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Assessment of Skin Absorption and Irritation Potential of
Arachidonic Acid and Glyceryl Arachidonate
Using In Vitro Diffusion Cell Techniques

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Abstract

The Cosmetic Ingredient Review Panel's final report on the safety assessment of arachidonic acid (AA) noted its use in cosmetic skin preparations although there is a lack of dermal absorption data. The safety concern of topical applied AA arises upon its metabolism to pro-inflammatory mediators. *In vitro* percutaneous absorption/metabolism studies were initiated in flow-through diffusion cells. AA, and its glycerin monoester, glyceryl arachidonate (GA), were each applied in an o/w emulsion to simulate cosmetic consumer use conditions. AA was analyzed for absorption/metabolism in viable rat and human skin while GA was tested in cultured (EpiDerm™), viable human, and human cadaver skin. Absorption data on both compounds was collected every 6hr over a 24hr period. The highest absorption was observed through EpiDerm™ (50%) and rat skin (20%). To a lesser extent, AA and GA penetrated human skin about 20%, of which ~2% absorbed. A reservoir of AA was observed in cadaver skin during an extended absorption study (72hr), in which absorption increased to 4%. Ester hydrolysis of GA occurred in each of the analyzed tissue samples.

Additionally, *in vitro* methods (as an alternative to the Draize test) were evaluated for their prediction of skin irritation potential in conjunction with flow-through diffusion cell absorption/metabolism measurements. Model compounds, with known irritation potential *in vivo*, were evaluated *in vitro* by measuring transepidermal water loss (TEWL), cytokine release (IL-1 α), and skin viability (MTT) of rat and cultured skin in flow-through diffusion cells. Irritation potential of the model compounds was less pronounced in the emulsion formulation compared to an aqueous vehicle. No significant

irritation from AA in an emulsion vehicle was observed with the alternative methods. As a supplementary assessment, TEWL was analyzed for its relationship to the $^3\text{H}_2\text{O}$ skin barrier integrity test, however no direct correlation was found to exist.

Absorption of topically applied AA and GA was observed, indicating a potential for systemic availability. Furthermore, ester hydrolysis of GA occurred during absorption. The in vitro alternative methods for measuring skin irritation in flow-through diffusion cells were able to differentiate between the potency of irritants in an aqueous but not an emulsion formulation.

This dissertation is dedicated in loving memory of my grandfather,

Garland Kenneth Uhlman,

.....

Who wanted me to take advantage of all the opportunities he never had.

Thank you for always being my loudest cheering section

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List of Abbreviations

AA = arachidonic acid

BCA = bicinchoninic acid

BSA = bovine serum albumin

cm = centimeter

DI H₂O = deionized water

ELISA = enzyme-linked immunosorbent assay

emul = emulsion vehicle

GA = glyceryl arachidonate

HETE = hydroxyeicosatetraenoic acids

HHBSS = Hepes buffered Hanks' balanced salt solution

³H₂O = tritium-labeled water

HpETE = hydroxyeicosatetraenoic acids

HPLC = high performance liquid chromatography

Hr = hour(s)

IL-1 α = interleukin-1 alpha

LT = leukotrienes

mg = milligram

min = minute

ml = milliliter

MTT = methylthiazoletetrazolium

n = number of replicates/samples

NSAIDs = non-steroid anti-inflammatory drugs

o/w = oil-in-water

PBS = phosphate buffered saline

pg = picogram

PG = prostaglandin

RH = relative humidity

rpm = rotations per minute

SA = surface area

SC = stratum corneum

sec = second

SEM = standard error mean

SLS = sodium lauryl sulfate

SPE = solid phase extraction

TEWL = transepidermal water loss

TLC = thin layer chromatography

Tx = thromboxanes

μg = microgram

μl = microliter

UV = ultraviolet

Introduction

The Skin

Skin, the largest organ of the human body, has many functions including protection, sensory, and thermoregulation (Menon 2002). A main function of the outer layer, the stratum corneum, is to defend the body against foreign materials and damage from the environment (Marks et al. 1998). Personal care, cosmetic, and pharmaceutical companies each devote much of their attention and owe some of their success to topical products (Menon 2002). Skin, however, can present many obstacles, such as allowing the absorption of undesirable compounds, while presenting a strong barrier against intended delivery of active ingredients (Menon 2002).

The skin can be divided into two sections, the dermis and epidermis. The epidermis is stratified into many layers (Leigh et al. 1994). The innermost layer is the stratum basal, followed sequentially by the stratum spinosum, stratum granulosum, and finally the stratum corneum (Leigh et al. 1994).

The underlying viable layers of epidermis are mainly hydrophilic. However, a lipid structure comprised mainly of phospholipids, cholesterol, and triglycerides exists (Leigh et al. 1994). Upward movement through the layers results in a change in the lipid composition (Leigh et al. 1994). The intercellular lipids present prior to desquamation of the cells are rich in ceramides, free sterols, and free fatty acids (Leigh et al. 1994).

Keratinocytes are located in the lower levels of the epidermis (Montagna and Parakkal 1974). These are differentiated into their dead counterpart, corneocytes, which no longer have a nucleus (Montagna and Parakkal 1974). These “cornified keratinocytes” (Jonca et al. 2002), located in the stratum corneum (SC) (Montagna and Parakkal 1974), are encased by a cornified cell envelope making them resilient and thus aiding in their function (Jonca et al. 2002).

Standard Test for Skin Irritation

For many years, skin irritation studies have been conducted on rabbits (Fentem et al. 2001). This process was termed the Draize Rabbit Skin Irritation Test (Basketter et al. 1997) after John H. Draize. In this procedure, the visual erythema and edema produced is evaluated by trained graders to determine the extent of irritation (Fentem et al. 2001). The test may cause the rabbit distress and pain therefore creating a desire for an alternative method to evaluate skin irritation (Fentem et al. 2001). In recent years alternative methods for measuring skin irritation have been developed. Some are currently being evaluated for their usefulness in reducing or replacing animal testing (Fentem et al. 2001).

Alternative Methods

The necessity for an *in vitro* alternative method to measure skin irritation is currently increasing, led by Europe’s intended banning of animal testing for cosmetic ingredients and products. Excised animal and human skin, which have been utilized as alternatives to *in vivo* studies, have their limitations (Stinchcomb 2003). Skin equivalents

attempt to mimic these tissues with added benefits such as minimizing animal use and limiting the potential loss of viability occurring with human skin from time of harvest to experiment (Stinchcomb 2003). With the increasing availability of cultured skin equivalents, there is heightened interest in their utility as a skin model as part of an alternative method for skin irritation.

When evaluating alternative methods to animal testing, two factors should be taken into consideration (Basketter et al. 1997). First, before trying to develop an alternate method to an *in vivo* animal test, verification of accurate results needs to be conveyed (Basketter et al. 1997). If the *in vivo* animal test was not providing accurate results, then there would be no need to create an alternate for it. This has already been confirmed for skin irritation based on the historical use of the Draize test as the standard (Fentem et al. 2001). Therefore, it is beneficial to discover an alternative method for this experiment. Secondly, the alternative method needs to be giving consistent results with the *in vivo* animal test in order for it to be considered an effective and validated test (Basketter et al. 1997).

Background/Justification

Arachidonic Acid (AA)

Arachidonic Acid ($C_{20}H_{32}O_2$), also referred to as 5,8,11,14-Eicosatetraenoic Acid (Wenninger and G.N. McEwen 1995), is used in cosmetics as a surfactant-cleansing and surfactant-emulsifying agent (1993). In lotions and creams, it has been used to pacify eczema and dermatitis (1993). As can be seen in Figure 1, AA is an omega-6 class polyunsaturated fatty acid (Zhu et al. 2002) with a hydrocarbon chain structure made up of 20 carbons (Nelson and Cox 2000). Within this chain, there are 4 double bonds (at carbons 5, 8, 11, & 14) and a terminal carboxyl group (Hardman and Limbird 1996). Due to the presence of multiple double bonds, the structure of this compound contains kinks and is rigid compared to saturated fatty acids (Nelson and Cox 2000). Arachidonic acid is a liquid at room temperature and absorbs on a spectrophotometer at wavelengths of 257nm, 268nm, and 315nm (1993).

Arachidonic acid is part of a group of chemicals, along with linoleic acid and linolenic acid, which together are referred to as Vitamin F (1993). Linoleate and linolenate are essential fatty acids and need to be acquired from one's diet (Stedman 1982; Nelson and Cox 2000). If a deficiency exists, arachidonate can be generated through elongation reactions from linoleate (Nelson and Cox 2000). AA is naturally found in liver, fish oil, bovine adrenal gland, butterfat, and lard (1993). In animals, it is located in the brain, glandular organs, and fat deposits (Windholz et al. 1976).

Figure 1. Structure of $^{14}\text{C}^$ Arachidonic Acid*

Arachidonic acid has been found to be beneficial to the neurological and neurophysiological development of infants (Zhu et al. 2002). It is naturally found in human mother's milk (Zhu et al. 2002) and for this reason is now being supplemented in infant formula (Hansen et al. 1997).

In 1992, twenty nine formulations for cosmetic use were reported to contain arachidonic acid (1993). The distribution was as follows: face, body, and hand skin care preparations 5; moisturizing skin care preparations 5; night skin care preparations 4; wrinkle smoothers 4; and other skin care preparations 11 (1993). Exact concentration of use values are not available, but it has been reported that arachidonic acid in cosmetic skin products is used at <5% (1993).

In the Cosmetic Ingredient Review Panel's Final Report on the Safety of Arachidonic Acid, it was noted that dermal absorption data is not available for this compound (1993). Therefore, the safety of its use cannot be determined until such data is available (1993). If it is determined that absorption does occur, other subsequent tests will need to be conducted to evaluate its overall safety in cosmetics based on its role in the inflammatory cascade (1993).

Glyceryl Arachidonate (GA)

Glyceryl arachidonate ($C_{23}H_{38}O_4$) is the glycerin monoester of arachidonic acid (Pepe et al. 2002). The chemical structure can be found in Figure 2. In cosmetics, it functions as a skin conditioning agent and emollient (Pepe et al. 2002). Through ester

Figure 2. Structure of $^3\text{H}^$ Glycerol Arachidonate*

hydrolysis (Figure 3), which is functional in skin, bioconversion of glyceryl arachidonate to arachidonic acid is possible (Hardman and Limbird 1996).

Figure 3. GA ester hydrolysis

Specific Aims

This study had 4 main focuses:

1. Measurement of absorption of arachidonic acid and glyceryl arachidonate in the skin
2. Measurement of ester hydrolysis of glyceryl arachidonate to arachidonic acid in the skin
3. Assessment of skin irritation markers caused by emulsion formulations of arachidonic acid and model compounds in diffusion cell studies
 - a. Inflammatory mediator release (IL-1 α)
 - b. Barrier damage (TEWL)
 - c. Tissue viability (MTT Assay)
4. The reliability of the *in vitro* alternative methods for prediction of a compound's skin irritancy in a diffusion cell study

Absorption

Skin absorption occurs by a process known as passive diffusion (Bronaugh et al. 1989). Passive diffusion occurs when a concentration gradient is present as a result of the solubility of the compound in the membrane (Hardman and Limbird 1996). The lower the solubility of the compound in the membrane, the slower the diffusion rate (Hardman and Limbird 1996). Diffusion will progress eventually reaching a steady state in which the concentration on either side of the membrane is the same, assuming infinite dose of a nonionic compound (Hardman and Limbird 1996). If the compound is ionic, then the

steady state concentrations will be influenced by the pH of the membrane (Hardman and Limbird 1996).

Together, hydrophilic corneocytes and their surrounding intercellular lipids make up the composition of the stratum corneum (Leigh et al. 1994). Bypass of the corneocytes is favored with topical application of a lipophilic compound, such as arachidonic acid (Hardman and Limbird 1996).

Arachidonic acid is known to be a precursor of inflammatory mediators. Percutaneous absorption of arachidonic acid is of importance because the degree of penetration will influence the potential for bioconversion. If it doesn't penetrate the skin, exposure to metabolizing enzymes can not occur (Lin and Hemming 1996). However, if penetration does occur to a sufficient extent, AA has the potential to initiate an irritation response. Therefore, *in vitro* studies were conducted to evaluate the absorption profile and distribution of arachidonic acid. The same was evaluated for glyceryl arachidonate due to the presence of ester hydrolysis activity in the skin.

Bronaugh has demonstrated good correlation between *in vivo* absorption data and that obtained with the *in vitro* flow-through diffusion cells utilizing compounds exhibiting various skin penetration rates (Bronaugh et al. 1982a). The ranking ability of penetration in addition to quantitative similarities were observed in the *in vitro* and *in vivo* comparison (Bronaugh et al. 1982a).

Due to the high vascularity at the dermal/epidermal junction, 4.5% of the body's blood volume can be found in the skin (Montagna and Parakkal 1974). If a compound is able to penetrate through to the dermis, its likelihood of being systemically absorbed is heightened (Klaasen 2001). During *in vitro* studies, the amount of material considered to be systemically available is frequently limited to that which reaches the receptor fluid. In this paper, the term absorption refers only to the aforementioned material which reaches the receptor fluid.

The term penetration refers to the total amount of compound entering the skin regardless of whether it is eventually systemically absorbed (Kraeling et al. 2004). Therefore, the penetration of a compound is defined as the amount that passes through the skin to the receptor fluid, plus that which remains in the skin at the end of the study (Bronaugh et al. 1989). The only amount considered to have not penetrated is that which is removed during the skin surface wash (Bronaugh 1996).

With *in vitro* diffusion cell studies, there is sometimes an artificial retention of absorbed lipophilic test compounds in the skin due to a lack of free partitioning from the skin into the aqueous receptor fluid (Bronaugh and Stewart 1984). The amount of test compound penetrating the skin may be a more accurate estimate of *in vivo* systemic absorption than exclusively considering the receptor fluid levels. Sometimes, additional data, such as that provided with an extended absorption study, may help determine the best estimate of systemic absorption (Hood et al. 1996).

Metabolism

Many chemicals are metabolized by skin enzymes during permeation (Roberts and Walters 1998). Metabolism in the skin is usually determined to be about 10 to 100 times less than that observed in the liver (Bronaugh et al. 1989). However, if the possible metabolites are toxic or can alter the skin's structure or function, the skin metabolism of the compound should be considered important.

Studies have reported a relationship between penetration and metabolism in which a higher amount of penetration is observed in cases where a highly lipophilic compound, such as arachidonic acid, is converted to more hydrophilic metabolites (Bando et al. 1997). In this study, ester hydrolysis in the skin of topically applied glyceryl arachidonate to arachidonic acid was investigated. Additionally, skin metabolism of topically applied arachidonic acid, the second major polyunsaturated fatty acid (PUFA) in skin, was of concern (Ziboh et al. 2000). Of all fatty acids found in human skin, arachidonic acid makes up about 9% (Ziboh et al. 2000). Enzymes responsible for the metabolism of PUFAs are highly active in skin epidermis (Ziboh et al. 2000). When AA is metabolized, a group of products called eicosanoids are formed (Hardman and Limbird 1996). Their name originates from the greek word *eikosi* meaning 'twenty' based on their 20-carbon parent compound, arachidonate (Nelson and Cox 2000).

When produced, eicosanoids are not made in excess and stored (Nelson and Cox 2000). Instead, they are synthesized as needed (Nelson and Cox 2000). When eicosanoids are required, hormones signal the calcium-activated phospholipase A₂

enzyme (Papirmeister et al. 1991) to release arachidonic acid from membrane phospholipids (Nelson and Cox 2000). This arachidonic acid is then available to be metabolized into the desired eicosanoids (Nelson and Cox 2000). Prostaglandins, thromboxanes, and leukotrienes comprise the three classes of eicosanoids (Nelson and Cox 2000). An example of each is displayed in Figure 4.

Prostaglandins, which were first discovered in the 1930s (Fitzpatrick et al. 1993), have a 5-carbon ring structure which is formed when the C-8 and C-12 carbons of arachidonate join (Nelson and Cox 2000). They are involved in the intracellular second messenger system during which they regulate the synthesis of cyclic AMP (cAMP) (Nelson and Cox 2000). As a result, cellular and tissue function are affected by the prostaglandins through cAMP regulated hormone activity (Nelson and Cox 2000).

With thromboxanes, like the prostaglandins, the C-8 and C-12 positions join, but this time, an oxygen atom is inserted to form a 6- member ring structure with an ether (Nelson and Cox 2000). The main function of thromboxanes is to participate in the formation of blood clots (Nelson and Cox 2000).

The third type of eicosanoids, which are reached through the linear pathway, are the leukotrienes. When molecular oxygen is incorporated, a structure is formed composed of 3 conjugate double bonds (Nelson and Cox 2000). Leukotrienes can also play a role in the inflammatory process by reacting with glutathione to produce anaphylaxis and chemotaxis mediators (Klaasen 2001).

Figure 4. Sample Structures of a Prostaglandin, Thromboxane, and Leukotriene

PGE₂

TxA₂

LtA₄

Arachidonic acid is the precursor of various pro-inflammatory mediators. The type of eicosanoid produced depends on the mechanism of metabolism utilized. Arachidonic acid is metabolized to produce eicosanoids through 1 of 3 pathways (1993). These are the cyclooxygenase, lipoxygenase, and the epoxygenase pathways (Figure 5), each named after their active enzyme (Marks et al. 1998).

Cyclooxygenase Pathway

The cyclooxygenase pathway involves the oxidation of AA to form the products E_2 , $F_{2\alpha}$, I_2 , D_2 , and A_2 (1993). The first four products are prostaglandins referred to as PGE_2 , $PGF_{2\alpha}$, PGI_2 , and PGD_2 , respectively (1993). The last product, A_2 , is a thromboxane designated by TxA_2 (1993). Cyclooxygenase (COX), also known as prostaglandin H_2 synthase, is the active enzyme for this pathway (Nelson and Cox 2000). In the metabolism of arachidonic acid to eicosanoids, cyclooxygenase is bifunctional (Nelson and Cox 2000). First, by adding molecular oxygen into the equation, cyclooxygenase converts arachidonic acid to prostaglandin G_2 (Fitzpatrick et al. 1993). The resulting compound is catalyzed into prostaglandin H_2 by the peroxidase activity of the cyclooxygenase molecule (Fitzpatrick et al. 1993). From this precursor, many other prostaglandins and thromboxanes can be created (Hardman and Limbird 1996).

The major AA metabolites in skin from the cyclooxygenase pathway are PGE_2 , $PGF_{2\alpha}$, and PGD_2 (Ziboh et al. 2000). PGE_2 has been shown to be a main metabolite in human keratinocytes following cytokine induction (Sjurse et al. 2000).

Figure 5. AA Metabolism

Isozymes of cyclooxygenase have been discovered (Nelson and Cox 2000). They are different enzymes in amino acid structure, substrate affinity, and physical properties, but they perform the same catalytic function (Nelson and Cox 2000). Mammals have two cyclooxygenase isozymes, which are denoted COX-1 and COX-2 (Nelson and Cox 2000). COX-1 mediates the secretion of gastric mucin whereas COX-2 controls inflammation, fever, and pain (Nelson and Cox 2000). Therefore, COX-2 is the active isozyme considered in this study dealing with skin inflammation.

The cyclooxygenase pathway can be inhibited at the first step by common non-steroid anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen (Nelson and Cox 2000). NSAIDs acetylate the serine residue of COX making the binding site unattainable to the substrate (Nelson and Cox 2000). This results in an irreversible form of the inactive enzyme, which cannot synthesize eicosanoids (Nelson and Cox 2000).

Lipoxygenase Pathway

Lipoxygenase, like cyclooxygenase, forms eicosanoids by first introducing molecular oxygen to arachidonic acid (Nelson and Cox 2000). Depending on the location of the resulting peroxide group, metabolism of AA through the lipoxygenase pathway can result in the formation of either leukotrienes (LTs) or hydroxy fatty acids (1993). Unlike the cyclooxygenase enzyme, lipoxygenase is not inhibited by NSAIDs (Nelson and Cox 2000).

The lipoxygenase pathway results in the formation of the intermediates 5-, 12-, and 15-hydroperoxyeicosatetraenoic acids (HpETEs) (Fitzpatrick et al. 1993). These are then further reduced to their corresponding products, hydroxyeicosatetraenoic acids (HETEs) (Fitzpatrick et al. 1993). A major skin metabolite from the lipoxygenase pathway is 15-hydroxyeicosatetraenoic acid (Ziboh et al. 2000).

Leukotrienes, found in the brain, heart, lung, spleen, and leukocytes, result from further metabolism of the 5-HpETE intermediate (Nelson and Cox 2000). LTB_4 is produced by keratinocytes, while LTC_4 , LTD_4 , and LTE_4 are released from mast cells (Papirmeister et al. 1991). Following cytokine induction, LTB_4 has been demonstrated to be a main AA metabolite in human keratinocytes (Sjurse et al. 2000).

Epoxygenase Pathway

As opposed to both the cyclooxygenase and lipoxygenase enzymes, the monooxygenase property of P450 only introduces a singular oxygen atom into arachidonic acid as opposed to molecular oxygen (Fitzpatrick et al. 1993). Molecular oxygen and NADPH strictly regulate the formation of these products (1993). The P450 metabolites have displayed vascular, endocrine, renal, and ocular effects (Hardman and Limbird 1996).

Metabolism of arachidonic acid by the epoxygenase pathway of the P450 enzyme creates epoxyeicosatrienoic acids (EETs) (Schaefer et al. 1997). The formation of EETs can be inhibited by the presence of carbon monoxide (Schaefer et al. 1997). EETs are

further metabolized through hydrolysis to form dihydroxyeicosatrienoic acids (DHETEs) (Schaefer et al. 1997). Hydroxylation products such as monohydroxyeicosatetraenoic acids additionally result from the P450 metabolism pathway (Fitzpatrick et al. 1993).

Two P450 isozymes, 2B12 and 2B19, which metabolize arachidonic acid have been located in the skin (Klaasen 2001). However, it has been observed that the degree of metabolism in skin as a result of P450 is relatively low (Nasseri-Sina et al. 1997). It has been determined that P450 metabolic activity is only 1-5% of that found in the liver (Merck et al. 1996).

Irritation

An inflammatory change occurring in the skin which is not permanent and therefore reversible is termed irritation (Fentem et al. 2001). Like other membranes throughout the body, when the skin is damaged, signals are sent to the cells (Nelson and Cox 2000). Signals can be either autocrine, paracrine, or endocrine (Nelson and Cox 2000) in nature. Autocrine signals occur when the cell being acted upon is the same one that sent the signal (Nelson and Cox 2000). Endocrine signals are sent through the bloodstream to distant cells (Nelson and Cox 2000). Nearby cells receive paracrine signals through interstitial fluid, rather than the bloodstream (Nelson and Cox 2000).

Once the signals are received through receptors, the cells interpret them and counteract against the damage (Nelson and Cox 2000). In the body, the T cells, monocytes, and keratinocytes are antigen receptors (Leigh et al. 1994). Whereas the T

cell and monocyte move throughout the blood stream looking for an antigen, the keratinocyte is immobile and must wait for the antigen to approach it (Leigh et al. 1994). When the T cell and monocyte are not actively involved in the inflammation process, they are in a resting state (Leigh et al. 1994). The keratinocyte on the other hand is constantly active, either involved in an inflammation reaction or working at growing and differentiating itself (Leigh et al. 1994). In addition to the keratinocyte, another epidermal cell, the Langerhans cell, participates in the skin's immune response (Cumberbatch et al. 1998).

Numerous inflammatory mediators have been documented as being present and active in the skin (Papirmeister et al. 1991). One response to damage is in the form of inflammatory cytokine expression (Marks et al. 1998). Cytokines are small polypeptide molecules required in picomolar concentrations to cause changes in cellular function (Leigh et al. 1994).

Keratinocytes produce two forms of the interleukin (IL)-1 cytokine, α and β (Leigh et al. 1994). The alpha form is biologically active following receptor binding, while beta needs to be processed prior to activation (Leigh et al. 1994). Keratinocytes mainly produce the alpha variety (Leigh et al. 1994). IL-1 is produced and stored intracellularly until it is needed (Leigh et al. 1994). IL-1 is released and becomes active during the irritation response as a consequence to various types of skin damage (Papirmeister et al. 1991).

This cytokine expression is triggered by the eicosanoids which are paracrine signaling hormones (Nelson and Cox 2000). Leukotrienes, produced by mast cells, in addition to prostaglandins result in an increase in vasodilation and vascular permeability of the skin (Papirmeister et al. 1991). Upon injection of arachidonic acid, PGE₂ and LTB₄ are produced within the first 5 minutes (Papirmeister et al. 1991). When these eicosanoids are introduced by injection they have resulted in the formation of edema and neutrophil infiltration (Papirmeister et al. 1991). Additionally, 12-HETE, a product of the 5-lipoxygenase pathway, has been found in elevated levels in inflamed skin (Papirmeister et al. 1991).

In previous studies, topical application of arachidonic acid to mouse skin has been shown to create dermal irritation and edema (1993). High topical doses of AA can also result in the formation of skin ulcers (1993). Early stages of irritation have been shown to be initiated by metabolites of arachidonic acid (Fitzpatrick et al. 1993). When a perfused bovine udder model was topically treated with arachidonic acid (5.6 % w/w) in an ointment cream, an increase in eicosanoid synthesis was observed (Baumer and Kietzmann 2000). The increase of the eicosanoids in the udder, specifically PGE₂, became noticeable at 60 minutes, and significant 120 minutes following application (Baumer and Kietzmann 2000). The ointment provides for continuous delivery of the arachidonic acid while in contact with the tissue, which the authors believed to be the reason why the increase in eicosanoids persisted longer than expected (Baumer and Kietzmann 2000).

Arachidonic acid's metabolites have been shown to play an active role in the inflammation response. Based on this information, the irritation promotion, in addition to absorption and metabolism, of topically applied AA was analyzed. Skin irritation was assessed in flow-through diffusion cells by developed alternative methods

Alternative Methods for Skin Irritation

Animal models and human skin have been historically utilized for absorption, metabolism, and irritation testing purposes. However, with the intended ban in Europe on animal testing of cosmetic ingredients and products (Benassi et al. 1999), and the trend towards minimization of the same in the United States, the development of *in vitro* alternative methods for evaluating skin irritation is highly valued.

History of Alternative Methods

Many attempts have been made to evaluate the utility of *in vitro* skin cultures for replacement of the current *in vivo* procedures. Among the skin models already considered include the commercially available Skin² (Osborne and Perkins 1994), Testskin (Osborne and Perkins 1994), EpiDermTM (Perkins et al. 1999; Robinson et al. 1999; Faller et al. 2002), and EPISKIN (Faller et al. 2002). Additionally, normal human epidermal keratinocytes (NHEK) (Korting et al. 1994; Osborne and Perkins 1994; Ward et al. 1998; Coquette et al. 1999; Lee et al. 2000), human epidermal equivalent (HEE) (Ward et al. 1998), and reconstituted human epidermis (RHE) (Heylings et al. 2001) have also been evaluated.

As far back as 1987, researchers were looking for alternative methods to animal research in regards to irritation (Gajjar and Benford 1987-88). Gajjar and Benford conducted studies utilizing cell cultures from rat sublingual epithelium. Following treatment with various surfactants, the toxicity endpoint was assessed with the acid phosphatase, neural red uptake, and kenacid blue assays (Gajjar and Benford 1987-88).

Since that time, numerous studies have been conducted evaluating other various *in vitro* endpoints of irritation. Cytotoxicity has been evaluated by means of an MTT assay (Korting et al. 1994; Osborne and Perkins 1994; Perkins et al. 1996; Ward et al. 1998; Coquette et al. 1999; Perkins et al. 1999; Robinson et al. 1999; Faller et al. 2002), neural red release (Korting et al. 1994), neural red uptake (Osborne and Perkins 1994; Lee et al. 2000), lactase dehydrogenase (Osborne and Perkins 1994; van Ruissen et al. 1998; Perkins et al. 1999; Faller et al. 2002), N-acetyl glucosaminidase release (Osborne and Perkins 1994), glucose utilization (Osborne and Perkins 1994), aspartate-aminotransferase (AST) (Perkins et al. 1999), and alamar blue (AB) (Lee et al. 2000).

Aside from cytotoxicity, other alternative endpoints include but are not limited to cytokine release, pro-inflammatory mediator release, cell morphology, and biochemical endpoints (Lee et al. 2000). Osborne and Perkins in 1994 investigated the release of the inflammatory mediator PGE₂. Cytokine release such as IL-1 α (Ward et al. 1998; Perkins et al. 1999; Robinson et al. 1999; Faller et al. 2002) and IL-8 (Coquette et al. 1999) have also been considered for irritation analysis. Still other means of evaluating irritation *in*

vitro include structure activity analysis (Lee et al. 2000) and histology (Perkins et al. 1996; Perkins et al. 1999).

Others have attempted to incorporate a non-invasive procedure into irritation studies (Heylings et al. 2001). Laser Doppler velocimetry (Zuang et al. 2000), evaporimetry (van Ruissen et al. 1998; Zuang et al. 2000), chromametry (Zuang et al. 2000), and corneometry (Zuang et al. 2000) have all been evaluated as means for determining irritation *in vivo*. Specifically noted was the detection of irritation before visual evidence by evaporimetry *in vivo* (Zuang et al. 2000). The main non-invasive test carried over for evaluation *in vitro* has been evaporimetry (Heylings et al. 2001; Zuang et al. 2002).

A prevalidation study, sponsored by the European Centre for the Validation of Alternative Methods (ECVAM), was conducted in 1999 and 2000 to evaluate *in vitro* methods for irritation assessment (Fentem et al. 2001). Skin samples tested during the study included EpiDerm™, EPISKIN, PREDISKIN, non-perfused pig ear, and mouse skin (Fentem et al. 2001). Among their studies included the evaluation of cytotoxicity (MTT) and TEWL of the skin samples (Fentem et al. 2001). The studies involving cultured skin were evaluated in culture plates. At the end of the prevalidation study, they concluded that “none of the tests evaluated in this prevalidation study are ready for inclusion in a formal validation study on *in vitro* tests for acute skin irritation” (Fentem et al. 2001). However, in a follow-up to the prevalidation study, it was observed for the EpiDerm™, EPISKIN, and mouse Skin Irritation Function Test methods that

modifications in the prediction model could result in an improvement in their utility as an *in vitro* model (Zuang et al. 2002). An ECVAM validation study is currently underway assessing these three methods.

The MTT cytotoxicity assay has proven its ability as an endpoint for irritation measurement in numerous studies. During a study on human foreskin keratinocytes, in which *in vivo* data was compared with *in vitro* data, MTT results were found to give a good correlation whereas the neural red release did not correlate as well (Korting et al. 1994). Additionally, Perkins et al. (1999) demonstrated success of the MTT in differentiating between various irritants with EpiDerm™. Their success with MTT was also demonstrated on a study conducted on Skin² and Testskin (Osborne and Perkins 1994). Robinson compared *in vivo* patch test results to MTT data obtained from EpiDerm™ studies and found a similarity in ranking ability of compounds with varying irritation potential (Robinson et al. 1999).

Some believe that IL-1 α is a biomarker of numerous types of dermal insult (Papirmeister et al. 1991). An increase in IL-1 α release in skin has been demonstrated following UV radiation exposure (Papirmeister et al. 1991). A study by Perkins evaluating alternative methods, determined the release of IL-1 α to be the most sensitive endpoint for distinguishing irritants from non-irritants with the use of skin cultures (Perkins et al. 1999). They showed that 14 day cumulative irritation index scores could be represented well with a 24hr endpoint (Perkins et al. 1999). High inter-study and inter-sample reproducibility was displayed (Perkins et al. 1999). Good discrimination

between treatments was found in multiple studies by analyzing the release of IL-1 α (Ward et al. 1998; Perkins et al. 1999; Robinson et al. 1999). It was additionally noted that IL-1 α could distinguish irritants over a large range of irritancy (Perkins et al. 1999; Robinson et al. 1999). Good correlation with *in vivo* data was also displayed (Ward et al. 1998; Robinson et al. 1999).

TEWL as a measure of skin irritation was analyzed by Heylings in glass diffusion cells (Heylings et al. 2001). Following a 20hr exposure to various aqueous concentrations of SLS, it was determined that mouse skin resulted in the most measurable degree of damage as evidenced by TEWL. In addition, it was the most reproducible specimen for TEWL measurements. Of the other tissue samples analyzed, rabbit whole skin and human reconstituted tissue gave the least observable change in TEWL following SLS treatment. In a study by van Ruissen, an increase in TEWL was observed following SLS treatment of skin cultures (van Ruissen et al. 1998).

At this time few cultured skin models have been able to closely reproduce the data that is achieved with *in vivo* irritation analysis (Lee et al. 2000). However, *in vitro* measurement of some skin irritation biomarkers have been able to closely mimic results observed *in vivo*, including assays which measure MTT conversion and PGE₂ release (Lee et al. 2000). It is important to remember that although skin cultures appear promising, they have their limitations just like any other tissue (Lee et al. 2000). Suggestions have been made to utilize the skin equivalents as a screening process prior to clinical studies (Mavon et al. 2004).

The majority of attempts at developing alternative methods for irritation have focused on treatments in aqueous formulations. Limited attempts have been made to analyze cosmetic formulations. A study evaluating aqueous based cosmetic and antiperspirant/deodorant formulations on EpiDerm™ in culture plates determined MTT, enzyme release, and cytokine release to be effective biomarkers of irritation (Perkins et al. 1999).

Alternative Method Development Approach

An alternative method for *in vitro* skin irritation assessment utilizing flow-through diffusion cells was developed. Most published attempts at developing studies of this kind have been conducted in culture plates. The use of flow-through diffusion cells in this study was in hopes of creating an alternative method which would allow for the measurement of absorption, metabolism, and irritation of a compound simultaneously. As markers for skin irritation, skin viability (MTT), transepidermal water loss (TEWL), and cytokine release (interluken-1 α) were evaluated. A cultured skin equivalent (EpiDerm™) and viable rat skin were evaluated as potential skin tissue sources for each irritation assay. Each alternative method consists of a combination of a specified tissue sample (EpiDerm™ or rat skin) and a corresponding irritation biomarker (IL-1 α , TEWL, or MTT).

Model surfactants with known skin irritancy promotion *in vivo* were selected (Muller-Decker et al. 1998; Benassi et al. 1999) as standards to evaluate the reliability of

the developed alternative methods. One strong irritant (sodium lauryl sulfate) (Fentem et al. 2001), one weakly-irritating/mild surfactant (Tween 80) (Coquette et al. 1999), and an appropriate control were utilized during initial studies. The predictability of the methods for their ranking potential of irritancy was determined by comparison of the acquired *in vitro* data with the acknowledged *in vivo* data (Muller-Decker et al. 1998). Initial studies were conducted evaluating the compounds in aqueous formulations against a distilled water control. Subsequent studies were conducted utilizing an oil-in-water (o/w) emulsion vehicle. Arachidonic acid in the emulsion vehicle was evaluated for skin irritation potential by the new methods.

The release of IL-1 α was quantified in this study as a potential means to assess irritation *in vitro*. In previous alternative method attempts using culture tissues, the underlying culture medium was assessed for cytokine release (Perkins et al. 1999). In this study, the intracellular IL-1 α in the skin was measured as opposed to extracellularly released cytokine. A normal flow rate used during a flow-through diffusion cell study resulted in excessive dilution of the extracellular IL-1 α in the receptor fluid (data not shown).

The cells of the stratum corneum are normally composed of 10-30% bound water (Leigh et al. 1994). The intercellular lipids in the stratum corneum work to maintain water in the skin (Hardman and Limbird 1996). Upon continual/repeated damage (irritation), the integrity of the stratum corneum as a barrier is diminished (Klaasen

2001). The result is an increase in H₂O escaping the skin (measurable by TEWL), in addition to unwanted compounds entering the skin.

TEWL was determined by comparing measurements prior to application (baseline) with those obtained at the end of a 24hr dosing period. Emulsion vehicles are occlusive in nature, and thus inhibit water loss through the skin. *In vivo* experiments have demonstrated a decrease in TEWL as a result of emulsion vehicle application. In order to utilize TEWL as an evaluation of *in vitro* skin irritation, the appropriate conditions for the terminal measurement needed to be determined. A time course study of TEWL measurements at the end of the 24hr dosing period was evaluated. This included the following TEWL measurements: 1) the treatment remained on the skin; 2) the treatment was recently removed from the skin surface; 3) interval time periods following the skin surface wash.

To support multiple endpoint analysis, in addition to the cytokine release and TEWL assessment, irritation was also assessed by means of the MTT assay. Initial viability was determined of skin samples prior to 24hr dosage. At the end of the 24hr period, the dosing site was isolated and the terminal percent of initial viability was determined for each treated cell.

Materials

Chemicals

The absorption/metabolism studies were conducted utilizing radio-labeled materials to increase analytical sensitivity (Bronaugh et al. 1982a). Arachidonic Acid-Carboxy- ^{14}C , specific activity 50mCi/mmol, was acquired from Sigma Chemical Co. (St Louis, MO 63178). Glycerol [5,6,8,9,11,12,14,15- ^3H] arachidonate, specific activity 60mCi/mmol, was synthesized by Research Triangle Institute (RTI), (Research Triangle Park, NC 27709). For barrier integrity evaluations, ^3H -Water was purchased from New England Nuclear (NEN), now Perkin Elmer (Boston, MA 02118).

Non-radiolabeled materials were utilized for HPLC method development. The arachidonic acid and glycerol arachidonate were both synthesized by Nu-Chek Prep, Inc. (Elysian, MN 56028). All HPLC-grade solvents were prepared by J.T. Baker (Water, Acetonitrile, Methanol, Acetic Acid).

PGE₂, PGD₂, and TxB₂ arachidonic acid metabolite standards were supplied by Sigma Chemical Co. (St. Louis, MO 63178). The remaining analyzed metabolite standards, HETEs and HpETEs, were acquired from Cayman Chemical Company (Ann Arbor, MI 48108).

Hanks' balanced salt solution (HBSS: powdered packets) was obtained from GibcoBRL, Life Technologies (Grand Island, NY 14072). Ultima Gold Scintillation

Cocktail was acquired from Perkin Elmer Life & Analytical Sciences (Boston, MA 02118).

The methylthiazoletetrazolium (MTT) utilized in the cytotoxicity assays was purchased from Sigma Chemical Co. The ELISA IL-1 α rat and human duo-sets were acquired from R&D Systems (Minneapolis, MN 55413). Bovine serum albumin (BSA), protease free grade utilized in ELISA assays, was received from Serological Proteins Inc. (Kankakee, IL 60901). The ELISA TMB substrate reagent set was purchased from Pharmingen (San Diego, CA 92121). BCA Protein Assay Kit was purchased from Pierce (Rockford, IL)

All other reagents unless individually noted were chemical grade and manufactured by either Sigma Chemical, Fisher Scientific (Fair Lawn, NJ) or J.T. Baker (Phillipsburg, NJ). An o/w emulsion was prepared as the dosing vehicle for arachidonic acid and glyceryl arachidonate (Table 1).

Skin Sources

Fuzzy Rat Skin

Female fuzzy rats, aged 10 weeks to 15 months, were obtained from Harlan Sprague Dawley (Indianapolis, IN 46229). Upon arrival, each rat was weighed and housed singly at Biosafety Level 1 in a polycarbonate shoe box equipped with a cage card for animal identification purposes. As specified by IACUC standards, a wire feeder

Table 1. Oil-in-Water Emulsion FormulationPROCEDURE

1. Heat Part A and B individually to 75-80°C
2. Pour Part A into Part B
3. Stir in Omni Mixer at setting 1 for 30 minutes
4. Turn Omni Mixer down to setting just below 1 and stir until reaches room temp
5. Add Part C
6. Stir in Omni Mixer at setting just below 1 for 1 hr.

Oil-in-Water Emulsion Formulation	
Part A (oil phase)	% weight/weight
Polyglyceryl-3 distearate	3
Mineral Oil	10
Cetearyl Alcohol	3
Part B (water phase)	
Propylene Glycol	5
pH 7.0 Buffer (NaOH/KH ₂ PO ₄)	78
Part C (preservatives)	
Methyl paraben	0.5
Propyl paraben	0.5

lid and 16oz. glass bottle was utilized to provide certified rodent chow and water to the animals ad libitum. Animal care was provided under IACUC supervision.

Viable Human Skin

Viable human skin was obtained from local clinics following a cosmetic surgical procedure. The skin was utilized the same day as excision in order to maintain viability. The surgery was elected by the patient and was not part of a research study; therefore the tissue would normally be discarded. The identity of the patient is undisclosed. Age and sex have been determined to be insignificant factors as determined in previous studies from this laboratory.

Human Cadaver Skin

Non-viable human cadaver skin is shipped overnight on dry ice from The National Disease Research Interchange (NDRI) (Philadelphia, PA). Upon arrival, the tissue is transferred to and stored in a -80°C freezer. Donor constraints consist of a 10-80 year old female of any race. No sepsis, skin diseases or scars should be present. HIV/Hepatitis status must be negative although chemotherapy & radiation therapy are okay. The full-thickness tissue ($>64\text{cm}^2$) was non-hairy, non-shaven abdominal skin and was excised within 12 hr post-autopsy or 3 hr post-abdominoplasty.

EpiDerm™ Skin Equivalent

EpiDerm™ Skin Equivalent (EPI-606-X; designed with an enhanced barrier function) was prepared by MatTek (Ashland, MA). These tissues were utilized for metabolism studies and evaluation of the alternative method for irritation. The kit

consists of 6 tissue samples in a 6-well culture plate. The tissues are cultured at the air-liquid interface and form a multi-layered, differentiated model representative of human epidermal tissue.

Methods / Experimental Design

In Vitro Penetration Studies

The purpose of this test is to evaluate the amount of the compound which penetrates the skin's barrier, resulting in absorption.

Arachidonic acid absorption and metabolism studies were conducted utilizing dorsal fuzzy rat skin and viable human skin. Due to the lack of availability of sufficient viable human skin, absorption/metabolism studies of glyceryl arachidonate were conducted on non-viable cadaver skin in correlation with cultured EpiDerm™ tissue in addition to the viable human skin. All handling of human tissue was conducted in accordance with the CFSAN Blood borne Pathogen Exposure Control Plan and Biosafety Level 2 conditions.

Prior to running the *in vitro* penetration studies, the diffusion cell system was set up and disinfected by pumping a 70% ethanol solution through the tubing (Collier et al. 1989) and allowing it to remain in the tubing for at least 30 minutes. Following disinfection, the ethanol solution was flushed out of the system and rinsed with receptor fluid (Kraeling 1997).

In order to maintain skin viability, a Hepes buffered Hanks' balanced salt solution (HHBSS, pH=7.4) was utilized in these experiments as the buffer (Collier et al. 1989). Skin viability maintenance is necessary for metabolite evaluation (Collier et al. 1989). Due to the high lipophilicity of the test material, 4% bovine serum albumin (BSA) was

added to the receptor fluid to aid in partitioning of the compound from the skin into the receptor fluid (Bronaugh 1996).

Albumin, a major component of circulating blood, is documented as a protein with high binding capacity for long chain fatty acids, such as arachidonic acid (Beck et al. 1998). BHT (0.01%) was also included in the receptor fluid as an anti-oxidant (Klaasen 2001). Prior to use, the buffer and receptor fluid were sterilized by vacuum filtration through a 0.2um filter.

The dorsal skin of the fuzzy rat serves as a good model for percutaneous absorption, metabolism, and irritation assays due to the presence of undeveloped hair follicles. However, it is important to remember that rat skin has been determined to be more permeable than human skin (Bronaugh et al. 1982b).

Skin Preparation

Fuzzy rats were euthanized with carbon dioxide (Bronaugh and Stewart 1986) using the Euthanex System as approved by IACUC. In order to ensure viability, full thickness dorsal rat skin was excised immediately prior to the experiment. The skin was then transported to the lab, on ice in buffer, for removal of subcutaneous fat. The remaining animal carcasses were disposed of accordingly.

When available, viable human skin was used for absorption/metabolism studies. Skin samples were obtained following abdominoplasty procedures from local clinics.

Excised human skin samples were transported, on ice, in buffer, from the surgery location to the lab for removal of subcutaneous fat. The skin was washed with a 10% liquid detergent solution (Palmolive), rinsed with distilled water, and patted dry in order to remove any foreign remnants of surgery.

Human cadaver skin was removed from the -80°C freezer and allowed to thaw. Excess subcutaneous fat was separated from the skin sample and disposed as biohazardous waste. As with the viable human skin, the sample was washed with a liquid detergent solution, rinsed, and patted dry.

The remaining skin specimen for fuzzy rat, viable human, and non-viable human cadaver skin was mounted epidermal side up on a Styrofoam block and held in place using hypodermic needles (Bronaugh and Stewart 1986). The top skin surface was dermatomed (Padgett Dermatome: Kansas, MO) to a thickness of 200-320µm (verified by micrometer measurement) (Bronaugh and Stewart 1986). After dermatoming, 17mm circular discs were cut with a brass punch and mounted dermal side down into flow-through diffusion cells (0.64cm² surface area). Depending on dermatoming accuracy, the skin from one fuzzy rat should provide enough disks for at least 6 diffusion cells. The upper stratum corneum surface of the diffusion cell is open and exposed to ambient air simulating normal skin exposure (Bronaugh et al. 1982a).

The EpiDerm™ culture plate is shipped out at 4°C on medium-supplemented agarose gels on Monday mornings and arrives at the laboratory on Tuesday mornings.

Upon arrival, the assay medium (provided in the kit) was warmed to 37°C in a hot water bath. As directed in MatTek's protocol, using sterile techniques, 6.3mL of the assay medium was pipetted into six sterile 100mm Petri dishes. Using forceps, each of the 6 EpiDerm™ sample inserts was transferred to an individual Petri dish. The samples were then placed in a humidified incubator at 37°C and 5% CO₂ overnight. To maintain consistency, all studies were initiated on that Wednesday.

EpiDerm™ samples were removed from the incubator following overnight storage. The cell was removed from the culture insert by cutting along the edge with a scalpel. Discs of cultured skin ~10mm in diameter were prepared freehand using a scalpel after which each disc was assembled in a flow-through diffusion cell (surface area =0.34cm²) (Bronaugh and Stewart 1985) dermal side down.

Flow-through Teflon diffusion cells are ideal because they allow for the determination of absorption and metabolism of compounds simultaneously (Bronaugh 1996). Assembly and cell design allow for contact between the dermal layer and the physiological receptor fluid in order to maintain skin viability (Collier et al. 1989). The setup of the flow-through diffusion cell can be seen in Figure 6.

Skin temperature was maintained at 32°C ± 1°C by pumping water from a circulating water bath at a temperature of 35°C ± 1°C through a diffusion cell holding block (Bronaugh and Stewart 1985). Skin surface temperature was verified by measurement with a laser thermometer. Receptor fluid was pumped through the system

Figure 6. Flow-through Diffusion Cell Setup

at a flow-rate of 1.5mL/hr (Kraeling et al. 1998). To aid in lipophilic partitioning, stir bars were assembled in the diffusion cell holding blocks which maintained stirring of the receptor fluid beneath the skin cell (Bronaugh 1996). However, it was concluded at the termination of the AA studies, that no increase in partitioning of AA was achieved with the use of the stir bars (data not shown). Therefore, stir bar use was discontinued prior to conducting the GA absorption/ metabolism studies. The cells were allowed to equilibrate at these conditions for a minimum of 30 minutes.

Skin Barrier Integrity Test

Human skin in diffusion cells, which was utilized for absorption/metabolism studies, was also simultaneously evaluated in a study comparing $^3\text{H}_2\text{O}$ absorption and TEWL. Baseline TEWL measurements were taken following a 30 minute equilibration period. Measurements were acquired according to the TEWL procedure outlined in the forthcoming irritation methods section. Prior to application of the test material, the barrier function of these cells was evaluated using the $^3\text{H}_2\text{O}$ skin barrier integrity test. The percent $^3\text{H}_2\text{O}$ absorption values obtained were compared against the measured baseline TEWL values for each individual cell, to evaluate the presence of any correlation.

Following equilibration and the baseline TEWL measurement, a tritiated water skin barrier integrity test as described by Bronaugh et al. in 1986 was conducted on human skin samples to ensure no barrier damage was incurred during surgery or preparation (Bronaugh et al. 1986). Prior to application of the test compound, each cell

was dosed with 200 μ l ($\sim$ 0.5uCi) of $^3\text{H}_2\text{O}$. After 20 minutes, any remaining, non-absorbed material was removed with a cotton swab. The cell was then washed once with 300 μ l of distilled water. The receptor fluid was collected for an additional 60 minutes. The total 80 minute fraction was analyzed for $^3\text{H}_2\text{O}$ absorption via liquid scintillation counting (LSC). If $<0.35\%$ of the applied $^3\text{H}_2\text{O}$ dose is absorbed into the receptor fluid, then the skin barrier integrity is intact and that skin cell may be utilized in the study.

Dosing Formulation Preparation & Application

Due to light sensitivity of AA and GA, the studies were conducted under minimal or yellow light conditions.

In order to simulate normal consumer use conditions, each compound was individually dosed in an o/w emulsion vehicle at 2mg/cm² ($\sim$ 0.5uCi/cell) for a 24 hr period. Both of the radio-labeled test materials were supplied in an alcohol solution (AA in ethanol; GA in methanol). To prevent breaking of the o/w emulsion upon addition of the compound, the stock samples were first concentrated either under a direct N₂ stream or in a dessicator filled with N₂. The concentrated stock was added to the vehicle until the desired dose of $\sim$ 0.5uCi/cell was achieved, resulting in a concentration of $\sim$ 1% AA and $\sim$ 0.4% GA. To create homogeneity, the dosing formulation was stirred by hand. Three dosing aliquots were taken and counted via LSC to determine the applied dose and to verify uniformity of the formulation. A positive displacement pipette was utilized to dispense and spread the formulation evenly over the entire dosing surface area (Perkins et al. 1996).

Experimental Procedure

Receptor fluid fractions were collected on a fraction collector in 6 hr intervals over the 24 hr dosing period, after which the cells were washed and the experiment terminated for sample analysis. Separate aliquots of each 6 hr receptor fluid fraction were collected and analyzed for absorption. This data provided information on the quantity of the compound which diffused through the barrier of the SC and is possibly systemically available. After aliquots were taken, the remaining receptor fluid fractions were stored at -20°C until metabolite analysis could be completed.

Following the 24hr dosing period, any remaining compound on the skin surface was removed and collected for analysis to determine the amount remaining on the surface of the skin. The skin surface was washed three times with 100 µl of a 10% soap solution, and rinsed twice with 100 µl of distilled water. After each wash or rinse step, the skin cells were gently rubbed with a cotton-tipped applicator to remove the wash/rinse solution and any unpenetrated test material. The cotton tips were compiled for each cell and kept for subsequent LSC analysis.

To determine the percent recovery, the diffusion cell setup was disassembled and cleaned. The remaining receptor fluid in the reservoir underneath the skin punch was collected as “cell contents”. The diffusion cell and cap were soaked overnight in a 10% soap solution. Aliquots of the wash solution were then analyzed for possible binding of the compound to the diffusion cell.

The stratum corneum was separated from the underlying viable tissue using cellophane tape stripping. This allows for the determination of the amount of the compound still remaining in the stratum corneum. Ten tape strips per cell were collected, unless there was evidence of separation of the epidermis from the dermis in which case the tape stripping ceased. Each tape strip was placed in an individual vial for analysis.

Quantification and composition analysis of the test material were possible through homogenization. To maintain the integrity of the metabolites, the remaining skin sample was stored at -20°C until homogenization. During homogenization, the frozen skin discs were re-hydrated in 1mL of cold HHBSS in a small beaker for ~5 minutes. The skin was then minced into small pieces with scissors. The resulting mixture was transferred to a small culture tube on ice. The beaker was rinsed once with 1mL HHBSS and added to the mixture in the tube. If there were any visible skin pieces remaining in the beaker, they were removed with a forceps and also transferred to the tube. The contents of the tube were homogenized using an Omni International Tissue Homogenizer (Warrenton, VA) (Yourick and Bronaugh 2000). A 10 second full-speed burst (Storm et al. 1990) was alternated with a 1min cooling off period (on ice) three times (Yourick and Bronaugh 2000). Following the last burst, the resulting homogenate was transferred into a scintillation vial. Twice 1mL of HHBSS was added to the homogenization tube, exposed to one 10 second burst, and then added to the scintillation vial for rinsing purposes.

An aliquot of the skin homogenate was digested in 3mL Scintigest tissue solubilizer and 1mL distilled water overnight in a 60°C oven (Yourick and Bronaugh 2000). The dissolved tissue solution was then analyzed for penetration. The remaining homogenate was stored at -20°C until metabolite analysis could be completed.

Extended Absorption Studies

Arachidonic acid is a highly lipophilic compound and hence its solubility in the aqueous receptor fluid is limited. To assist in partitioning, BSA was added to the receptor fluid. Even with this accommodation, it is possible that the solubility of AA in the receptor fluid is saturated during each 6hr fraction thus creating a reservoir effect (Yourick et al. 2004). The formation of a reservoir and continual delivery of the compound was observed in previous studies (Hood et al. 1996; Mavon et al. 2004). For highly lipophilic compounds, a depot can develop in the skin allowing further material to be released into the body long after exposure to the chemical has been terminated (Klaasen 2001). To evaluate for the presence of this reservoir effect, extended absorption studies (72 hr) were conducted. The skin sample was dosed and removed at 24hr as with the previous studies. However, 6hr receptor fluid fractions were continually collected for a total of 3 days.

Absorption Analysis

To evaluate the amount of absorbed radioactivity, Ultima Gold scintillation cocktail (Packard, Meriden, CT) was mixed with receptor fluid aliquots, cell contents, stratum corneum tape strips, and digested homogenized skin aliquots. Cocktail was

additionally added to the cotton tipped applicators and diffusion cell wash aliquots to determine the amount which was not penetrated. These samples were then analyzed via liquid scintillation counting on a Beckman LS 6500 (Beckman, Fullerton, CA). Skin absorption was defined as the amount of the applied dose appearing in the receptor fluid: Skin penetration was considered to be the sum of the receptor fluid levels and skin levels of test compound determined at the end of the experiment.

Assay for Arachidonic Acid, Glycerol Arachidonate, and their Products

The purpose of this test is to determine if the compound is metabolized to any of its various metabolites by enzymes found in the skin. The metabolism or lack thereof could influence the overall safety of the compound for skin application.

The test material was radio-labeled in order to increase the sensitivity of the method, making parent compound metabolites identifiable. The animal, human, and cultured skin samples used in these studies were the same as those used previously in the skin absorption studies. Hydrolysis of glycerol arachidonate to arachidonic acid in EpiDerm™ receptor fluids was analyzed by a high performance liquid chromatography (HPLC) System (Waters; Milford MA) in conjunction with a radioisotope detector (Radiomatic Flo-One Series A-500, Packard; Meriden, CT). Metabolism of arachidonic acid and glycerol arachidonate was investigated by analysis of extracted homogenized skin samples using a Bioscan AR-2000 (Bioscan, Washington DC).

Solid Phase Extraction

Initial studies utilizing an ethyl acetate extraction followed by concentration under a N₂ stream resulted in a large amount of loss observed. Therefore, extractions utilized for metabolism analysis were prepared with solid phase extraction (SPE) columns. C18, 3mL, 15mg solid phase extraction columns (Ansys Technologies/Varian Inc. Palo Alto, CA 94304), were assembled on a standard vacuum manifold with a vial underneath to collect the waste. The column was prepared by allowing 300µl of methanol and 300µl of distilled water to individually flow to waste by gravity. An aliquot of either homogenate or receptor fluid was diluted in a 1:1 ratio with distilled water to create a diluted sample. Once the distilled water had passed through the column, the diluted sample was applied to the column under 1-2" Hg vacuum pressure. After the solution flowed to waste, 600µl of distilled water followed it. To ensure a thorough column washing, 15" Hg pressure was applied to the column for 1 minute. At this point, the vacuum was discontinued and the manifold disassembled after which it was setup for elution and collection. 300µl of a filtered 50/50 chloroform- methanol mixture was used to elute the column.

HPLC Analysis

The extracted EpiDerm™ receptor fluid fractions were filtered and injected onto an HPLC in tandem arrangement with a radioisotope detector. Reverse phase high performance liquid chromatography was performed using a C18 Alltima 5µm, 250 X 4.6mm column (Alltech; Deerfield IL) with a flow rate of 1.0mL/min. The attached radioisotope detector simultaneously pumped Scintillation Flo-M cocktail at a rate of 3.0mL/min. The solvent system was comprised of acetonitrile (ACN) (mobile phase A),

and 0.1% acetic acid (mobile phase B). The gradient table conditions are described in Table 2.

TLC Analysis

The plate developing solution consisted of Petroleum Ether 75: Chloroform 80: Methanol 20: Ether 10: Acetic Acid 1: Hydrochloric Acid 20 drops. Following mixture, 150ml of the solution was added to the TLC tank, which was allowed to equilibrate for at least 3 hr. 300µl volumes of samples and standards were spotted 10µl at a time onto 500mm coated silica TLC plates (Whatman; Maidstone England). Once the plates were spotted, they were allowed to thoroughly dry before development in the TLC tank.

Following development, the plate was dried prior to visualization under iodine vapor. Once visualization was achieved, the metabolite locations were marked prior to evaporation of the iodine. When the iodine had nearly all evaporated off (to minimize contamination of the detector), the plate was analyzed on the Bioscan AR-2000. Samples were counted for 600 minutes due to the low efficiency of ^3H . Due to the high concentration values of the stock solutions, they were counted for 5 minutes. Resulting GA sample chromatograms were compared against each other and the equivalent AA sample chromatograms to determine the presence of AA metabolites in the samples.

Iodine visualization was utilized for location of chosen metabolites of arachidonic acid. PGE₂, PGD₂, TxB₂, 5-HETE, 5-HpETE, 15-HETE, and 15-HpETE were each

**Table 2. High Performance Liquid Chromatography (HPLC)
Analysis Conditions**

Time	Flow Rate	% A	%B	Curve
	1.0	40	60	
12	1.0	46	54	6
13	1.0	75	25	1
60	1.0	40	60	11

evaluated under the same TLC conditions utilized for the samples. An average of 10µg of each standard was spotted onto the plates. Following iodine vapor exposure, the resulting location was noted for comparison with sample chromatograms.

***In Vitro* Skin Irritation Study**

Two standards (sodium lauryl sulfate and Tween) were dosed in an aqueous vehicle against a distilled (DI) H₂O control. The same standards were subsequently dosed in a lotion vehicle with an o/w emulsion control. The test compound, arachidonic acid, is highly lipophilic and thus was tested solely in the emulsion vehicle at various concentrations. All cells were terminated at the 24hr endpoint based on previous *in vitro* studies reporting it as giving the best indication of actual irritation generation in human skin (Perkins et al. 1999).

Flow-through Teflon diffusion cells were used to conduct the *in vitro* skin irritation study to determine whether irritation could be determined in the same manner in which absorption and metabolism are studied. Initial irritation studies were conducted utilizing excised rat skin and EpiDerm™. Of these skin samples, that which proved to be the most sensitive for each individual biomarker, was utilized for further studies. Purchased colorimetric assay kits were utilized for the MTT and IL-1α assessments. The same skin cells utilized during either the MTT or IL-1α studies were also utilized for the TEWL analysis.

MTT Assay

The purpose of this test is to determine the skin viability after application of the compound. Skin viability has been found to be affected by irritants. Therefore, the MTT assay was utilized to evaluate the viability status of the cells post-application of the compound. This viability test has commonly been used in previous skin irritation experiments. Due to light sensitivity of MTT, the studies were conducted under minimal or yellow light conditions.

A 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used to confirm the maintenance of skin viability during the experiment. This test assays for the presence of metabolically active mitochondria indicating that those cells are viable. A water-insoluble blue formazan derivative results when the water-soluble yellow MTT substrate is reduced by the mitochondria. Therefore, there is a directly proportional relationship between the quantity of viable cells and the amount of the blue formazan derivative produced.

For initial viability control values, additional untreated skin samples of the freshly dermatomed rat skin and EpiDerm™ were analyzed by the MTT assay in the same manner as the treated samples as described below. Prior to analysis, the skin samples were allowed to soak in HHBSS for at least 20 minutes to equate the equilibration period of the samples in the diffusion cells.

MTT in skin from diffusion cells was assayed by a modified method originally described by Hood and Bronaugh (Hood and Bronaugh 1999). MTT was dissolved in HHBSS at a concentration of 2mg/ml. The resulting solution was centrifuged at 1500g for 5 minutes to remove any remaining un-dissolved crystals. Immediately prior to analysis, the MTT solution was warmed to 37°C in a water bath. Following the wash, the dosing site of the treated samples was isolated (using a 10mm diameter punch) and immediately analyzed for viability. The skin sections were placed into individual wells of a 6-well tissue culture plate (Corning Costar Corporation, Cambridge, MA) with 2ml of the MTT solution (2mg/ml). The plate was incubated for 2hr at 37°C and 90% humidity on a rotating platform (150rpm), after which the MTT solution was aspirated from the wells. The remaining skin section was washed twice with 1ml of phosphate-buffered saline for 2 minutes each followed by an aspiration. To dissolve and extract the blue MTT-formazan crystal from the skin, 4mL of isopropanol was added to each well prior to plate agitation on a rotating platform for 2hr at room temperature (Perkins et al. 1996). The resulting blue extract (2mL) from each well was transferred to individual vials and diluted in a 1:1 ratio with 2ml of isopropanol. UV spectral absorbance was measured at 540nm, using isopropanol as a reference.

Interleukin -1 alpha (IL-1 α) Release

The purpose of this test is to evaluate the quantity of the IL-1 α cytokine released upon irritation by the compounds of interest. Upon disruption, membranes release some of their cytokines. The Interluken-1alpha is one main cytokine released in response to skin irritation. Therefore, an ELISA assay for IL-

1 α was incorporated. The higher the presence of the cytokine observed, the greater the irritation caused by the compound.

A modification of a method originally described by Kabbur was utilized to assess intracellular levels of IL-1 α in skin (Kabbur et al. 2001). Following treatment, the diffusion cell was disassembled, and the dosing site of the cell was isolated with a 10mm punch. The resulting skin sample and pulverizer were individually cooled in liquid nitrogen prior to skin pulverization. The pulverized skin sample was then transferred to a vial where it was sonicated for 1 minute in 2.5ml HHBSS (gentamycin omitted to limit interference with ELISA kit) and centrifuged for 30 minutes at 6780 rpm, 4°C. The supernatant was collected and stored at -80°C until further analysis. The release of the cytokine IL-1 α was quantified according to a purchased ELISA kit (R&D Systems; Minneapolis, MN).

96 well plates were prepared for ELISA analysis the day prior by coating the wells with 100 μ l of the capture antibody, sealing the plate, and incubating overnight at room temperature. An aspiration/wash step was conducted which involved aspirating the previous solution and then washing each well 3X with 300 μ l of the wash buffer (0.05% Tween 20 in PBS, pH 7.2-7.4) ensuring complete removal of the liquid at each step by inverting the plate and blotting against paper towels and aspirating following the final wash. The plates were blocked for 1hr at room temperature with 300 μ l /well block buffer (1% BSA, 5% Sucrose in PBS). Another aspiration/wash step is conducted prior to addition of 100 μ l of sample/standard (diluted in HHBSS without gentamycin) to each

well. Various dilutions of the samples and standards were allowed to incubate for 2hr at room temperature in the sealed plate. Standards were analyzed in 2-fold dilutions with a high standard value of 1000 pg/ml (rat) and 500 pg/ml (human). The plate was aspirated/washed for a 3rd time followed by addition of 100µl of detection antibody (rat assay required addition of 2% heat inactivated normal goat serum) and incubation for 2hr at room temperature. Again, an aspiration/wash step was conducted prior to 100µl addition of the Streptavidin-HRP which incubated for 20 minutes at room temperature out of direct light. A final aspiration/wash step was performed on the plate after which 100µl of the substrate solution (1:1 H₂O₂ and tetramethylbenzidine) was added to the wells. After 20 minute incubation at room temperature out of direct light, the substrate solution results in formation of a blue solution whose concentration is related to the amount of IL-1α present. 50µl of a stop solution (2N H₂SO₄) was added to each well to extend the available time to analysis. Addition of the stop solution changes the blue solution to yellow upon gentle tapping of the plate. The plate was immediately counted on a microplate reader where readings at 540nm were subtracted from readings at 450nm as indicated by the purchased kit.

In addition to the IL-1α ELISA assay, a BCA (bicinchoninic acid) Protein Assay (Pierce, Rockford, IL) was conducted alongside to analyze and correct for the protein concentration in the samples. Bovine serum albumin standards ranging from 0 to 2000µg/mL were prepared immediately prior to the assay. 25µl of each standard/sample was pipetted into individual wells of a 96 well plate. 200µl of a working reagent was subsequently added to each sample/standard. The working reagent consisted of a 50A:

1B ratio where B was 4% cupric sulfate and A was a solution containing sodium carbonate, sodium bicarbonate, bichinchonic acid, and sodium tartrate in 0.1M sodium hydroxide. The plate was mixed on a plate shaker for 30 seconds before the plate was covered and incubated at 37°C for 30 minutes. After the plate was allowed to cool to room temperature, the absorbance was measured on a microplate reader at 562nm.

Transepidermal Water Loss (TEWL)

The purpose of this test is to evaluate the barrier properties of the skin after application of the compound. Some irritating compounds have been shown to disrupt the barrier properties of the skin. It was determined whether the compounds used in this study also result in barrier property disruption.

The TEWL evaporimetry test is an example of a skin integrity function test (SIFT). The generated data reflects the integrity of the skin at various stages of damage by the compound. This method has commonly been used *in vivo* to evaluate the integrity of the barrier function of the skin.

TEWL *in vivo* has been demonstrated to give a good correlation with other *in vivo* methods of determining skin irritation, such as visual scoring and colorimetry (Korting et al. 1994). Following irritation, a decrease in barrier function can occur, which in turn is reflected by an increase in TEWL. Theoretically, the more irritating a compound is, the greater its increase in TEWL will be. Therefore, evaporimetry can potentially be used to distinguish between an irritant and a non-irritant. This method is helpful in diagnosing

early stages of irritation because the integrity of the stratum corneum (SC) is altered before inflammatory mediators are released or visual evidence of irritation is obvious (Zuang et al. 2000)

The DermaLab Evaporimeter (Cyberderm, Media, PA) probe is composed of 2 individual sensors designed to measure relative humidity, temperature, and vapor pressure at that point. One sensor, positioned near the skin, measures skin surface conditions whereas the other sensor, located away from the skin, measures environmental conditions. The calculated evaporative water loss (EWL) rate is proportional to the water vapor at the sensors.

An environment check is conducted prior to each use of the TEWL probe to measure ambient conditions. Under these conditions, the vapor pressure at both of the probes should theoretically be identical resulting in an EWL value of 0.

In vivo measurements of transepidermal water loss are conducted by directly placing the TEWL probe (open end) onto the skin. Steady-state conditions are reached when the standard deviation values remain low. This is usually achieved in 25 seconds. For *in vitro* studies, the TEWL probe can not directly be placed onto the skin surface, but rather it lies on top of the diffusion cell. The increased distance between the skin surface and the sensor causes the time to steady state to increase up to a few minutes. To improve the time to and achievement of steady-state conditions *in vitro*, an adapter was created which resulted in a better seal between the diffusion cell and the TEWL probe.

To evaluate TEWL using the diffusion cells the skin was maintained at a constant temperature and relative humidity (limited by facility conditions), because diffusion and water loss have been determined to be dependent upon these factors (Montagna and Parakkal 1974). The probe is operated under continuous mode in which four measurements are taken per second. After steady state conditions were achieved, a 180 sec measurement was made, from which the 20 sec mean value is reported. Values obtained are expressed in terms of grams per square meter per hour (Zuang et al. 2000).

Skin cells utilized for the TEWL irritation assays were the same as those used either in the IL-1 α or the MTT assay studies. This was possible due to the non-invasive nature of transepidermal water loss analysis.

Baseline TEWL measurements were taken after the 30 minute equilibration of the diffusion cells, but prior to dosage. After the 24hr treatment period, additional TEWL measurements were taken prior to and immediately following the skin surface wash and rinse. A time course study was conducted for 2hr following the wash and rinse with measurements acquired every 40 minutes, to determine the appropriate terminal endpoint.

Statistical Analysis

The results obtained were evaluated statistically using ANOVA (analysis of variance). Subsequent pairwise comparisons were then conducted as deemed appropriate. Comparisons were made between similar formulations, either emulsion or

aqueous vehicles. For two test conditions, the student t- test was used to evaluate the data using a p-value <0.05 to determine statistical significance.

Results and Discussion

³H₂O Barrier Integrity Test vs. TEWL

During the course of evaluating skin barrier integrity prior to absorption studies, the ability of TEWL to equate to the ³H₂O test was investigated. The baseline TEWL values of human skin in diffusion cells which failed the ³H₂O test ranged from 6.4 g/m²/hr to 22.5 g/m²/hr, the average of which was 11.9 g/m²/hr (Table 3). The average TEWL value observed with passing cells was 9.6 g/m²/hr, while the average for cells which failed was 11.9 g/m²/hr. The largest percent ³H₂O absorption determined was 25.1% which had a corresponding TEWL value of 22.5 g/m²/hr. However, another cell which gave a baseline TEWL reading of 22.85 g/m²/hr passed the ³H₂O test with only 0.13% absorption. Comparison of the raw data of these values yielded no significant difference (p=0.063) in TEWL values between the cells which passed and those that failed the ³H₂O test (Table 3).

A scatter plot of the ³H₂O vs. TEWL data acquired from all experiments is shown in Figure 7. This plot gives a clear representation of the lack of direct relationship between these two factors. It can be seen that the cells which failed the ³H₂O test occupy the same range of TEWL values as those cells which passed the ³H₂O test. It was determined that no linear correlation could be achieved between TEWL and ³H₂O ($r^2 = 0.1059$ from linear plot). These results coincide with previous unpublished data from our lab in addition to studies conducted by others (Chilcott et al. 2002a).

Table 3. Average $^3\text{H}_2\text{O}$ Absorption and TEWL Values of Cells which Passed and Failed the $^3\text{H}_2\text{O}$ Skin Barrier Integrity Test

	Average Percent $^3\text{H}_2\text{O}$ absorption	Average TEWL value
Passed $^3\text{H}_2\text{O}$ Test ^a	0.173 ± 0.0075	9.61 ± 0.52
Failed $^3\text{H}_2\text{O}$ Test ^b	3.21 ± 1.63	11.75 ± 1.34

* TEWL values are not significantly different for those cells which passed vs. failed (p=0.063)

^a Passed $^3\text{H}_2\text{O}$ Test n = 73

^b Failed $^3\text{H}_2\text{O}$ Test n =17

Skin specimens from separate donors were utilized for each individual experiment. Based on potentially high inter-sample variability of skin specimens, it may be more accurate to compare data in individual experiments instead of looking at the combined results. When the data is broken up in this manner, a potential trend can be observed. Individual graphs of these experiments can be seen in Figure 8a-f. Based on the range of % $^3\text{H}_2\text{O}$ observed, the axis in these plots are different for each individual experiment.

Although the pattern isn't as obvious in Figures 8b, 8d, and 8e, a potential tendency towards a high TEWL measurement occurrence with high $^3\text{H}_2\text{O}$ absorption is apparent in Figures 8a, 8c, and 8f. However, it is evident that the opposite (high TEWL leads to high $^3\text{H}_2\text{O}$ absorption) cannot be said. Multiple samples gave relatively large ($>15 \text{ g/m}^2/\text{hr}$) values while still passing the $^3\text{H}_2\text{O}$ test, sometimes well under the 0.35% limit. Various examples of this exist, one of which occurred in Experiment #1 (Figure 8a) during which a TEWL value of $16.54 \text{ g/m}^2/\text{hr}$ resulted in a percent $^3\text{H}_2\text{O}$ absorption value of only 0.26%.

***In Vitro* Penetration Studies**

Arachidonic Acid

^{14}C Arachidonic acid was dosed at 2mg/cm^2 onto viable human and fuzzy rat skin. At the end of 24hr, a normal consumer dosing period for lotion vehicles, the distribution of the applied dose was evaluated. Following the treatment of fuzzy rat skin, it was determined that 19.8% of the applied dose was absorbed into the receptor fluid.

Figure 8 (a). Experiment #1
Percent $^3\text{H}_2\text{O}$ Absorption vs TEWL

FIG. 8(a). Experiment #1- Percent $^3\text{H}_2\text{O}$ Absorption vs. TEWL. Baseline TEWL measurements obtained from a single human cadaver skin donor which either passed ($<0.35\%$) or failed the $^3\text{H}_2\text{O}$ skin barrier integrity test.

Figure 8 (b). Experiment #2
Percent $^3\text{H}_2\text{O}$ Absorption vs TEWL

FIG. 8(b). Experiment #2- Percent $^3\text{H}_2\text{O}$ Absorption vs. TEWL. Baseline TEWL measurements obtained from a single human cadaver skin donor which either passed ($<0.35\%$) or failed the $^3\text{H}_2\text{O}$ skin barrier integrity test.

Figure 8 (c). Experiment #3
Percent $^3\text{H}_2\text{O}$ Absorption vs TEWL

FIG. 8(c). Experiment #3- Percent $^3\text{H}_2\text{O}$ Absorption vs. TEWL. Baseline TEWL measurements obtained from a single human cadaver skin donor which either passed ($<0.35\%$) or failed the $^3\text{H}_2\text{O}$ skin barrier integrity test.

Figure 8 (d). Experiment #4
Percent $^3\text{H}_2\text{O}$ Absorption vs TEWL

FIG. 8(d). Experiment #4- Percent $^3\text{H}_2\text{O}$ Absorption vs. TEWL. Baseline TEWL measurements obtained from a single human cadaver skin donor which either passed (<0.35%) or failed the $^3\text{H}_2\text{O}$ skin barrier integrity test.

Figure 8 (e). Experiment #5
Percent $^3\text{H}_2\text{O}$ Absorption vs TEWL

FIG. 8(e). Experiment #5- Percent $^3\text{H}_2\text{O}$ Absorption vs. TEWL. Baseline TEWL measurements obtained from a single human cadaver skin donor which either passed ($<0.35\%$) or failed the $^3\text{H}_2\text{O}$ skin barrier integrity test.

Figure 8 (f). Experiment #6
Percent $^3\text{H}_2\text{O}$ Absorption vs TEWL

FIG. 8(f). Experiment #6- Percent $^3\text{H}_2\text{O}$ Absorption vs. TEWL. Baseline TEWL measurements obtained from a single human cadaver skin donor which either passed ($<0.35\%$) or failed the $^3\text{H}_2\text{O}$ skin barrier integrity test.

Additionally, 30.5% was found in the skin (12.9% in the stratum corneum and 17.6% in the underlying viable skin layers). Therefore, 52.3% of the applied dose penetrated the skin and was not removed under the wash conditions (Table 4).

Viable human skin was subjected to an identical study, with the addition of a $^3\text{H}_2\text{O}$ barrier function test. The human skin utilized for the evaluation of arachidonic acid percutaneous absorption passed with a $^3\text{H}_2\text{O}$ absorption range of 0.11 to 0.23% of the applied dose. Of the applied dose of AA, 19.5% penetrated the skin. The amount in the skin accounted for 18.2% with the remaining 1.3% partitioning into the receptor fluid (Table 4). Of the 19.5% of the applied dose of AA which penetrated the skin, 61% remained in the stratum corneum at the end of the 24hr study. This illustrates the importance of the stratum corneum reservoir, particularly with human skin, for lipophilic compounds.

Additional comparison of the arachidonic acid penetration distribution in both rat and viable human skin can be seen in Figure 9. Viable skin levels and receptor fluid levels are both elevated in the rat skin compared to the lower levels found in the human skin. It is also noteworthy that the percent amount of compound found in each of the 10 analyzed layers of stratum corneum appears to be almost identical in both the rat and human skin (Figure 10). There is a large amount of AA in the first, uppermost, stripped layer, but then the amounts drastically fall. Additionally, a similar pattern is observed between the skin samples which displayed a uniform amount of compound absorbing into the receptor fluid in any given 6hr collection period. Although the receptor fluid levels of

Table 4. Arachidonic Acid Absorption/Penetration from Lotion Formulation (% of applied dose)*

Application Time = 24 hours

Application Amount = 2mg/cm²

	Rat Skin ^a	Viable Human ^b
Receptor Fluid	19.8 ± 2.52	1.3 ± 0.09
Stratum Corneum	12.9 ± 1.01	11.8 ± 1.22
Viable Epidermis/Dermis	17.6 ± 1.42	6.5 ± 0.44
Total Penetration	52.3 ± 3.00	19.5 ± 1.32
Wash & Rinse	42.6 ± 2.18	66.3 ± 1.32
Recovery	97.6 ± 3.54	91.2 ± 1.33

* Values are mean ± SEM

^a Rat Skin n= 24

^b Viable Human n=15

Figure 9. Arachidonic Acid Penetration in Viable Rat and Viable Human Skin

FIG. 9. Arachidonic Acid Penetration in Viable Rat and Viable Human Skin. Penetration of arachidonic acid determined in individual recovery components. Values are mean $\pm$ SEM. Number of diffusion cells are 24 (Rat) and 15 (Human). Indistinguishable error bars are contained within the symbol.

Figure 10. Percent of Applied AA Dose in Individual Stratum Corneum Layers

FIG. 10. Percent of Applied Dose in Individual Stratum Corneum Layers. Penetration of arachidonic acid determined in individual layers of stratum corneum acquired during tape stripping. Values are mean $\pm$ SEM. Number of diffusion cells are 24 (Rat) and 15 (Human). Indistinguishable error bars are contained within the symbol.

rat skin are higher than those for the human skin, each individually remains the same from one 6hr fraction to the next (Figure 11).

Glyceryl Arachidonate

^3H Glyceryl Arachidonate was applied to viable human, human cadaver skin, and cultured skin equivalent (EpiDerm™) assembled in diffusion cells. The human skin samples following the $^3\text{H}_2\text{O}$ barrier test passed with absorption ranges of 0.08 to 0.32% (cadaver) and 0.12 to 0.18% (viable) of the applied $^3\text{H}_2\text{O}$ dose. GA absorption through viable human skin was 3.2% compared to 2.2% through human cadaver skin (Table 5). Total GA penetration (receptor fluid plus skin levels) in viable human and human cadaver skin was 20.2% and 17.5%, respectively. The absorption of GA through viable human and human cadaver skin was determined to not be significantly different ($p=0.18$), confirming that the barrier properties are still intact even after viability of the tissue has been lost. On the other hand, barrier properties of EpiDerm™ were dissimilar in comparison to the human skin samples. Total absorption of GA through EpiDerm™ was found to be 51.0% with a total penetration of 55.8%.

The same trend observed between rat skin and human skin in regard to the stratum corneum levels in the AA studies can be seen when comparing GA levels in both viable human and human cadaver samples (Figure 12). Additionally, when the data is compiled from the AA and GA studies, there is similarity in the stratum corneum levels and distribution in the viable human, human cadaver, and rat skin (Figure 13). Due to its

Figure 11. Arachidonic Acid Absorption
in Viable Fuzzy Rat and Human Skin

FIG. 11. Arachidonic Acid Absorption in Viable Fuzzy Rat and Human Skin. Percent of applied dose of arachidonic acid determined in individual 6hr receptor fluid fractions. Values are mean $\pm$ SEM. Number of diffusion cells are 24 (Rat) and 15 (Human). Indistinguishable error bars are contained within the symbol.

Table 5. Glyceryl Arachidonate Absorption/Penetration from Lotion Formulation (% of applied dose)*

Application Time = 24 hours

Application Amount = 2mg/cm²

	Viable Human ^a	Human Cadaver ^b	EpiDerm TM ^c
Receptor Fluid	3.2± 0.18	2.2 ± 0.29	51.0 ± 10.60
Stratum Corneum	9.8 ± 0.77	10.3 ± 1.00	N/A
Viable Epidermis/Dermis	7.2 ± 0.69	5.0 ± 0.31	6.6 ± 1.35
Total Penetration	20.2 ± 1.13	17.5 ± 1.28	55.8 ± 9.81
Wash & Rinse	76.3 ± 3.42	73.3 ± 2.93	37.5 ± 6.58
Recovery	99.9 ± 2.46	87.8 ± 2.41	97.8 ± 4.67

*Values are Mean ± SEM

^a Viable Human skin n=9

^b Human Cadaver skin n=17

^c EpiDermTM n=16

Figure 12. Percent of Applied GA Dose in Individual Stratum Corneum Layers

FIG. 12. Percent of applied dose in Individual Stratum Corneum Layers. Penetration of glyceryl arachidonate determined in individual layers of stratum corneum acquired during tape stripping. Values are mean $\pm$ SEM. Number of diffusion cells are 9 (Viable) and 17 (Cadaver). Indistinguishable error bars are contained within the symbol.

Figure 13. Percent of Applied Dose of AA and GA in Individual Stratum Corneum Layers

FIG. 13. Percent of applied dose in Individual Stratum Corneum Layers. Arachidonic acid and glyceryl arachidonate penetration into individual layers of stratum corneum acquired during tape stripping. Values are mean $\pm$ SEM. Number of diffusion cells are 9 (Viable Human –GA), 17 (Human Cadaver-GA), 15 (Viable Human-AA) and 24 (Viable Rat-AA). Indistinguishable error bars are contained within the symbol.

incomplete stratum corneum layer, tape stripping of EpiDerm™ was not possible. Therefore, stratum corneum data for EpiDerm™ could not be included in this comparison.

As can be seen on Figure 14, absorption patterns seem relatively similar and consistent for studies involving either viable human, human cadaver, or rat skin. However, with the studies conducted on EpiDerm™ there is a large spike in absorption levels noted during the first 6hr fraction. Also as noted in Table 5, there was a lot of variability observed in the absorption of GA through EpiDerm™. For this reason, it is believed that although strides have been made to improve the barrier of skin equivalents, their barrier properties are still not at a level comparable to human skin.

A graph encompassing all absorption data for each of the test compounds and their corresponding skin samples can be found in Figure 15. From this graph, it is very difficult to deny the enhanced permeability observed in the EpiDerm™ tissue. Additionally, similarities between the 3 human skin absorption profiles can be detected.

Extended Studies

One of the most important concerns with *in vitro* skin absorption studies is the “systemic” fate of the penetrated compound remaining in the skin at the end of a 24hr study. Absorption values for arachidonic acid in human cadaver skin were determined in studies terminated at either 24hr or 72hr, following a 24hr exposure period. Receptor fluid values increased significantly ($p=0.014$) from 1.9% at 24hr to 4.0% in the extended study (Table 6).

Figure 14. Percent of Applied Dose Absorbed in Individual Receptor Fluid Fractions

FIG. 14. Percent of Applied Dose Absorbed in Individual Receptor Fluid Fractions. Values are mean $\pm$ SEM. Number of replicates are 9 (Viable Human – GA), 17 (Human Cadaver-GA), 15 (Viable Human-AA) and 24 (Viable Rat-AA). Indistinguishable error bars are contained within the symbol.

Figure 15. Penetration Distribution of Arachidonic Acid and Glyceryl Arachidonate

FIG. 15. Penetration Distribution of Arachidonic Acid and Glyceryl Arachidonate. Percent of applied dose of either arachidonic acid or glyceryl arachidonate distributed in the receptor fluid, stratum corneum, and skin components. Values are mean $\pm$ SEM. Number of diffusion cells are 9 (Viable Human –GA), 17 (Human Cadaver-GA), 15 (Viable Human-AA) and 24 (Viable Rat-AA).

Table 6. Extended Study of Arachidonic Acid Absorption/Penetration from Lotion Formulation (% of applied dose)*

Application Time = 24hr vs. 72hr Application Amount = 2mg/cm²

	Human Cadaver ^a (24hr)	Human Cadaver ^b (72hr)
Receptor Fluid	1.9 $\pm$ 0.21	4.0 $\pm$ 0.53
Stratum Corneum	12.9 $\pm$ 2.93	13.4 $\pm$ 1.81
Viable Epidermis/Dermis	7.4 $\pm$ 0.91	9.4 $\pm$ 1.14
Total Penetration	22.3 $\pm$ 3.36	24.4 $\pm$ 1.94
Wash & Rinse	69.4 $\pm$ 2.70	69.0 $\pm$ 2.65
Recovery	97.7 $\pm$ 2.56	97.0 $\pm$ 1.60

* Values are Mean $\pm$ SEM

^a 24hr Human Cadaver n = 5

^b 72hr Human Cadaver n = 8

The percent of applied dose which absorbed into the receptor fluid during each 6hr fraction of the 24hr studies, continued to absorb for each 6hr fraction following the skin surface wash until the 72hr termination point (Figure 16). This data supports the hypothesis that a reservoir effect could occur with a lipophilic compound, such as arachidonic acid. Based on these findings, the percent of applied dose found in the receptor fluid following a 24hr study could significantly underestimate the actual amount which could be systemically available *in vivo*.

Assay for Arachidonic Acid, Glyceryl Arachidonate, and their Products

Metabolism of absorbed arachidonic acid and glyceryl arachidonate was observed in each of the skin samples used in these studies. There are numerous potential metabolites of these test compounds in skin resulting in some major peaks, but also small peaks that were difficult to distinguish from background levels of radioactivity.

TLC analysis of arachidonic acid in viable human skin tissue yielded ~70% byproducts. By investigation of the metabolite peaks of the resulting AA chromatograms, four main peaks were evident. However, the chromatograms indicate that these peaks may be mixtures of more than one compound. Therefore, they have been labeled according to increasing lipophilicity and identified as “Unidentified Byproduct A”, “Unidentified Byproduct B”, “Unidentified Byproduct C”, and “Unidentified Byproduct D”.

Figure 16. Arachidonic Acid Absorption in Individual Receptor Fluids During 72hr Extended Study

FIG. 16. Percent of Applied Dose Absorbed in Individual Receptor Fluid Fractions. Values are mean $\pm$ SEM. Number of replicates for human cadaver skin is 8.

Arachidonic acid metabolism data was analyzed in viable human skin from 2 separate donors. The metabolism data from one of the donors had ~ 45% un-metabolized AA (high AA), while the other donor had ~10% un-metabolized AA remaining (low AA) (Table 7). It was noted that when the un-metabolized AA levels were low, the amount of Unidentified Byproduct A and B increased.

The locations of the assayed AA metabolite standards, with the exception of TxB_2 , did not coincide with any of the 4 main metabolites determined following sample analysis. The chromatogram location of TxB_2 coincided with the peak denoted as Unidentified Byproduct B, however when samples were exposed to iodine, this area was not visible. Therefore, it can not be confirmed that this metabolite is indeed TxB_2 . The peak determined to be arachidonic acid was easily visualized post-iodine exposure. This is consistent with other studies which have utilized iodine as a means to visualize AA (Daniels et al. 1999). Additionally, iodine visualization was apparent in the region where the Unidentified Byproduct D was located. However, this region did not correlate with either of the tested standards and therefore remains unidentified.

The four main peaks found in the analysis of AA metabolism, also were distinguishable peaks on the GA chromatograms. Metabolism of arachidonic acid and glyceryl arachidonate are compared in Table 8. Only half as much of Byproduct A and B are present in GA metabolism analysis compared to AA analysis. The resulting amount of Byproduct D is significantly more following GA metabolism versus AA

Table 7. Metabolism/Breakdown of Arachidonic Acid (% of compound in skin)		
Application Time = 24hr		Application Amount = 2mg/cm ²
	High AA levels ^a	Low AA levels ^b
Unidentified Byproduct A	17.0 $\pm$ 2.98	26.7 $\pm$ 11.40
Unidentified Byproduct B	19.8 $\pm$ 3.84	33.4 $\pm$ 2.20
Unidentified Byproduct C	8.2 $\pm$ 0.99	4.2 $\pm$ 0.15
Arachidonic Acid	45.5 $\pm$ 3.75	10.4 $\pm$ 0.37
Unidentified Byproduct D	0.53 $\pm$ 0.05	0.98 $\pm$ 0.71

* Values are Mean $\pm$ SEM

^a High AA levels n=2

^b Low AA levels n=2

Table 8. Metabolism/Breakdown Distribution of AA and GA in Viable Human Skin (% of compound in skin)

Application Time = 24hr

Application Amount = 2mg/cm²

	Arachidonic Acid ^a Viable Human	Glycerol Arachidonate ^b Viable Human
Unidentified Byproduct A	21.8 ± 5.56	12.6 ± 1.47
Unidentified Byproduct B	26.6 ± 4.33	14.6 ± 3.27
Unidentified Byproduct C	6.2 ± 1.22	5.6 ± 0.76
Arachidonic Acid	27.9 ± 10.23	27.8 ± 2.86
Unidentified Byproduct D	0.8 ± 0.32	9.2 ± 2.88

* Values are Mean ± SEM

^a Arachidonic Acid n = 4

^b Glycerol Arachidonate = 5

metabolism. The average amount of remaining arachidonic acid following GA metabolism is similar to the amount remaining following AA treatment.

In GA treated human cadaver skin, less arachidonic acid was observed following metabolism. However, under these circumstances, there was more unidentified byproduct B present (Table 9). This is the same correlation noted during AA metabolism analysis. As the amount of intact arachidonic acid decreases, the amount of unidentified byproduct B increases. In each tissue sample, notable amounts of Byproduct A and B resulted from GA metabolism.

Hydrolysis of glyceryl arachidonate to arachidonate acid was observed to be about 3% following absorption through EpiDerm™ to the underlying receptor fluid. The majority of the remaining material was unmetabolized glyceryl arachidonate (Figure 17). Even though absorption through EpiDerm™ was shown to be rapid during absorption studies, under these conditions, hydrolysis was still possible.

The hydrolysis of glyceryl arachidonate in EpiDerm™, viable human, and human cadaver skin can be seen in Table 10. Metabolism of GA was very similar between the EpiDerm™ and viable human samples (Table 9). In these samples, about 26% was metabolized to arachidonic acid, but was not metabolized further. Since the viability and utility of cultured equivalents such as EpiDerm™ are thought by others to represent that observed with viable skin, it is conceivable that the metabolism profile observed with viable human skin is similar to that seen with EpiDerm™.

**Table 9. Metabolism/Breakdown of Glycerol Arachidonate
(% of compound in skin)***

Application Time = 24 hours Application Amount = 2mg/cm²

	Viable Human ^a	Human Cadaver ^b	EpiDerm™ ^c
Unidentified Byproduct A	12.6 ± 1.47	17.3 ± 2.14	14.2 ± 1.71
Unidentified Byproduct B	14.6 ± 3.27	28.0 ± 0.82	11.8 ± 2.21
Unidentified Byproduct C	5.6 ± 0.76	4.1 ± 1.05	7.8 ± 0.98
Arachidonic Acid	27.8 ± 2.86	19.6 ± 1.34	25.1 ± 5.22
Unidentified Byproduct D	9.2 ± 2.88	3.7 ± 0.74	6.5 ± 1.48

*Values are Mean ± SEM

^a Viable Human skin n= 5

^b Human Cadaver skin n= 4

^c EpiDerm™ n= 4

Figure 17. HPLC Chromatogram of GA Hydrolysis in EpiDerm™ Receptor Fluid

FIG. 17. HPLC Chromatogram of GA Hydrolysis in EpiDerm™ Receptor Fluid. Glyceryl Arachidonate's Corresponding Retention Time is 34.00 min. Arachidonic Acid's Corresponding Retention Time is 44.80.

Table 10. Glycerol Arachidonate Hydrolysis from Lotion Formulation (% of total peak area from chromatogram)*

Application Time = 24 hours

Application Amount = 2mg/cm²

	Viable Human ^a	Human Cadaver ^b	EpiDerm TM ^c
Glycerol Arachidonate	Trace Amounts	Trace Amounts	Trace Amounts
Arachidonic Acid	27.8 $\pm$ 2.86	19.6 $\pm$ 1.34	25.1 $\pm$ 5.22
Other Major AA Byproducts	42.0 $\pm$ 1.78	53.1 $\pm$ 1.16	40.2 $\pm$ 4.97

* Values are Mean $\pm$ SEM

^a Viable Human n = 5

^b Human Cadaver n = 4

^c EpiDermTM n = 4

In order to examine the potential safety concerns from the use of GA in topically applied cosmetic products, it was necessary to estimate the amount of free arachidonic acid that would be formed in skin by ester hydrolysis of absorbed GA. It was not however necessary to specifically identify the various AA metabolites formed. Total GA ester hydrolysis observed (as calculated by addition of AA and its 4 unidentified metabolites peaks) was about 70% in each of the 3 tissue samples, even though the distribution of metabolites is unique to the skin specimen (Table 8).

***In Vitro* Skin Irritation Study**

Transepidermal Water Loss

Transepidermal Water Loss (TEWL) measurements have been used *in vivo* to evaluate the integrity of the skin and analyzed *in vitro* as an endpoint for irritation studies. Therefore, TEWL was analyzed for its utility at measuring irritation promotion during *in vitro* diffusion cell studies. Viable rat skin and EpiDerm™ samples were analyzed during initial studies to determine the more sensitive tissue for this type of analysis.

Initial studies utilizing rat skin were subjected to a time course study to evaluate the appropriate time to take final TEWL measurements. Twenty four hours after dosing, measurements were evaluated prior to the wash, immediately after the wash, and every 40 minutes following the wash for a total of 2hr. The first TEWL measurement taken prior to the wash only displayed an increase in TEWL values for those cells which were treated with aqueous vehicles. For the emulsion vehicle treatments, their TEWL values

either remained the same or decreased. The decrease in TEWL was likely due to the occlusivity of the lotion formulation. This same occurrence is seen with TEWL studies conducted *in vivo* evaluating the improvement in the skin integrity following treatment with a lotion vehicle. Due to occlusion, final TEWL measurements should not be taken prior to removal of the emulsion vehicle. When the next potential TEWL endpoint was evaluated, a large spike in TEWL values was observed between the measurement taken prior to the skin surface wash and that immediately following it (Figure 18). Based on the high probability that the evaporation of the wash solution is actually what is being measured immediately following the wash, a later time point was determined to be necessary. Within forty minutes following the wash, the TEWL measurements had decreased an average of 8.4 g/m²/hr. Over the subsequent two readings at 80 min and 120 min post-wash, average decreases of 1.27 and 0.41, respectively were observed. During this point, for emulsion vehicle treatments, their TEWL values had returned to near baseline measurement (Figure 18).

Based on the initial time course studies, final studies compared baseline TEWL measurements with those recorded 60 minutes following the skin surface wash (24hr after initial dosing). The largest Δ TEWL observed on rat skin for the emulsion formulations was observed with the 5% SLS treatment (4.4 g/m²/hr), while the smallest Δ TEWL observed was the 5% AA treatment (1.6 g/m²/hr) (Table 11). No significant difference was observed between the emulsion vehicle standard treatments ($p=0.169$) (Figure 19). The Δ TEWL following topical application of AA in the emulsion vehicle was not

Figure 18. TEWL Time Course Study

FIG. 18. TEWL Time Course Study. TEWL measurements in relation to skin surface wash conducted 24hr following application of rat skin. Values are mean $\pm$ SEM. Number of diffusion cells are 5 (o/w emulsion; 5% SLS in emulsion), 4 (10% Tween in emulsion), and 2 (DI H₂O; 5% SLS in H₂O). Indistinguishable error bars are contained within the symbol.

Table 11. Comparison of Δ TEWL in EpiDerm™ and Rat SkinApplication Time = 24hr Application Amount = 10mg/cm²

	EpiDerm™ ^a	Rat Skin ^b
o/w Emulsion	0.1 $\pm$ 0.47	3.3 $\pm$ 0.65
10% Tween in Emul	0.1 $\pm$ 0.22	1.7 $\pm$ 1.04
5% SLS in Emul	-0.2 $\pm$ 0.70	4.4 $\pm$ 0.51
2 ½ % AA in Emul	1.6 $\pm$ 0.57	N/A
5% AA in Emul	-0.6 $\pm$ 0.40	1.6 $\pm$ 0.35
10% AA in Emul	-0.5 $\pm$ 0.62	2.9 $\pm$ 2.21
100% AA	1.29 $\pm$ N/A	N/A
5% SLS in H ₂ O	3.27 $\pm$ N/A	13.2 $\pm$ 0.77
15% SLS in H ₂ O	N/A	19.5 $\pm$ 0.74
DI H ₂ O	N/A	4.5 $\pm$ 1.13

* Values are Mean $\pm$ SEM

^a EpiDerm™ n = 1 (100% AA, 5% SLS in H₂O); n = 3 (2 ½ % AA in emul); n = 4 (10% Tween in emul); n=5 (5% SLS in emul); n = 6 (o/w emul); n= 8 (5% AA in emul, 10% AA in emul)

^b Rat Skin n = 3 (10% Tween in emul, 15% SLS in H₂O); n = 6 (5% AA in emul, DI H₂O); n= 8 (5% SLS in H₂O); n = 12 (o/w emul, 10% AA in emul); n = 5% SLS in emul)

Figure 19. Change in TEWL as a measure of skin irritation in EpiDerm and Rat Skin Following Emulsion Treatment

FIG. 19. Change in TEWL measurements as a measure of skin irritation in EpiDerm™ and rat skin following emulsion treatment. Measurements taken 60 minutes post-wash (24hr after dosing) were compared with baseline values prior to application with both rat skin and EpiDerm™. Values are mean $\pm$ SEM. n-values ranged from 3 to 13 diffusion cells. Indistinguishable error bars are contained within the symbol.

significantly different ($p=0.487$) than the control, o/w emulsion. Aqueous sodium lauryl sulfate caused a significant increase in TEWL of 13.2 and 19.5 for the 5% and 15% concentrations, respectively. The 3 aqueous treatments were significantly different ($p<0.001$) from each other when compared with the Δ TEWL of 4.5 g/m²/hr observed with the DI water control (Figure 20). Previous studies have demonstrated an increase in TEWL following treatment with 5% SLS on dorsal hairless rat skin *in vivo* (Okuda et al. 2002) and dorsal full-thickness pig skin *in vitro* (Chilcott et al. 2002b) both of which were published following initiation of this study. It was determined that TEWL evaluation was able to properly rank the aqueous treatments on rat skin according to irritation promotion.

It is important to note that aqueous vehicle treatments, especially those including a strong irritant such as 5% SLS, were not subjected to the occlusivity prior to the wash which was observed with the emulsion formulations. For this reason, TEWL measurements taken prior to the wash were similar to those measured 60 minutes after the wash.

Baseline TEWL values for EpiDerm™ and rat skin were 19.1 g/m²/hr (n=36) and 6.1 g/m²/hr (n=147), respectively. Comparing the baseline rat TEWL values with that previously observed *in vivo* (Kanikkannan et al. 2002; Kanikkannan and Singh 2002; Agren et al. 2003) gives relatively good agreement. The elevated baseline value for EpiDerm™ made changes in TEWL, due to application of the treatment, difficult to

Figure 20. Change in TEWL as a Measure of Skin Irritation on EpiDerm and Rat Skin Following 24hr Treatment

FIG. 20. Change in TEWL as a measure of skin irritation on EpiDerm™ and rat skin following 24hr treatment. Measurements taken 60 minutes post-wash (after 24hr treatment) were compared with baseline values prior to application with both rat skin and EpiDerm. Values are mean $\pm$ SEM. n-values ranged from 3 to 13 diffusion cells. Indistinguishable error bars are contained within the symbol. Aqueous 15% SLS (n=3) was significantly different (ANOVA, $p < 0.001$; Tukey, $p < 0.05$) than 5% SLS (n= 8) treatment. Additionally, each aqueous SLS treatment was significantly different (ANOVA, $p < 0.001$; Tukey, $p < 0.05$) than its control (n=6).

discern (Figure 20). The average Δ TEWL for all treated EpiDerm™ cells was 0.03 g/m²/hr with final values measured 60 minutes post skin surface wash. The greatest Δ TEWL observed during the EpiDerm™ studies was 3.27 g/m²/hr caused by treatment with 5% SLS in H₂O (Table 11). The emulsion vehicle treatments however only resulted in a Δ TEWL ranging from -0.6 to 1.6 g/m²/hr. With EpiDerm™, there was not a statistically significant difference ($p=0.685$) between the standard emulsion treatments which could be utilized for irritation ranking purposes. The AA emulsion treatments were not significantly different from the o/w emulsion ($p=0.128$).

Cytotoxicity (MTT)

As a second potential endpoint for irritation evaluation of skin membranes assembled in diffusion cells, the change in cell viability following 24hr treatment was considered. To measure the initial and terminal viability of skin in the cell, an MTT assay was utilized. As with the TEWL analysis, rat skin and EpiDerm™ were compared to determine the more suitable tissue for this assay.

Rat skin showed a significant difference between the aqueous vehicle treatments (Table 12). There was a distinct decrease in viability observed with the 5% SLS in H₂O treatment which resulted in a terminal viability average of 4.6%. This Δ viability was significantly different ($p<0.05$) from both the 5% Tween in H₂O treatment and the DI H₂O control. Although as observed in previous analysis (TEWL) the 5% Tween in H₂O was not significantly different from the DI H₂O control ($p>0.05$).

Table 12. Percent of Initial Viability in EpiDerm™ and Rat Skin Measured by MTT Analysis

Application Time = 24hr Application Amount = 10mg/cm²

	EpiDerm™ ^a	Rat Skin ^b
o/w Emulsion	40.2 ± 3.19	69.7 ± 8.28
10% Tween in Emul	39.8 ± 4.80	87.1 ± 1.82
5% SLS in Emul	21.9 ± 2.23	84.2 ± 4.55
2 ½ % AA in Emul	43.4 ± 7.00	N/A
5% AA in Emul	38.9 ± 9.28	N/A
10% AA in Emul	34.9 ± 3.72	N/A
5% SLS in H ₂ O	N/A	4.6 ± 2.02
DI H ₂ O	N/A	100.9 ± 11.47
5% Tween in H ₂ O	N/A	107.5 ± 7.13

* Values are Mean ± SEM

^a EpiDerm™ n = 5 (2 ½ % AA in emul, 5% AA in emul); n = 6 (10% AA in emul); n = 8 (5% SLS in emul); n = 9 (10% Tween in emul); n = 14 (o/w emul)

^b Rat Skin n = 3 (10% Tween in emul); n=5 (5% Tween in H₂O); n = 7 (o/w emul, 5% SLS in emul); n = 11 (5% SLS in H₂O); n = 12 (DI H₂O)

The o/w emulsion, 10% Tween in emulsion, and 5% SLS in emulsion treatments respectively decreased viability of rat skin by 30.3%, 12.9%, and 15.8% (Figure 21). Of the emulsion vehicle treatments, the o/w emulsion control was most effective in reducing skin viability. Comparing the decrease in viability for the 5% SLS in emulsion versus its control, the o/w emulsion, they were determined not to be significantly different ($p = 0.149$). Furthermore, no significant difference ($p = 0.311$) was observed between any of the emulsion treatments.

When the MTT assay was conducted on EpiDerm™, the standards dosed in the emulsion vehicle were proven to be significantly different. The o/w emulsion control and 10% Tween in emulsion both decreased viability about 60% while a 78% decrease was observed with the 5% SLS in emulsion treatment (Figure 22). The 5% SLS in emulsion was proven to be significantly different from the o/w emulsion ($p = 0.005$) and the 5% Tween in emulsion ($p = 0.014$). For this reason, AA was dosed on the EpiDerm™ tissue for irritation analysis.

10%, 5%, and 2 ½ % AA in emulsion were not significantly different ($p = 0.671$) from each other causing an average percent of initial viability of about 39% (Table 12). Also, the AA treatments were not significantly different from either of the other emulsion treatments, including the control.

Figure 21. Percent of Initial Viability as a Measure of Skin Irritation of Emulsion Treatments on Epiderm and Rat Skin

FIG. 21. Percent of initial viability as a measure of skin irritation of emulsion treatments on EpiDerm™ and rat skin. Terminal viability, assessed by the MTT assay, was measured following 24 hr treatment. Values are mean $\pm$ SEM. n-values ranged from 3 to 14 repetitions. A significant difference (ANOVA, $p=0.003$; Tukey, $p<0.05$) was observed between the 5% SLS emulsion treatment ($n=8$) and its control ($n=14$) on EpiDerm™.

Figure 22. Percent of Initial Viability (MTT) as a Measure of Skin Irritation in Epiderm and Rat Skin

FIG. 22. Percent of initial viability (MTT) as a measure of skin irritation in EpiDerm™ and rat skin. Terminal viability, assessed by the MTT assay, was measured following 24 hr treatment. Values are mean $\pm$ SEM. n-values ranged from 3 to 14 repetitions. Aqueous 5% SLS (n=11) was found to be significantly different (ANOVA, $p < 0.001$; Dunn's, $p < 0.05$) than both aqueous 5% Tween (n=5) and the control (n=12)

A recent study, published after this work had begun, supports the data found here in that no significant difference could be obtained with cytotoxicity values from an MTT assay in regard to emulsion/cream formulations (Lampen et al. 2003).

Cytokine Release (IL-1 α)

The final endpoint analyzed as a marker of *in vitro* irritation was the release of the cytokine IL-1 α . The skin samples were analyzed to evaluate the amount of intracellular IL-1 α present. Lower amounts of IL-1 α were observed in cases of strong irritation, such as aqueous 5% SLS, while non-irritants maintained higher IL-1 α concentrations. This is consistent with what is expected because as irritation occurs, IL-1 α is released into the extracellular fluid, in this case the receptor fluid. Initial analysis of the receptor fluid proved it too dilute to analyze (data not shown). Following 24hr treatment, 1:10 dilutions of the pulverized skin samples were determined to provide accurate data values based on the standard curve. Data obtained from the BCA protein analysis made no significant contribution to the IL-1 α data and therefore was not reported.

Analysis of rat skin as a tissue source for the cytokine release assay showed a significant difference in the 5% SLS in H₂O and its control (p=0.003) (Table 13). The aqueous 5% SLS and DI H₂O had respective IL-1 α concentrations of 924.7 pg/mL and 4,724.2pg/mL. Conversely, emulsion vehicle IL-1 α concentrations for 5% SLS (1692.4 pg/mL), 10% Tween (3124.8 pg/mL), 10% AA (2478.5 pg/mL), and the control (2701.3 pg/mL) were not significantly different (p=0.249).

Table 13. Concentration of IL-1 α in EpiDerm™ and Rat SkinApplication Time = 24hr Application Amount = 10mg/cm²

	EpiDerm™ ^a	Rat Skin ^b
o/w Emulsion	1251.0 $\pm$ 93.0	2701.3 $\pm$ 457.9
10% Tween in Emul	1374.6 $\pm$ 110.3	3124.8 $\pm$ 1401.1
5% SLS in Emul	1522.0 $\pm$ 200.9	1692.4 $\pm$ 305.0
5% AA in Emul	1347.5 $\pm$ 128.4	2228.3 $\pm$ 162.6
10% AA in Emul	1365.3 $\pm$ 243.4	2478.5 $\pm$ 326.4
5% SLS in H ₂ O	N/A	924.7 $\pm$ 276.1
DI H ₂ O	N/A	4724.2 $\pm$ 432.4

* Values are Mean $\pm$ SEM^a EpiDerm™ n = 4 (10% Tween in emul) and n = 5 (o/w emul, 5% SLS in emul, 5% AA in emul, and 10% AA in emul)^b Rat Skin n = 3 (10% Tween in emul, DI H₂O); n=5 (5% AA in emul, 5% SLS in H₂O); n = 9 (o/w emul, 5% SLS in emul, and 10% AA in emul)

When the emulsion treatments were analyzed on EpiDerm™, there was a minimal difference in the IL-1 α concentrations for each treatment (Table 13). An IL-1 α concentration range of 1251-1522 pg/mL was observed with 5% SLS at the high end and the o/w emulsion at the low end. In between were 10% Tween and 10% AA with values of 1374.6pg/mL and 1365.3pg/mL. There was no significant difference observed between the emulsion vehicles treated on EpiDerm™ ($p=0.848$) (Figure 23). Treatments of 10% and 5% AA in the emulsion vehicle were additionally not distinguishable from each other with IL-1 α concentrations of 1365.3pg/mL and 1347.5pg/mL.

A graphical comparison of the treatments in rat skin and EpiDerm™ can be seen in Figure 24. It is obvious from this representation the low sensitivity observed between the treatments analyzed with the skin culture. Although the rat skin gave more variability between cytokine values following various treatments, the ranking potential was not precise enough to evaluate for irritation.

Figure 23. IL-1 α Concentration in Epiderm and Rat Skin Following Emulsion Treatment

FIG. 23. IL-1 α Concentration in EpiDerm™ and rat skin following emulsion treatment. Intracellular concentration of IL-1 α (pg/mL) were assessed by ELISA assay following 24hr emulsion vehicle treatment. Values are mean $\pm$ SEM. n-values ranged from 3 to 9 repetitions.

Figure 24. Concentration of IL-1 α in Rat and Human Skin Following 24hr Treatment

FIG. 24. Concentration of IL-1 α in rat and human skin following 24hr treatment. Intracellular concentration of IL-1 α (pg/mL) was assessed by ELISA following 24hr treatment. Values are mean $\pm$ SEM. n-values ranged from 3 to 9 diffusion cells. A student's t-test displayed a significant difference ($p \leq 0.001$) between the aqueous 5% SLS treatment (n=5) and its control (n=3).

Conclusions/Summary

The purpose of this research was to determine the absorption, metabolism, and irritation promotion of the compounds arachidonic acid and glyceryl arachidonate. This study was intended to help in the evaluation of the safety of their use in cosmetics. In addition, an *in vitro* alternative method for irritation was developed and evaluated.

Overall efficiency of TEWL as a means to replace the $^3\text{H}_2\text{O}$ skin barrier integrity test could not be shown. There was some indication that high $^3\text{H}_2\text{O}$ absorption values lead to high TEWL values. However, there were numerous instances during this study which proved the opposite untrue. If TEWL is utilized as a means to eliminate skin samples from use in absorption studies, it appears as if many suitable cells (as determined by $^3\text{H}_2\text{O}$) would be discarded. One specific TEWL value above which all cells were found to fail the $^3\text{H}_2\text{O}$ test could not be determined. Therefore, the concept of a universal TEWL value as a cutoff point for barrier integrity does not appear to be supported by this data.

Through percutaneous absorption/metabolism studies, it was determined that both arachidonic acid and glyceryl arachidonate permeate to some degree each type of skin sample evaluated. Furthermore, AA and GA were absorbed through the skin samples into the receptor fluid indicating a potential for systemic availability of these compounds.

Human skin was found to have a better barrier to arachidonic acid penetration when compared with rat skin. In the glyceryl arachidonate studies, EpiDerm™ was

significantly more permeable than human skin. When evaluating the penetration of the 3 tissue samples by lipophilic compounds, the resulting ranking was observed: Human skin < Rat skin < EpiDerm™.

For treatment with either arachidonic acid or glyceryl arachidonate, the amount which absorbed into the receptor fluid in human skin (~2%) is significantly less than the total amount penetrated (~20%). In rat skin, AA penetration accounted for more than 50% of the applied dose, a substantial amount of which partitioned into the receptor fluid.

Percutaneous absorption of arachidonic acid and glyceryl arachidonate in human skin showed a similar absorption/distribution pattern. Total penetration of each compound was about 20%, half of which permeated through the upper 10 layers of stratum corneum and was found in either the lower layers of the skin or the receptor fluid.

The amount of arachidonic acid penetrating the skin remained relatively constant between each 6 hr fraction in both the fuzzy rat and human skin. The same trend was observed during the glyceryl arachidonate studies, with the exception of EpiDerm™. Due to the increased permeability of EpiDerm™, most of the absorbed material appeared in the receptor fluid within the first 6hr.

This tendency could be due to the reservoir effect which has previously been observed during *in vitro* studies of lipophilic compounds. The partitioning of the lipophilic compound into the receptor fluid is limited by its solubility in the aqueous

buffer. Although 4% BSA was added to aid in partitioning from the skin, saturation of the compound in the receptor fluid may still have been reached. This is supported by the extended studies (72hr) which showed a relatively consistent amount of compound continued to partition into the receptor fluid for each 6hr period over the course of the study, even after the skin surface wash at 24hr. By increasing the study from 1 to 3 days, the amount of compound in the receptor fluid increased by a factor of 2.

Based on the 72hr extended studies, it appears as if a reservoir effect is indeed occurring due to the significant (p value <0.001) difference in the amount found in the receptor fluid after 72hr compared to 24hr. Therefore, using the receptor fluid values alone as an estimate of systemic absorption of arachidonic acid would likely underestimate the total amount that would be absorbed.

Topical application of glyceryl arachidonate to skin samples resulted in almost complete metabolism/degradation. At least 70% of the dose remaining in the skin is believed to have been hydrolyzed to arachidonic acid and its metabolites. The remainder was either metabolized to undistinguishable AA metabolites or other GA metabolism by-products. A trivial amount of GA was found to be un-metabolized.

GA metabolism in EpiDerm™ and viable human skin tissue gave similar profiles under these test conditions indicating a potential usefulness in metabolism studies. However, since the extent of skin metabolism is known to be affected by permeation rate, the comparability of GA metabolism values observed with EpiDerm™ to human skin

metabolism during percutaneous absorption is uncertain. Previous studies have also confirmed that the metabolism properties of existing skin equivalents could be beneficial, even though they do not appear to have the highly defined barrier required for *in vitro* penetration studies (Stinchcomb 2003; Mavon et al. 2004). It was suggested that if not used as a total replacement for human skin metabolism, at minimum skin equivalents could be utilized as a supplementary test (Stinchcomb 2003).

Upon TLC analysis of viable human skin, it was observed that more than 50% of the penetrated arachidonic acid had been metabolized through the arachidonic acid cascade. Four obvious metabolite peaks were observed.

Due to the occlusive property of an emulsion vehicle, its presence on the skin surface interferes with the measurement of transepidermal water loss. To utilize TEWL as a means to evaluate skin irritation of emulsion vehicles *in vitro*, it was determined to be necessary to utilize terminal TEWL measurements 60 minutes after the skin surface wash. Both immediately prior to and immediately following the wash either gave inaccurate occluded or elevated values.

The average baseline TEWL value measured *in vitro* with rat skin was 6.1 g/m²/hr. This data coincides with similar published *in vivo* values of 9 g/m²/hr (Agren et al. 2003), ~ 5 g/m²/hr (Kanikkannan and Singh 2002), and ~ 8 g/m²/hr (Kanikkannan et al. 2002). EpiDerm™'s initial TEWL values were elevated in comparison to the baseline values obtained with rat skin. These elevated values were in the region of TEWL values

observed on rat skin post treatment with a strong irritant in an aqueous vehicle. For this reason, changes in TEWL caused by irritation to the skin were not as easily observed in EpiDerm™ as with rat skin. Thus, when comparing Δ TEWL for irritation promotion evaluation, rat skin was determined to be the more sensitive tissue sample. Although, even with the rat skin model, it was determined that TEWL measurements were not sensitive enough to rank irritancy of emulsion vehicle treatments to skin membranes in diffusion cells. However, analysis of a strong irritant in an aqueous vehicle (ex. 5% SLS) on rat skin can be distinguished with TEWL measurements and is significantly different from its control treatment as was also observed in previous studies (Okuda et al. 2002).

Cytokine release as a means to evaluate irritation *in vitro* seemed to be favorable in terms of aqueous treatments. However, when evaluating a cosmetic formulation such as an emulsion formulation, its ability to differentiate irritants diminished. This tendency was observed both in the treatment of EpiDerm™ and rat skin. The inability of the IL-1 α assay to distinguish irritants *in vitro* from cosmetic formulations was also observed in a previous study conducted in EpiDerm™ (Faller et al. 2002).

Cytotoxicity evaluation through utilization of the MTT assay gave more promising results in respect to emulsion formulations. However, only a strong irritant (5% SLS) was able to be significantly differentiated in only the EpiDerm™ tissue. MTT analysis of rat skin provided no difference between emulsion treatments. Nevertheless, as previously seen before, 5% SLS in an aqueous vehicle could be determined as an irritant in rat skin with the use of an MTT assay.

Glyceryl arachidonate was shown when topically applied in a cosmetic formulation to penetrate the skin and hydrolyze so as to introduce free arachidonic acid into the body. At that point, arachidonic acid was available for metabolism to its corresponding eicosanoids. Based on the compiled absorption and metabolism data, it is determined to be possible that topical application of either arachidonic acid or glyceryl arachidonate could initiate the arachidonic acid irritation cascade in skin.

This work demonstrates that the vehicle used to apply an irritant chemical to the skin can substantially determine the magnitude of the irritant effect observed. A cosmetic emulsion formulation may result in reduced irritation as compared to the aqueous solutions often used in irritation assays.

Future Directions

Absorption / Metabolism

As occurs with most in depth research projects, more questions arise through the course of a study than those which are answered. This study has demonstrated that both arachidonic acid and glyceryl arachidonate penetrate skin and to some degree absorb into the underlying receptor fluid. The occurrence of absorption leads to the likelihood of systemic availability of these compounds. This study has initiated the investigation into the fate of these chemicals following topical application, and has shown ester hydrolysis of glyceryl arachidonate. However, continued research into the metabolism/degradation of these compounds is encouraged.

Multiple byproducts are isolated from skin following topical application of AA. Due to a limit of time and resources, identification of these products was not possible. However, further research analyzing the pathway of metabolism or degradation which generated the resulting products would be of interest. An effort to further minimize degradation of AA during the experiment would also be essential. Additionally, an attempt to isolate products produced as a result of skin metabolism from those resulting from environmental exposure should be included.

$^3\text{H}_2\text{O}$ vs. TEWL *in vitro*

The use of TEWL to measure barrier integrity *in vivo* has been readily documented and utilized by many researchers. It would therefore be ideal to take advantage of this technology and transfer it to an *in vitro* experimental design. However,

the utility of TEWL as an assessment/replacement for barrier integrity in an *in vitro* setting is a controversial topic. Based on its reliable data *in vivo*, many would like to believe the same could be achieved *in vitro*. However, there is a lack of data in publication confirming this to be true.

Barrier integrity *in vitro* has traditionally been evaluated with the $^3\text{H}_2\text{O}$ skin barrier integrity test. To prove utility of TEWL *in vitro*, a direct correlation between transepidermal water loss measurements and $^3\text{H}_2\text{O}$ absorption would be ideal. If $^3\text{H}_2\text{O}$ barrier tests could be replaced with a non-invasive measurement such as TEWL, the use of radioactivity during the course of a study could be limited or even eliminated altogether. However, this study has provided data indicating a lack of a direct relationship between these two factors.

During this study a time course evaluation of terminal TEWL measurements was conducted for the purpose of irritation evaluation. However, a time course study investigating baseline measurements may give more information regarding the relationship, or lack thereof, of TEWL to $^3\text{H}_2\text{O}$ absorption. A minimal amount of moisture on the surface of the skin could result in an elevated TEWL measurement. Investigating a longer equilibration time prior to baseline measurements could eliminate any residual moisture on the skin surface.

Due to facility limitations, relative humidity and temperature could not be maintained at constant values from one experiment to the next. A study investigating the

effect of the environment on TEWL measurements in an *in vitro* setting could be informative. If the use of an environmental chamber proves to be the limiting factor of TEWL *in vitro*, the practicality of its use in a variety of labs is diminished.

The incorporation of an additional measurement for comparison with TEWL and $^3\text{H}_2\text{O}$ could prove beneficial in understanding why the technology of TEWL has not been shown to be transferable to *in vitro* studies in regard to barrier integrity assessment. There are bioelectrical non-invasive measurements which are utilized during clinical studies which could be tested for correlation to $^3\text{H}_2\text{O}$.

Irritation

It was necessary to evaluate the alternative methods using an emulsion vehicle because of the lipophilicity of arachidonic acid. Unfortunately, model irritants when dosed in an emulsion formulation do not result in the irritation promotion which is observed following aqueous vehicle application. A deeper investigation into the comparison of *in vitro* irritation assessment in diffusion cells and that conducted in culture plates needs to be undertaken. Most of the existing *in vitro* studies have analyzed skin irritation in culture plates using aqueous solutions. In order to compare the diffusion cell method and culture plate method, studies utilizing the same parameters (and test formulations) would need to be conducted. We have already established that extracellular measurement of IL-1 α by means of the diffusion cell method is not applicable due to the increased dilution factor in the receptor fluid. However other parameters such as MTT may equate the two types of studies, thus proving the flow-

through diffusion cell system to be a reliable alternative method for other dosing formulations (such as aqueous).

References

Agren, J., S. Zelenin, et al. (2003). "Transepidermal Water Loss in Developing Rats: Role of Aquaporins in the Immature Skin." Pediatric Research **53**(4): 558-565.

Bando, H., S. Mohri, et al. (1997). "Effects of Skin Metabolism on Percutaneous Penetration of Lipophilic Drugs." J. Pharm Sci **86**(6): 759-761.

Basketter, D. A., M. Chamberlain, et al. (1997). "The Classification of Skin Irritants by Human Patch Test." Food and Chemical Toxicology **35**: 845-852.

Baumer, W. and M. Kietzmann (2000). "The isolated Perfused Bovine Udder as a Model of Dermal Eicosanoid Release." ATLA **28**: 643-649.

Beck, R., S. Bertolino, et al. (1998). "Modulation of Arachidonic Acid Release and Membrane Fluidity by Albumin in Vascular Smooth Muscle and Endothelial Cells." Circulation Research **83**: 923-931.

Benassi, L., G. Bertazzoni, et al. (1999). "In vitro testing of tensides employing monolayer cultures: a comparison with results of patch tests on human volunteers." Contact Dermatitis **40**(1): 38-44.

Bronaugh, R. L. (1996). "In vitro viable skin model." Pharm Biotechnol. **8**: 375-86.

Bronaugh, R. L. and R. F. Stewart (1984). "Methods for in vitro percutaneous absorption studies III: hydrophobic compounds." J. Pharm Sci **73**(9): 1255-1258.

Bronaugh, R. L. and R. F. Stewart (1985). "Methods for in vitro percutaneous absorption studies IV: The flow-through diffusion cell." J. Pharm Sci **74** (1)(January): 64-7.

Bronaugh, R. L. and R. F. Stewart (1986). "Methods for in vitro percutaneous absorption studies. VI: Preparation of the barrier layer." J. Pharm Sci **75** (5): 487-91.

Bronaugh, R. L., R. F. Stewart, et al. (1982b). "Methods for In Vitro Percutaneous Absorption Studies II: Animal Models for Human Skin." Toxicol Appl Pharmacol **62**(3): 481-8.

Bronaugh, R. L., R. F. Stewart, et al. (1982a). "Methods for in Vitro Percutaneous Absorption Studies I: Comparison with in Vivo Results." Toxicol Appl Pharmacol **62**(3): 474-480.

Bronaugh, R. L., R. F. Stewart, et al. (1986). "Methods for in vitro percutaneous absorption studies. VII: Use of excised human skin." J. Pharm Sci **75** (11): 1094-7.

Bronaugh, R. L., R. F. Stewart, et al. (1989). "Extent of Cutaneous Metabolism during Percutaneous Absorption of Xenobiotics." Toxicology and Applied Pharmacology **99**: 534-543.

Chilcott, R. P., C. H. Dalton, et al. (2002a). "Transepidermal Water Loss Does Not Correlate with Skin Barrier Function In Vitro." Journal of Investigative Dermatology **118**(5): 871-875.

Chilcott, R. P., A. Patel, et al. (2002b). The effects of chemical damage on TEWL. In: Marks, R., J.L. Leveque, R. Voegeli (Eds), The Essential Stratum Corneum, CRC.

CIR (1993). "Final Report on the Safety Assessment of Arachidonic Acid." Journal of the American College of Toxicology **12**(5): 481-559.

Collier, S., N. Sheikh, et al. (1989). "Maintenance of skin viability during in vitro percutaneous absorption/metabolism studies." Toxicol Appl Pharmacol **99** (3)(Jul): 522-33.

Coquette, A., N. Berna, et al. (1999). "Differential Expression and Release of Cytokines by an In Vitro Reconstructed Human Epidermis Following Exposure to Skin Irritant and Sensitizing Chemicals." Toxicology in Vitro **13**: 867-877.

Cumberbatch, M., R. J. Dearman, et al. (1998). Characterization of Epidermal Langerhans Cells. Dermatotoxicology Methods. F. N. Marzulli and H. I. Maibach, Taylor & Francis: 207-215.

Daniels, I., J. Fletcher, et al. (1999). "Role of p38 in the Priming of Human Neutrophils by Peritoneal Dialysis Effluent." Clinical and Diagnostic Laboratory Immunology **6**(6): 878-884.

Faller, C., M. Bracher, et al. (2002). "Predictive Ability of Reconstructed Human Epidermis Equivalents for the Assessment of Skin Irritation of Cosmetics." Toxicology in Vitro **16**: 557-572.

Fentem, J. H., D. Briggs, et al. (2001). "A prevalidation study on in vitro tests for acute skin irritation: results and evaluation by the Management Team." Toxicology in Vitro **15**: 57-93.

Fitzpatrick, T. B., A. Z. Eisen, et al. (1993). Dermatology in General Medicine. New York, McGraw-Hill, Inc.

Gajjar, L. and D. J. Benford (1987-88). "Irritancy testing in cultured keratinocytes." Mol Toxicol **1**(4): 513-23.

Hansen, J., D. Schade, et al. (1997). "Docosahexaenoic acid plus arachidonic acid enhance preterm infant growth." Prostaglandins, Leukotrienes and Essential Fatty Acids **57**(2): 196.

Hardman, J. G. and L. E. Limbird (1996). Goodman & Gilman's The Pharmacological Basis of Therapeutics. New York, McGraw-Hill.

Heylings, J. R., H. M. Clowes, et al. (2001). "Comparison of tissue sources for the skin integrity function test (SIFT)." Toxicology in Vitro **15**(4-5): 597-600.

Hood, H. L. and R. L. Bronaugh (1999). "A Comparison of Skin Viability Assays for In Vitro Skin Absorption/Metabolism Studies." In Vitro and Molecular Toxicology **12**(1): 3-9.

Hood, H. L., R. R. Wickett, et al. (1996). "In Vitro Percutaneous Absorption of the Fragrance Ingredient Musk Xylol." Food and Chemical Toxicology **34**: 483-488.

Jonca, N., M. Guerrin, et al. (2002). "Corneodesmosin, a Component of Epidermal Corneocyte Desmosomes, Displays Homophilic Adhesive Properties." The Journal of Biological Chemistry **277** (7)(February 15): 5024-5029.

Kabbur, M. B., J. V. Rogers, et al. (2001). "Effect of JP-8 Jet Fuel on Molecular and Histological Parameters Related to Acute Skin Irritation." Toxicol Appl Pharmacol **175**: 83-88.

Kanikkannan, N., B. R. Locke, et al. (2002). "Effect of jet fuels on the skin morphology and irritation in hairless rats." Toxicology **175**: 35-47.

Kanikkannan, N. and M. Singh (2002). "Skin Permeation Enhancement Effect and Skin Irritation of Saturated Fatty Alcohols." International Journal of Pharmaceutics **248**: 219-228.

Klaasen, C. D. (2001). Casarett & Doull's Toxicology: The Basic Science of Poisons. New York, McGraw-Hill.

Korting, H. C., S. Schindler, et al. (1994). "MTT-assay and neutral red release (NRR)-assay: relative role in the prediction of the irritancy potential of surfactants." Life Sci **55**(7): 533-40.

Kraeling, M. E. K. (1997). "In vitro Percutaneous Absorption of Alpha Hydroxy Acids in Human Skin." J. Soc. Cosmet. Chem **48**: 187-197.

Kraeling, M. E. K., G. Reddy, et al. (1998). "Percutaneous Absorption of Trinitrobenzene: Animal Models for Human Skin." Journal of Applied Toxicology **18**: 387-392.

Kraeling, M. E. K., J. J. Yourick, et al. (2004). "In vitro human skin penetration of diethanolamine." Food and Chemical Toxicology **42**: 1553-1561.

- Lampen, P., W. Pittermann, et al. (2003). "Penetration studies of vitamin E-acetate applied from cosmetic formulations to the stratum corneum of an in-vitro model using quantification by tape stripping, UV-spectroscopy and HPLC." J. Cosmet. Sci **54**: 119-131.
- Lee, J. K., D. B. Kim, et al. (2000). "In vitro cytotoxicity tests on cultured human skin fibroblasts to predict skin irritation potential of surfactants." Toxicology in Vitro **14**: 345-349.
- Leigh, I. M., E. B. Lane, et al. (1994). The Keratinocyte Handbook. Great Britain, Cambridge University Press.
- Lin, G. H. Y. and M. Hemming (1996). "Ocular and Dermal Irritation Studies of some Quaternary Ammonium Compounds." Food and Chemical Toxicology **34**(2): 177-182.
- Marks, F., G. Furstenberger, et al. (1998). "Arachidonic Acid metabolism as a reporter of skin irritancy and target of cancer chemoprevention." Toxicology Letters **96, 97**: 111-118.
- Mavon, A., V. Raufast, et al. (2004). "Skin absorption and metabolism of a new vitamin E prodrug, gamma-tocopherol-glucoside: in vitro evaluation in human skin models." Journal of Controlled Release **100**: 221-231.

Menon, G. K. (2002). "New insights into skin structure: scratching the surface."

Advanced Drug Delivery Reviews **54 Suppl.**(1): S3-S17.

Merck, H. F., F. K. Jugert, et al. (1996). Biotransformations in the Skin. Washington DC, Taylor & Francis.

Montagna, W. and P. F. Parakkal (1974). The Structure and Function of Skin. New York, Academic Press.

Muller-Decker, K., T. Heinzelmann, et al. (1998). "Arachidonic Acid metabolism in primary irritant dermatitis produced by patch testing of human skin with surfactants."

Toxicol Appl Pharmacol **153**(1): 59-67.

Nasseri-Sina, P., S. A. M. Hotchkiss, et al. (1997). "Cutaneous Xenobiotic Metabolism: Glycine Conjugation in Human and Rat Keratinocytes." Food and Chemical Toxicology **35**: 409-416.

Nelson, D. L. and M. M. Cox (2000). Lehninger Principles of Biochemistry. New York, Worth Publishers.

Okuda, M., T. Yoshiike, et al. (2002). "Detergent-induced epidermal barrier dysfunction and its prevention." Journal of Dermatological Science **30**: 173-179.

Osborne, R. and M. A. Perkins (1994). "An approach for development of alternative test methods based on mechanisms of skin irritation." Food and Chemical Toxicology **32**(2): 133-142.

Papirmeister, B., A. J. Feister, et al. (1991). Medical Defense Against Mustard Gas: Toxic Mechanisms and Pharmacological Implications. Boca Raton, CRC Press.

Pepe, R. C., J. A. Wenninger, et al. (2002). International Cosmetic Ingredient Dictionary and Handbook. Washington DC, The Cosmetic, Toiletry, and Fragrance Association.

Perkins, M. A., R. Osborne, et al. (1996). "Development of an in Vitro Method for Skin Corrosion Testing." Fundamental and Applied Toxicology **31**: 9-18.

Perkins, M. A., R. Osborne, et al. (1999). "Comparison of in Vitro and in Vivo Human Skin Responses to Consumer Products and Ingredients with a Range of Irritancy Potential." Toxicological Sciences **48**: 218-229.

Roberts, M. S. and K. A. Walters (1998). Dermal Absorption and Toxicity Assessment. New York, Marcel Dekker.

Robinson, M. K., R. Osborne, et al. (1999). "Strategies for the assessment of acute skin irritation potential." Journal of Pharmacological and Toxicological Methods **42**: 1-9.

Schaefer, W. R., K. Werner, et al. (1997). "Cytochrome P450 Metabolites of Arachidonic Acid in Human Placenta." Prostaglandins **54**(4): 677-687.

Sjurse, W., O. L. Brekke, et al. (2000). "Secretory and cytosolic phospholipase A(2) regulate the long-term cytokine-induced eicosanoid production in human keratinocytes." Cytokine **12**(8): 1189-94.

Stedman (1982). Illustrated Stedman's Medical Dictionary. Baltimore, Williams & Wilkins.

Stinchcomb, A. L. (2003). "Xenobiotic Bioconversion in Human Epidermis Models." Pharm Res. **20**(8): 1113-8.

Storm, J. E., S. Collier, et al. (1990). "Metabolism of Xenobiotics during Percutaneous Penetration: Role of Absorption Rate and Cutaneous Enzyme Activity." Fundamental and Applied Toxicology **15**: 132-141.

van Ruissen, F., M. Le, et al. (1998). "Differential effects of detergents on keratinocyte gene expression." J. Invest Dermatol **110**(4): 358-363.

Ward, R. K., A. W. Hubbard, et al. (1998). "Human Keratinocyte Cultures in an In Vitro Approach for the Assessment of Surfactant-induced Irritation." Toxicology in Vitro **12**: 163-173.

Wenninger, J. A. and J. G.N. McEwen, Ph.D, J.D, Eds. (1995). International Cosmetic Ingredient Dictionary. Washington DC, The Cosmetic, Toiletry, and Fragrance Association.

Windholz, M., S. Budavari, et al., Eds. (1976). The Merck Index. Rahway, Merck & Co., Inc.

Yourick, J. J. and R. L. Bronaugh (2000). "Percutaneous Penetration and Metabolism of 2-Nitro-p-phenylenediamine in Human and Fuzzy Rat Skin." Toxicol Appl Pharmacol **166**: 13-23.

Yourick, J. J., M. L. Koenig, et al. (2004). "Fate of Chemicals in Skin after Dermal Application: Does the In Vitro Skin Reservoir Affect the Estimate of Systemic Absorption?" Toxicol Appl Pharmacol.

Zhu, M., P. P. Zhou, et al. (2002). "Extraction of lipids from *Mortierella alpina* and enrichment of arachidonic acid from the fungal lipids." Bioresource Technology **84**(1): 93-95.

Ziboh, V. A., C. C. Miller, et al. (2000). "Metabolism of polyunsaturated fatty acids by skin epidermal enzymes: generation of antiinflammatory and antiproliferative metabolites." American Journal for Clinical Nutrition **71**(1 Suppl): 361S-6S.

Zuang, V., M. Balls, et al. (2002). "Follow-up to the ECVAM Prevalidation Study on In Vitro Tests for Acute Skin Irritation." ATLA **30**: 109-129.

Zuang, V., C. Rona, et al. (2000). "Detection of Skin Irritation Potential of Cosmetics by Non-Invasive Measurements." Skin Pharmacology and Applied Skin Physiology **13**: 358-371.