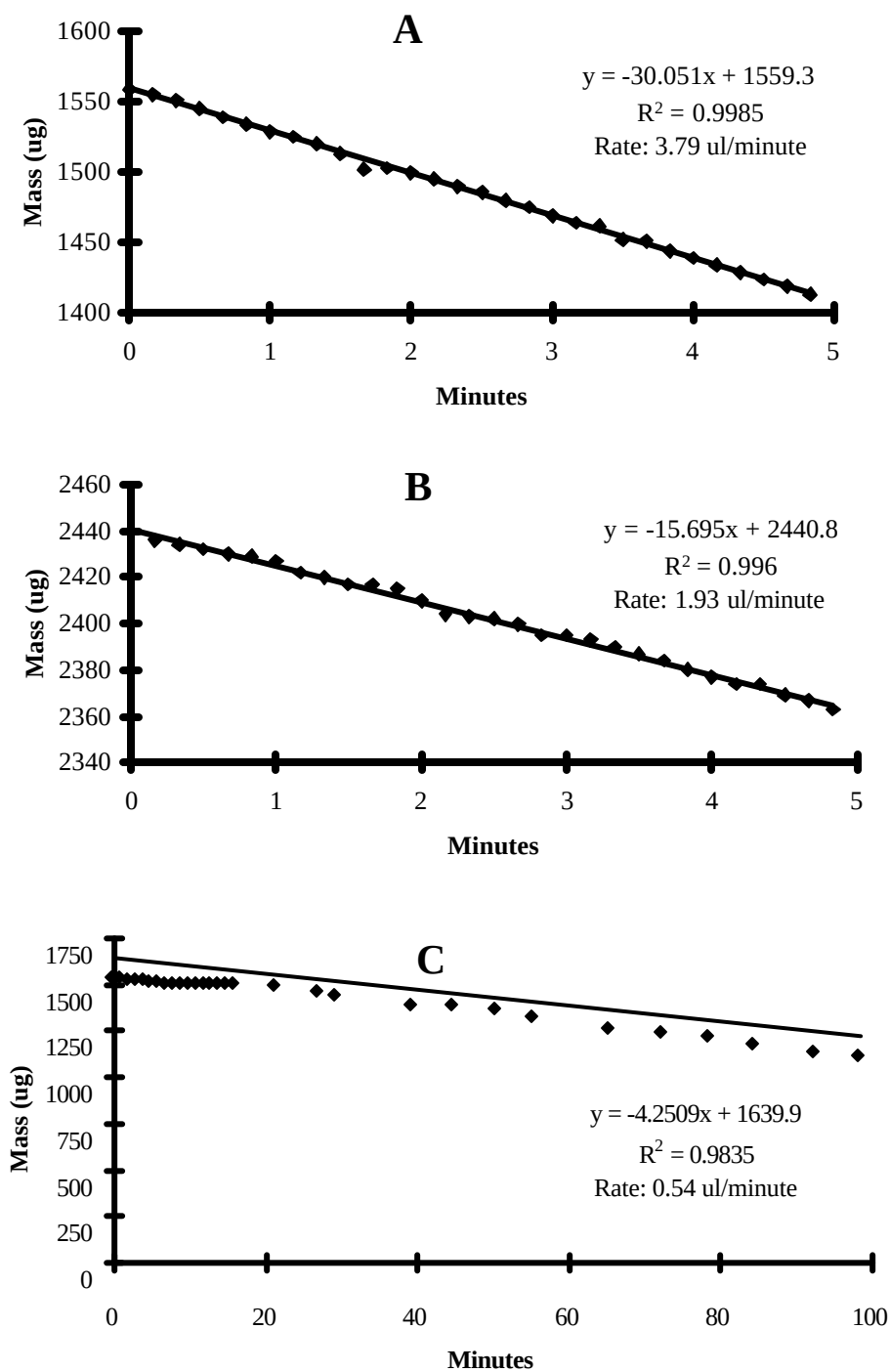


Figure 6.3. Evaporation rates of acetone (A), ethanol (B) and 2-propanol (C) from flow-through cells at 32°C and 30 % relative humidity. Linear trend lines, trend line equations, R^2 – values of the trend line/data correlations and evaporation rates in terms of volume/time are displayed.

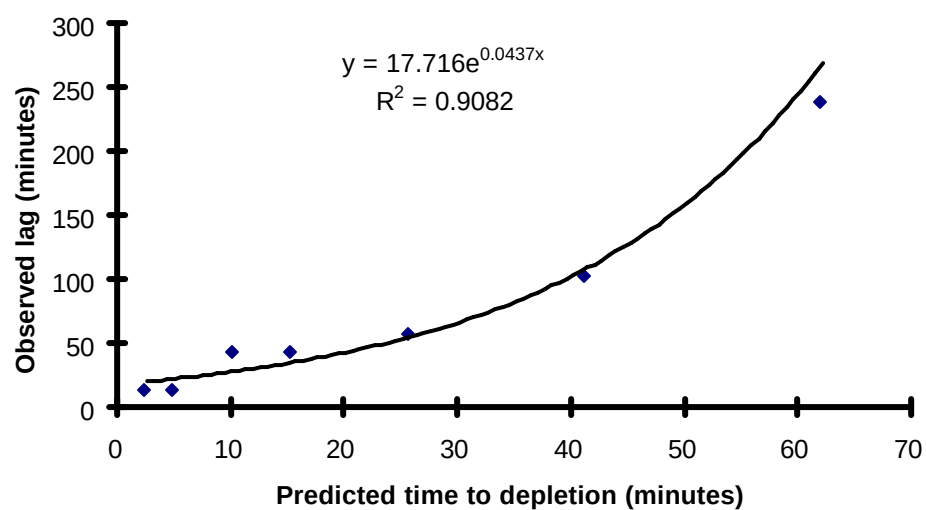


Figure 6.4. The predicted time to solvent depletion based on an estimated evaporation rate of 1.93 $\mu\text{l}/\text{minute}$; compared to the observed lag time of parathion absorption from 5, 10, 20, 30, 50, 80 and 120 μl ethanol.

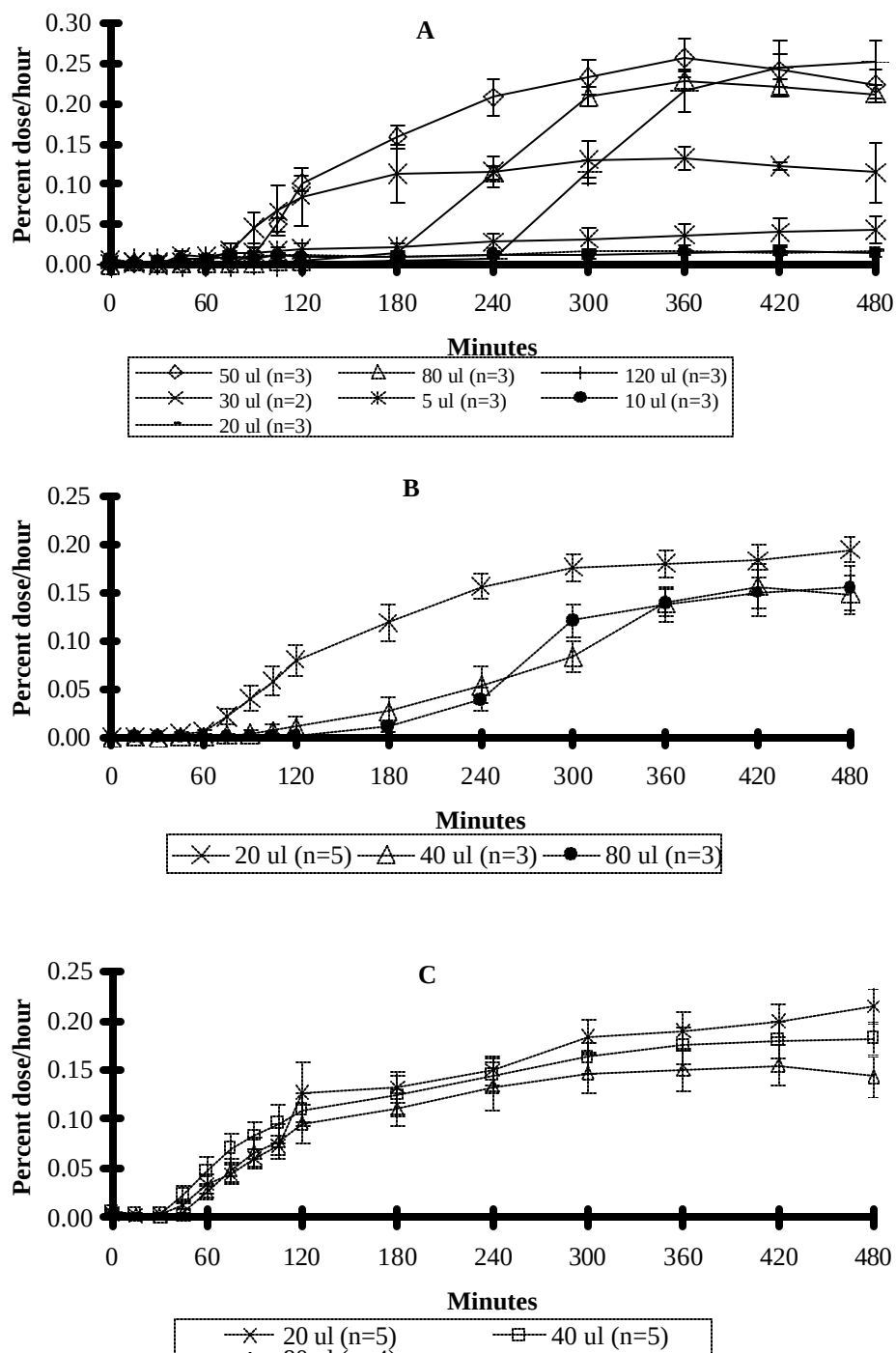


Figure 6.5. The effects of different non-occluded solvent volumes on observed flux/time curves: 20.2 $\mu\text{g}/\text{cm}^2$ (SE=0.35) parathion dosed in 5 μl , 10 μl , 20 μl , 30 μl , 50 μl , 80 μl and 120 μl ethanol (A); 25.1 $\mu\text{g}/\text{cm}^2$ (SE=0.15) parathion dosed in 20 μl , 40 μl and 80 μl 2-propanol (B) and 24.7 $\mu\text{g}/\text{cm}^2$ (SE=0.01) parathion dosed in 20 μl , 40 μl and 80 μl acetone (C).

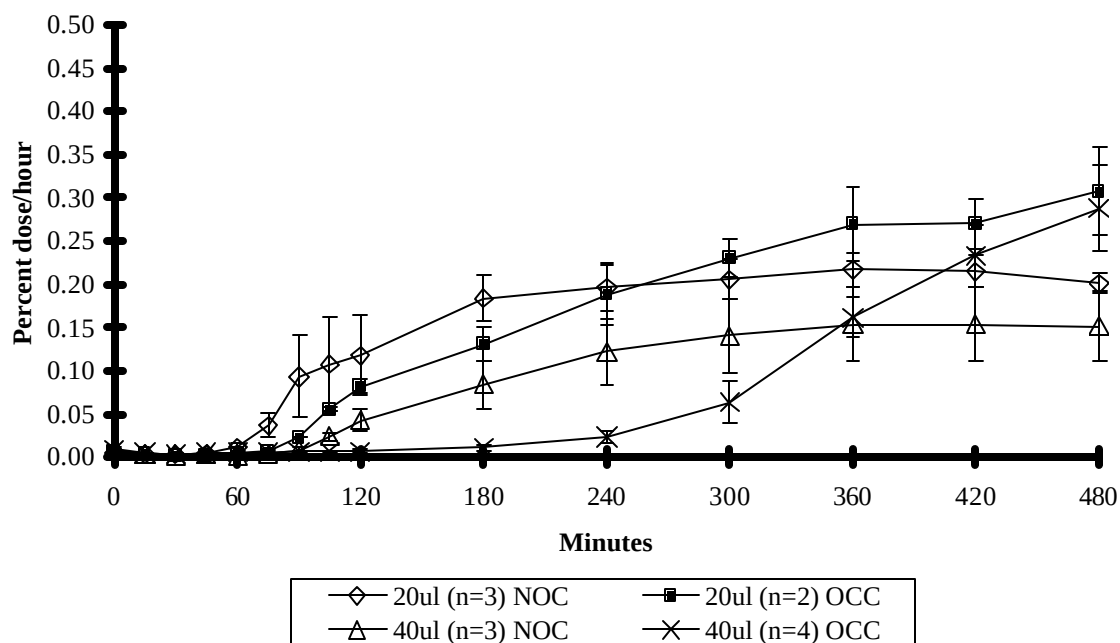


Figure 6.6. Observed flux/time curves of 20.9 $\mu\text{g}/\text{cm}^2$ (SE=0.03) parathion dosed in 20 μl and 40 μl ethanol under occluded (OCC) and non-occluded (NOC) conditions.

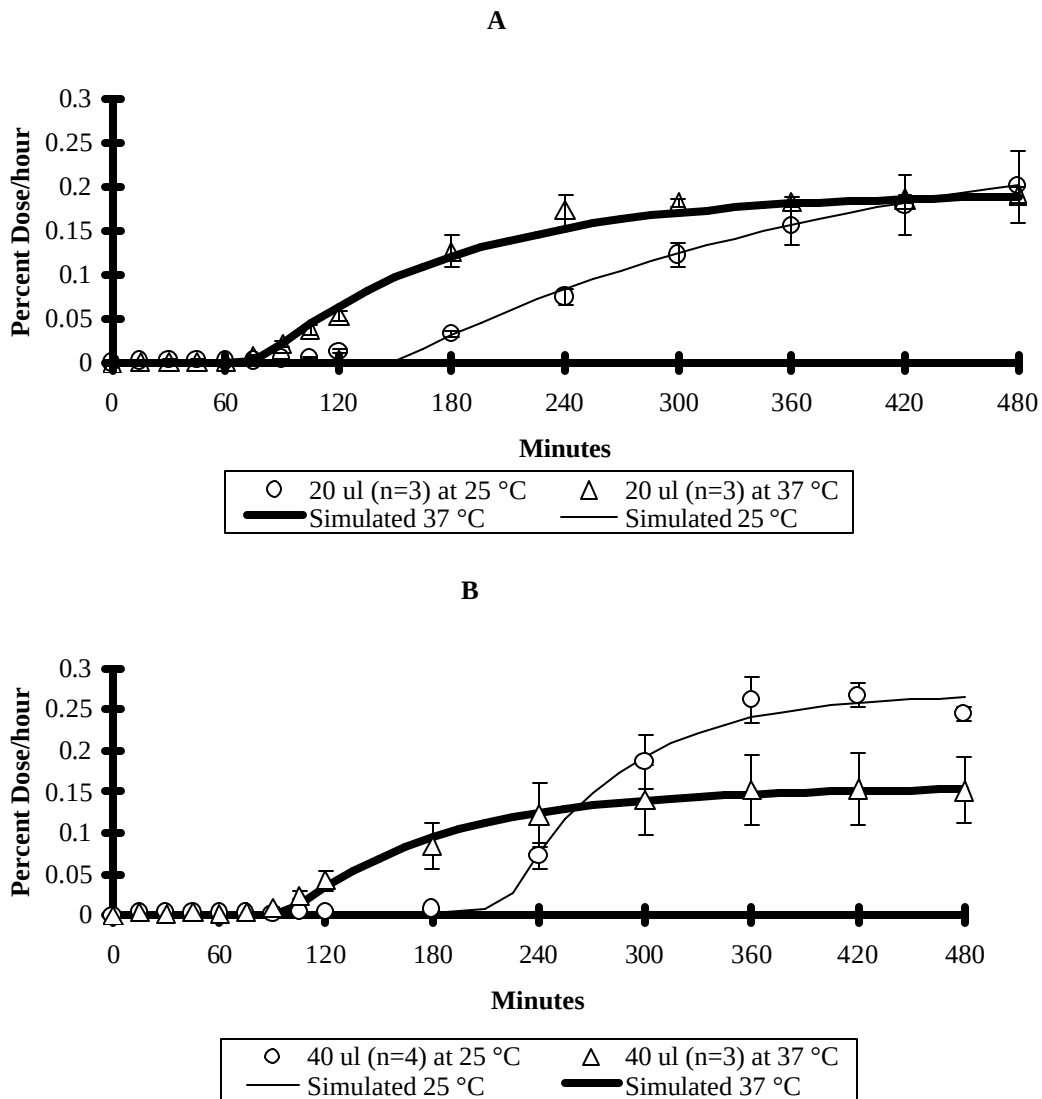


Figure 6.7. Observed flux/time curves of $20.5 \mu\text{g}/\text{cm}^2$ ($\text{SE}=0.31$) parathion dosed in $20 \mu\text{l}$ (A) and $40 \mu\text{l}$ (B) ethanol at a water bath temperature of 25°C , $20.4 \mu\text{g}/\text{cm}^2$ ($\text{SE}=0.18$) parathion dosed in $20 \mu\text{l}$ (A) and $40 \mu\text{l}$ (B) ethanol at a water bath temperature of 37°C and simulated flux/time curves of parathion at 25°C and 37°C

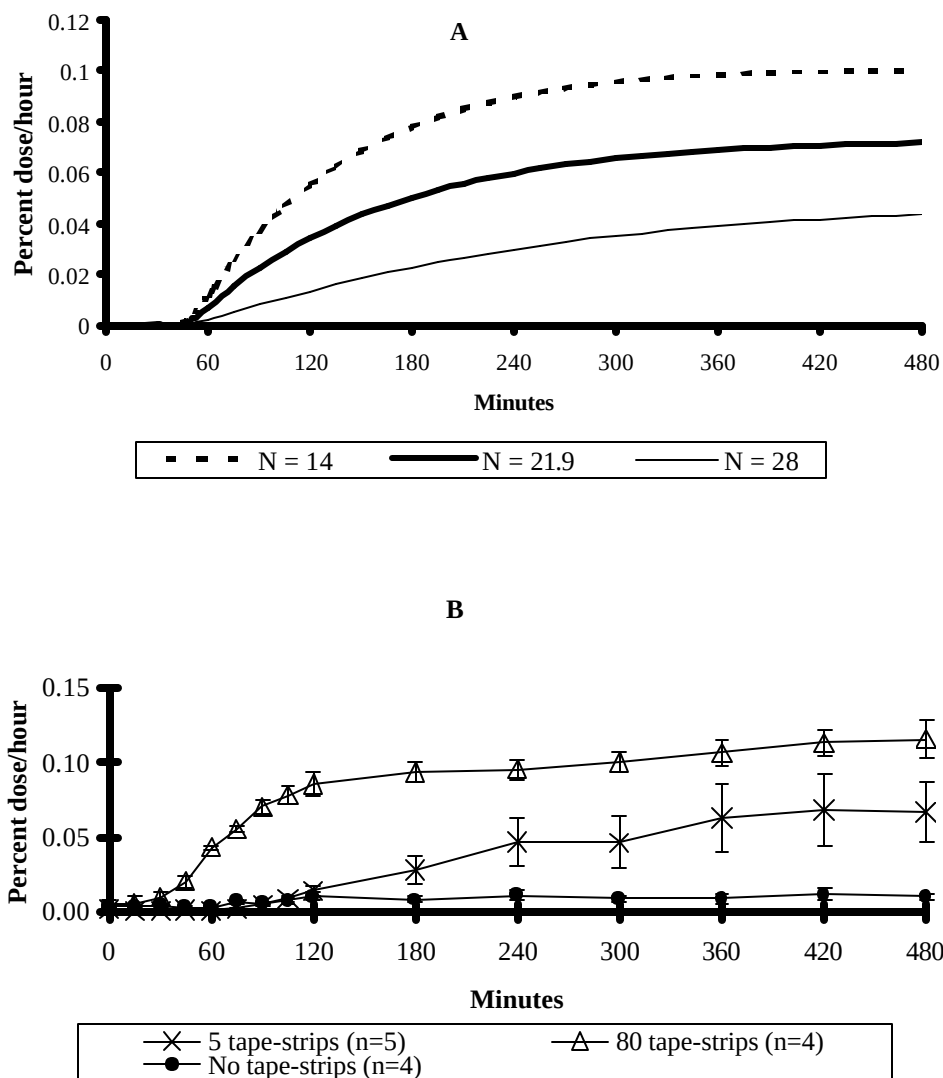


Figure 6.8. Simulated flux/time curves of parathion with 14, 21.9 and 28 corneocyte layers (N) in the stratum corneum (A) and observed flux/time curves of $19.4 \mu\text{g}/\text{cm}^2$ (SE=0.00) parathion dosed in $20 \mu\text{l}$ ethanol on control skin, skin that was tape-stripped 5 times and skin that was tape-stripped 80 times (B).

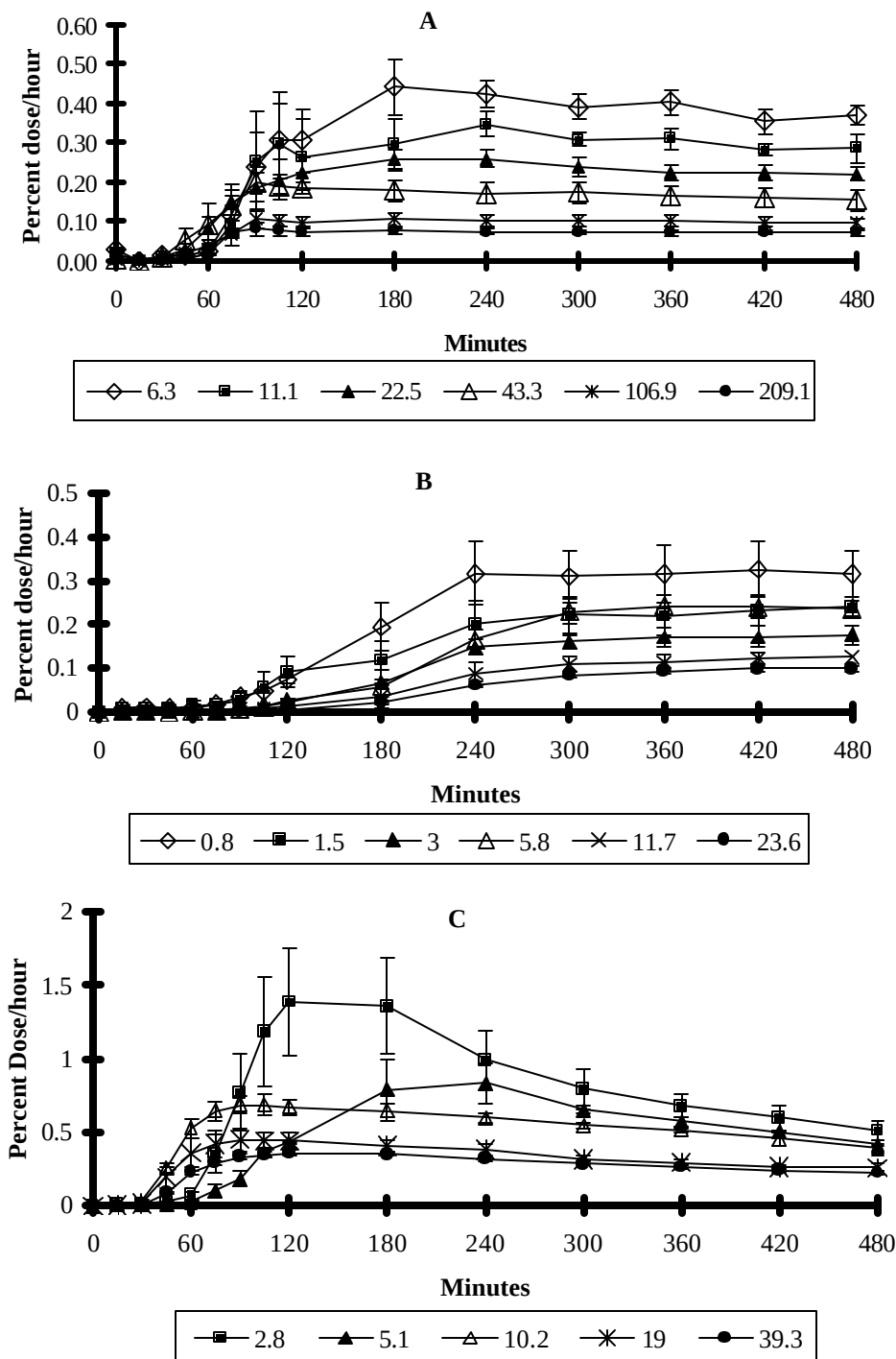


Figure 6.9. Observed flux/time curves of parathion (A), fenthion (B) and methyl parathion (C) dosed in 20 μ l ethanol. The doses used were: 6.3 $\mu\text{g}/\text{cm}^2$ (n=5), 11.1 $\mu\text{g}/\text{cm}^2$ (n=4), 22.5 $\mu\text{g}/\text{cm}^2$ (n=6), 43.3 $\mu\text{g}/\text{cm}^2$ (n=3), 106.9 $\mu\text{g}/\text{cm}^2$ (n=4) and 209.1 $\mu\text{g}/\text{cm}^2$ (n=4) for parathion; 0.8 $\mu\text{g}/\text{cm}^2$ (n=4), 1.5 $\mu\text{g}/\text{cm}^2$ (n=3), 3.0 $\mu\text{g}/\text{cm}^2$ (n=4), 5.8 $\mu\text{g}/\text{cm}^2$ (n=5), 11.7 $\mu\text{g}/\text{cm}^2$ (n=5) and 23.6 $\mu\text{g}/\text{cm}^2$ (n=4) for fenthion; and 2.8 $\mu\text{g}/\text{cm}^2$ (n=5), 5.1 $\mu\text{g}/\text{cm}^2$ (n=4), 10.2 $\mu\text{g}/\text{cm}^2$ (n=4), 19.0 $\mu\text{g}/\text{cm}^2$ (n=5) and 39.3 $\mu\text{g}/\text{cm}^2$ (n=4) for methyl parathion.

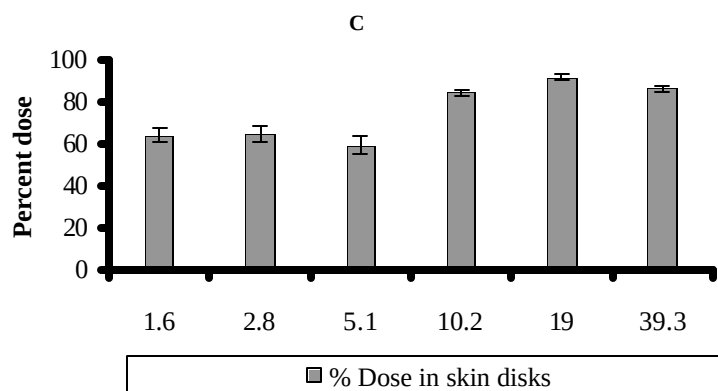
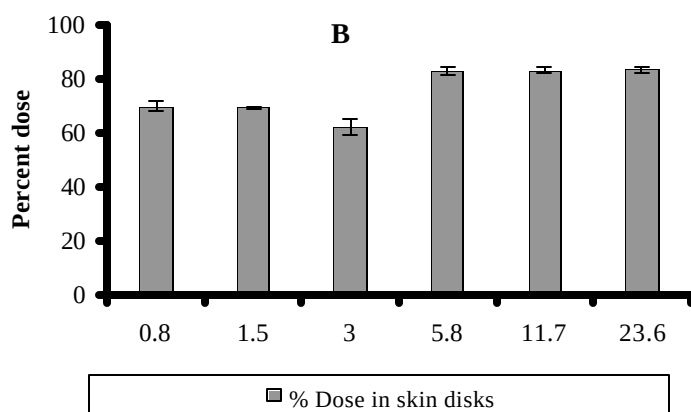
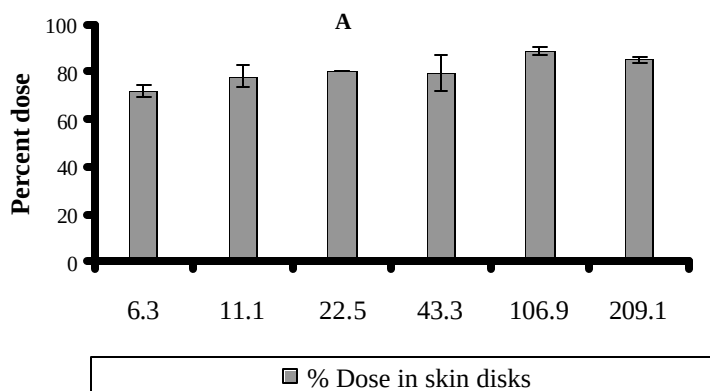


Figure 6.10. The fraction of parathion (A), fenthion (B) and methyl parathion (C) doses remaining in the skin and the disintegrations per minute (DPM) counted in the skin at the conclusion of 8 hour experiments. Doses were: $6.3 \mu\text{g}/\text{cm}^2$, $11.1 \mu\text{g}/\text{cm}^2$, $22.5 \mu\text{g}/\text{cm}^2$, $43.3 \mu\text{g}/\text{cm}^2$, $106.9 \mu\text{g}/\text{cm}^2$ and $209.1 \mu\text{g}/\text{cm}^2$ for parathion; $0.8 \mu\text{g}/\text{cm}^2$, $1.5 \mu\text{g}/\text{cm}^2$, $3.0 \mu\text{g}/\text{cm}^2$, $5.8 \mu\text{g}/\text{cm}^2$, $11.7 \mu\text{g}/\text{cm}^2$ and $23.6 \mu\text{g}/\text{cm}^2$ for fenthion; $1.6 \mu\text{g}/\text{cm}^2$, $2.8 \mu\text{g}/\text{cm}^2$, $5.1 \mu\text{g}/\text{cm}^2$, $10.2 \mu\text{g}/\text{cm}^2$, $19.0 \mu\text{g}/\text{cm}^2$ and $39.3 \mu\text{g}/\text{cm}^2$ for methyl parathion.

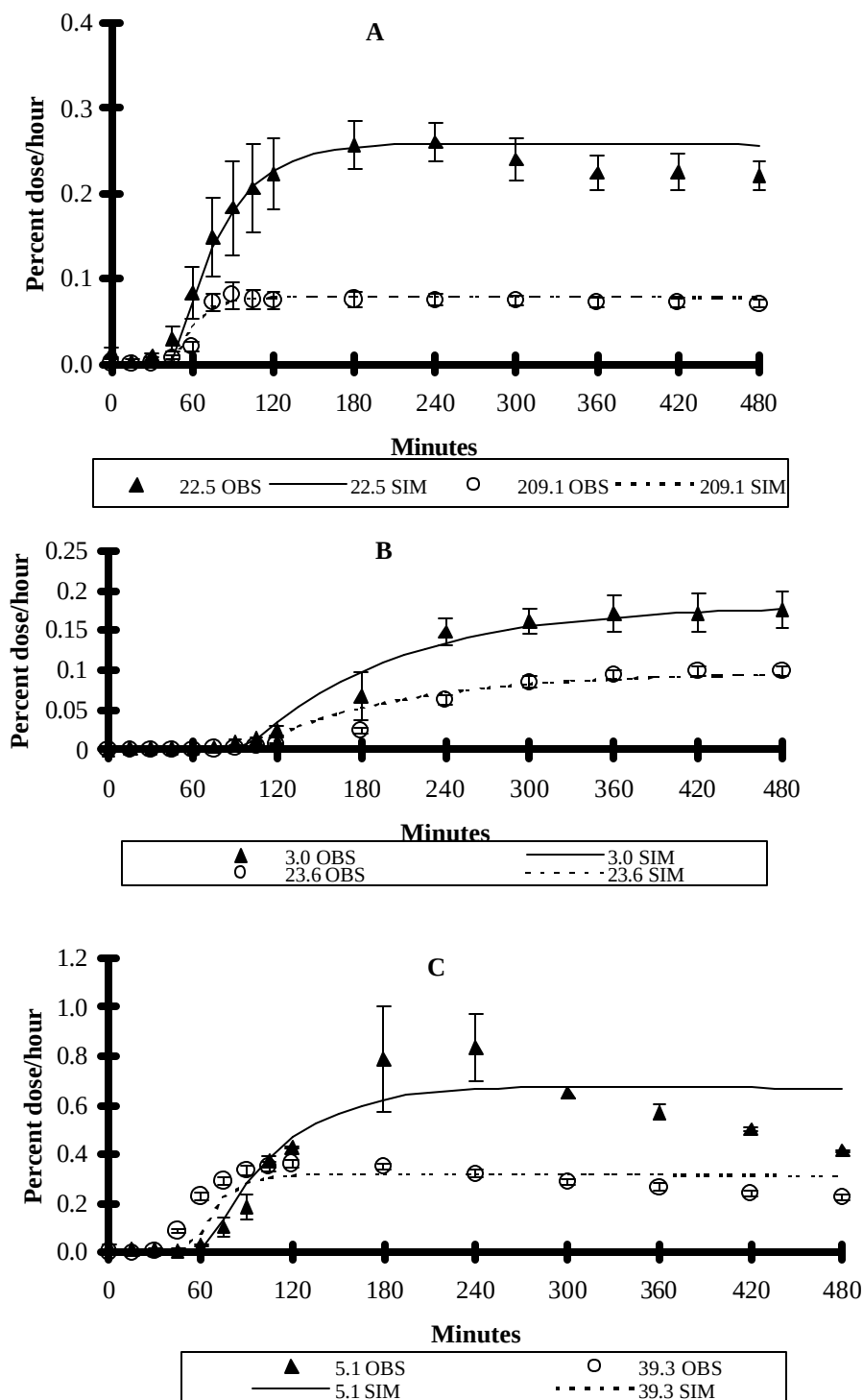


Figure 6.11. Simulated (SIM) and observed (OBS) flux/time curves: parathion (A) dosed at 22.5 $\mu\text{g}/\text{cm}^2$ (n=5) and 209.1 $\mu\text{g}/\text{cm}^2$ (n=4) in 20 μl ethanol; fenthion (B) dosed at 3.0 $\mu\text{g}/\text{cm}^2$ (n=4) and 23.6 $\mu\text{g}/\text{cm}^2$ (n=4) in 20 μl ethanol and methyl parathion (C) dosed at 5.1 $\mu\text{g}/\text{cm}^2$ (n=5) and 39.3 $\mu\text{g}/\text{cm}^2$ (n=4) in 20 μl ethanol.

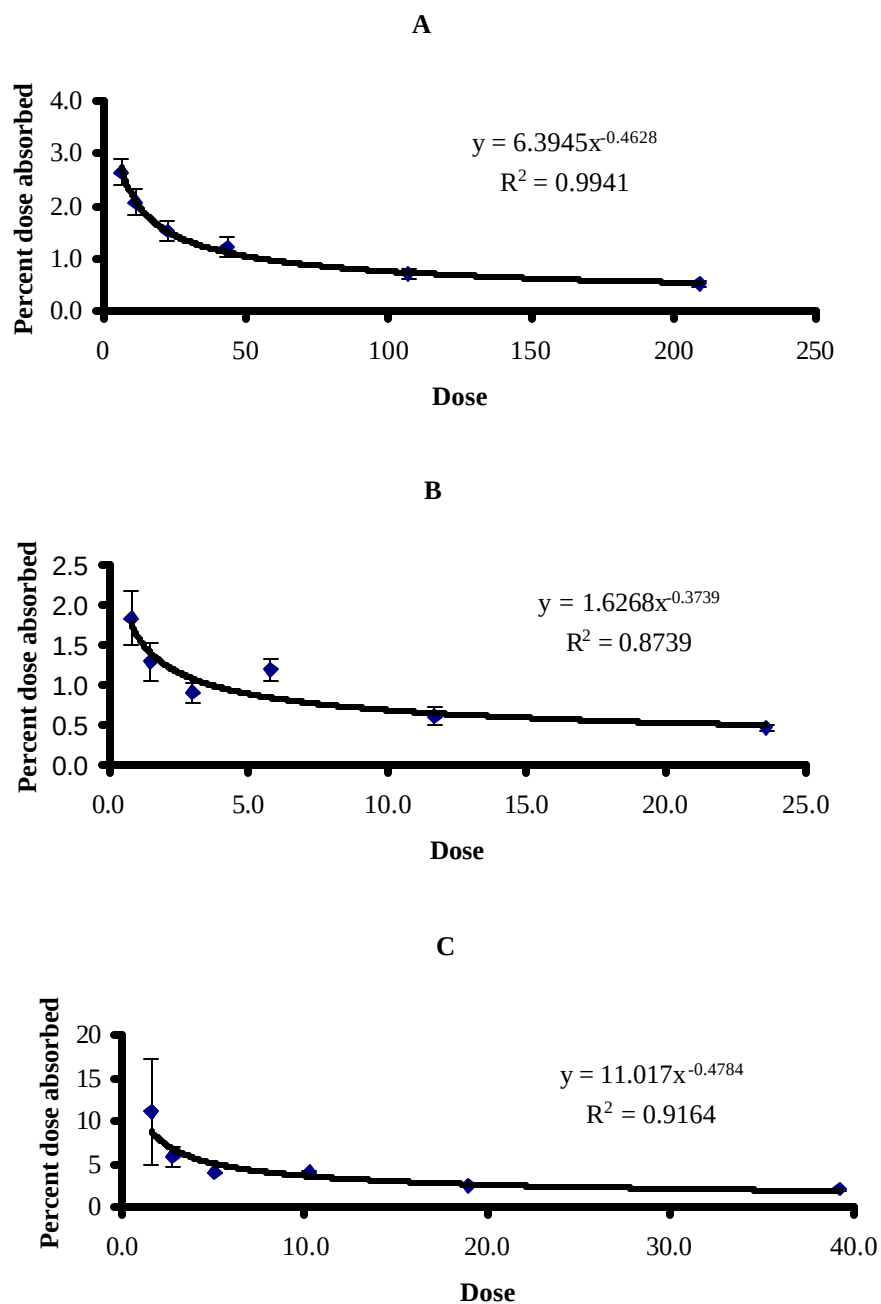


Figure 6.12. Scatter plots of the dose ($\mu\text{g}/\text{cm}^2$) and the percent of the dose absorbed after eight hours for parathion (A), fenthion (B) and methyl parathion (C) including power function trendlines, power function equations and their associated R^2 -values.

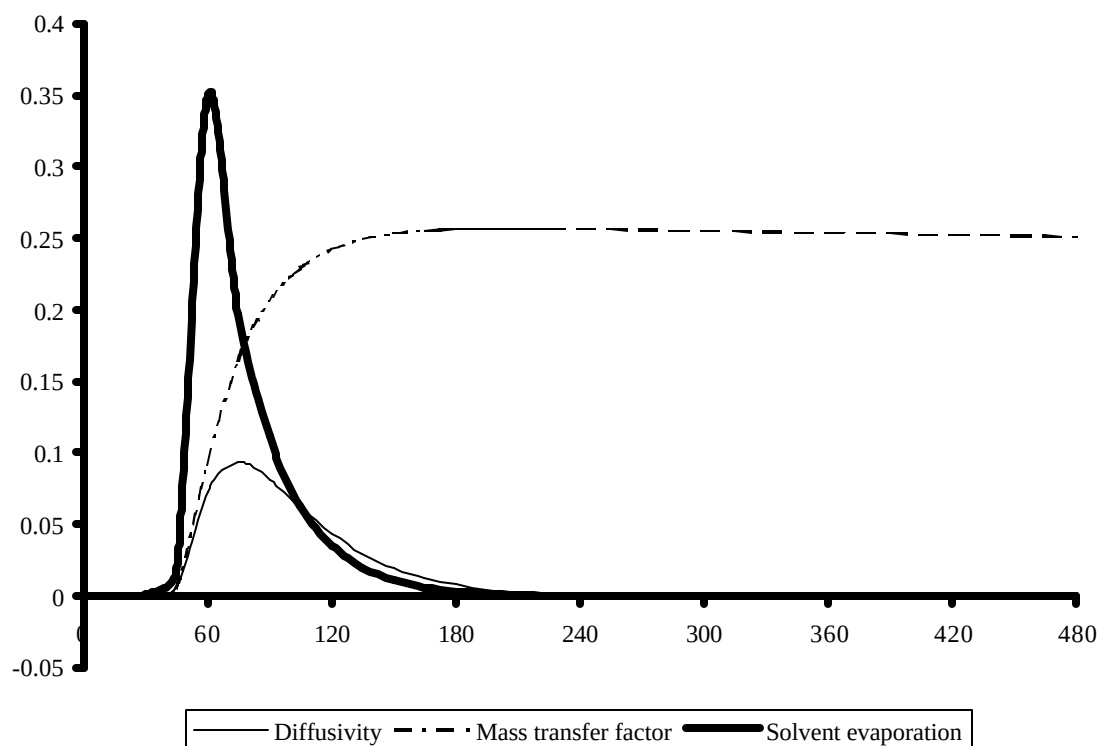


Figure 6.13. Sensitivity analysis of parameters used to optimize the simulation of $22.5 \mu\text{g}/\text{cm}^2$ parathion absorption from $20 \mu\text{l}$ ethanol, including diffusivity, mass transfer factor and solvent evaporation rate.

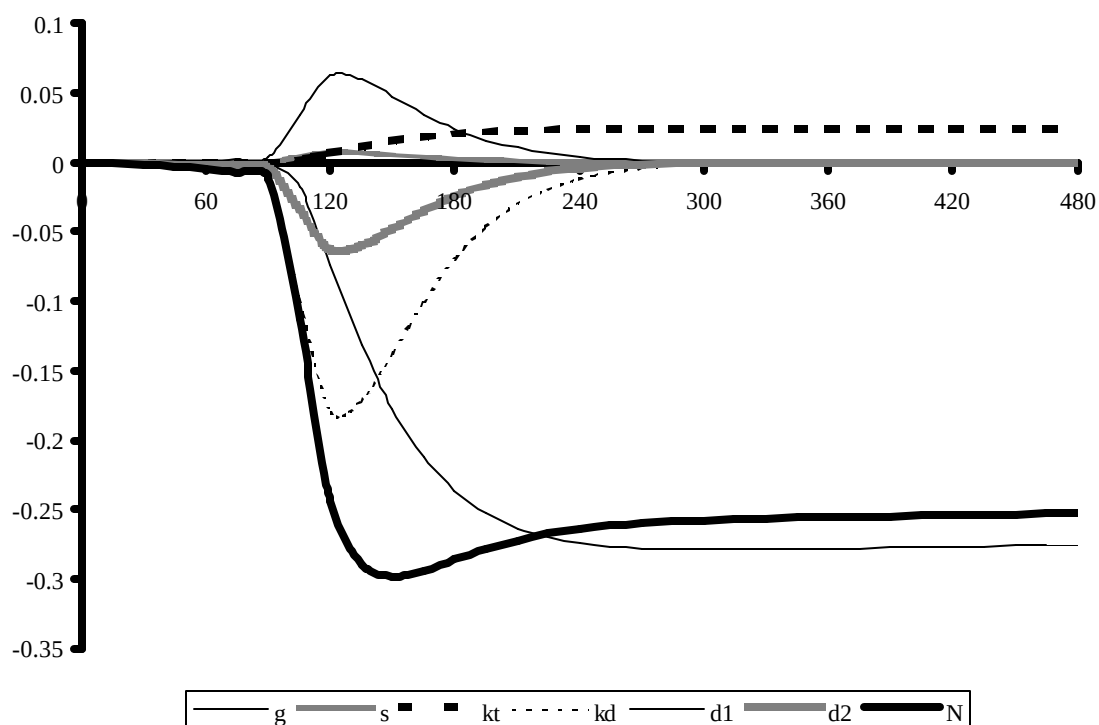


Figure 6.14. Sensitivity analysis of parameters used to describe the stratum corneum and determine effective tortuosity associated with $22.5 \mu\text{g}/\text{cm}^2$ parathion absorption from $20 \mu\text{l}$ ethanol – including vertical gap between corneocytes (g), lateral gap between corneocytes (s), corneocyte thickness (kt), corneocyte diameter (kd), long leg of corneocyte overlap (d1), short leg of corneocyte overlap (d2) and number of corneocyte layers (N).

7. CONCLUSIONS AND FUTURE DIRECTIONS

The studies included in this dissertation were aimed at reaching two general objectives:

- To understand the principles that govern absorption from chemical mixtures; and
- To develop a model of dermal absorption that is adaptable to a wide range of exposure conditions.

The studies on the effects of water, ethanol and water/ethanol mixtures on partitioning into silastic membranes and porcine stratum corneum included in Chapter 3 demonstrated a determining influence of solvents on partitioning – an essential initial process of dermal absorption. It showed that octanol/water partitioning could be predictive of solvent/stratum corneum partitioning if the solvent has physical-chemical properties similar to water. It suggested that octanol/water partitioning would not be a useful predictor of dermal absorption when used in models that do not have a polar solvent system.

The studies on the effects of the addition of sodium lauryl sulphate (SLS) to water, ethanol and propylene glycol on partitioning and permeability, reported in Chapter 4, expanded on the findings of Chapter 3. It indicated a relationship between solvent/stratum corneum partitioning and the relative solubility of the solute between the solvent and the stratum corneum lipids. It also demonstrated that permeability is not predictable from a simple relationship with partitioning, because permeability is also dependent on processes of solute transport through the stratum corneum. These studies suggested that permeability parameters determined from neat chemical exposures may not be predictive of permeability from chemical mixtures and that the physical-chemical interactions between the molecules in experimental solvent mixtures should be defined to determine its similarity to a specific real-world exposure scenario before it is used in risk assessment models.

The determining influence of solvent polarity on partitioning was also supported by the partitioning and permeability data structure analyses reported in Chapter 5. It indicated that solvents could have effects on diffusivity that might be strong enough to mask solvent effects on partitioning. It also showed a relationship between solvent volatility, which leads to solute super saturation and markedly increased solute transdermal concentration gradients when volatile solvents are used, and permeability. The use of multivariate cluster analysis of large datasets to generate and test hypotheses was demonstrated. Although exact predictions are not

possible when using these methods, it could be used with success to identify generally consistent trends associated with solute and solvent physical-chemical characteristics. This can be used to target additional work to investigate the mechanisms of broadly repeatable solvent effects on dermal absorption. Expansion of such datasets to include wider ranges of solute and solvent physical-chemical characteristics should be useful.

The validated physiological-based pharmacokinetic (PBPK) model of organophosphate pesticide dermal absorption reported in Chapter 6 demonstrated that such models could be used for *in silico* hypotheses generation and limited hypotheses testing if important aspects of dermal absorption are captured in the model. A dependence of the observed apparent absorption lag time on solvent volatility was shown. The number of corneocyte layers in the stratum corneum was a significant determinant of the stratum corneum's barrier function. For the compounds tested, the rate of transfer through the stratum corneum appeared to be the limiting factor in the rate of absorption – not partitioning. The results also suggested that these compounds could form depots in the stratum corneum that could potentially be absorbed over extended periods of time. An important consequence of efforts to validate the model over wide dose ranges was that dermal absorption over time was not a constant fraction of the dose - as predicted by Fick's first law of diffusion. This has implications for the use of permeability constants in risk assessment. The model was based on current understanding of the processes of dermal absorption. A key advantage of PBPK models is that it can be adapted as our understanding evolves.

These studies demonstrated the potential of using large data sets to identify consistent solvent and chemical mixture effects on the dermal absorption of a range of chemical permeants. It offers a workable approach to reduce uncertainty in the risk assessment of real-world dermal exposure to chemicals. Additional work is needed to fully understand the mechanisms by which broadly repeatable solvent and chemical mixture effects function. Broadening the database to include more chemical mixtures and permeants will increase our confidence in defining the broad principles that allow accurate predictions of dermal absorption.

The importance of factors affecting dermal absorption, other than those related to the permeant and the skin only, were clearly shown. These factors include solvent effects, chemical interactions and environmental conditions influencing solvent evaporation. It is essential that these factors should be considered when risk analyses are conducted.

The factors contributing to the need for the use of a mass transfer factor in the PBPK model need to be elucidated. Although the PBPK model was shown to be a useful tool in deciphering the various contributing processes resulting in typical flux/time curves, resolving the mass transfer factor will increase the predictive power of the model.

The PBPK model should be expanded to other species. This will increase our ability to apply data obtained from one species to predictions in other species. It could be used to reduce the need for *in vivo* experimentation in general, which is an important ethical and practical consideration. It could also be of particular value where data cannot be produced in the target species - such as determining the absorption of highly toxic substances in humans or rare species.

Fick's First Law of diffusion could not be used to fully explain the absorption of organophosphate pesticides across wide concentration ranges. Alternative models of diffusion in the stratum corneum should be investigated. Applying single molecule tracking techniques to the skin could potentially be of great value if methods can be developed with adequate resolution to track molecules within the inter-corneocyte lipid matrix.

Finally, the PBPK model could be linked to full body PBPK models for use in predicting the concentrations and duration above critical levels of drugs or toxins at target tissues.

APPENDIX

Example of acslXtreme code used to simulate parathion dermal absorption in a flow-through cell

```

!-----
! Surface block
!-----
!
global Cs
global Csc
global Cvs
global Crf
global Jf1
global Jf2
global Jf3
global Jf4
global h
global D
global minpath
global dose
global area
global ah
global P
global kt
global g
global dermisdepth
!
constant tz = 0.0      ! start time
constant endt = 480    ! end time
constant dose = 22.5    ! amount of permeant deposited onto skin surface (ug)
constant doseevap = 0.0001 ! rate of permeant evaporation from skin surface (ug/min)
constant insolventvol = 20.0 ! solvent volume deposited onto skin surface (ul)
constant solventevaprate = 0.4 ! rate of solvent evaporation from skin surface (ul/min)
constant logP = 1.090    ! Log [stratum corneum]/[solvent]
constant D = 0.002      ! diffusivity in stratum corneum lipid matrix (cm**2/min)
constant area = 0.64    ! skin surface area (cm**2)
constant e = 2.718282    ! base of natural logarithm
constant bb = 0.1       ! minimum solvent volume on skin surface (ul)
!
!-----
!
! Tortuosity calculator
!
!-----
!
constant noimped = 1    ! resistance to transport through membrane with no impediments
constant kt = 0.302619048 ! corneocyte thickness (um)
constant kd = 32.0      ! corneocyte diameter (um)
constant g = 0.033222922 ! vertical gap between corneocytes (um)
constant s = 0.033222922 ! lateral gap between corneocytes (um)
constant N = 21.9       ! number of corneocute layers
constant d1 = 27.6      ! long leg of corneocyte overlap (um)
constant d2 = 4.4       ! short leg of corneocyte overlap (um)
!
!----- Actual SC thickness (um)
ah = ((kt+g)*(N-1))+kt
!

```

```

!-----Corneocyte aspect ratio
ar = kd/kt
!
!-----Lipid/Corneocyte thickness ratio
br = g/kt
!
!-----Slit shape term
ss = kt/g
!
!-----Corneocyte volume fraction of SC
cvol = (kt+kd)/(kt+kd+g+s)
!
!-----Corneocyte offset ratio
w = d1/d2
!
!-----Geometric tortuosity (Talreja et al 2001)
Tg = (d2/((N/(N-1))*kt+g))+1
!
!-----Corneocyte diameter/2*junction gap
term = kd/(2*s)
!
!-----Resistance associated with necking down into gaps between corneocytes
necking = log(term)*((2*g)/ah)
!
!-----Resistance of transport through corneocyte junction gaps
junctionresist = (N*kd*kt)/(s*ah)
!
!-----Resistance of transport in the parallel gaps between corneocytes
parallelresist = (kd/(1+w))*(kd/(1+w))*(w/(ah*g))*(N-1)
!
!-----Effective tortuosity
tortuosity = noimped + necking + junctionresist + parallelresist
!
!-----Effective skin thickness (cm)
h = ((ah*tortuosity)/10000)+(dermisdepth-(ah/10000))
!
!-----Minimum pathway (um)
minpath = ((kt+g+d2)*(N-1))+kt
!
!end of tortuosity calculator
!-----
!
! Fractional movement into SC and surface concentration calculation

!-----
!
! -----SC/Solvent partition coefficient
P = (10**logP)
!
! -----Rate of solvent evaporation (ul/min)
solventevap = solventevaprate
!
!-----Fraction of solute moving into stratum corneum per minute
Jf1 = (P*D*area)/(h/2)
!
!-----Fraction of solute returning to surface per minute

```

```

Jf2 = (1/P*D*area)/(h/2)
!
!-----Solventvolume (Note: never reaches zero)
solventvol = bound(bb, insolventvol, (insolventvol-(solventevap*t)))
!
!-----Rate of change of solute on surface (ug/min)
achangesurface = Cs*(-Jf1)+Csc*Jf2-doseevap
!
!-----Amount of solute on surface (ug)
Asurface = integ(achangesurface, dose)
!
!-----Solute concentration on surface
Cs=Asurface/solventvol
!
!End of surface block
!-----

!-----
! Stratum corneum block
!-----
!
global Cs
global Csc
global Cvs
global Crf
global Jf1
global Jf2
global Jf3
global Jf4
global D
global minpath
global Qb
global P
global kt
global g
global area
global ah
global VSconc
!
constant LogPsw = 2.952      ! LogP [stratum corneum]/[water]
constant MTf = 20           !Mass transfer factor
!
!-----Stratum corneum/water partition coefficient
Psw = (10**logPsw)
!
!-----Stratum corneum lipid volume (ul)
SClipidvol = (g/kt)*area*ah*1000
!
!-----SC/viable skin partition coefficient
SCepipart = 1/Psw
!
!-----Fraction of solute moving into viable skin per minute
Jf3 = Scepipart*MTf
!
!-----Fraction of solute returning to stratum corneum per minute
Jf4 = 1/Scepipart

```

```

!
!-----Rate of change of solute in stratum corneum (ug/min)
achangesc = Cs*Jf1 - Csc*Jf2 - Csc*Jf3 + Cvs*Jf4
!
!-----Amount of solute in stratum corneum (ug)
Asc = integ(achangesc, 0.0)
!
!-----Concentration of solute in SC lipid (ug/ul)
Csc = Asc/SCLipidvol
!
!End of stratum corneum block
!-----

!-----
! Viable skin block
!-----
!
global Cs
global Csc
global Cvs
global Crf
global Jf1
global Jf2
global Jf3
global Jf4
global D
global minpath
global Qb
global P
global Kt
global g
global area
global ah
global Rvol
global dose
global dermisdepth
!
constant Vmax = 0.001      ! maximum rate of metabolism (ug/min)
constant Km = 1000.0      ![permeant] at 50 % of Vmax (ug/ul)
constant dermisdepth = 0.05 ! dermatome depth setting (cm)
!
!-----Rate of metabolism (ug/min)
Rm = (Vmax*Cvs)/(Km + Cvs)
!
!-----Volume of VS and receptor fluid chamber (ul)
VSvol = area*(dermisdepth-(ah/1000))*1000 + Rvol
!
!-----Lag time (Note: 1.0E10 is a unit conversion factor)
lag = (minpath**2)/(6*D*1.0E10)
!
!-----Rate of change of solute in viable skin/receptor fluid without lag (ug/min)
achangevs = Csc*Jf3 - Cvs*Jf4 - Rm - (Qb*Cvs)
!
!-----Rate of change of solute in viable skin/receptor fluid with lag (ug/min)
achangevslag = delay(achangevs, 0.0, lag, 100, 1)
!

```

```

!-----Amount of solute in viable skin/receptor fluid
Avs = integ(achangevslag, 0.0)
!
!-----Concentration of solute in viable skin/receptor fluid
Cvs = Avs/VSvol
!
!-----Rate of exit (flux) in terms of % dose/hour
percentflux = (achangevslag*100*60)/dose
!
!End of viable skin block
!-----

!-----
! Receptor fluid block
!-----
!
constant Rdepth = 0.48 ! receptor chamber depth (cm)
constant Qb = 67      ! receptor fluid flow rate (ul/min)
!
global Qb
global RVol
!
!-----Volume of receptor fluid chamber (ul)
RVol = area*Rdepth*1000
!
!End of receptor fluid block
!-----

```