1 THEORETICAL BACKGROUND

1.1 HUMAN SKIN

Basic skin functions

The skin is a complex organ, which covers the entire body surface as a flexible shield with the primary function to protect the organism and act as permeability barrier to the environment. In terms of chemical composition, the skin is made up from about 70% water, 25% proteins and 3% lipids. The reminder includes minerals, nucleic acids, glycosamines, proteoglycans and numerous other chemicals. Skin has several protective functions (Wagner *et al.*, 2002):

1. A chemical barrier in two directions, controlling the loss of water, electrolytes, and other body constituents while barring the entry of harmful or unwanted compounds from the environment.
2. A microbiological barrier preventing the penetration of microorganisms through the intact *stratum corneum*.
3. A radiation barrier against UV light - as a result of producing melanin by the melanocytes in the basal layer upon ultraviolet light stimulation.
4. The temperature regulation of the body. The skin is responsible for maintaining the body temperature at approximately 37°C.

Other important functions of the human skin are sensation, storage and synthesis of important molecules, excretion and absorption.

Basic structure of the skin

The skin is composed of two layers: the outer epidermis and the underlying dermis. The two layers are firmly associated and together form a barrier that ranges in thickness from 0.5 mm (e.g. eyelid) to 4 mm (e.g. palm or sole). The epidermis and dermis are separated by a basement membrane.

A third subcutaneous layer is to be found below the dermis and is composed mainly of adipocytes. Although this layer is not technically part of the skin, it plays an integral role by acting as a heat insulator and shock absorber (Figure 1).

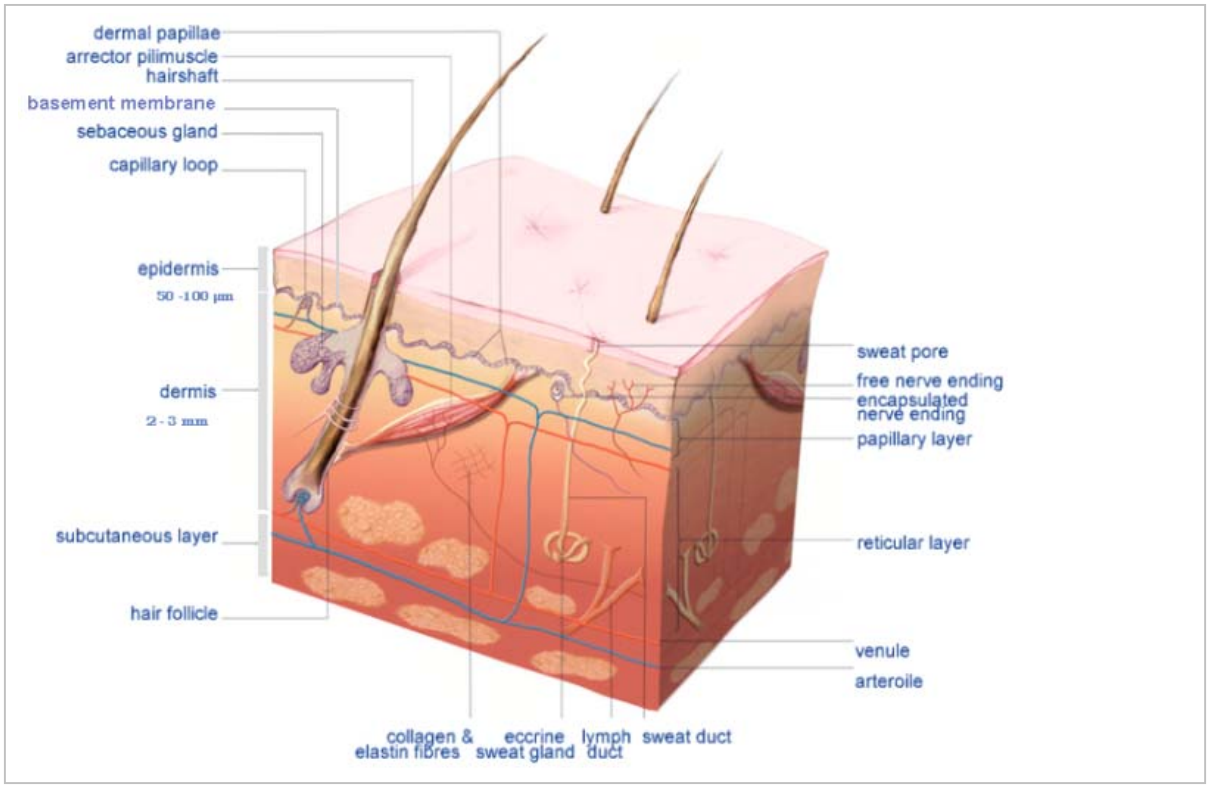


Figure 1. Basic structure of the human skin.
Downloaded from: http://vrc.belfastinstitute.ac.uk/resources/skin/skin.htm

The Epidermis

The epidermis is the superficial layer of the skin and provides the first barrier of protection from the invasion of foreign substances into the body (Snell, 1967). Epidermis consists of stratified squamous epithelium with an underlying basement membrane. The principle cell of the epidermis is the keratinocyte. Other constituents of the normal epidermis are Langerhans cells, melanocytes, Merkel cells and sporadic T-lymphocytes.

Epidermis is subdivided into the following strata (beginning with the outermost layer): *stratum corneum* (SC), *stratum granulosum* (SG), *stratum spinosum* (SS) and *stratum basale* (SB). In the thick skin on the soles of the feet and the palms of the hands, the *stratum lucidum* (SL) can be found (Figure 2).

The protection of the skin is provided primarily by the *stratum corneum*, the superficial region which is 10-40 µm thick (15-20 layers, at palms and soles 5-10 fold). The SC provides primary barrier to percutaneous absorption of compounds as well as to water loss. Underlying the SC is the viable epidermis (40-150 µm thick), which is responsible for generation of the *stratum corneum*.

The epidermis is a perpetually renewing tissue whereby keratinocytes arise from the basal layer (*stratum basale*) and move through a series of cellular differentiation events until they are finally sloughed off from the outer *stratum corneum*. This process is called desquamation, and takes in healthy skin about 14 - 30 days.

The Dermis

The dermis is 2-3 mm thick and contains a number of structures including blood vessels, nerves, hair follicles, smooth muscle, glands and lymphatic tissue. The main cell types of the dermis are fibroblasts, macrophages, and mast cells. Fibroblasts are responsible for secreting collagen, elastin and proteoglycanes that give the support and elasticity of the skin (Eckert, 1992). Both, the cells and the matrix have an influence on the rate and extent of differentiation of the keratinocytes and the formation of the epidermal layer (Eckert, 1989; Schäfer *et al.*, 1996).

The Dermis is structurally divided into two areas: a superficial area adjacent to the epidermis, called the papillary region, and a deep thicker area known as the reticular region (Figure 1). The reticular layer of the dermis consists of dense irregular connective tissue, whereas the papillary layer is mainly made up of loose connective tissue. The reticular layer of the dermis is important in giving the skin its overall strength and elasticity, as well as housing other important epithelial derived structures such as glands and hair follicles.

The Subcutaneous Tissue

The subcutaneous tissue is underlying the dermis, and is composed of a network of adipocytes (fat cells) arranged in lobules, fibroblasts, lymphocytes and mast cells. The subcutaneous fat layer serves as a heat insulator, mechanical cushion and stores readily available high-energy chemicals (Schäfer *et al.*, 1996; Wagner *et al.*, 2002).

Skin Appendages

The skin appendages present about 1% of the total skin surface layer (Schäfer *et al.*, 1993) and include mainly sweat glands, hair follicles, sebaceous glands and nails.

Sweat glands (apocrine and eccrine) are present in large numbers (about 400 glands/cm^2) in the human skin (Schäfer *et al.*, 1996). Apocrine glands emerge into follicular duct and are located in the axilla and pernial regions in adults. Eccrine sweat glands are smaller than apocrine glands and are spread over the whole body surface exept from mucosal tissue. The glands excrete sweat via the sweat duct to the skin surface.

Hairs are distributed over the entire body except the palms and soles. Hair consists of the hair shaft, which is the visible part of the hair, and the hair follicle. The growing hair is formed by dermal papillae cells located at the base of hair follicle. Hair follicles may be an important site for percutaneous penetration, although their surface area accounts for only 0,1 - 1% of the total surface area of the skin (Illel *et al.*, 1991).

Sebaceous glands are holocrine glands. They are found in all regions covering human body at a density of 1-2 glands per hair follicle. The activity of these glands varies

according to site and age (Blume *et al.*, 1991). Together, a hair follicle and its associated sebaceous gland are called a pilosebaceous unit.

Appendages are often envisaged as channels bypassing the *stratum corneum* barrier and are generally thought to facilitate the dermal absorption of topical agents.

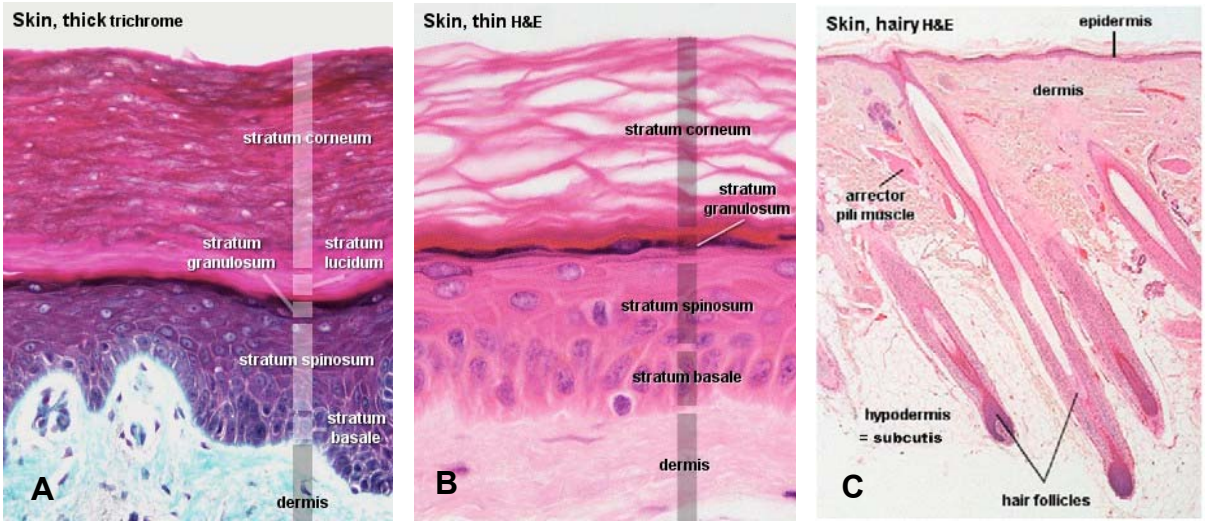


Figure 2. The characteristics of the skin with regard to location on the body.

A) Thick skin is present on the soles of the feet and the palms of the hands. It has a larger *stratum corneum* with a higher keratin content.

B) Thin skin is present on the bulk of the body and has a smaller *stratum corneum* and fewer papillae ridges. It contains hair follicles and is softer and more elastic.

C) Hairy skin is present on the scalp and genitals. It contains many hair follicles and increased number of sebaceous glands.

Pictures downloaded from: http://www.lab.anhb.uwa.edu.au/mb140/CorePages/Integumentary/Integum.htm

1.1.1 FUNDAMENTALS OF THE CUTANEOUS BARRIER

The principal role and function of the skin is to act as a two-way barrier, preventing the loss of water, electrolytes and other body constituents as well as the entry of substances and microorganism from the environment. Before a chemical penetrant can reach the dermis and thus enter the blood circulation, it must pass several potential barriers. These are surface hair (if any), the *stratum corneum*, the remainder of the viable epidermis and parts of the dermis. Using water diffusion to indicate permeability, the resistance of isolated epidermis appeared almost as good as that of whole skin. Furthermore, this and other experiments identified the SC as the principal barrier layer against trans-epidermal water loss (TEWL) (Fartasch, 1995, Salminen and Roberts, 2000, Leblanc, 2004).

The highly resistant structure of the SC is determined by the chemical composition of protein-enriched corneocytes and the surrounding extra-cellular lipids. Elias (1981, 1983, 1992) described the architecture of the *stratum corneum* as a two-compartments system, composed of a series of "bricks" (corneocytes) bounded in "mortar" (lipids) (Figure 3).

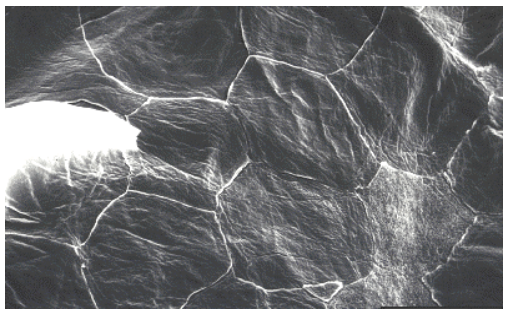


Figure 3. The architecture of the *stratum corneum.* Corneocytes paving in the scanning electron microscopy. Downloaded from: www.skin-science.com

Composition of corneocytes:

The corneocytes consist of about 80% of different types of keratin. Keratin mix provides them structural stability, strength and elasticity (Vicanova, 1997; Eckert, 1989). The major keratins presented in corneocytes are Keratin K1 and Keratin K10, which are produced in suprabasal layers of epidermis. In the lower portion of the *stratum corneum*, the keratin filaments are associated with filaggrin, which is in the upper parts of the SC completely degraded to free amino acids, serving as moisturizing components (Elias 1992, Dale *et al.*, 1994, Vicanova, 1997).

The corneocytes are surrounded by a chemically highly resistant envelope (also called cornified envelope), which basically consists of three layers and contains mainly proteins loricin, small proline rich proteins 1 and 2, and fillagrin (Vicanova, 1997).

Composition of *stratum corneum* lipids:

The lipids of *stratum corneum* are known to be important regulators of skin permeability. An essential role in the lipid barrier formation play characteristic ovoid organelles (lamellar bodies) appearing in the cells of *stratum granulosum*. These organelles are enriched mainly in polar lipids and catabolic enzymes, which deliver the lipids required for the generation of the *stratum corneum* (Grayson *et al.*, 1985; Wertz *et al.*, 1989, Bouwstra *et al.*, 2003).

After the extrusion of lamellar bodies at the *stratum granulosum*/*stratum corneum* interface, the polar lipid precursors are enzymatically converted into nonpolar products and assembled into lamellar structures surrounding the corneocytes. Hydrolysis of glycolipids generates ceramides (CER), while phospholipids are converted into free fatty acids (FFA) (Figure 4).

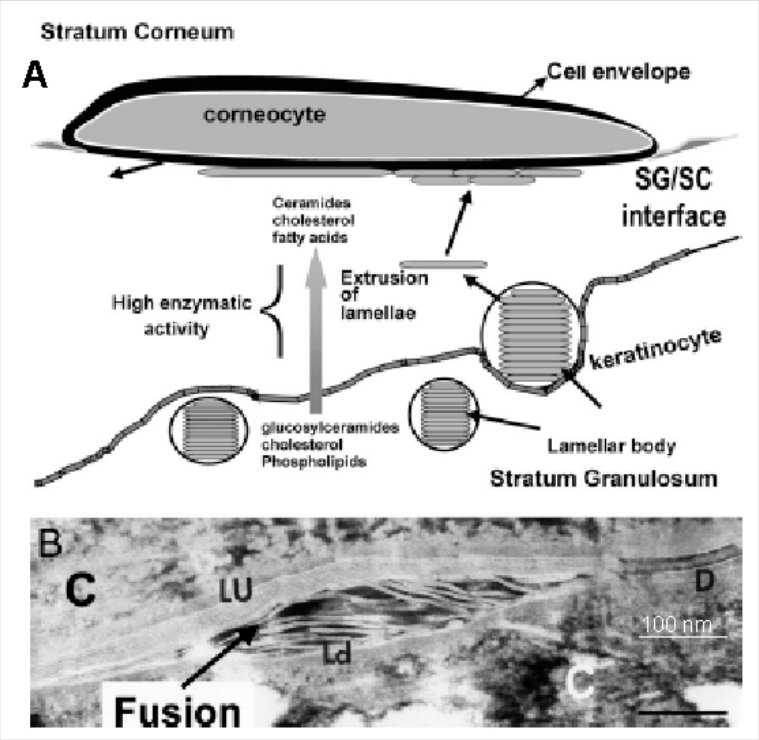


Figure 4. The lamellar extrusion process (Bouwstra *et al.*, 2003).
At the interface between stratum granulosum and stratum corneum lamellar bodies are extruded into the intercellular regions. The lipid content is rearranged into long lipid lamellae filling the intercellular regions in the stratum corneum. Simultaneously major changes in the lipid composition occur.
(A) Schematic drawing of the lamellar body extrusion process at the stratum granulosum-stratum corneum interface (SG/SC).
(B) An electron micrograph of the fusion of the lipid lamellae along the corneocyte.
C = corneocyte; D = desmosome; LU = Landmann unit consisting of a broad-narrow-broad sequence; Ld = lipid disk

It is generally accepted that the intercellular lipids are composed mainly of cholesterol, free fatty acids and ceramides. Furthermore, cholesterol esters and small amounts of triglycerides, glucosphingolipids and cholesterol sulphate were detected in human stratum corneum (reviewed in details in Yerdley 1986; Elias and Menon, 1991; Schürer and Elias 1991, Vicanova 1997).

Ceramides

Nine free ceramides have been identified in human *stratum corneum* (Bouwstra *et al.*, 2003). They differ in molecular structure and polarity. In general, ceramides are composed of a long-chain sphingosine or phytosphingosine base (varying in the position and number of hydoxyl groups, double bounds and chain lenght) with an amide-linked nonhydroxy fatty acid or a hydroxy fatty acid. Hydroxyceramides and dihydroxyceramides, covalently bound to the proteins surrounding cornified envelopes, are also important components of the *stratum corneum*. The site of covalent attachment of the lipid is unknown, though it has been hypothesized to be the cornified protein involucrin (Wertz *et al*., 1989, Vicanova, 1997).

Free Fatty Acids

Free fatty acids account about 10% of the *stratum corneum* lipids. This lipid fraction consists predominantly of saturated fatty acids, having more than 20 carbon atoms. Lipid analysis of free fatty acids isolated from human SC revealed presence of lignocerin acid (C24:0), hexacosanoic acid (C26:0) and stearic acid (C18:0), and small amounts of palmitic (C16:0), arachidic (C20:0), docosanoic (C22:0) and hexacosanoic (C23:0) acid. Oleic (C18:1) and linoleic (18:2) acid were the only unsaturated fatty acids detected in the *stratum corneum* (Vicanova, 1997).

Cholesterol, cholesteryl esters and cholesteryl sulphate

Other important lipids in the *stratum corneum* are cholesterol and cholesterol sulphate. Free cholesterol is the second most abundant lipid in the SC, amounting to 25% of the extractable lipids. In addition, the SC lipids contain about 5 % of cholesterol sulphate and small amounts (typically 2 – 5% w/w) of fatty acid esters of cholesterol (Downing, 1992).

Although cholesteryl sulphate is present only in small amounts in the human epidermis, it is important for the desquamation process of *stratum corneum* (Lampe *et al.*, 1983, Downing, 1992; Bouwstra *et al.*, 2003).

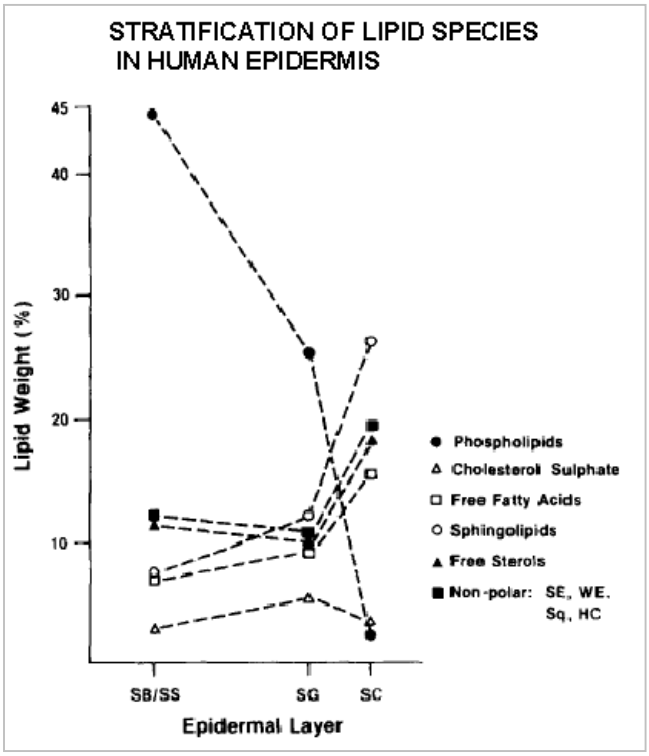


Figure 5. The major fluxes in lipid content occuring during epidermal differentiation (Lampe *et al.*, 1983).
SB/SS - stratum basale/stratum spoinosum, SG - straum granulosum, SC Stratum corneum
Sq - squalene, WE - wax esters, SE- sterol, HC- n-alkanes

1.1.2 PENETRATION PATHWAYS THROUGH THE SKIN

When a diffusing compound gets into contact with intact skin, two major routes of penetration are feasible:

a) through the skin appendages, like hair follicles and sweat ducts,
b) across the intact *stratum corneum* in between these appendages via:
   - intercellular route and /or
   - transcellular route

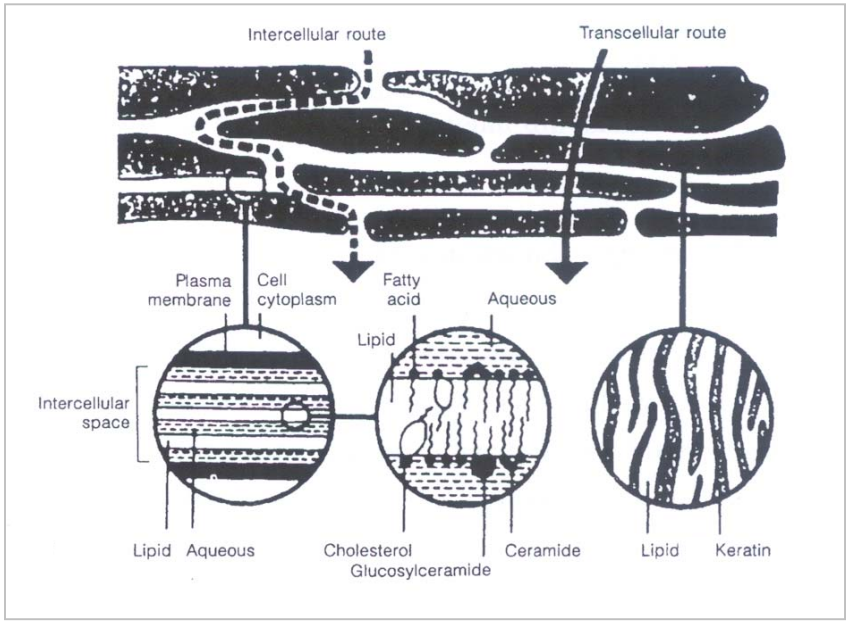


Figure 6. Model of penetration pathways.
Permeation through the *stratum corneum* (trans-corneal permeation) may be considered to occur through the intercellular lipid domain or through corneocytes (transcellular route) (according to Elias 1983 and Schaefer, 1996).

Penetration route via skin appendages

Skin appendages cover less than 1% of the total skin area (Moghimi *et al.*, 1999) and usually this route does not contribute considerably to the steady state flux. However, in the early stages of penetration, and in case of large polar substances, diffusion trough the appendages may be relevant as these agents cross the intact *stratum corneum* only with difficulties (Wagner *et al.*, 2002).

Intercellular and transcellular routes of penetration

Due to the relatively impermeable character of the cornified envelope, the major route of penetration resides in the tortuous pathway between the corneocytes as revealed by confocal laser scanning microscopy and X-ray analysis studies (Bouwstra *et al.*, 2003).

Several factors influence the rate of diffusion of compounds across the *stratum corneum*. The following parameters are relevant to the invasion rate:

- physicochemical characteristics of the permeant (e.g. molecular weight, chemical structure, lipopholicity etc.),
- concentration of the permeant,
- vehicle used,
- diffusivity of the compound within the *stratum corneum*.

In general, lipophilic compounds of low molecular weight can permeate the skin better than can those that are hydrophilic or of high molecular weight. This is due to the low water and high lipid content of the *stratum corneum*, which allows lipophilic agents to penetrate more readily (Salminen and Roberts, 2000).

However, if the skin becomes hydrated on prolonged exposure to water, its effectiveness as a barrier to hydrophilic substances declines. Often the skin of laboratory animals is covered with plastic wrap to enhance the hydration of the skin and increase the rate of uptake of agents applied to the surface of the skin (Schaefer, 1996; Salminen and Roberts, 2000).

The rate of diffusion through the epidermis varies among anatomical sites and is not solely a function of skin thickness. In wounded or sick skin, the barrier is compromised and direct access to the systemic circulation is available.

1.1.3 EPIDERMAL RESPONSE TO EXOGENOUS SKIN BARRIER DISTURBANCE

Skin exposure to xenobiotics (including chemicals, drugs or cosmetics) can lead to a variety of skin reactions. The most frequently seen responses to skin barrier disturbance at early stage is skin dryness (loss of the water) and skin irritation manifested by formation of erythema and oedema. Adverse reactions as sensitisation leading to contact allergy and pigmentation are frequently observed.

Substances entering the *stratum corneum* may cause delipidation and protein denaturation (Welss *et al.*, 2004). Once the balance between different lipid classes is disturbed, the barrier function of the skin decreases (Ponec, 1992; Bouwstra *et al.*, 2003). Damage of the epidermal barrier leads to enhanced transepidermal water loss and, more seriously, enhanced penetration of toxic compounds to viable keratinocytes.

Many compounds penetrating SC are able to disrupt membranes of viable cells, resulting in the release of cytoplasm into extracellular spaces (Osborne and Perkins, 1994). The cytoplasm of keratinocytes of all epidermal layers contains the pro-inflammatory cytokine IL-1α, which induces an inflammatory cascade shown in Figure 7 (Luger, 1989; Nickoloff and Naidu, 1994; Corsini and Galli, 1998, Van de Sandt *et al.*, 1999, Welss *et al.*, 2004). The release of IL-1α induce secondary molecular responses, followed by morphological alterations and finally the onset of typical symptoms of contact dermatitis (e.g. oedema, erythema, itching and pain).

Irritation may also be induced by oxidative stress (Rogers *et al.*, 2001; Willis *et al.*, 1998; Camera, 1998). Reactive oxidative species (ROS) cause cellular damage by oxidising nucleic acids, proteins and membrane lipids, which may lead in altered gene expression or direct cytotoxicity (Camhi *et al.*, 1995; Allen and Tresini, 2000, Welss *et al.*, 2004).

Another mechanism by which xenobiotics may affect skin barrier is the modulation of the lipid bilayers of cell membranes (Wells *et al.*, 2004). At sub-cytotoxic concentrations, the interaction of compounds with cellular membranes may change membrane fluidity (Fulbright *et al.*, 1997) which may consequently have an impact receptor-mediated signal transduction (Rossette and Karin, 1996) and thus induce irritating responses.

In summary, numerous mechanisms are involved in the epidermal response to exogenous barrier disturbance. Better understanding of these complicated processes is important for the development of adequate toxicological tests.

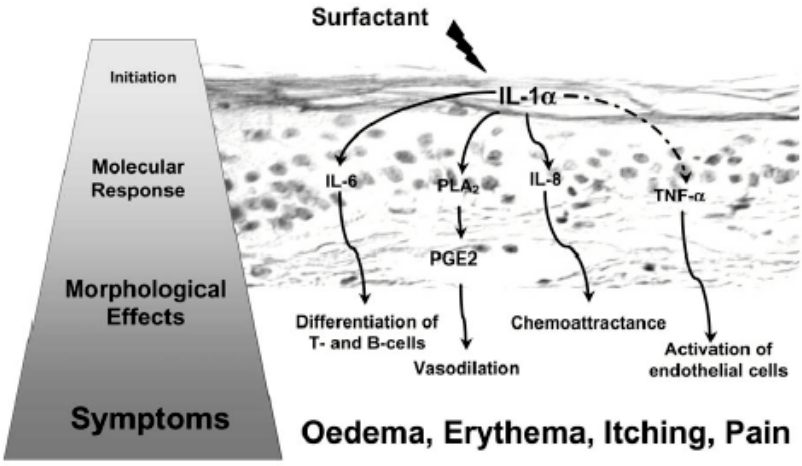


Figure 7. Skin irritation induced by surfactants (Wells *et al.*, 2004).
Surfactants initiate the release of IL-1α, subsequently leading to the induction of secondary mediators (molecular responses), followed by morphological alterations and, finally, the onset of typical symptoms of contact dermatitis.
(IL-1α, inflammatory cytokine; TNF-α, tumour necrosis factor; IL-8, chemotactic cytokine; PLA_2, phospholipase A_2, PGE_2 - prostaglandin E_2)

1.2 RECONSTRUCTED HUMAN SKIN AS AN ALTERNATIVE TEST MODEL IN TOXICITY TESTING

The prime objective of toxicology tests is to identify the nature of any hazard that may be associated with a substance and to use the evidence as a part of risk assessment for man (Chamberlain and Parish, 1990). The use of animals in toxicity testing is the primary method of assessing human hazard. Although animal studies have a variety of limitations, their main advantage lies in the broad experience of using the results of animal studies for predicting human hazard (Purchase, 1990).

As a part of the overall philosophy of reducing the number of animals used for toxicological experiments, *in vitro* and *ex-vivo* methods have particular value. The most frequently used *in vitro* tests in toxicology are based on immortalised cell lines, which allow for a high level of test standardisation, reduce of experimental costs and number of test animals. However, the use of cell lines is limited. Mainly due to the lack of barrier, monolayer cell cultures frequently tend to overpredict toxic effects.

For the evaluation of skin lesions after exposure to xenobiotics, ex-vivo human skin might be regarded as the ideal (and preferred) alternative test model. However, the supply of fresh human skin by surgical departments is minimal and irregular. Despite the difficulties associated with being at the bottom of the priority list, there are also legal and ethical issues linked to the use of human tissues for commercial purposes, such as routine testing for commercial purposes (Benford *et al.*, 1997). Therefore, a considerable interest has focused on three-dimensional reconstructed human skin models that became available in 1980's.

These models have been initially developed for treatment of patients with severe burns. Some of them have been later adapted for testing the potential dermato-toxic effects of chemicals and products (Roguet and Schaefer, 1997). Today several reconstructed human skin models are commercially available, e.g. the EPISKIN™ model (EPISKIN-SNC, France), the EpiDerm™ model (MatTek Corporation, MA, USA) the SkinEthic™ model (SkinEthic Laboratories, France), EST-1000™ (Cell Systems GmbH, Germany) and Phenion ™ (Phenion GmbH, Germany).

In principle, reconstructed human skin models can be used for almost all domains of topical toxicity testing, e.g. skin corrosion, skin irritation, skin penetration and phototoxicity. An extensive research has been focused on exploring possibilities of incorporating various cell types or additional skin structures into the skin reconstructs.

Nowadays, epidermal models (containing keratinocytes), "full thickness" models (containing keratinocytes and fibroblasts) and reconstructed models containing melanocytes are commercially available. Special reconstructed human tissues were developed also for ocular toxicity testing (e.g. EpiOcular ^{TM} model from MatTek or SkinEthic's reconstructed corneal model). Advanced skin equivalents containing Langerhans cells (intended for skin sensitisation and allergy testing) are currently under development.

The advantages and limitation of the reconstructed human skin models are briefly presented in Table 1. In summary, although the reconstructed human skin models at current stage of development do not resemble the human skin by 100%, they can be used e.g. in skin research or in *in vitro* toxicology and pharmacology.

Table 1: Advantages and limitations of the reconstructed humans skin models and human skin as test models. (With modification according van de Sandt *et al.*, 1999).

Parameter	**Human skin ex-vivo**	**Reconstructed human skin model**	**Monolayers**
Barrier function	Present for limited period	Present, but less developed	Absent
Presence of cell types	All resident cell types	Incomplete, but sufficient for certain type of tests	Incomplete
Recovery assessment	Limited	Limited	No
Variability of results	Medium	Low due to the high standardisation of the manufacturing process and standardisation of the test techniques (GMP and GLP)	Low due to the high standardisation of cell culture techniques (GLP)
Type of variability	Between donors	Between batches	Between batches
Availability	Limited	Good	Good
Cost	Low	Medium / High	Low / Medium
Predictive value	Good, but rarely used	Medium to high (in case of validated methods)	Medium to high (in case of validated methods)

1.2.1 ESTABLISHMENT OF RECONSTRUCTED HUMAN SKIN MODELS

The first reconstructed human (dermal) model was introduced by Bell *et al.* in 1979. It consisted of a mixture of living fibroblasts embedded in a gel composed of collagen fibrils. Another dermal equivalent, nylon meshes populated with fibroblasts, was developed by Naughton *et al.* in 1989. The complete epidermal differentiation, typical for native epidermis, was achieved by exposure of the keratinocyte culture to the air (Pruniéras *et al.*, 1983). Since then, various advanced technologies have been developed to reconstitute human skin *in vitro.* However, the fundamentals of the skin reconstruction from primary cells did not change (Roguet and Schäfer, 1997) and are described in following paragraphs.

In order to obtain human skin cell cultures keratinocytes and fibroblasts are isolated from skin biopsies. Commonly juvenile foreskin or adult breast skin from surgery is used; the subcutaneous fat is removed and the biopsies are subsequently cut into small pieces. After separation of epidermis from dermis (mechanically or enzymatically, e.g. using trypsine or combination of trypsin and dispase) each layer is digested to obtain a single cell suspension (Figure 8) (Prunieras *et al.*, 1976, Ghalbzouri 2004).

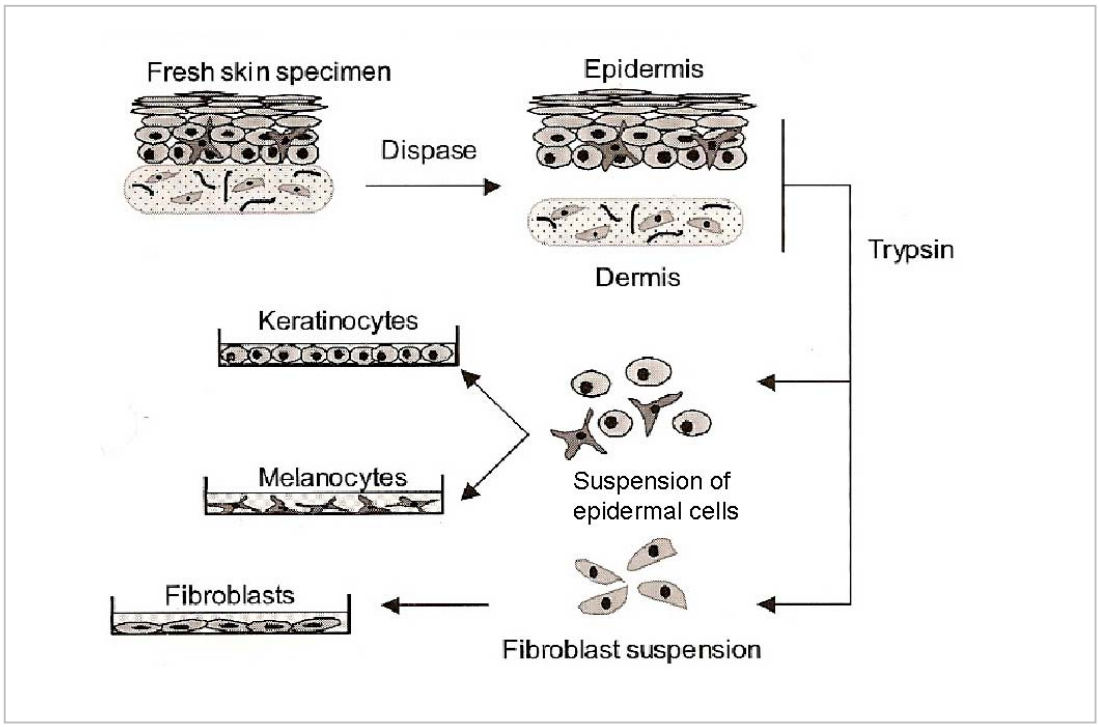


Figure 8. Schematic overview of monolayer cell culture establishment from the fresh skin biopsies (Ghalbzouri, 2004). Skin specimens are cut and incubated with dispase to separate the epidermis from the dermis. After a short incubation period with trypsine, an epidermal cell suspension is obtained for establishment of keratinocyte or melanocyte cultures. Fibroblasts can be harvested from outgrowing dermal fragments or after enzymatic treatment of the dermis (Ghalbzouri, 2004).

Reconstructed human skin models are generated by seeding dissociated keratinocytes on an appropriate substrate, e.g. fibroblast populated collagen matrix, de-epidermised dermis, collagen or artificial membrane (Figure 9). After attachment of cells to the substrate, the cultures are kept submerged in culture medium for several days to stimulate proliferation and formation of multilayer tissue (Asselineau *et al.*, 1985; Boddé *et al.*, 1990; Ponec *et al.*, 1988; Regnier *et al.*, 1993; Boelsma *et al.*, 1999).

Subsequently, the cultures are exposed to the air (at the air-liquid interface) to induce further differentiation. Exposure of the epidermal cells to the air is crucial for the appearance of a multi-layered tissue with a coherent *stratum corneum* (Asselineau *et al.*, 1985; Boddé *et al.*, 1990; Ponec *et al.*, 1988; Regnier *et al.*, 1993; Boelsma *et al.*, 1999).

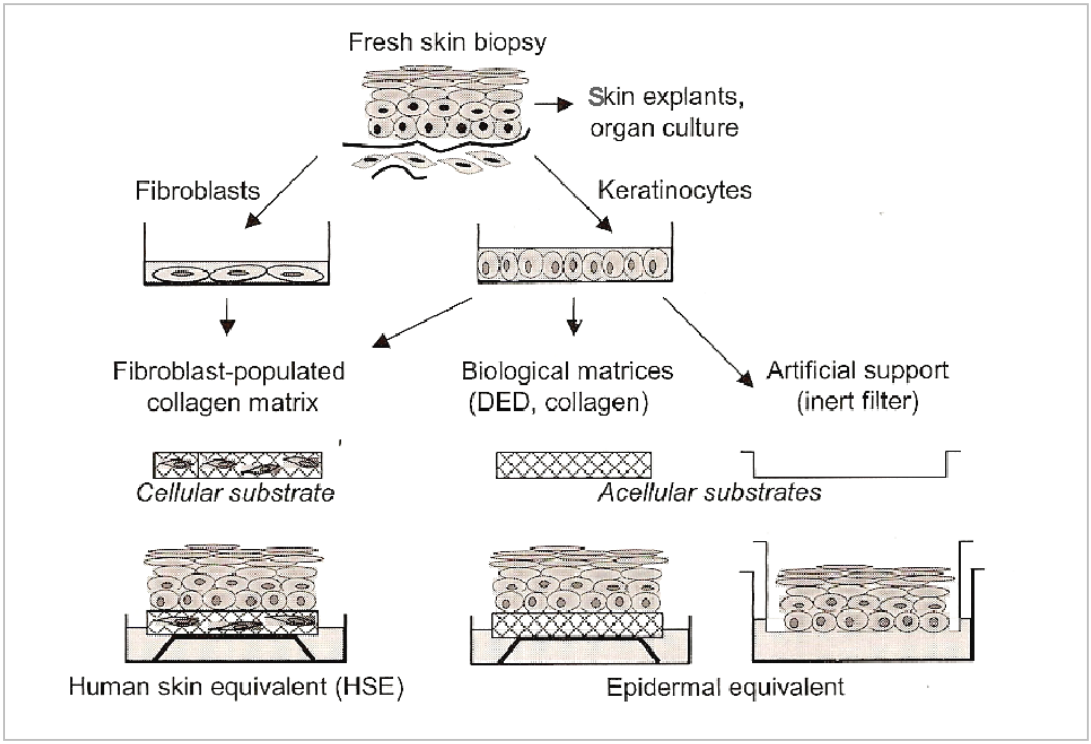


Figure 9. Schematic overview of the generation of three-dimensional skin equivalents (Ghalbzouri, 2004).
Keratinocyte and fibroblast cultures are established from fresh skin biopsies. After trypsinisation, keratinocytes are seeded on a cellular or acelular substrate (Ghalbzouri, 2004).

The reconstructed human skin models can be generated in serum-containing or serum-free media supplemented with various growth-promoting agents, such as hormones, vitamins, growth factors and fatty acids. The medium types and supplements used for cultivation of skin recombinants are summarised in Table 2.

Table 2: Commonly used culture media (according to Boelsma, 1997).

Basal media	
Eagle's Basal Medium (BME), Dulbecco's Modified Eagle's Medium (DMEM), DMEM/Ham's F-12 Nutrient Mixture, Eagle's Minimal Essential Medium, RPMI Medium 1640, Medium 199 (M199), MCDB 153	
Supplementation*	
Serum	calf, human
Hormones	insuline, hydrocortisone, bovine pituitary extract
Growth factors	EGF, KGF, TGFα, IGF, FGF
Vitamines	lipid soluble (e.g. retinoids), water soluble (e.g. ascorbic acid)
Binding proteins	albumin
Trace elements	Cu, Fe, Mn, Ni, Se, Zn
Lipids	fatty acids

***** *Supplementation is dependent on nutritional requirements of the cell types under study and on the presence or absence of serum in medium.*

Different types of reconstructed human skin models

Various human skin equivalents can be produced, depending on the substrate used for the model construction (Figure 10). The three basic types are:

- reconstructed epidermis (keratinocytes grown on acellular substrate),
- reconstructed dermal equivalents (dermal substrates populated with fibroblasts),
- reconstructed "full thickness models" = models composed from epidermal and dermal compartment (contain keratinocytes grown on dermal substrate populated with fibroblasts).

Substrates used for cultivation of keratinocytes may consist of artificial or biological components or their combinations. The most frequently used substrates are:

- de-epidermised dermis obtained from human cadaver skin with preserved basement membrane components (Freeman *et al.*, 1976; Ponec *et al.*, 1988; Régnier *et al.*, 1986, 1988; Ghalbzouri *et al.*, 2002),
- inert filters composed of cellulose acetate or polycarbonate, e.g. EpiDerm (MatTek USA; Cannon *et al.*, 1994), SkinEthic (SkinEtic laboratories, France; Rosdy *et al.*, 1990), EST-1000 (Cell Systems, Germany; Hoffmann *et al.*, 2005),
- sheets composed of collagen e.g. EPSKIN, Lyon, France (Tionis *et al.*, 1991)
- fibroblast populated collagen type I gels (Asselineau *et al.*, 1985; Bell *et al.*, 1991)
- fibroblasts grown on nylon meshes (Naughton *et al.*, 1989)
- lyophilised collagen-glycosaminoglycan membranes (Boyce *et al.*, 1990).

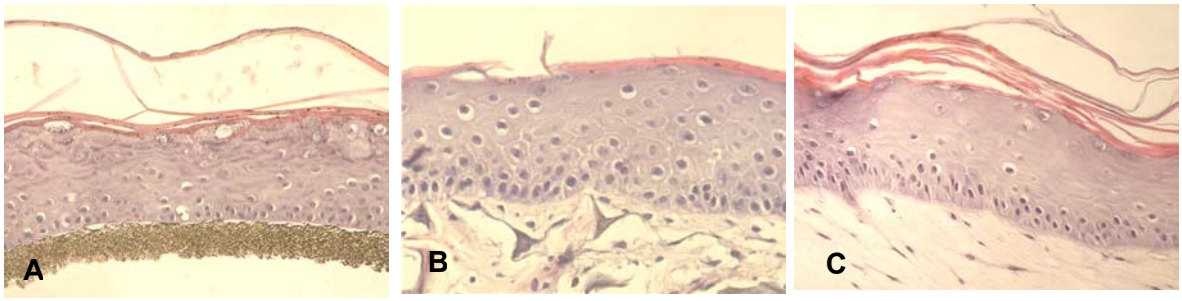


Figure 10. Different types of the reconstructed human skin models.
A) reconstructed human epidermis model produced on acellular substrate (inert filter insert - EpiDerm EPI-200)
B) reconstructed human skin equivalent produced on artificial substrate populated with fibroblasts (Phenion model)
C) reconstructed human skin equivalent produced on collagen substrate populated with fibroblasts (EpiDerm FT-model)

The ideal reconstructed human skin model should possess all important structural elements and functions of normal human skin (see Figure 1 and Figure 2). Moreover, the models should be highly standardised, resulting in low variability within a testing batch and over the time.

1.2.2 RECONSTRUCTED HUMAN SKIN MODELS AND THEIR VALUE IN REGULATORY TOXICOLOGY

The development of new alternative toxicity testing methods (including those using reconstructed human skin models) has progressed rapidly over the last ten years. However, their acceptance in regulatory toxicology does not proceed quickly. The main obstacle is the fact that regulators accept new tests only if they allow classification and labelling of chemicals in the same way as the results of currently used animal tests. Moreover, the OECD has indicated that *in vitro* toxicity tests can be accepted for regulatory purposes only after a successful experimental validation study (Liebsch and Spielmann, 2002).

To approach the problem scientifically, European and American scientists interested in the validation of toxicity tests met twice in Amden (1990 and 1994), Switzerland, to agree on a definition of experimental validation and to define the essential steps in this process.

Validation was defined as the process by which reliability and relevance of a procedure are established for a particular purpose (Balls *et al.*, 1990), regardless whether the method is an *in vitro* or *in vivo* test.

In addition, the essential steps of the experimental validation process were defined. It was agreed that the process of development and validation of an alternative method

should consist of:

- test development in a single laboratory,
- the definition of a biostatistically based prediction model (PM),
- pre-validation stage,
- experimental validation under blind conditions in several laboratories in a ring trial,
- independent assessment of the results of the validation trial,
- regulatory acceptance (in case of successful validation) and definition of minimum performance standards of the method.

The importance of the efficient and experienced study management team, thorough selection of the test laboratories (preferably working according to the GLP rules) and the organisation of their work during the validation process was recognised as well (Fentem *et al.*, 1995; Balls and Fentem, 1997).

The remaining problem is the quality and availability of reference *in vivo* data as almost no *in vivo* assay has been validated in the past. In many cases, the data were generated without concerns about predictive power and applicability domain of the test. Consequently, insufficient *in vivo* data complicate development of relevant alternative methods and their validation.

Another, very special "post-validation problem" arose when several commercial products disappeared from the market after successful validation. In other words, a specific test assay was experimentally validated, but the product was not available after finalisation of the study. A clear example is reconstructed human skin model Skin^2 ZK1350^{TM}, where the production of the model was completely terminated already during the skin corrosion validation study. Another example was the commercial unavailability of the EPISKIN model, which lasted several years, after a change of the company ownership.

This situation led to re-consideration of the validation approach and selection of assays into the validation trials. It was endorsed to support only validation of these methods, for which endpoints and test protocols are clearly described and which could be applied from one model to another one (Balls, 1997). This idea, supported by a successful "catch-up" skin corrosion validation study with EpiDerm model (Liebsch *et al.*, 2000), resulted in a "common protocol concept".

This concept is based on the assumption that reconstructed human skin models will perform comparably in a robust test protocol if they share similar structural and functional attributes. Therefore, the OECD Test Guideline 431 - *In vitro skin corrosion: Human skin model test* (OECD, 2004a), defines structural and performance criteria to allow new test systems (meeting these criteria) being considered as scientifically valid for skin corrosion testing.

The basic inquiries of the OECD TG 431 (OECD, 2004a) for a skin model quality are following:

"General Model Conditions":

1. Human keratinocytes should be used to construct the epithelium.
2. Multiple layers of viable epithelial cells should be present under a functional *stratum corneum*.
3. The *stratum corneum* should be multi-layered with a lipid profile necessary to produce a functional barrier with robustness to resist rapid penetration of cytotoxic markers.
4. The containment properties of the model should prevent the passage of material around the *stratum corneum* to the viable tissue. Passage of test chemicals around the *stratum corneum* will lead to poor modelling of the exposure to skin.
5. The skin model should be free of contamination with bacteria (including mycoplasma) or fungi.

"Functional Model Conditions":

1. The magnitude of viability is usually quantified by using MTT or other metabolically converted vital dyes. In these cases the optical density (OD) of the extracted (solubilized) dye from the negative control tissue should be at least 20-fold greater than the OD of the extraction solvent alone.
2. The negative control tissue should be stable in culture (provide similar viability measurements) for the duration of the test exposure period.
3. The *stratum corneum* should be sufficiently robust to resist the rapid penetration of certain cytotoxic marker chemicals (e.g. 1% Triton X-100). This property can be estimated by the exposure time required to reduce cell viability by 50% (ET50).
4. The tissue should demonstrate reproducibility over time and preferably between laboratories. Moreover, the model should be capable of predicting the corrosive potential of reference chemicals when used in the selected testing protocol.

By now, the skin corrosion test *in vitro* is the only, regulatory accepted method using reconstructed human skin models. However, several studies are currently performed which might extend the use of these unique tools in regulatory toxicology. These are mainly: the skin absorption pre-validation study - performed within a BMBF project (Schäefer-Korting *et al.*, 2006), the ECVAM Skin Irritation Validation study and the ECVAM Photopotency Feasibility Study.

2 OBJECTIVES OF THE THESIS

Advances in bioengineering of reconstructed human skin models have led to the development of standardised and highly reproducible models closely resembling native human skin. They can be widely used for many purposes, e.g. studies of basic skin reactions, metabolism, wound healing, skin penetration, transdermal drug delivery, assessment of efficacy of compounds and products etc. Reconstructed human skin models are currently used for screening purposes mainly in cosmetology and pharmacology. However, it has been shown that reconstructed human skin models may be also very useful in testing for regulatory purposes (e.g. *in vitro* skin corrosion test).

The overall aim of the work was to evaluate several reconstructed human skin models with regard to their use in regulatory toxicity - namely for skin corrosion, skin irritation and phototoxicity testing. Thus, the thesis aimed at the following tasks:

1. Evaluation of present-day quality of several commercially available reconstructed human skin models applying recommendations and criteria as described by the OECD Test Guideline 431.
2. Prove of transferability of the existing protocol for *in vitro* skin corrosion to various reconstructed human skin models in order to evaluate the idea of the “common protocol concept” (as described in chapter 1.2.2).
3. Based on the common protocol concept, to optimise and evaluate skin irritation protocols for EpiDerm and SkinEthic models for skin irritation validation studies.
4. Evaluation of applicability of the EpiDerm Phototoxicity assay as an adjunct test for testing of substances "over-predicted" in the validated 3T3 NRU-PT test (OECD TG 432).

3 INTRODUCTION INTO THE EXPERIMENTAL PART

3.1 CHARACTERISATION OF RECONSTRUCTED HUMAN SKIN MODELS

For the characterisation of reconstructed human skin models various methods are currently available. The most frequently used are summarised in Table 3.

Based on recommendation of OECD Test Guideline 431 (OECD, 2004a), the morphology of the models should be one of the first parameters verified. Well-developed reconstructed human skin models should show a stratified epidermis, consisting of *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum*. The model should exhibit at least four layers of viable cells. For a general evaluation of the morphology of the reconstructed human skin model, hematoxylin-eosin staining of vertical sections can be used. Detailed information about the level of the model development can be obtained when studying expression of differentiation markers, e.g. keratins, involucrin, loricin, fillagrin.

Attention should be focused on structure of the *stratum corneum* (SC) and its lipid composition. It has been demonstrated that differences exist between SC of reconstructed human skin models and SC of healthy human skin. Irregularities in the lipid composition of SC of reconstructed human skin models may explain higher permeation rate of some compounds, when compared to healthy human epidermis (Regnier *et al.*, 1990; Ponec, 1992; Bell *et al.*, 1991; Boelsma *et al.* 2000; Ponec *et al.*, 2002).

For the semi-quantitative analysis of the SC lipids an efficient and relatively simple method based on high performance thin layer chromatography (HPTLC) technique can be used. However, attention must me paid to a standardised extraction procedure, which presents the most critical step in the entire assay.

The OECD TG 431 states the *stratum corneum* should be sufficiently robust to resist the rapid penetration of certain cytotoxic marker chemicals (e.g. Triton X-100). Thus, the quality of the tissue barrier can be evaluated in a time-course assay, where the exposure time required to reduce cell viability e.g. by 50% is determined (= determination of ET50 value). Unfortunately, the test guideline does not describe, or gives reference, how the procedure should be performed.

Measurement of transepidermal electrical resistance (TEER) was further recommended as one of the suitable parameters to evaluate the barrier function. However, only a few studies with reconstructed human skin models employing this parameter were performed until now.

In this thesis, three reconstructed human skin models (EpiDerm, EPISKIN and SkinEthic) were evaluated, taking into account recommendations of the OECD TG 431. Tissue morphology was evaluated using light and confocal laser scanning microscopy, lipid content was determined by HPTLC technique. Barrier function of the *stratum corneum* was evaluated in a time-course assay, by determination of the ET 50 value after exposure to Triton X-100. In addition, TEER was measured with the aim to evaluate applicability of this endpoint as an additional quality test parameter.

Table 3: Methods for characterisation of reconstructed human skin models (according to Boelsma, 1999, with minor modifications).

	Method	**Endpoint**
Tissue morphology	Light microscopy	
	- Hematoxylin/eosin staining	- overall tissue morphology
	- Immunohistochemistry	- specific differentiation markers
	Electron microscopy (EM) techniques	
	- transmission EM	- overall detailed tissue morphology (OsO_4 post-fixation), intrcelullar lipid lamellae (RuO_4 post-fixation)[1,2]
	- freeze-fracture EM	- inter-corneocyte organisation (lipids, desmosomes)[3]
	- particle microprobes	- ion distribution[4]
Lipid analysis	High performance thin layer chromatography	- evaluation and semi-quantitative analysis of individual lipid classes[5]
	Gas chromatography	- determination of free fatty acid profile[6]
	X-ray diffraction (small and wide angle)	- stacking of lipid lamellae and repeat distances in the SC lamellar phase[7]
Permeability properties (quality of SC and barrier function of the model)	Confocal laser scanning microscopy	- visualisation of penetration pathways in time and depth[8]
	Franz cell techniques	- penetration speed and quantitative determination of the penetrated compound (permeability of the models)[9]
	TEER (transepidermal electrical resistance)	- changes in the electrical resistance indicating the barrier quality[10]
	TEWL (transepidermal water loss)	- changes in the in transepidermal water loss[11, 12]

1. Fartasch et al., 2003; 2. Van der Meulen, et al. 1996; Bodde et al., 1990; Forslindt et al., 1997; 5. Ponec and Weerheim, 1990; 6.Christie, 1989; 7. Bouwstra et al., 1995; 8. Shotton et al. 1989; 9. Franz 1978; 10. Kim, 2002. 11. Heylings et al. 2001; Heylings et al., 2003.

3.2 SKIN CORROSION TESTING

According to the definition given by the OECD Test Guideline 404 (OECD, 2002), skin corrosion is defined as production of irreversible damage to the skin; namely, visible necrosis through the epidermis and into the dermis following the application of a test substance for up to four hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of observation at 14 days, by discoloration due to bleaching of the skin, complete areas of alopecia, and scars.

The potential of chemicals to cause skin corrosion is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals (Botham *et al.*, 1998). According to current international regulatory requirements, assessment of skin corrosion is mandatory for all chemicals placed on the market.

3.2.1 SKIN CORROSION TEST *IN VIVO*

Testing of substances for skin corrosion in laboratory animals is not permitted anymore in Europe as validated alternative *in vitro* methods exist. However, for completeness, a brief explanation of the *in vivo* test is given below.

Depending on the reaction of the animal on exposure the *in vivo* test covers both skin corrosion as well as skin irritation with one test protocol. The test is performed on adult albino rabbits. Approximately 24 hours before the test, fur is removed from the test area. It is recommended to use only animals with healthy, intact skin. The test substance (a dose of 0.5 ml of liquid or 0.5 g of solid or paste) is applied in a single dose to a small area (approximately 6 cm^2) of skin and covered with a gauze patch. Untreated skin areas of the test animal serve as a control. It is strongly recommended to perform the test initially using one animal, especially when the substance is suspected to have corrosion potential.

Up to three test patches are applied sequentially to the animal. The first patch is removed after three minutes. If no serious skin reaction was observed, a second patch is applied at a different site and removed after one hour. If the observations at this stage indicate, that exposure can humanely be allowed to be extended a third patch is applied and removed after four hours. The responses are graded at each observation time.

If a corrosive effect (e.g. ulcers or bleeding) is observed after any of the three sequential exposures, the test is immediately terminated. If a corrosive effect was not observed after the last patch is removed, the animal is observed for 14 days, unless corrosion develop at an earlier time point.

Recently, general refinement provisions were included in the OECD Test Guideline 404 (OECD, 2002). This updated version recommends to apply sequential testing strategy prior to undertake any *in vivo* test. Example of decision-making in the testing strategy is given in Figure 11.

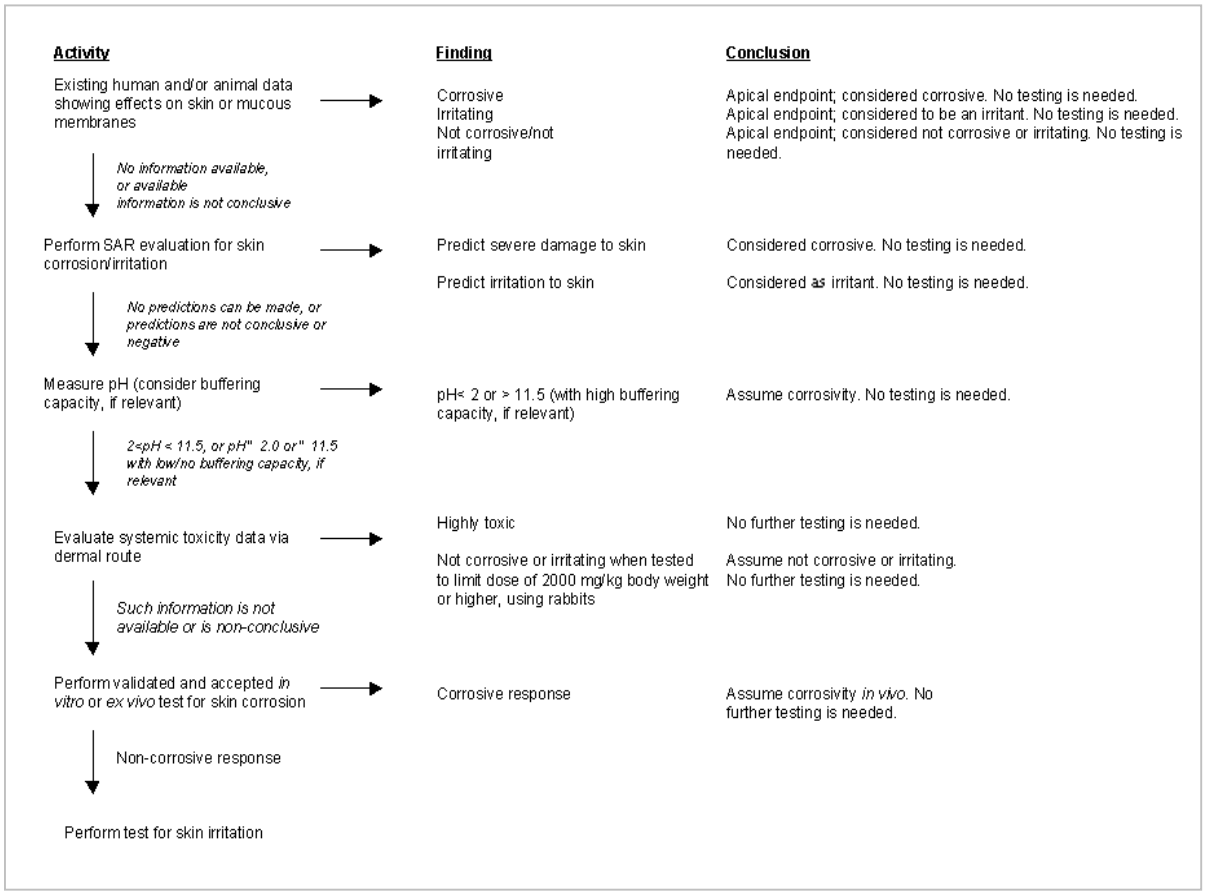


Figure 11. Testing and evaluation strategy for dermal corrosion according to OECD TG 404 (OECD, 2002).
SAR = structure activity relationship

3.2.2 VALIDATION HISTORY OF *IN VITRO* SKIN CORROSION TESTS

Because testing skin corrosion properties *in vivo* may cause severe discomfort or pain to laboratory animals, many attempts have been made to replace this procedure by an alternative method.

A prevalidation study on three *in vitro* tests for skin corrosivity was performed in 1994, and a formal validation study initiated and sponsored by European Centre for Validation of Alternative Methods (ECVAM) was conducted in 1996-1997. Four tests were evaluated in this validation trial: the rat skin transcutaneous electrical resistance test (TER), CORROSITEX, the Skin² ZK1350 and EPISKIN tests (Fentem *et al.*, 1998).

Finally, two assays were accepted as a full replacement of the *in vivo* procedure: the TER assay, based on measuring of the transcutaneous electrical resistance in the *ex vivo* rat skin, and the EPISKIN assay using the reconstructed human epidermal model EPISKIN (EPISKIN SNC, France) (Fentem *et al.*, 1998). Later, another reconstructed human epidermal model - EpiDerm (produced by MatTek Corporation, MA, USA) was also successfully validated in an ECVAM "catch-up" validation study performed during 1999 and 2000 (Liebsch *et al.*, 2000).

In 2000, the TER and *in vitro* skin model corrosion tests were adopted as Method B.40 in Annex V to Directive 67/548 EWG. These two methods were also accepted at the OECD level and adopted in 2004 as OECD Test Guideline 430 (TER Test) and OECD TG 431 (Human Skin Model Test).

To allow other test systems (e.g. new reconstructed human skin models) to be used for the skin corrosion testing, experts preparing the OECD TG 431 agreed on definition of *general* and *functional* model conditions that need to be evaluated prior the test system use (for details see chapter 1.2.2). In addition, the capability for the correct classification of twelve reference chemicals specified in the OECD TG 431 must be demonstrated (OECD, 2004a). Any new test system that is meeting these criteria should be considered as scientifically valid and acceptable after an independent confirmatory study.

Skin corrosion study using the reconstructed human skin model Skinethic

The SkinEthic reconstructed epidermal model (SkinEthic Laboratories, Nice, France) is commercially available since 1992. It has been evaluated in many scientific studies that were extensively published. In the context of OECD TG 431, the validity of the endpoint "metabolic conversion" MTT reduction has been demonstrated by Boelsma *et al.* (2000), Faller and Bracher (2002) and Faller *et al.* (2002), and the tissue histology, ultrastructure, lipid composition and lipid organisation by Ponec *et al.* (2000a, 2000b, 2001 and 2002). Therefore, in the current study, the SkinEthic model was regarded sufficiently well characterised and comparable to the models EpiDerm and EPISKIN. To obtain regulatory acceptance for the SkinEthic RHE model in skin corrosion test, it was necessary to evaluate if the model correctly predicts the corrosion potential of reference chemicals specified in OECD TG 431.

Based on the knowledge that reconstructed human skin models perform similarly in toxicological studies (e.g. Perkins *et al.*, 1996; Liebsch *et al.*, 1997; Liebsch *et al.*, 1999, Liebsch *et al.*, 2000) the EpiDerm skin corrosion protocol (and its prediction model) was adopted for the SkinEthic model.

The study was divided into the two phases. In the first phase screening of the model performance and its predictive ability with EpiDerm protocol was performed. For this purpose chemicals were selected with specific properties included in previous ECVAM validation studies (Barratt *et al.*, 1998; Fentem *et al.*, 1998; Liebsch *et al.*, 2000). After minimal optimisation of the protocol, results obtained with SkinEthic RHE were comparable to published results of EpiDerm and EPISKIN models.

Therefore, it was decided to further evaluate the model and to assess the intra- and inter-laboratory variability in a validation trial. Three laboratories, BASF AG (Germany); SafePharm Laboratories (United Kingdom) and ZEBET at the BfR (Germany) participated in an inter-laboratory study. Although not required by TG 431, the study was conducted with coded test materials to increase credibility of the data. An independent statistician evaluated predictivity and reliability of the new test method.

Results of the study were published in Toxicology *In vitro* (Kandárová *et al.*, 2006a). The new method is currently evaluated by ESAC (ECVAM Scientific Advisory Committee).

Skin corrosion study using the reconstructed human skin model EST-1000

In 2004 Cell Systems GmbH (Germany) has introduced a new reconstructed human epidermal model - EST-1000. The model is intended to being used for prediction of skin corrosion and skin irritation potential of chemicals (Hoffman *et al.*, 2005). Therefore, it was decided to evaluate this model in a similar study as previously performed for SkinEthic RHE model.

Initially, the twelve OECD reference test chemicals were tested to demonstrate the predictive ability of the model. A multi-centre study was planed and initiated in 2005, however, due to the unexpected difficulties in the production of the model, the study was be postponed to spring 2006.

3.3 SKIN IRRITATION TESTING

Dermal irritation is defined as the production of reversible damage of the skin following the application of a test substance for up to 4 hours (OECD, 2002). It is generally assessed by the potential of a test substance to cause erythema and/or oedema after a single topical application on rabbit skin (OECD, 2002).

Similarly as for skin corrosion, the information about the potential of a chemical to cause irritation is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals. In addition, testing of skin irritation potential is required for safety assessment of cosmetic compounds and products.

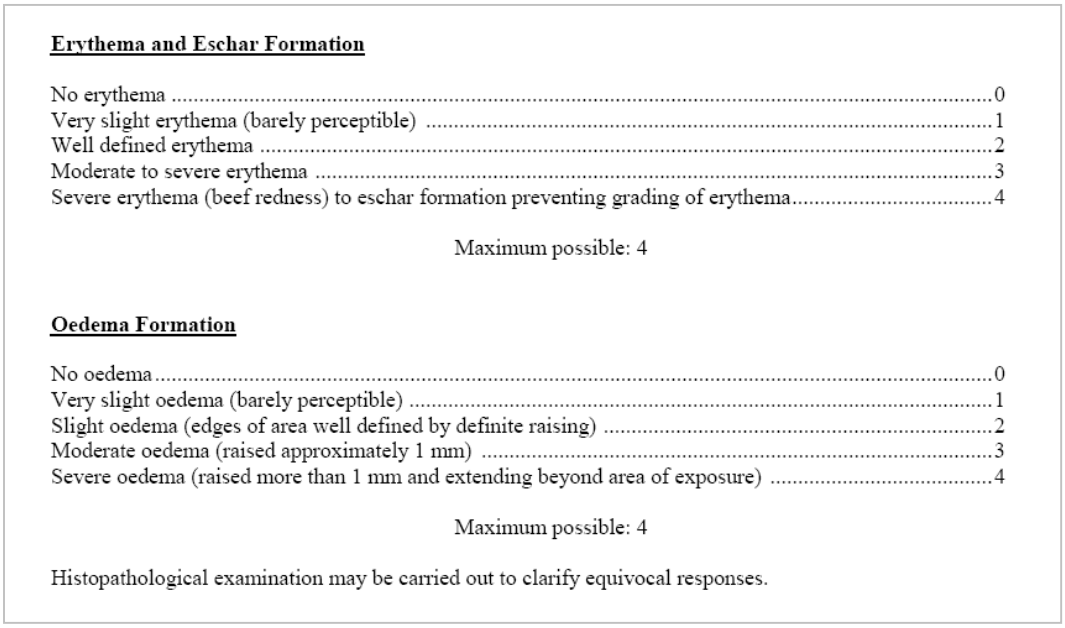
3.3.1 SKIN IRRITATION TESTING *IN VIVO*

The *in vivo* skin irritation test is performed according to OECD TG 404 (OECD, 2002). According to the amended version of the test guideline, only non-corrosive substances should be tested for skin irritation properties *in vivo* (see Figure 11).

According to OECD TG 404 the test substance is applied in a single dose (0.5 mL of liquid or 0.5 g of solid or paste) to a defined area of skin of albino rabbit and covered with a gauze patch for four hours. Untreated skin areas of the test animal serve as the control. Usually three animals are used to determine the skin irritation potential of a test substance. At the end of exposure, the patches are removed and the formation of typical irritation effects (oedema/erythema) is scored using the score system given in Figure 12. All animals are examined for signs of erythema and oedema, and the responses are scored at 60 minutes, and then at 24, 48 and 72 hours after patch removal.

If there is damage to skin which cannot be identified as irritation at 72 hours, observations may be needed until day 14 to determine the reversibility of the effects. In addition to the observation of irritation, all local toxic effects, such as defatting of the skin, and any systemic adverse effects (e.g. clinical signs of toxicity and changes of body weight), should be fully documented (OECD, 2002).

Although the OECD TG 404 was recently refined, there are to date no validated alternative methods replacing the classical Draize test for predicting the acute skin irritation of cosmetic ingredients or chemicals (Botham *et al.*, 1998; Zuang *et al.*, 2005).



Erythema and Eschar Formation

No erythema 0
Very slight erythema (barely perceptible) 1
Well defined erythema 2
Moderate to severe erythema 3
Severe erythema (beef redness) to eschar formation preventing grading of erythema 4

Maximum possible: 4

Oedema Formation

No oedema 0
Very slight oedema (barely perceptible) 1
Slight oedema (edges of area well defined by definite raising) 2
Moderate oedema (raised approximately 1 mm) 3
Severe oedema (raised more than 1 mm and extending beyond area of exposure) 4

Maximum possible: 4

Histopathological examination may be carried out to clarify equivocal responses.

Figure 12. Grading of skin reaction according to the OECD TG 404 (OECD, 2002).

3.3.2 VALIDATION HISTORY OF *IN VITRO* SKIN IRRITATION TESTS

To replace the skin irritation test in rabbits, several *in vitro* test systems have been developed up to date. The best results were obtained with three-dimensional human skin models, which are regulatory accepted systems for the prediction of skin corrosion potential of chemicals. Therefore, in the ECVAM-funded prevalidation study on *in vitro* skin irritation tests, two human epidermal models EPISKIN (EPISKIN SNC, Lyon, France) and EpiDerm (MatTek, Ashland, USA) were evaluated amongst other systems.

However, the prevalidation study revealed insufficient predictive performance of the of both models (Fentem *et al.*, 2001), and further studies were required to improve the test protocols. A retrospective analysis of results obtained with the EpiDerm and EPISKIN models revealed that the two models showed a similar sensitivity although despite test protocols had been used (Zuang *et al.*, 2002).

As a result of this analysis and experience gained in other studies with human skin models (Liebsch *et al.*, 1997; Liebsch *et al.*, 2000), the ECVAM Skin Irritation Task Force suggested a collaboration between L'ORÉAL (Paris, France) and ZEBET (Berlin, Germany) with the aim to develop a common skin irritation protocol for EPISKIN and EpiDerm (Zuang *et al.*, 2002).

The EPISKIN protocol was refined in 2002 by L'Oreal. A new test design was proposed, which consisted of a short exposure time to the test substances (15 minutes)

followed by a post-incubation period of 18 hours which would enable the development of cytotoxic effects or tissue recovery after slight injury (Portes *et al.*, 2002). Further improvement of the test performance was obtained when the post-incubation period was increased to 42 hours (J. Cotovio, personal communication, data presented at the Stakeholder Workshop on the Skin Irritation Validation Study, May 7–8th, 2003, Ispra, Italy). With this second improvement, probability for correct classification has been increased by a better separation of irritants and non-irritants from the classification border separating irritant from non-irritants.

The protocol also fulfilled all requirements defined by the management team of the validation study and was regarded to be ready for a formal validation.

Skin irritation study using the reconstructed human skin model EpiDerm

Following recommendation of the ECVAM Skin Irritation Task Force about common skin model protocol development, the first refined EPISKIN protocol (Porters *et al.*, 2002) was applied without changes on EpiDerm model. However, only negligible improvement of the test performance was achieved.

The second protocol (based on 15-min exposure time and 42 hours post-incubation), provided significantly better results. In addition, increase of the application volume in the EpiDerm assay and improvements in washing techniques improved the test performance, and helped to reduce variability.

Since the studies on improving the EPISKIN and EpiDerm protocols were performed using the same set of test chemicals (20 samples from pre-validation studies), confirmation of the test optimisation with a new set of chemicals became necessary. It was therefore decided that both laboratories (L'ORÉAL and ZEBET) should generate data with new chemicals to confirm the promising performance the test.

The predictive power (sensitivity, specificity and accuracy), and the probability for correct classification was calculated for each chemical tested in the EpiDerm test. An additional statistical analysis was performed to evaluate whether the prediction model (PM) defined for EPISKIN (Portes *et al.*, 2002) could be applied without alteration to data obtained with EpiDerm, and whether the PM was sufficiently robust when new test chemicals were used.

Results of the studies obtained with the EpiDerm model and the common protocol were published in ALTEX (Kandárová *et al.*, 2004) and ATLA (Kandárová *et al.*, 2005).

Skin irritation study using the reconstructed human skin model SkinEthic

In parallel to ECVAM skin irritation validation study, the “common protocol concept” for different reconstructed human skin models was evaluated with SkinEthic reconstructed human epidermal model (SkinEthic Laboratories, Nice, France). This approach was recommended by the Skin Irritation Task force in 2004, when the proposed SkinEthic protocol (developed mainly for testing of cosmetic products) was not meeting the acceptance criteria of the management team of the ECVAM skin irritation validation study.

The Skin Irritation Task force recommended to share experience obtained during the development of the common EpiDerm and EPISKIN protocol with SkinEthic Laboratories, and to investigate if the SkinEthic model could provide comparable results to EpiDerm and EPISKIN when the same set of reference chemicals would be tested. It was further recommended to evaluate the inter-laboratory transferability of the "common protocol" adapted to SkinEthic RHE model.

Therefore, the twenty chemicals, extensively evaluated in all skin irritation studies were used for the optimisation of the SkinEthic assay and assessment of intra-laboratory variability. Protocol transferability was evaluated in collaboration with department of experimental toxicology, SCHERING AG, Berlin. In addition, the usefulness of quantifying interleukin (IL-1α) release, as an additional endpoint, was also assessed.

Results of the skin irritation study with reconstructed human skin model SkinEthic were accepted for publication in ATLA (Kandárová *et al.*, 2006b). An ECVAM catch-up validation study is in preparation.

3.4 PHOTOTOXICITY FEASIBILITY STUDY WITH RECONSTRUCTED HUMAN SKIN MODEL

Phototoxicity is defined as an acute toxic response that is elicited after initial exposure of the skin to certain chemicals and subsequent exposure to light, or that is induced by skin irradiation after the systemic administration of a chemical substance (OECD, 2004b).

The assessment of phototoxicity is necessary for all substances that sufficiently absorb certain parts of UV and visible light and that are intended for human use (including pharmaceuticals, cosmetic ingredients, and food additives).

None of more than ten different animal tests used for predicting acute phototoxicity in humans have been scientifically validated. However, it was proven in an international EU/ECVAM/COLIPA validation study (Spielmann *et al.*, 1994; Spielmann *et al.*, 1998a; Spielmann *et al.*, 1998b) that the phototoxicity of chemicals can correctly be predicted by the *in vitro* phototoxicity test (3T3-NRU-PT) which involves the use of the permanent mouse fibroblast cell line, Balb/c 3T3.

This test gained regulatory acceptance in all EU Member States in June 2000 as Method No. 41 in Annex V to *Directive 67/548/EEC*, and was accepted as the new OECD Test Guideline (OECD TG 432) in 2004. Due to its excellent sensitivity, the 3T3-NRU-PT is regarded as a basic method for identifying acute phototoxic potential and is widely used by the pharmaceutical and cosmetic industries.

If a chemical provides a *negative* result in the 3T3 NRU PT, no further testing is required in most instances. However, if the result is positive, the chemical may be still applied topically to the skin at safe concentrations, given the chemical is not significantly absorbed and accumulated in the skin.

Thus, in addition to the information on phototoxicity, which is assessed in the 3T3-NRU-PT, additional testing may be required to obtain combined information on the phototoxicity and bioavailability of the compound in the skin, i.e. its phototoxic potency (Liebsch *et al.*, 2005).

Ideally, a photopotency test should be performed *in vivo* in human volunteers, but this is often not acceptable for ethical reasons, especially if the chemical may be a photoallergen. Reconstituted human skin models could therefore offer an effective means of avoiding the need for confirmatory testing *in vivo*, especially since such models are characterised by having both viable, metabolising primary skin cells and skin barrier functions.

Based on the promising outcome of an ECVAM-funded prevalidation study on the EpiDerm™ model (Liebsch *et al.*, 1999), the European Medicine Evaluation Agency (EMEA) suggested in a recent Guidance Document on Photosafety Testing, that confirmatory testing can be performed on a skin model phototoxicity test. However, the assay should undergo a validation trial for that purpose (EMEA, 2002).

The aim of current feasibility study (supported by ECVAM) was to assess whether the potencies of phototoxic substances can correctly be predicted when subjected to the pre-validated *in vitro* EpiDerm Phototoxicity Test. In other words, to test whether the lowest topical phototoxic dose (concentration) determined *in vitro* in the human skin model test correlates well with the lowest phototoxic dose (concentration) measured *in vivo* in human volunteers.

Two laboratories were involved in this study: ZEBET at the BfR, Berlin, Germany and National Institute of Public Health, Prague, Czech Republic. In both laboratories 3T3 NRU PT and *in vitro* EpiDerm Phototoxicity test were performed. In addition, the National Institute of Public Health, Prague performed photo-patch tests on human volunteers to confirm correlation of the skin model results and response of human volunteers.

The results obtained within the study were submitted in February 2006 to ECVAM in form of an interim report. Parts of the study were already published (Jírová *et al.*, 2005a) and presented as posters (Jírová *et al*., 2005b; Kandárová *et al.*, 2005b). The final, summarising publication is in preparation.