TOPICAL BIOAVAILABILITY OF GLUCOCORTICOSTEROIDS

Dermatopharmacokinetic and dermatopharmacodynamic of topically applied triamcinolone acetonide in humans

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INDEX

Abbreviations 1

Summary 2

Aim of the thesis 5

THEORETICAL SECTION

1. Human skin 9

1.1. Skin anatomy and physiology 9

1.2. Stratum corneum, the skin barrier 11

1.3. Skin metabolism 12

2. Percutaneous absorption and topical bioavailability 13

2.1. Definitions 13

2.2. Routes of penetration into the skin 14

2.3. Mathematical models 15

2.4. Factors affecting percutaneous absorption 18

3. Assessment of topical bioavailability 24

3.1. Regulatory requirements 24

3.2. Tape stripping 25

3.3. Further techniques 28

4. Stratum corneum reservoir 29

4.1. Definition 29

4.2. Localization of the reservoir 29

4.3. Factors influencing the reservoir 31

4.4. Relevance of the reservoir 32

5. Topical glucocorticosteroids 33

5.1. Overview 33

5.2. Concentration and dose 37

5.3. Application frequency 39

5.4. Occlusion 42

EXPERIMENTAL SECTION

6. Project I: Validation of the methodology and proof of concept.....47

6.1. Introduction..... 48

6.2. Materials and methods..... 49

6.3. Validation of the HPLC method..... 54

6.4. Proof of concept..... 63

6.5. Discussion..... 67

6.6. Conclusions..... 70

7. Project II: Effect of dose and application frequency.....71

7.1. Introduction..... 72

7.2. Subjects and methods..... 73

7.3. Results..... 77

7.4. Discussion..... 82

7.5. Conclusions..... 84

8. Project III: Effect of occlusion.....85

8.1. Introduction..... 86

8.2. Subjects and methods..... 86

8.3. Results..... 90

8.4. Discussion..... 93

8.5. Conclusions..... 94

9. Project IV: Efficacy of low-dose corticosteroids in atopic dermatitis.....95

9.1. Introduction..... 96

9.2. Subjects and methods..... 98

9.3. Results..... 100

9.4. Discussion..... 105

9.5. Conclusions..... 106

10. Final conclusions and perspectives.....107

11. Appendix.....111

12. References.....175

13. Curriculum vitae.....185

Abbreviations

AD	Atopic Dermatitis
ANDA	Abbreviated New Drug Application
ANOVA	ANalysis Of VAriance
CFR	Code of Federal Regulations
DHA	Dihydroxyacetone
DMAC	Dimethylacetamide
DMSO	Dimethyl sulfoxide
DPK	Dermatopharmacokinetic
EMEA	European Agency for the Evaluation of Medicinal Products
FDA	Food and Drug Administration
HPLC	High Performance Liquid Chromatography
ICH	International Conference on the Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
MW	Molecular Weight
NDA	New Drug Application
NMF	Natural Moisturizing Factors
rpm	revolutions per minute
RSD	Relative Standard Deviation
SC	Stratum corneum
SCORAD	SCOring Atopic Dermatitis
SD	Standard Deviation $SD = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}}$
TACA	Triamcinolone acetonide
TEWL	Transepidermal Water Loss
TS	Tape Stripping
TTS	Transdermal Therapeutic System
UV	Ultraviolet
VIS	Visible

Summary

The aim of the present thesis was the *in vivo* investigation of the topical bioavailability of a model glucocorticosteroid, triamcinolone acetonide (TACA), using tape stripping. The layer by layer removal of the stratum corneum by tape stripping enables the quantification of drug amounts penetrated into the stratum corneum over time. This dermatopharmacokinetic (DPK) approach has been subject of fervent discussions in the past years, and concern about adequacy and reproducibility of the technique has been expressed. Yet, the successful performance of reliable and reproducible tape stripping investigations highly depends on the use of a standardized methodology and suitable analytical methods. This thesis proposed a standardized tape stripping protocol in combination with carefully validated analytical methods (Project I). After a proof of concept, the set of methods was applied in an *in vivo* investigation of the influence of different factors on topical bioavailability. Both pharmacokinetic and pharmacodynamic aspects ultimately determining the successful therapy outcome were investigated: the effect of dose and application frequency (Project II), the effect of occlusion (Project III), and the efficacy of a low-dose TACA formulation (Project IV). Concomitantly, the corticosteroid accumulation within the stratum corneum (reservoir development) was monitored, since a reservoir can considerably affect the therapy outcome and is particularly advantageous to prevent systemic side effects.

In **Project I**, the tape stripping technique was standardized and an HPLC method for TACA quantification on tapes after extraction was validated. The standardized tape stripping protocol included the use of a template (ensured the removal of stratum corneum samples from the same skin site) and a hand roller (ensured a constant pressure on the tape before stripping), and, most importantly, the removal of the entire stratum corneum of one skin site to cope with inter- and intra-individual differences in stratum corneum thickness. The HPLC method for TACA quantification was successfully validated and proved to have suitable specificity, linearity, accuracy, precision, and robustness in the working range. The combination of 1) standardized tape stripping as sampling method, 2) UV/VIS-spectroscopy for quantification of corneocytes (previously validated), and 3) the new validated HPLC method for quantification of TACA was then applied in a proof of concept with 6 healthy volunteers. TACA was applied on their forearm skin in either an acetonic solution or an ethanolic gel, and stratum corneum samples were removed by tape stripping after 0.5 h, 3 h, and 24 h. A clear vehicle effect on the TACA penetration could be observed. Whereas TACA deeply penetrated into the stratum corneum after application of the acetonic solution, the penetration after application of the ethanolic gel was only superficial (development of a skin surface reservoir). The method set proved to be suitable for the investigation of the TACA penetration into stratum corneum and was applied in a pharmacokinetic clinical trial with healthy volunteers (Projects II and III).

In **Project II**, the effect of dose and application frequency on the *in vivo* penetration of TACA into stratum corneum was investigated in 15 healthy volunteers. Dose and application frequency of topical corticosteroids are recurrently debated topics. Multiple-daily applications are common, although a superior efficacy compared to once-daily applications is not unequivocally proven. In the dose experiment, higher TACA amounts were quantified within the stratum corneum after application of a high dose (300 μg/cm^2 vs. 100 μg/cm^2; acetonic solution). However, this difference was only significant immediately after application, and no difference was recorded at 4 h and 24 h. The application frequency experiment showed slightly higher TACA amounts within the stratum corneum after multiple application (3x100 μg/cm^2) than after single application of the total dose (1x300 μg/cm^2). As a result of multiple applications, the skin was periodically reloaded with new drug, thus achieving temporary higher amounts within the stratum corneum and redissolving potential TACA crystals. The still well quantifiable TACA amount retained within the stratum corneum at 24 h was rather due to the slow diffusion through the stratum corneum barrier than to a classical reservoir formation. The performance of a mass balance showed that a high TACA dose could result in faster stratum corneum permeation and higher systemic exposure, unwelcome in topical therapy. Thus, a low dose applied once daily may be preferable to higher doses.

In **Project III**, the effects of occlusion before (pre-occlusion) and after (post-occlusion) TACA application (100 μg/cm^2; acetonic solution) were investigated on the forearms of 10 healthy volunteers. Occlusion is known to enhance skin hydration and can induce the formation of a stratum corneum reservoir. Moreover, occlusion is clinically used to improve the efficacy of topical corticosteroids in severe forms of skin diseases. Pre-occlusion showed no effect on the TACA penetration into stratum corneum. In contrast, post-occlusion enhanced the TACA penetration by a factor of 2, favoring the development of a 24 h-lasting reservoir.

The efficacy of low-dose TACA in the treatment of atopic dermatitis was proved in **Project IV**, a double-blind, vehicle-controlled, randomized pharmacodynamic explorative study with half-side comparison in 14 patients. Low-dose TACA was added to a marketed skin care cream (Lichtena®) in a concentration which was 40 times lower than typical therapeutical corticosteroid concentrations (25 vs. 1000 μg/g). Twice-daily application of the low-dose TACA formulation reduced the severity of the lesions (assessed by SCORAD) already after 1 week. In contrast, the cream base alone had no significant influence on the severity of atopic dermatitis measured for 1 month. These findings indicate that some corticosteroids may already be effective at much lower concentrations than usually used therapeutically, and that marketed corticosteroid formulations may contain a much higher concentration than necessary.

The investigations described in this thesis show how tape stripping, correctly performed, asserts itself as a valuable technique for topical bioavailability assessment. The DPK approach can be applied for the investigation of topical bioavailability of other compounds as well, provided that specific analytical methods for their quantification are developed and validated. Re-implementation of the DPK approach on regulatory level could be considered.

Aim of the thesis

The aim of this thesis was to investigate *in vivo* the topical bioavailability of a model glucocorticosteroid, triamcinolone acetonide (TACA), using tape stripping. The layer by layer removal of the stratum corneum by tape stripping enables the quantification of drug amounts penetrated into the stratum corneum over time. This DPK approach has been subject of fervent discussions in the past years, and concern about adequacy and reproducibility of the technique has led to the withdrawal of the corresponding FDA draft guidance "Topical dermatological drug products NDAs and ANDAs – *In vivo* bioavailability, bioequivalence, *in vitro* release, and associated studies" [1]. Currently, no technique is advised at regulatory level for the specific assessment of topical bioavailability.

Since the successful performance of reliable and reproducible tape stripping investigations highly depends on the techniques used, this thesis proposes a standardized tape stripping protocol in combination with carefully validated analytical methods (UV/VIS-spectroscopy, HPLC) to possibly rehabilitate the tape stripping technique (Project I). After proof of concept with 6 healthy volunteers, the set of methods was applied to investigate *in vivo* different factors which influence percutaneous penetration and which thus, ultimately, influence the successful outcome of a topical therapy. The therapeutical class of corticosteroids was chosen because it is still the gold standard for the therapy of several dermatological affections. Among the wide palette of corticosteroids, TACA as a commonly used and moderately potent steroid was chosen as model. Both pharmacokinetic and pharmacodynamic aspects were investigated.

The effect of the following pharmacokinetic parameters on the TACA penetration into stratum corneum was assessed in a clinical trial with 25 healthy volunteers: dose, application frequency, and occlusion. Dose and application frequency (Project II) of topical corticosteroids are recurrently debated topics. Multiple-daily applications are common, although a superior efficacy compared to once-daily applications is not unequivocally proven. Occlusion (Project III) is known to enhance the percutaneous penetration of many but not all drugs. Moreover, occlusion can induce the formation of a drug reservoir within the stratum corneum. A skin reservoir is desired in topical therapy, since the drug should remain for a long time at the site of action, exerting a local and not a systemic action. Finally, the efficacy of a low-dose TACA formulation was assessed in a pharmacodynamic clinical trial with 14 patients suffering from atopic dermatitis (Project IV).

This thesis is structured into a theoretical and an experimental section. The theoretical section gives an overview of: anatomy and physiology of the skin (Chapter 1); principles of percutaneous absorption and topical bioavailability (Chapter 2); techniques for the assessment of topical bioavailability and regulatory requirements (Chapter 3); skin reservoir (Chapter 4); and topical corticosteroids (Chapter 5). In this last chapter, both pharmacodynamic and pharmacokinetic aspects of dose, application frequency, and occlusion are reviewed. The experimental section describes the validation of the methodology (Project I) and the results of the *in vivo* investigations (Projects II-IV), submitted for publication in different scientific journals.

THEORETICAL SECTION

1. Human skin

1.1. Skin anatomy and physiology

The human skin (integumentum commune) is the barrier between the organism's internal environment and its surroundings. With an area of 1.5-1.8 m^2, it is our biggest organ. The following vital functions are ensured by the skin: mechanical and chemical protection, protection against UV radiation and micro-organisms, thermoregulation, and sensory perception. Anatomically, the skin can be divided into subcutis and cutis (Fig.1-1). The **subcutis** (tela subcutanea) is formed of small lobes of fat (panniculus adiposus) separated by septa of connective tissue. The fat is responsible for thermo-insulation, and the connective tissue incorporates lymph and blood vessels reaching into the dermis. The **cutis** is divided into dermis (corium) and epidermis, which are firmly bound together in the dermo-epidermal junction by hemidesmosomes on the epidermal side and by anchoring collagen fibrils on the dermal side [2].

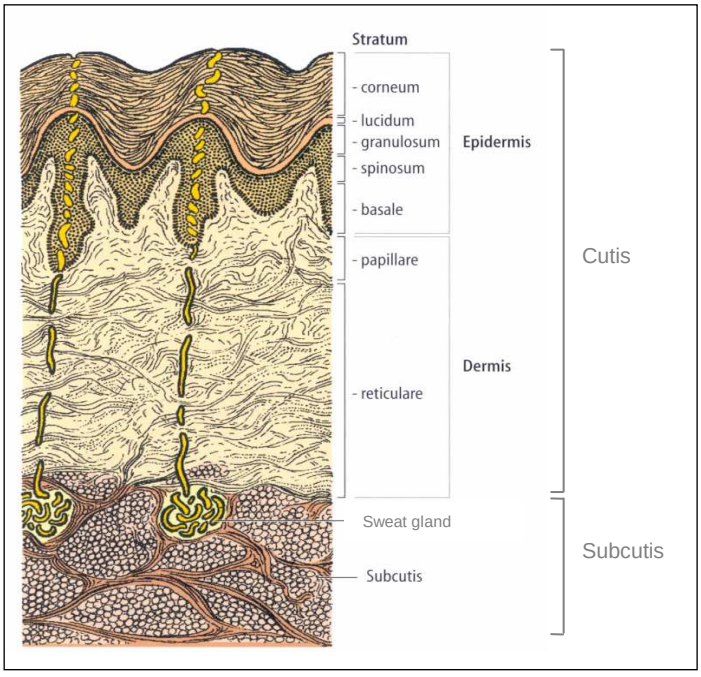


Fig.1-1: Schematic representation of the different skin layers (from [2]).

The **dermis** is about 1-3 mm thick, and consists of cells (fibroblasts, inflammatory cells) and fibers (collagen, elastic, reticular) embedded in an amorphous matrix consisting of mucopolysaccharides produced by the fibroblasts. Also present in the dermis are: blood and lymph vessels, free nerves endings for the perception of temperature, itching and pain, encapsulated nerve endings such as the Vater-Pacini corpuscles (sensitive to pressure and vibration) and the Meissner's corpuscles (sensitive to touch), nerves for the vegetative innervation, and muscles (M. arrector pili, mimetic muscles). Skin appendages (hair, sebaceous glands, sweat glands, nails) originate in the dermis or in the upper subcutis (sweat glands).

Structurally, the dermis comprises the deeper situated, thicker stratum reticulare (few cells except fibroblasts, many fibers) and the stratum papillare (many cells, capillaries, nerves), located just below the epidermis [2].

The **epidermis** is the outermost skin layer and is a vessel-free, nerve-free, stratified, squamous epithelium with a water content of 70%. It is nourished by the underlying capillary loops of the stratum papillare. The thickness of the epidermis varies depending on the anatomical region, with mean values of 77 μm at the forearm [3], minimal values of 30 μm at the eye lid and maximal values of 1.6 mm at the plantar region. Two kinds of cells make up the epidermis. First, the keratinocytes (90%), which are responsible for keratin production and are kept together by desmosomes. Second, the dendritic cells (10%): melanocytes (pigment cells), Langerhans cells (immunocompetent cells), and Merkel's cells (responsible for the perception). The following layers characterize the epidermis: the stratum basale (basal layer) with one cell layer, the stratum spinosum (prickle cell layer) with 2-5 cell layers, the stratum granulosum (granular layer) with 1-3 cell layers, the stratum lucidum (in palmar and plantar skin only), and the stratum corneum (corneal layer) with 10-20 cell layers. In a cycle of about 1 month, new keratinocytes originate in the stratum basale, differentiate in the stratum spinosum, produce keratohyalin-containing granules and lipid/enzymes-containing lamellar bodies (Odland bodies), which are then exocyted in the stratum granulosum and are finally transformed into the stratum corneum [2].

Within the **stratum corneum**, the keratinocytes undergo complete keratinization, forming the nucleus-devoid, flattened, hexagonal corneocytes of about 0.5-3 μm thickness and 30-40 μm width [4]. The bottom part of the stratum corneum (stratum compactum) is very firmly bound together by corneo(desmo)somes and intercellular lipids and has an important protective function. The top part is looser in its structure (stratum disjunctum) and undergoes desquamation by enzymatic digestion of the corneo(desmo)somes [5]. The thickness of the stratum corneum depends, like the thickness of epidermis and dermis, on the anatomical region. Mean values of 15 μm (16 ± 4 cell layers) were recorded at the flexor forearm and maximal values of 1 mm (86 ± 36 cell layers) at the heel [3, 6-8] .

1.2. Stratum corneum, the skin barrier

The stratum corneum consists of 15% water, 70% proteins, and 15% lipids. According to the brick-and-mortar model [9, 10], two compartments can be discerned: keratinous, lipid-devoid corneocytes as bricks and intercellular, continuous lamellar bilayers of lipids as mortar. The **corneocytes** are built out of an insoluble protein complex consisting of highly organized keratin macrofibrils, and they contain natural moisturizing factors (NMF), low-molecular-weight, water-soluble compounds responsible for water retention. The NMF are mainly derived from the protein filaggrin and are composed of amino acids (40%), pyrrolidon carboxylic acid (12%), lactic acid (12%), and urea (7%) [11]. Each corneocyte is encapsulated in an insoluble tough protein shell of 10 nm thickness, the cornified cell envelope, which is covalently bound to an outer lipid envelope consisting of a layer of long-chain ceramides [12]. The free **intercellular lipid bilayers** of the stratum corneum have a unique composition compared to other epithelial lipid bilayers and consist of ceramides (50%), cholesterol (25%), and fatty acids (10-20%, highly enriched in linoleic acid). No phospholipids are present in healthy stratum corneum, and more than one third of the lipids have chain lengths longer than 22 carbons (vs. 16-18 carbons in other mammalian cell membranes) [13]. Most lipids of the lamellar bilayer are derived from the Odland bodies, extruded as phospholipids, sphingolipids, and plasma membrane constituents at the interface stratum granulosum / stratum corneum and then enzymatically cleaved [14].

It was not until the 1940's that the stratum corneum clearly emerged as the specific site of the **skin barrier** for both endogenous and exogenous compounds [15, 16]. In the 70's, the intercellular lipids were recognized as the primary site of the barrier [17]. The qualitative and quantitative organization of the intercellular lipid lamellae is determinant for the barrier function. Several models such as the domain-mosaic [18], the sandwich [19], and the single-gel-phase model [20] have been proposed to explain their molecular organization [21]. An appropriate moisturization level of the stratum corneum, regulated by the presence of NMF, is also important for the maintenance of an effective skin barrier [11].

The stratum corneum is very resistant to physical (mechanical, thermic, actinic) and chemical (acids, to a lesser extent bases) damage. The barrier is more sensitive to organic solvents which can extract the intercellular lipids and to detergents which can damage the cell membrane [22].

Lipophilic compounds usually pass more easily through the lipophilic stratum corneum barrier than hydrophilic compounds. However, the passage through the hydrophilic (epi)dermis may then become the rate limiting step for a lipophilic compound. Furthermore, hydrophilic substances may penetrate the barrier also by follicular pathways [23]. The topic of percutaneous penetration is extensively discussed in chapter 2.

1.3. Skin metabolism

The maturation process of the stratum corneum requires the involvement of different enzymes, for example for the formation of the cornified envelope (e.g., C kinase, acid phosphatase) and intercellular lipid lamellae (e.g., β-glucocerebrosidase, phospholipase A_2, sphyngomyelinase, cholesterol sulfatase) as well as for the corneodesmosome hydrolysis during desquamation (e.g., trypsine, chymotrypsine-like serine proteases) [24]. These enzymes have been detected even in the upper layers of the stratum corneum, and their activity shows a high pH-dependence [25].

The presence of a wide spectrum of enzymes outlines the highly metabolic activity of the skin, which comprises both phase-1 and phase-2 metabolism. Among the phase-1 enzymes, reductases, esterases, oxidases (e.g., cytochrome P-450 monooxygenases), and hydrolases (e.g., proteases) have been localized within the skin. Among the phase-2 enzymes, different transferases needed for conjugation to glucuronic acid, sulphur, glutathione, and glycine have been detected. The skin activity of the CYP 450 enzymes can be as high as 1-5% of the liver activity and the one of the transferases even 10% [26, 27].

Most of the metabolizing enzymes are located in epidermis, sebaceous glands, and hair follicles. The resident skin microflora (e.g., Staphylococcus epidermidis) can exert additional metabolic activity. Staphylococcus aureus, for example, is responsible for the metabolization of the steroid ester betamethasone 17-valerate to betamethasone in psoriatic and eczematous skin [28].

Cutaneous metabolism has been observed for different xenobiotics such as corticosteroids, β-blockers, nitrates, theophylline, polycyclic aromatic hydrocarbons, and pesticides [26-28]. The metabolism of xenobiotics can lead to the inactivation of the compound and/or to the formation of toxic metabolites (irritant, allergenic, or even carcinogenic). Yet, the metabolic activity of the skin can also be profitably commercialized, for example with the design of inactive compounds undergoing metabolic transformation in the skin to form the active drug (**prodrug concept**). This can be useful for parent drugs displaying inappropriate lipophilicity, high toxicity, or chemical instability. An esterification of glucocorticosteroids (e.g., 17-monoester, 21-monoester, or 17,21-diester) leads to a higher lipophilicity and consequently to an enhanced penetration rate and a prolonged dermal retention. By contrast, the **antedrug concept** is defined by the design of locally highly potent drugs undergoing rapid metabolic deactivation as soon as the systemic circulation is reached (also known as “soft drugs”). Systemic side effects are minimized and the safety profile is improved. A single compound can consist of a combination of both concepts (**pro-antedrugs**) [29]. Prednicarbate (prednisolone-17-ethylcarbonate-21-propionate), for example, is cleaved within the skin to the pharmacologically active prednisolone 17-ester and further metabolized to the less active prednisolone in the systemic circulation.

2. Percutaneous absorption and topical bioavailability

2.1. Definitions

The EMEA (European Agency for the Evaluation of Medicinal Products) defines the term **bioavailability** as the "rate and extent to which the active substance or active moiety is absorbed from a pharmaceutical form and becomes available at the site of action" [30]. The "absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives[1] becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriate designed study" is termed **bioequivalence** [31].

Topical dermatological drug products belong to the class of locally acting drug products [32]. In this case, the site of pharmacological action is the skin. Stratum corneum and skin surface are considered to be the compartments of invasion, whereas the blood system represents the compartment of excretion [33]. Therefore, two different types of bioavailability have to be distinguished for topical application. The **topical bioavailability** reflects the rate and extent to which the active moiety becomes available at the site of action, i.e. the skin. The **systemic bioavailability**, instead, may not properly reflect the cutaneous bioavailability for medications intended to treat local skin disorders but becomes important for the toxicological evaluation of the body burden and for transdermal therapeutic systems (TTSs) [34].

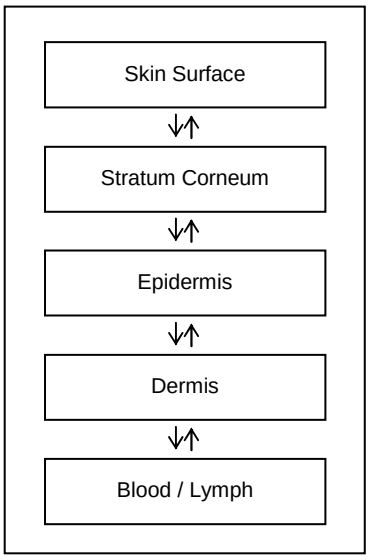


Fig. 2-1: Different compartments that a compound has to pass through during percutaneous absorption.

Percutaneous absorption is the uptake of a compound into the systemic circulation after topical application and describes the movement through the various layers of the skin with respect to both rate and extent (Fig. 2-1). The percutaneous absorption process can be divided into the following 3 steps [35]. **Penetration** is the entry of a substance into a particular layer. **Permeation** is the passage through one layer into another layer. **Absorption** is the uptake of a substance into the vascular system (blood and/or lymph vessel), which acts as the central compartment, and reflects the systemic bioavailability.

[1] Drug products are **pharmaceutical equivalents** if they contain the same amount of the same active substance(s) in the same dosage forms that meet the same or comparable standards and **pharmaceutical alternatives** if they contain the same active moiety but differ in chemical form (salt, ester, etc) or in dosage form or strength [30].

2.2. Routes of penetration into the skin

There are 3 potential routes of penetration from the skin surface into the epidermis (Fig. 2-2) [36]: 1) the intercellular route, 2) the transcellular route, and 3) the transappendageal route through either the eccrine (sweat) glands or the hair follicles with their associated sebaceous glands.

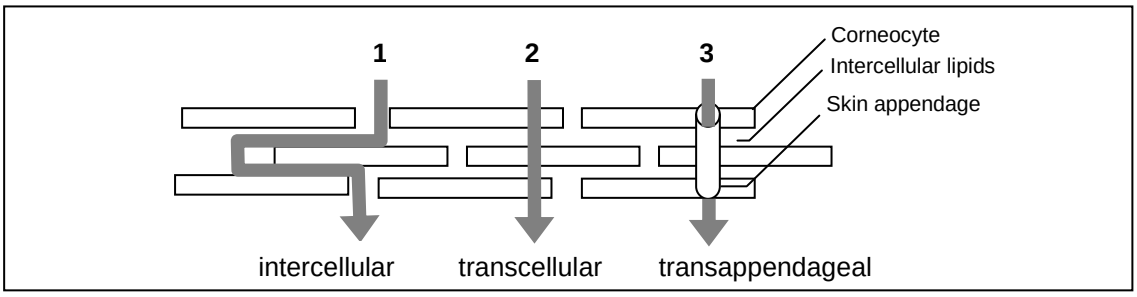


Fig. 2-2: The 3 possible routes of penetration into the stratum corneum: 1) intercellular, 2) transcellular, 3) transappendageal route. The stratum corneum is depicted according to the brick-and-mortar model [9, 10]: the corneocytes represent the bricks, the intercellular lipids represent the mortar.

Under normal circumstances, the predominant is the **intercellular route**, which consists of a tortuous route along the cornified envelope-armored corneocytes through the structured intercellular lipid bilayers [37, 38]. The tortuous diffusional path length has been estimated to be as long as 300-500 μm [39, 40] in contrast to a mean stratum corneum thickness of just 20 μm. The transport process involves sequential diffusion and partitioning between the polar head group regions and the long alkyl chains of the lipids [41]. The **transcellular route** is possible for small hydrophilic substances like water [38]. Waibler observed an *in vivo* intracellular distribution of hydrophilic dyes (patent blue, sodium fluoresceine) applied in water, and a prevalent intercellular distribution of the lipophilic dye curcumin applied in liquid paraffin [42].

The **transappendageal route** was, in the past, considered to play a subordinate role during percutaneous penetration, since the skin surface of the appendages yields only a maximal of 0.1% of the total skin surface[2]. The contribution of the appendages was regarded as an initial "shunt" diffusion, whereas the main "bulk" diffusion took place through the stratum corneum [33]. Yet, the transappendageal route can be relevant for polar steroids showing a low diffusion through the stratum corneum [44, 45] as well as for lipophilic compounds [43]. A higher impact of the transappendageal route on percutaneous absorption has to be expected on the forehead, where the follicle density is very high (292 follicles/cm^2 vs. 14-22 /cm^2 in other skin regions) [46].

[2] *De facto*, the actual area for potential transfollicular absorption is much higher than the mere skin surface area because the hair follicle is an invagination of the epidermis extending deep into the dermis [43]

2.3. Mathematical models

2.3.1. Fick's laws of diffusion

After application of a topical formulation, the active compound has to be released from the vehicle, partition between vehicle and stratum corneum, and diffuse through (and partition between) the different layers of the skin before it can exert its pharmacological action, finally being "excreted" into the systemic circulation (Fig. 2-3). Diffusion is a passive kinetic process taking place along a concentration gradient from a region of higher concentration to a region of lower concentration. The diffusion through the skin can be described by **Fick's first law**:

$$J = -D\frac{dc}{dx} = \frac{dm}{dt} \cdot \frac{1}{A} \qquad \textbf{(Equation 1)}$$

where J is the steady state flux of the compound mass (m) through the stratum corneum per unit area (A) and unit time (t) (μg/cm^{2}s), D is the diffusion coefficient of the compound in the stratum corneum (cm^2/s), c is the drug concentration, and x is the position [47]. The solution of the equation with the appropriate boundary conditions gives:

$$J = \frac{K\,D}{h} \cdot \Delta c = k_p \cdot \Delta c \qquad \textbf{(Equation 2)}$$

where K is the partition coefficient of the compound between vehicle and stratum corneum, h is the diffusional pathlength (cm), k_p is the permeability coefficient, and Δc (= c_{appl} - c_{rec}) is the concentration difference (μg/cm^2) across the stratum corneum between applied concentration (c_{appl}) and concentration below the stratum corneum (*in vivo*) or in the receptor phase (*in vitro*, c_{rec}) [36, 48]. Under normal circumstances, the applied concentration (c_{appl}) is much larger than the concentration in deeper skin layers, and Δc can be replaced with c_{appl}. The real diffusional pathlength (h) is the tortuous pathway along the intercellular lipids, which is longer than the mere stratum corneum thickness. However, the stratum corneum thickness is mostly used, since it is easily measurable.

If the steady state is not attained, the diffusional flux can be explained by **Fick's second law**, which describes the concentration change over time at a definite position x within the membrane [47]:

$$\frac{\partial J_{(x)}}{\partial x} = -\frac{\partial c_{(x,t)}}{\partial t} = -D\frac{\partial^2 c_{(x)}}{\partial x^2} \qquad \textbf{(Equation 3)}$$

Different solutions of this equation with appropriate boundary conditions have been proposed [49-51].

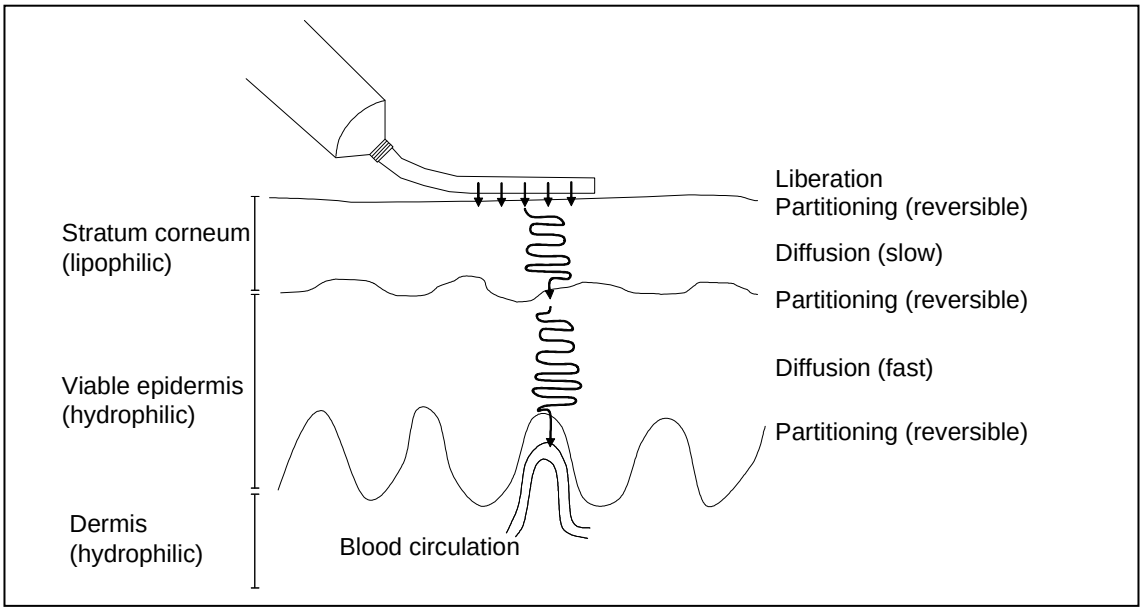


Fig. 2-3: Schematic depiction of percutaneous absorption (modified from [52]).

2.3.2. Solubility

The concentration difference of the compound across the skin or the membrane (concentration gradient Δc) is the driving force for diffusion. A balance has to be found between good solubility within the vehicle and good release from the vehicle [48]. The maximal stable concentration is achieved at saturation solubility (thermodynamic activity or leaving potential = 1) [48]. Particles suspended within the vehicle do not directly contribute to the concentration gradient but constitute a reservoir maintaining saturation conditions in the vehicle for a prolonged time.

2.3.3. Partition coefficient (K)

The partition coefficient is a thermodynamic, time-independent factor and displays the relative preference of a compound to stay either within the vehicle or within the membrane. The degree of partitioning is dependent on the relative solubility (affinity) of the compound in both vehicle and membrane. A hostile vehicle environment, and thus a higher affinity to the membrane, favors partitioning [48].

2.3.4. Diffusion coefficient (D)

The degree of diffusion within a specific medium (vehicle, membrane) is described by the diffusion coefficient. The diffusion coefficient depends on: the physicochemical properties of both the diffusing compound (size, radius) and the diffusional medium (viscosity), temperature, and pressure. The **Einstein diffusion equation** points out the dependency on these factors [47]:

$$D = \frac{R\,T}{N_A\,6\pi\eta\,r} = \frac{k_B\,T}{6\pi\eta\,r}$$ **(Equation 4)**

where
- R = universal gas constant = $k_B \cdot N_A$ = 8.314472 J/mol K
- k_B = Boltzmann constant = R/N_A = $1.3806505 \cdot 10^{-23}$ J/K
- N_A = Avogadro's number = $6.0221415 \cdot 10^{23}$ /mol
- η = viscosity of the medium (vehicle, membrane)
- r = radius of the compound particle
- T = absolute temperature

2.3.5. Permeability coefficient (k_p)

The permeability coefficient is a heterogeneous rate constant characteristic for a membrane and a compound and is expressed as depth of diffusion per unit time (cm/h):

$$k_p = \frac{K \cdot D}{h}$$ **(Equation 5)**

This constant can be calculated from experimental results by dividing the flux J with the concentration difference Δc (see Equation 2), whereas it is more difficult to calculate the terms K and D separately [41].

Permeability is influenced to a greater extent by variation of the partition coefficient than by variation of the diffusion coefficient. This can be observed in the analysis of a homologous series (e.g., of n-alkanols), where partition coefficient and permeability coefficient increase with increasing chain length, whereas the diffusion coefficient remains more or less constant (Table 2-1) [35].

Table 2-1: Average permeability data for n-alkanols. The diffusion coefficients were calculated from Equation 2 using 40 µm for stratum corneum thickness (data from [35]).

Alkanol	Partition Coefficient K (vehicle/stratum corneum)	Diffusion Coefficient D [cm^2/s]	Permeability Coefficient kp [cm/h]
Methanol	0.6	0.9×10^{-9}	0.5×10^{-3}
Ethanol	0.9	1.0×10^{-9}	0.8×10^{-3}
Propanol	1.1	1.2×10^{-9}	1.2×10^{-3}
Butanol	2.5	1.1×10^{-9}	2.5×10^{-3}
Pentanol	5.0	1.4×10^{-9}	6.1×10^{-3}
Hexanol	10	1.4×10^{-9}	13.0×10^{-3}
Heptanol	30	1.2×10^{-9}	32.1×10^{-3}
Octanol	Not available	1.1×10^{-9}	50.4×10^{-3}
Nonanol	Not available	Not available	61.2×10^{-3}
Decanol	Not available	Not available	79.2×10^{-3}

2.4. Factors affecting percutaneous absorption

2.4.1. Overview

Factors affecting percutaneous absorption and topical bioavailability of topically applied compounds are:

- the physicochemical characteristics of the compound
- the physicochemical characteristics of the vehicle
- the application conditions
- the skin conditions.

2.4.2. Physicochemical characteristics of the compound

Molecular weight (size), degree of ionization (charge), and lipophilicity are important factors determining the partition and diffusion coefficients [35, 53]. The molecular weight is inversely proportional to percutaneous absorption and seems to particularly influence the diffusion coefficient. Molecules larger than 500 Daltons have usually more difficulty to pass through the healthy stratum corneum [54]. It is a common presumption that only non-ionized compounds are able to diffuse through the lipophilic intercellular regions of the stratum corneum. Yet, ionized compounds have been reported to permeate through human skin through the intracellular and transappendageal pathway, albeit at a slower rate [35]. Moreover, the formation of ion pairs between compound ions and ions present in the skin can lead to neutral compounds [41]. As most drugs are either weak acids or weak bases, the pH of aqueous vehicles determines the ionization state (compare chapter 2.4.3) [48, 55].

The best percutaneous absorption would be achieved by an amphiphilic compound showing both high solubility in the lipophilic stratum corneum (maximal input into the stratum corneum) and high aqueous solubility in the hydrophilic viable epidermis (maximal output into deeper layers). In general, compounds with a $\log K_{octanol/water}$ of about 1-3 have optimum partition behavior [56]. High penetration into the stratum corneum but limited penetration into the viable epidermis (observed for very lipophilic compounds) may induce a reservoir into the stratum corneum (see chapter 4). The same can occur for compounds with small diffusivity in the stratum corneum and for compounds binding to specific tissue components [35].

2.4.3. Physicochemical characteristics of the vehicle

The vehicle can both influence drug release as well as alter the stratum corneum structure. Drug release is affected by the viscosity of the vehicle (alteration of diffusion coefficient) and by the solubility of the compound in the vehicle. Alteration of the stratum corneum structure includes extraction of intercellular skin lipids and occlusive effects. The pH of the vehicle, the state of the compound in the vehicle (dissolved, suspended), the concentration of the compound, and the presence of cosolvents (e.g., propylene glycol), penetration enhancers (e.g., urea, DMSO[3]), and surfactants are all factors influencing percutaneous absorption. Among the penetration enhancers, two types can be distinguished: those influencing diffusion (e.g., Azone®[4], oleic acid, surfactants) and those influencing partitioning (most solvents, e.g., propylene glycol) [41].

After application of a topical formulation to the skin, the vehicle undergoes important structural changes also known as “metamorphosis of the vehicle”. The partitioning between vehicle and stratum corneum, and thus the penetration into stratum corneum, is different for each component of the formulation. In addition, evaporation of the different ingredients is possible. After application of a compound in a volatile vehicle (e.g., ethanol, acetone), the rapid evaporation of the vehicle subsequently increases the concentration and the saturation degree of the compound, thus altering the driving force of diffusion (enhanced thermodynamic activity). The maximum drug penetration into the skin is known to take place when the drug is in a saturated state [57]. In some cases, supersaturation can occur and thus further enhance percutaneous penetration [58].

After complete evaporation of the solvent, the remnant drug precipitates onto the skin as ”solvent deposited solid”. In this case, percutaneous penetration becomes a dissolution rate limited process [59, 60]. Percutaneous absorption following application in a volatile vehicle is quite different than percutaneous absorption from non-volatile vehicles and is not a steady-state process [61]. Akther et al. observed, after application of flurbiprofen and ibuprofen in acetone, an initially low percutaneous penetration which then increased as soon as evaporation of the vehicle took place [60]. Different mathematical models of percutaneous absorption kinetics after application of finite vehicle volumes and solvent deposited solids have been proposed [49].

[3] DMSO is dimethyl sulfoxide.

[4] Azone® is 1-dodecylaza cycloheptan-2-one.

2.4.4. Application conditions

Dosing technique, dose, and application under occlusion are the most important application conditions affecting percutaneous absorption.

Two main dosing techniques are known - the infinite and the finite. The **infinite dose technique** concerns the application of an amount of material much higher than is expected to penetrate into the skin. The surplus of material forms a surface reservoir which appears to be “infinite”. The steady-state flux after application of an infinite dose follows Fick’s first law of diffusion (described in chapter 2.3.1). The **finite dose technique** is more closely related to clinical settings. A definite, low amount of material is applied. With time, the amount of material on the skin surface is depleted and the flux into the skin decreases. No steady-state flux can be observed. In this case, the diffusion has to be explained with an appropriate solution of Fick’s second law (chapter 2.3.1), and several models have been proposed [62-64]. A special case at the boundary between finite and infinite dosing is observed after finite application of poorly absorbed compounds (e.g., corticosteroids). In this case, the amount recovered on the skin surface after penetration is usually high (ca. 60-95% of the dose applied) and forms a not completely depleted (i.e. infinite) surface reservoir. Table 2-2 outlines the different dosing techniques [65].

Table 2-2: Dosing techniques for topical drug application (modified from [65]).

Dosing technique	Loading	Estimated bioavailability	Comment
Infinite dose	high (> 10 mg/cm^2)	< 5% (typically 1-2%)	Mostly for experimental settings
Finite/Infinite dose	clinical use (0.5-5 mg/cm^2)	< 5% (typically 1-2%)	Poorly absorbed molecules in clinical situations (e.g., corticosteroids)
Finite dose	clinical use (0.5-5 mg/cm^2)	> 10% (typically 25-50%)	Well absorbed molecules in clinical situations (e.g., nitroglycerin)

After application of a single **dose**, the following parameters have been shown to influence percutaneous absorption: concentration of the dose, applied film thickness, area on which the dose is applied, and duration of the application. The effect of concentration on the percutaneous absorption of topical corticosteroids is explained in detail in chapter 5.2. Other effects have been reviewed by Surber and Davis [65].

Occlusion is defined as the external insulation of the skin with a water evaporation limiting barrier [66] and can be performed either by covering the skin with an impermeable wrap (e.g., plastic film, tape or wound wrap, gloves, impermeable textiles, diapers) or by applying topical vehicles containing fats or oils (e.g., petrolatum or paraffin). Because of the inhibition of the transepidermal water loss (TEWL) from the skin surface by occlusion, the normal water content of the stratum corneum (10-20%) can be increased by up to 50%. Even short-time occlusion

(e.g., 30 min) results in a overhydration of the skin [67, 68]. Water has been shown to accumulate either within the corneocytes, inducing their swelling[5] [69], or within the intercellular lipids, forming intercellular water pools [70]. Moreover, occlusion has an effect on: skin surface temperature (increase from 32℃ to 37℃), blood flo w, composition of epidermal lipids, DNA synthesis, epidermal morphology and turnover, composition of the microbial skin flora, pH, and activity of sweat glands and Langerhans cells. In general, the barrier function of the stratum corneum is reduced by occlusion [67, 71]. The increased stratum corneum hydration and increased temperature can positively alter the partitioning of applied compounds between vehicle, stratum corneum, and viable epidermis, thus enhancing percutaneous absorption in a simple manner. After occlusion, the hydrated stratum corneum and the viable epidermis appears more similar (facilitated partitioning), with a greater effect for lipophilic compounds [72, 73]. The specific effect of occlusion on the penetration of topically applied compounds with a focus on corticosteroids is described in chapter 5.4.

[5] The swelling of the corneocytes is more pronounced in the thickness: 25-30% increase in thickness and 3-5% increase in length have been observed after occlusion [69].

2.4.5. Skin conditions

The main skin conditions affecting percutaneous absorption are: the anatomic site, the temperature, the blood flow, and the health state. Minor factors are: age, skin type, race, gender, and the circadian rhythm of the barrier function.

Percutaneous absorption in man varies depending on the **anatomic site** (reviewed in Wester and Maibach [74]). Experiments with hydrocortisone resulted in the following rank order of penetration: scrotum > forehead > scalp > back > forearm > palm > plantar skin [75]. There are 3 main theories which attempt to explain the regional differences in percutaneous absorption: first, the variation of stratum corneum thickness and especially the lipid composition [76]; second, the different distribution of skin appendages acting as shunts; third, the differences in cutaneous blood flow [23]. Increased **blood flow** and **skin hydration** can be caused by an increased **skin temperature** and occlusion. A higher skin temperature increases the compound solubility and diffusion within vehicle and stratum corneum, thus enhancing percutaneous absorption [65].

Physical (weather, sunlight, occlusion), chemical (solvents, detergents, acids, alkalis), and pathological factors (mechanical damage, skin diseases) all influence the **state of the skin**. Many skin disorders (e.g., atopic dermatitis, psoriasis, ichthyosis, xerosis) are characterized by an imbalance of the lipid composition and structure, especially of the stratum corneum ceramides. In addition, a diminished water-binding property, a lack of NMF, an incomplete maturation of the cornified envelope, and a defective corneocyte cohesion and desquamation have been reported [14]. The impaired barrier function of atopic and psoriatic skin is reflected by the increased TEWL [77]. The penetration into diseased or experimentally disrupted skin (e.g., by phonophoresis, electroporation, or tape stripping) can significantly differ from the penetration into healthy skin, even allowing the penetration of larger molecules, which would normally not penetrate (Fig. 2-4) [54].

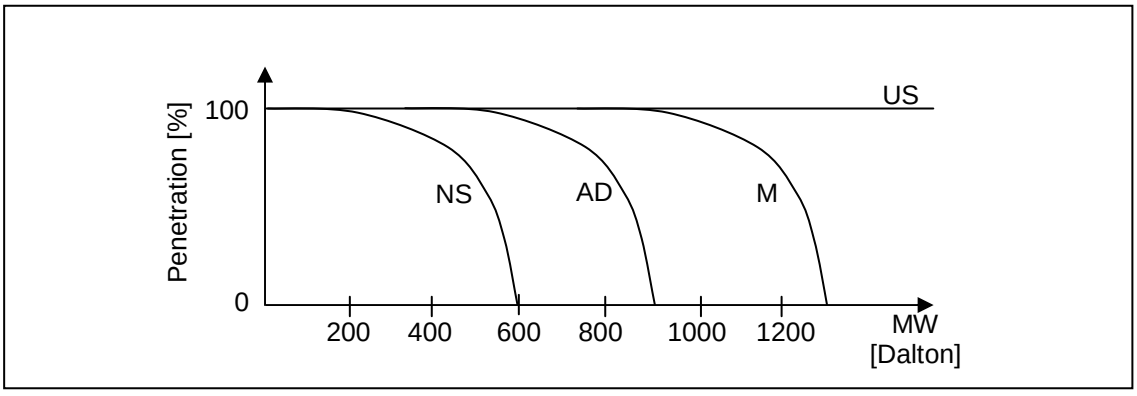


Fig. 2-4: Influence of the molecular weight and the state of the skin on human skin penetration. Normal human skin (NS), atopic dermatitis skin (AD), mucosa (M), and ultrasound treated skin (US) [54].

Turpeinen et al. observed a statistically significant enhanced percutaneous absorption after application of hydrocortisone cream in children [78, 79] and adults [80] suffering from atopic dermatitis. The percutaneous absorption correlated with the severity of the dermatitis, and diminished with the restoration of the skin. With patients suffering from psoriasis, Schaefer et al. observed an enhanced TACA penetration after application of ointment and cream preparations. TACA concentrations 3-10 times higher than in healthy skin were quantified within epidermis and dermis [81]. Yet, Wester el al. observed similar hydrocortisone absorption in stable psoriatic plaques and healthy skin [82].

Age seems to exert a minor effect on percutaneous absorption. Whereas the stratum corneum barrier of premature infants is not completely mature, the skin of term infants seems to possess barrier properties already comparable to that of adult skin, and a similar percutaneous absorption can be expected [83]. Reports of drug toxicity after topical drug applications in children are mainly due to the higher surface area-to-weight ratio [84]. With increasing age, the skin undergoes important morphological, physiological, and biochemical changes. The number of dermal capillary loops, the skin lipid content, and the hydration status of stratum corneum decrease, and the dermis undergoes atrophy [85]. This leads to a decreased percutaneous absorption, particularly observed for hydrophilic compounds [86].

Racial differences in skin properties have been reported. Black skin has higher TEWL, variable blood vessel reactivity, decreased skin surface pH, and larger mast cell granules compared to white skin. The results about Asian and Hispanic skin are inconclusive [87]. Reed et al. observed that the **skin type** is a more relevant determinant of barrier function than race itself. Despite a comparable stratum corneum thickness, darkly pigmented skin (type V/VI) requires more tape strips to remove the stratum corneum by tape stripping, showing a superior barrier capacity. The permeability barrier function in skin type II/III does not seem to be affected by race or **gender** [88]. Also independent of gender are stratum corneum thickness, sebum rate, hydration status, and TEWL [7, 89], and a similar percutaneous absorption can be expected in men and women. In conclusion, percutaneous absorption seems to be more influenced by inter-individual differences than by racial or gender related differences.

The reported **circadian rhythm** in TEWL (highest in the night), skin surface pH (highest in the afternoon), and skin temperature (highest in the night) suggest higher skin permeability in the evening and the night [90]. Moreover, many endogenous compounds follow a circadian rhythm. In the specific case of corticosteroids, the time period with the lowest circulating concentration of endogenous cortisol is between 8 p.m. and 4 a.m. Consideration of this factor could be critical for bioequivalence assessment of topical corticosteroids using the vasoconstriction assay (see chapter 3.1), since the vasoconstrictive effect of the endogenous cortisol could interfere with the effect of the topically applied steroid [91].

3. Assessment of topical bioavailability

3.1. Regulatory requirements

In the US, the following general approaches, listed in order of preference, are regarded as acceptable by the Code of Federal Regulations (CFR) of the FDA for bioavailability and/or bioequivalence assessment: 1) pharmacokinetic approach based on measurements of the concentration of the active moiety and/or metabolites in blood, plasma, serum, or other appropriate biological fluid as a function of time; 2) pharmacokinetic approach based on the measurement of the urinary excretion of the active moiety and/or metabolites as a function of time (only appropriate if urinary excretion is a significant mechanism of elimination); 3) pharmacodynamic approach based on the measurement of an appropriate acute pharmacological effect of the active moiety and/or metabolites (particularly appropriate to dosage forms not intended to deliver the active moiety to the bloodstream for systemic distribution); 4) comparative clinical trials; and 5) *in vitro* studies (21CFR320.24). For drug products that are not intended to be absorbed into the bloodstream, bioavailability may be assessed by measurements intended to reflect the rate and extent to which the active moiety becomes available at the site of action (21CFR320.23) [31].

In the EU, the "Note for guidance on investigation of bioavailability and bioequivalence" released by the EMEA states that for products for local use (after oral, nasal, inhalative, ocular, dermal, rectal, vaginal administration) intended to act without systemic absorption, the approach to determine bioequivalence based on systemic measurements is not applicable and pharmacodynamic or comparative clinical trials are required. The determination of the systemic exposure resulting from locally applied products is only relevant if there is a risk of systemic adverse reactions [30].

Comparative clinical efficacy trials are relatively insensitive, highly variable, time consuming, costly, and need a high number of volunteers. Pharmacodynamic investigations can only be performed if the topically applied compound produces a measurable pharmacodynamic response, and this is not always the case. Nevertheless, there are two outstanding examples. Corticosteroids induce a vasoconstriction, and the degree of skin blanching can be correlated with the efficacy and potency of the steroid. A relevant guidance ("Topical dermatological corticosteroids: *in vivo* bioequivalence") has been released by the FDA [92]. Furthermore, retinoids induce an enhanced TEWL, which can be used as pharmacodynamic measure for bioavailability assessment.

An explicit approach for measurements intended to reflect the rate and extent to which the active moiety becomes available at the site of action is still missing in the regulatory specifications. It was against this background that the FDA proposed in 1998 a draft guidance for the bioavailability assessment of topical dermatological drug products ("Topical dermatological drug products NDAs and ANDAs – *In vivo* bioavailability, bioequivalence, *in vitro*

release, and associated studies") [1]. The guidance focused on the DPK approach, which is based on the measurement of the active moiety in the stratum corneum. The DPK approach is comparable to a blood, plasma, or urine pharmacokinetic approach applied to the stratum corneum. Generally, DPK studies are performed in healthy volunteers, since diseased skin is highly variable and changes over time. Among different models, tape stripping showed the highest potential for the DPK characterization of topically applied drugs, since the percutaneous penetration was assessable without invasive procedures. Yet, the draft guidance was withdrawn in 2002 because of the following two reasons[6]. The first concern considered the adequacy of the DPK method to assess the bioequivalence of topical dermatological drug products, because the products are used to treat a variety of diseases in different part of the skin, not just the stratum corneum. Yet, despite the fact that the target site for topical dermatological drug products may not always be the stratum corneum, the drug must still pass through the stratum corneum barrier to reach deeper sites of action. In certain cases, the stratum corneum itself is the site of action (e.g., for antimykotika). For antiacne drug products, the target sites are the hair follicles and the sebaceous glands, and it has been shown that there is a positive correlation between stratum corneum and follicular concentrations. The second concern considered the reproducibility of the DPK method between laboratories. The detailed presentation of the tape stripping technique in the next chapter will provide a better understanding of this second concern.

3.2. Tape stripping

Tape stripping is a technique which enables the removal of the stratum corneum layer by layer. Adhesive tapes are sequentially pressed onto the same skin region, and then stripped off [93]. Using a standardized technique, Jacobi et al. showed that 66% of the stratum corneum is removed with 20 tapes (using Tesa® Multi-Film Crystal-Clear tape), and nearly the complete stratum corneum (95%) is removed with 50 tapes [94]. Yet, depending on the adhesive tapes used, up to 100 tapes may be required to remove the entire stratum corneum from one skin site [95] (Fig. 3-1). With the tapes, corneocytes and substances previously applied on the skin are removed and can be quantified with an appropriate analytical method (e.g., HPLC, spectroscopy, scintillation counting).

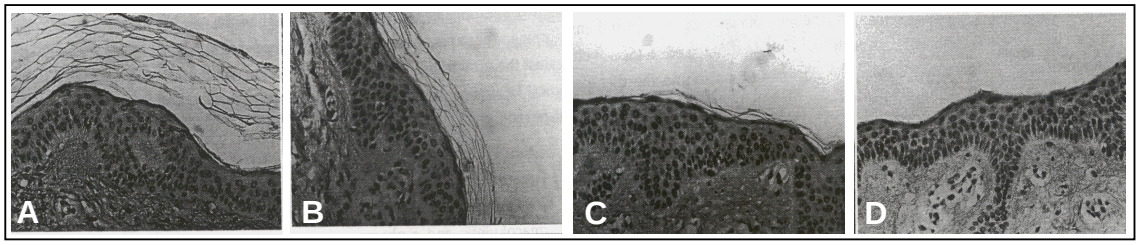


Fig. 3-1: Microscopic depiction of the skin. A) Epidermis with intact stratum corneum; B) Epidermis after stripping off 10 tapes; C) Epidermis after stripping off 50 tapes; D) Epidermis after stripping off 100 tapes [95].

[6] The report of withdrawal is available at the site: www.fda.gov/OHRMS/DOCKETS/98fr/98d-0388-nwl0001-vol1.pdf

The tape stripping technique was introduced in the early 40's by Wolf. He investigated the topography of the skin by removing layers of corneocytes with transparent adhesive tapes and by examining them microscopically [96, 97]. In the 50's, Pinkus applied the new technique to investigate the epidermal regeneration process. By taking punch biopsies of stripped skin sites, he observed that tape stripping stimulated the mitotic rate in the stratum basale [98, 99]. The mitotic rate was maximal after 3 days, and a couple of weeks were required for complete skin renewing. Further studies investigating the epidermal growth kinetic after standardized injury of healthy [100-102] and atopic dermatitis skin [103] by tape stripping were performed. Recently, Choi et al. showed that tape stripping induces the production of cytokines such as interleukins, tumor necrosis factor α, and γ-interferon [104], enhances the enzymatic activity, and increases the ceramides synthesis [105]. The artificial removal of the stratum corneum barrier by tape stripping has become a frequently used model for simulating diseased skin [106], and for assessing the efficacy of skin care products in restoring the barrier [107, 108]. Moreover, a percutaneous penetration enhancement of macromolecules like peptides and oligonucleotides, which usually do not penetrate healthy skin, was observed after removal of the stratum corneum, thus opening the possibility of topical vaccinations [104, 109].

In the 80's, the methodology was standardized by Dupuis et al. [110, 111], making of tape stripping a method widely used in dermatological research to non-invasively investigate the topical bioavailability and percutaneous penetration of topically applied substances. The investigation of the topical activity of antiviralia [112, 113] and antimykotika [114, 115], the investigation of vehicle effects [116, 117], barrier function and reservoir formation within the stratum corneum [118-120], and the investigation of the percutaneous penetration and absorption of topical corticosteroids [91, 121-123] are some important examples of application areas. Furthermore, tape stripping has been used to investigate stratum corneum lipids [124] and epidermal enzymes [125].

A prerequisite for making the tape stripping technique a capable, robust, accurate, and reproducible method to investigate the bioavailability of topically applied drugs is the strict standardization and a clear definition of the stripping parameters. The technique is susceptible to numerous confounding factors, both intrinsic and extrinsic. Among the intrinsic factors, the anatomical site, the hydration status of the skin, the cohesion of the corneocytes (influenced as well by the vehicle used [126]), and the presence of furrows have to be mentioned [127]. Van der Molen observed that topically applied titanium dioxide could still be detected after removal of 40 tapes because of an accumulation in superficial skin furrows, thus falsifying the results [128]. In addition, interseasonal differences have been observed [129]. Among the extrinsic factors, the type of tape used (e.g., cellophane, polyester, polypropylene, polyethylene, rayon) [130, 131], the pressure with which the tape is applied onto the skin, the duration of the pressure, and the force of removal (slow/quick) influence the stratum corneum removal [127]. These are the parameters which can and must be standardized. Surber et al. outlined a standard protocol for tape stripping experiments [93]. The protocol implies the use of a template

to delineate the stripping area, and the exertion of a definite pressure (e.g., with a roller [132]) on each tape before removal. The use of a roller has been shown to prevent artifacts due to furrows and wrinkles [133]. Because of inter-individual differences in stratum corneum thickness, the complete stratum corneum of one skin site should be stripped. In newer investigations, Weigmann et al. used the tape stripping method to calculate horny layer profiles, in which the amount of drug penetrated is correlated not only to the tape number stripped but also to the depth of the stratum corneum. This correlation enables the localization of the drug within the stratum corneum layers. For this purpose, the removal of the entire stratum corneum and the quantification of both corneocytes and drug on the tapes are required [134]. Recently, Jacobi et al. reported a procedure for the estimation of the removed stratum corneum amount on each tape, thus providing a possibility to avoid the complete removal of the stratum corneum [94].

After a review of all the influencing and confounding factors affecting the tape stripping technique, the withdrawal of the DPK draft guideline for the bioavailability assessment of topical dermatological drug products [1] becomes more comprehensible. The concern of inadequate reproducibility between laboratories is not at all amazing. The FDA draft guidance recommended the application of the test formulation on ≥ 8 skin sites on each forearm, with tape stripping performed at incremental times after application. Only 12 tapes, without specification of the type of tape to be used, had to be stripped. Moreover, the first 2 tapes were discarded from evaluation and the remaining 10 tapes pooled and extracted together. The protocol described in the draft guidance was not yet mature and did not satisfy the regulatory requirements for bioavailability/bioequivalence assessment at the time of withdrawal. In the future, the proposal of a more realistic and elaborated protocol could ensure a successful regulatory basis for a promising and already widely used DPK method like tape stripping.

3.3. Further techniques

Other approaches for the assessment of the drug concentration profile in the skin following topical application do exist (reviewed in [65]). The most invasive method is represented by the direct excision of skin tissue. The **punch biopsy** is performed with round disposable knives (diameter 2-10 mm), and the biopsy contains epidermis, dermis, and part of the subcutis. The **shave biopsy** is more superficial, contains epidermis and part of the dermis, and is usually performed with a razor blade [135]. The **skin scraping** (curettage) technique usually removes the superficial stratum corneum with a dermal curette, but deeper parts of the epidermis can be sampled as well with a more vigorous scraping [136]. The **skin surface biopsy** uses quick drying cyanoacrylate glue to remove the stratum corneum in few samplings. The glue is applied to a glass slide, pressed onto the skin surface, and then removed after polymerization of the glue [137].

Cutaneous microdialysis enables the real-time assessment of cutaneous drug delivery in a minimally invasive manner. A probe, consisting of a semipermeable dialysis membrane tube and simulating a blood vessel, is inserted into the dermis and perfused by a physiological solution, which equilibrates with the extracellular fluid of the surrounding tissue. Topically applied compounds can thus be determined in the dermal extracellular water [138].

The **suction blister** technique is usually performed to assess drug levels in the skin after systemic administration. Intra-epidermal blisters are artificially produced by placing a chamber with several holes onto the skin and applying a vacuum. The suction blister fluid displays approximately the same composition of the interstitial fluid [139].

In addition to these invasive techniques, several imaging methods have been applied to the skin in the last years. The most promising optical methods seem to be the **confocal scanning laser microscopy** [140] and the **confocal Raman microspectroscopy** [141, 142]. These new techniques allow to focus a beam to a given depth within the skin and to determine the concentration of the topically applied compound at this level in an absolutely non-invasive manner.

4. Stratum corneum reservoir

4.1. Definition

The term “reservoir” referred to the skin can be defined as an accumulation of a topically applied compound within the skin or within a particular skin layer for a longer time period. This accumulation can be due to: 1) temporary sequestration of compound because of binding to specific structures of the skin (e.g., to keratin, proteins, amino acids [143], collagen fibers [144], stratum corneum lipids [145]); or 2) high partitioning into a specific skin layer (e.g., into the intercellular lipids of the stratum corneum) and relatively slow release into the next layer. The consequence is either a hindered diffusion to deeper skin layers in the first case, or a low diffusional flux of minimal amounts into deeper tissues in the second case [53, 146]. In both cases, the reservoir is not a time-stable state, and the factors rate and extent have to be considered [42].

The formation and the duration of a reservoir can affect the topical application in terms of dose and application frequency and should be considered when assessing DPK. During topical therapy with corticosteroids, it is desiderable that the drug remains for a long time at the site of action, i.e. within the skin, and that it exerts a local and not a systemic action. In this case, the formation of a stratum corneum reservoir and a slow drug release to deeper skin tissues over a longer time period is advantageous and desired.

4.2. Localization of the reservoir

The ability of the skin to store topically applied drugs was already suggested in the 50’s, when Malkinson and Ferguson detected ^{14}C-hydrocortisone in the urine of 2 volunteers 7 days after topical application [147]. Possible localizations of the reservoir were: the upper dermis, the viable epidermis, the stratum corneum, the skin appendages, or a drug film on the skin surface.

In 1963, Vickers demonstrated the existence of a reservoir for corticosteroids within the stratum corneum of healthy volunteers [148]. After application of fluocinolone acetonide and TACA in 95% ethanol and 16 h-occlusion, he observed the typical blanching effect due to the vasoconstrictive property on the dermal blood vessels. After simple re-occlusion, the vasoconstriction could be observed for 14 days. Vickers concluded that a depot of corticosteroid was present within the skin and that it could be re-activated by occlusion. Tape stripping experiments demonstrated that it was not possible to induce a reservoir on stripped skin, and that a reservoir induced on intact skin could be eliminated by tape stripping the stratum corneum [149]. The participation of the drug film on the skin surface could be excluded by washing the skin with several methods (soap and water, ether, 95% ethanol) [148, 150, 151]. Vicker’s reservoir was confirmed and quantified in 1966 by Carr and Wieland with ^{14}C-labeled TACA applied in 95% ethanol under occlusion. More than 75% of the steroid was detected in the upper stratum corneum [152]. Schaefer et al. showed that less than 5% of topically applied

TACA (in both an ointment and cream vehicle) was systemically absorbed. The healthy stratum corneum was able to retain up to 30% of the steroid as a reservoir, and 70-90% remained on the skin surface [81].

McKenzie and Stoughton excluded the participation of the dermis to the reservoir development by intradermally injecting TACA in one arm and topically applying under occlusion a comparable amount on the contralateral arm of 12 volunteers [153]. Initially, a vasoconstriction was observed on both arms, but the vasoconstriction on the “intradermal-injection-arm” faded after a couple of hours and did not reappear, whereas a re-occlusion of the “topical-application-arm” still developed a vasoconstriction.

Yet, the participation of epidermis, dermis, and underlying tissues to a reservoir formation has recently been revised [146]. Binding to specific skin structures as well as high partitioning in one skin layer and low release to the next layer is not only possible for the stratum corneum, but also for epidermis and dermis. A high binding to dermal tissue has been observed for diclofenac in the Wistar rat [154]. Cross et al. observed with human skin *in vitro* a decreased dermal permeation for the more lipophilic alcohols of a homologous series (C2-C10), which resulted in an (epi-)dermal accumulation. They postulated that the dermis may represent a more lipophilic compartment than originally believed [53]. In an *in vitro* investigation of the fate of dihydroxyacetone (DHA) in human and rat skin, Yourick et al. observed that 24 h after application of a realistic DHA dose, 22% of the applied dose was still within the whole skin, with half the amount residing within the stratum corneum and half within the viable epidermis/dermis. Approximately 5% of the DHA was covalently bound to proteins, and the majority of DHA was probably noncovalently bound to free amino acids [155]. Jacobi et al. measured high amounts of flufenamic acid, which remained constantly high for 21 h, in porcine dermis *in vitro* after infinite dosing in wool alcohol ointment [156]. An accumulation of dexamethasone and hydrocortisone was observed in dermis, deeper epidermis, and hair follicles after application of a Transcutol® (diethylene glycol monoethyl ether) containing vehicle on human and rat skin *in vitro* [144, 157]. In newer studies, the participation of skin appendages, especially the follicles, to the reservoir formation was also reconsidered [158, 159]. A higher lipophilicity of the applied compound [155, 160] as well as the application in polar vehicles like propylene glycol [161] and ethanol [162] seem to improve the follicular targeting. The formation of a follicular reservoir can become relevant in skin regions with high follicular density like the forehead [46].

In conclusion, the accumulation of topically applied compounds within the skin and thus the formation of a reservoir is possible for both lipophilic and hydrophilic compounds. The reservoir is usually located within the stratum corneum, but can sometimes occur also in viable epidermis, dermis, and follicles. Though the reservoir formation was initially studied with corticosteroids, it is not restricted to this drug class. A stratum corneum reservoir has been observed for several other compounds, for example antimicrobials (hexachlorophene [151], fusidic acid [149]), vitamin E [163], nicotine [164], caffeine [165], cationic β-blockers [166], sunscreen agents [167, 168], hair dyes [169], surfactants in soaps [170], and carcinogenic aromatic amines [171].

4.3. Factors influencing the reservoir

The factors promoting a reservoir formation are substantially the same as those influencing their percutaneous penetration (see chapter 2.4) [172].

Among the physicochemical properties of the **drug**, a high lipophilicity and protein affinity is favorable for a reservoir development in the stratum corneum [53, 173]. A minimal, defined **amount** of drug has to be applied to induce a reservoir. Clarys observed the formation of a 5-day-lasting halcinonide reservoir after application in an oil-in-water vehicle, but only for the highest tested concentration (0.2% vs. 0.05% and 0.005%) [174]. Carr and Tarnowsky obtained similar findings with TACA ointments (0.1% vs. 0.01%) [175].

The ability to develop a reservoir is highly dependent on the **vehicle**. Vickers observed *in vivo* that the application of steroids in alcoholic solutions always induced a reservoir (lasting 9.6 days), hydrophilic creams induced a reservoir in 92% of the volunteers (lasting 7.4 days), and ointments induced a reservoir in 68% of the volunteers (lasting 4.6 days). Presumably, the intercellular lipids of the stratum corneum were partly damaged by the alcoholic solvent, thus facilitating drug penetration [149]. In contrast, with fluocinolone acetonide *in vitro*, Munro observed the highest stratum corneum retention after application in white petrolatum [172]. Particularly, the presence of **penetration enhancers** like urea [174], dimethyl sulfoxide (DMSO) [150], dimethylacetamide (DMAC) [151], dimethylformamide [176], Transcutol® (diethylene glycol monoethyl ether) [144, 157], or propylene glycol [177] has been shown to promote a reservoir formation. Stoughton observed that the addition of 40% DMSO to different formulations could induce an accumulation of 15-100% more steroid (hydrocortisone, fluocinolone acetonide) within the stratum corneum compared to a DMSO-free vehicle. Even without the need of an application under occlusion, a 16-day-lasting reservoir was formed [150]. The addition of urea to an oil-in water vehicle induced a 5-day-lasting reservoir after application of low amounts of halcinonide (0.005%). This was not observed after application of the urea-free formulation [174]. The addition of Transcutol® to a gel vehicle increased the penetration of hydrocortisone into rat skin, decreased its permeation across the skin, and induced a skin depot lasting 7 days after a single infinite application under occlusion [144].

As already described in chapter 4.2, a reservoir is often induced after application under **occlusion**. The enhanced hydration can considerably change the affinity of the applied compound to the stratum corneum and its partitioning between the different skin layers (stratum corneum, epidermis, dermis) [153]. The duration of occlusion seems to play an important role: an occlusion ≤ 8 hours does not induce a reservoir or induces a reservoir of short duration [149].

4.4. Relevance of the reservoir

The therapeutical and physiological relevance of the reservoir formation has been the subject of discussions since its discovery. In 1966, Carr and Tarnovsky doubted its relevance. They observed that the percutaneous absorption out of the stratum corneum reservoir after application of a TACA ointment on the whole body under occlusion was not sufficient to suppress the pituitary-adrenal axis [175]. Yet, the suppression of the pituitary-adrenal axis is not really a phenomenon which can be correlated with the topical clinical effect. In a therapeutically more relevant study, Stoughton showed that the induction of a hexachlorophene reservoir after application of a DMAC-containing formulation increased and prolonged the bacteriostatic efficacy of hexachlorophene compared to a commercial formulation [151].

The main problem during the investigation of topical bioavailability or percutaneous absorption of topically applied compounds is the application on usually healthy skin, whereas clinically the compounds are usually applied on diseased skin. In atopic dermatitis, eczema, or psoriasis, the skin displays an impaired stratum corneum barrier and a different composition of the stratum corneum lipids. An enhanced penetration and systemic absorption have to be expected if an intact stratum corneum barrier is not guaranteed. The induction of a reservoir in diseased skin was not successful in several experiments, for example with volunteers displaying psoriasis or Lichen simplex [149]. Schäfer et al. measured a 3-10 times higher TACA concentration in psoriatic epidermis and dermis after application of TACA in ointment and cream formulations, and a marked higher systemic absorption compared to healthy skin [81]. Yet, in patients suffering from atopic dermatitis, a hydrocortisone reservoir could be formed, and the steroid could be released from the reservoir after 12 h by application of a propylene glycol-containing moisturizer [177].

During therapy with corticosteroids, the reservoir formation seems to have a more preventive than therapeutical relevance [149]. For the formation of a long-lasting reservoir, a more or less intact stratum corneum is required, and this is in contradiction with the acute therapeutical indication for topical corticosteroids. Nevertheless, already after a partial regeneration of the stratum corneum, the development of a reservoir becomes relevant again. Several established application regimens of corticosteroids take advantage of the fact that “stored” steroids can be remobilized out of the reservoir by a simple application of moisturizing cream (compare the interval and tandem therapy outlined in chapter 5.1, “Application regimen”).

5. Topical glucocorticosteroids

5.1. Overview

Classification

Topical corticosteroids are classified according to their efficacy into four potency classes[7] where class I represents mildly potent, class II moderately potent, class III potent, and class IV very potent corticosteroids. The same drug can be classified into a different potency class depending on the concentration and the vehicle (Table 5-1). This classification is based upon the vasoconstrictive properties of the corticosteroids, macroscopically visible as a paling of the skin ("blanching effect") [178].

Table 5-1: Potency of selected glucocorticosteroids (modified after Niedner [180]).

Corticosteroid	mild (class I)	moderately strong (class II)	strong (class III)	very strong (class IV)
Hydrocortisone	0.25-2.5%			
Prednisolone	0.4%			
Clobetasole butyrate	0.05%			
Dexamethasone	0.01%	0.05-0.1%		
Triamcinolone acetonide		0.01-0.1%		
Hydrocortisone butyrate		0.1%		
Betamethasone valerate		0.05%		
Fluocinolone acetonide		0.01%	0.025%	
Betamethasone dipropionate			0.05%	
Halcinonide			0.1%	
Diflucortolon valerate				0.3%
Clobetasol propionate				0.05%

Independently of the potency classification, topical corticosteroids can be classified into four generations. Starting from hydrocortisone and prednisolone (1st generation), several chemical modifications have been performed to enhance the desired pharmacological effects (e.g., anti-inflammatory, anti-proliferative effects) and to reduce the adverse effects (e.g., the mineralocorticoid effect). The halogenation of the compounds led to a higher efficacy but also to a higher rate of side effects (2nd and 3rd generation). The design of ester prodrugs led to highly effective compounds with a better side effect profile [180].

[7] In the Anglo-American region, the potency classification includes 7 classes, where class 1 represents the superpotent, and class 7 the mildly potent corticosteroids [178].

Glucocorticoid effects and indications

The antiphlogistic, immunosuppressive, and antipruriginous effects of corticosteroids are used for the treatment of the atopic dermatitis, of the seborrhoeic, nummular, and allergic contact dermatitis, as well as of the dishydrotic eczema. More potent corticosteroids additionally exerting an antiproliferative effect are used in the treatment of psoriasis, lichenifications, Lichen planus or sclerosus, and discoid lupus erythematodes [181].

The molecular effects result by binding of the corticosteroid to the intracellular gluco-corticosteroid receptor. After molecular transformation, the complex is translocated into the nucleus, where it represses or activates the expression of various genes. Moreover, non-genomic effects result by binding of the steroid to membrane-associated receptors [182, 183]. Table 5-2 summarizes the effects observable at the cellular level.

Table 5-2: Cellular effects of topical glucocorticosteroids [184].

Cell type	Cellular effect
Keratinocytes	Inhibition of proliferation and normalization of the hornification process
Fibroblasts	Inhibition of the synthesis of collagene and mucopolysaccharides
Lymphocytes, granulocytes	Inhibition of immigration and proliferation
Mast cells, basophils	Inhibition of the release of inflammatory mediators
Langerhans cells	Inhibition of proliferation and function
Melanocytes	Inhibition of pigment formation
Adipocytes	Inhibition of proliferation
Blood vessels	Vasoconstriction

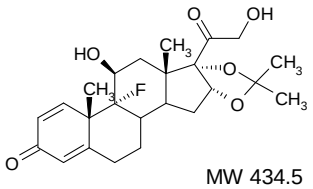
Adverse effects and contraindications

The antiproliferative effect of potent corticosteroids can lead to local effects such as atrophy of epidermis and dermis. This has been reported with very potent steroids, after a long treatment period, and after application under occlusion. The atrophogenic potential of the very potent corticosteroids is clinically used for the treatment of keloids, where the steroid is directly injected into the skin. Furthermore, the development of striae, folliculitis, hypertrichosis, hypopigmentation, perioral dermatitis, steroid-inducted acne, and a disturbed wound healing are possible. Skin infections can occur because of the immunosuppressive effect. Allergy to the steroid or to the vehicle has been reported, as well as the development of glaucoma if potent corticosteroids (class 3 and 4) are applied near the eye. Tachyphylaxis and rebound phenomena have been described after prolonged therapy and abrupt discontinuing of the therapy. Therefore, a short-term therapy and a gradual withdrawal of the steroid are indicated. Systemic side effects are possible if the percutaneous penetration of the topical corticosteroid is enhanced, e.g. by occlusion, in intertriginous skin regions, or during acute skin affection where the skin barrier is highly damaged. The worst case can result in an adrenocortical suppression and in a cushing-syndrome, which, however, is very rare and has been reported only with very potent corticosteroids [185]. Contraindications for a therapy with topical corticosteroids are skin infections, rosacea, perioral dermatitis, urticaria, corticosteroid allergy, pregnancy, and breast feeding.

Triamcinolone acetonide (TACA)

TACA (9α-fluoro-11β,21-dihydroxy-16α,17α-isopropylidenedioxy-1,4-pregnadiene-3,20-dione) is a synthetic, moderately potent glucocorticosteroid with pronounced antiallergic, antiphlogistic, antipruriginous, and vasoconstrictive properties. Common topical concentrations are 0.025-0.5%. The derivatization of triamcinolone (orally available, logP 1.02) to the more lipophilic acetonide (logP 2.27) confers on TACA topical bioavailability [186] and a 200 times higher affinity to the glucocorticosteroid receptor [187]. The compound is almost insoluble in water and more easily soluble in organic solvents. Table 5-3 displays its chemical structure and the solubility in different solvents [188].

Table 5-3: Chemical structure of TACA ($C_{24}H_{31}FO_6$) and solubility in different solvents.

Triamcinolone acetonide (TACA)	Solubility [mg/ml]	Solvent
	0.04	water (pH 7)
	25	chloroform
	40	isopropyl alcohol
	50	95% ethanol
	90	acetone
MW 434.5	250	dimethylformamide

The acetonide group protects TACA against oxidative degradation by epidermal enzymes [189]. Absorbed TACA is extensively metabolized in the liver (6β-hydroxy-TACA, 21-carboxy-6β-hydroxy-TACA, 21-carboxy-TACA) and excreted mainly by the kidney (85% as metabolites, 15% unchanged) and, to a minor extent, by the bile [190].

Application regimen

The efficacy of corticosteroids depends on several factors. The most relevant are: the concentration of the applied formulation (and simultaneously the applied dose), the frequency of application, the potency of the corticosteroid, the properties of the vehicle (e.g., occlusive, emollient, drying, volatile), the application conditions (e.g., under occlusion or extreme rubbing), the contact time, and the skin disease [191]. The effect of concentration, application frequency, and occlusion on the percutaneous penetration and the efficacy of low dose topical corticosteroids are outlined in detail in chapters 5.2, 5.3, and 5.4, and were investigated experimentally in Projects II, III, and IV. First, an overview of different application regimens is given.

In current dermatopharmacotherapy, a **step-down therapy** is generally advised (Fig. 5-1). The treatment of an acute dermatosis should begin with a potent corticosteroid (class III, occasionally class IV) until a good clinical response is observed. Then, the potent corticosteroid can be replaced with a moderately potent (class II) one, and finally a mildly potent (class I) can be used. This procedure prevents local side effects and rebound [192].

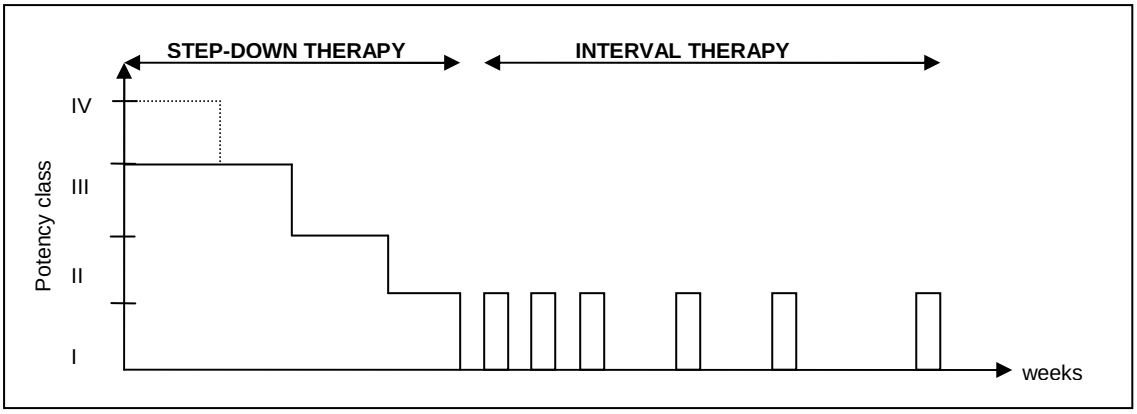


Fig. 5-1: Therapy scheme of topical corticosteroids. Step-down and interval therapy (modified from Hatz [192]).

A valuable alternative is the **interval therapy** (Fig. 5-1). Instead of abruptly stopping the therapy, corticosteroid-free intervals during which only the cream base is used are alternated with the corticosteroid applications [192]. The duration of the corticosteroid-free intervals can be quite variable, and every-other-day (alternate-day), twice-weekly, or weekend-only (pulse therapy) corticosteroid applications are not uncommon [193]. In a double-blind, randomized, vehicle-controlled, bilateral-paired comparison with 32 psoriatic patients treated with betamethasone dipropionate cream, Singh did not observed any significant difference between alternate-day vs. once-daily corticosteroid applications. On the contrary, the application every third day or weekly was less effective than the once-daily application [194]. In patients suffering from atopic dermatitis, the twice-weekly application of fluticasone propionate combined with the daily emollient significantly reduced the risk of a relapse, and was thus paradoxically steroid sparing [195].

Corticosteroid applications often follow a **tandem therapy**: the corticosteroid is alternated with the cream base, for example the corticosteroid is applied in the evening, the cream base in the morning. The adjunctive skin care with the cream base is of extreme importance in most dermatoses. Moreover, the intermittent steroid application has proved useful in preventing tachyphylaxis, observed after prolonged and intensive therapy, especially after multiple-daily applications [196, 197].

A more specific treatment regimen is the **topical rotational therapy** [198], known especially from the psoriasis treatment: corticosteroid applications are alternated with applications of other topical agents (e.g., calcipotriol, tazarotene). This offers certain advantages, since monotherapy with topical corticosteroids for psoriasis generally results in a limited total clearance of the lesions (4-36%) [199]. Singh et al observed that the use of betamethasone and calcipotriol on alternate weeks was more effective than the daily application of the corticosteroid alone. This new treatment strategy seems to prevent or minimize tachyphylaxis [198].

5.2. Concentration and dose

Pharmacodynamic aspect

Potency and efficacy of corticosteroids are usually assessed using the skin blanching assay, which estimates the potency based on the skin whitening side effect due to vasoconstriction [153]. The blanching assay is suited for bioequivalence testing of different proprietary formulations and for efficacy testing of different concentrations of the same glucocorticosteroids in the same vehicle [92].

The dose-response curves obtained by blanching assessment of topically applied corticosteroids can usually be divided into 2 parts (Fig. 5-2). The first part displays a relatively steep dose-response relationship where small changes in concentration lead to a high potency increase. The second part displays a rather flat dose-response relationship where large increases in concentration lead only to small changes in potency. The typical concentration of proprietary corticosteroid formulations usually resides in the flat, second part of the dose-response curve, and often a 10-to-20-fold dilution is needed to achieve a detectable change in potency [191]. In the case of TACA, the 4-fold dilution of different proprietary formulations (0.5%-0.025%) did not change its efficacy significantly [200].

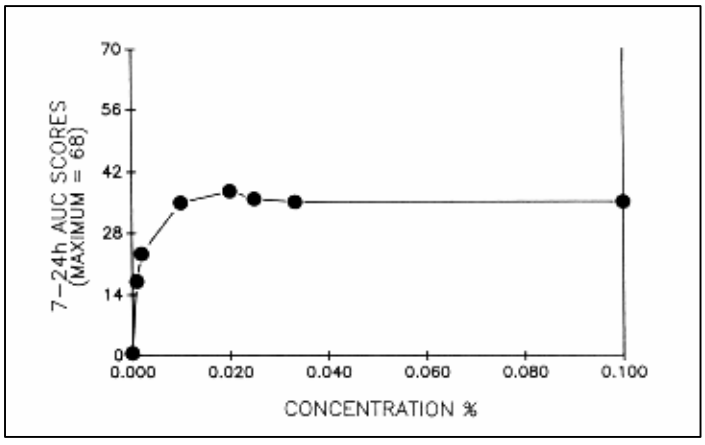


Fig. 5-2 : AUC / concentration curve for a betamethasone valerate ointment. The AUC scores are a measure of the vasoconstrictive potency, and were obtained from blanching response profiles from 7 to 24 h after application of the formulation [191].

For this reason, it could be assumed that marketed corticosteroid formulations contain much more drug than necessary for their efficacy. Actually, in clinical practice, proprietary formulations are often diluted with the cream base to decrease the corticosteroid concentration.

The topic concerning the efficacy of low-dose corticosteroid formulations as well as advantages and risks of dilution has been investigated and discussed in the experimental section (Project IV: Efficacy of low-dose corticosteroids in atopic dermatitis).

Pharmacokinetic aspect

Increasing the concentration of the active compound in a topical formulation does increase its percutaneous penetration, provided that the compound is dissolved. The relationship between dose applied and absolute absorption can be defined as roughly linear, but only over a finite range. After surpassing the threshold, a surplus of the compound may not be absorbed from the skin because of skin saturation or because of compound precipitation. In case of skin saturation, the compound diffusion through the skin is not expected to follow Fick's law of diffusion [201, 202].

Wester and Maibach thoroughly investigated the percutaneous penetration of hydrocortisone, testosterone, benzoic acid, and nitroglycerine in a wide concentration range (Table 5-4) [203]. After application of a 10-fold increased concentration of hydrocortisone (4 vs. 40 µg/cm^2), only a 4-fold increase in hydrocortisone absorption was observed. After application of increasing concentrations of nitroglycerin, the absolute percutaneous absorption increased roughly linearly in the concentration range of 10-7000 µg/cm^2, but then decreased abruptly at 10000 µg/cm^2. As shown in Table 5-4, the application of high drug concentrations always results in a decreased relative absorption [204]. In contrast, low-dose formulations are absorbed more efficiently (higher relative absorption). Moreover, the saturation degree of the drug within the formulation plays a determinant role [196]

Table 5-4: Percutaneous absorption of different topical doses of hydrocortisone, testosterone, benzoic acid, and nitroglycerin in the rhesus monkey model and in human volunteers (data for hydrocortisone, testosterone, and benzoic acid from [203, 204]; data for nitroglycerine from [205]).

Compound	Dose applied	Percutaneous absorption (monkey)		Percutaneous absorption (human)	
	[µg/cm^2]	Absolute [µg]	Relative [%]	Absolute [µg]	Relative [%]
Hydrocortisone	4	0.12	2.9	0.06	1.6
	40	0.84	2.1	0.24	0.6
Testosterone	3	-	-	0.35	11.8
	30	-	-	2.6	8.8
	40	2.7	6.7	-	-
	250	7.2	2.9	-	-
	400	8.8	2.2	11.2	2.8
	1600	46.4	2.9	-	-
	4000	56.0	1.4	-	-
Benzoic acid	3	-	-	1.1	37.0
	4	2.4	59.2	-	-
	400	134.4	33.6	102.8	25.7
	2000	348.0	17.4	288.0	14.4
Nitroglycerine	10	-	-	0.004	41.8
	100	-	-	0.04	43.5
	1000	-	-	0.4	36.6
	7000	-	-	1.9	26.6
	10000	-	-	0.8	7.8

Similar results were observed with other compounds, e.g. parathion and lindane [203], phenol [202], and 4-cyanophenol [61]. The percutaneous penetration of TACA after application of a higher and a lower concentration was experimentally investigated in Project II: Effect of dose and application frequency.

5.3. Application frequency

Pharmacodynamic aspect

The application frequency of topical corticosteroids is a recurrently debated topic. Traditionally, the application of topical corticosteroids followed a multiple-daily scheme, with the application of the product up to 3-4 times a day. Yet, this habit had no scientific basis [206]. Newer guidelines usually recommend once- or twice-daily applications [193], and most clinicians follow the twice-daily application schedule until a clinical response is obtained and then reduce the application frequency [179].

Two systematic reviews of atopic dermatitis treatment revealed a similar clinical efficacy of once-daily vs. more frequent applications. There was no clear evidence to support the multiple-daily corticosteroid use [207, 208]. In a review involving both atopic dermatitis and psoriasis patients, Lagos et al. found no significant difference in the treatment response of once vs. twice daily applications with very potent corticosteroids, and no difference or only a slight difference with potent or moderately potent corticosteroids [209]. The once-daily application may also be preferable to minimize the risk of side effects and tachyphylaxis [196, 197], to improve patient compliance, and to reduce the therapy costs.

The individual circumstances have always to be considered to ensure an optimal therapy. Relevant factors determining the choice of application frequency are: the severity, localization, and type of the dermatosis, as well as the potency and type of the corticosteroid. Whereas once-daily applications may be sufficient to cure mild or moderate skin affections, a more frequent application might be needed in severe forms to achieve a higher total clearance of the lesions [210] and an accelerated onset of action [211-213]. Severe psoriasis, for example, may require a more frequent application compared to atopic dermatitis [214]. For very potent corticosteroids, once-daily applications are usually recommended, and the new generation corticosteroids like mometasone furoate [215, 216] and fluticasone propionate [217, 218] are marketed as “once-daily” preparations.

Table 5-5 gives an overview of the most important pharmacodynamic studies comparing the treatment efficacy of once-daily and multiple-daily applications of the same corticosteroid formulation. The once-daily application schedule always followed the tandem therapy (see chapter 5.1), with applications of the corticosteroid in the evening alternating with applications of an emollient in the morning and in the afternoon. On the whole, the results show that a once-daily application can be as effective as multiple-daily applications, but that a twice- or thrice-daily application may accelerate the onset of action. The adjunctive skin care with emollients increases the efficacy of the treatment and has steroid-sparing quality [199, 219].

Table 5-5: Overview of the pharmacodynamic studies investigating the efficacy of once-daily vs. multiple-daily topical corticosteroid applications. All the studies are randomized, prospective and vehicle-controlled (additional applications of emollient cream in case of the once-daily schedule), and are displayed in chronological order.

Author	Study design	Disease	Corticosteroid	Preferred regimen	
				Once daily	Multiple daily
Fredriksson et al. (1980) [211]	Double-blind bilateral-paired multi-centered 3 weeks	Psoriasis (n=35) Atopic dermatitis (n=60)	Halcinonide cream 0.1%	X	
Sudilovsky et al. (1981) [214]	Double-blind bilateral-paired multi-centered 3 weeks	Psoriasis (n=194) Atopic dermatitis (n=149)	Halcinonide cream 0.1%		X thrice daily
Gärtner et al. (1984) [220]	Double-blind parallel 2-centered 2 weeks	Atopic dermatitis (n=100)	Betamethasone dipropionate cream 0.05%	X	
English et al. (1989) [212]	Double-blind parallel 2-centered 3 weeks	Atopic dermatitis (n=63) Psoriasis (n=34)	Betamethasone dipropionate cream 0.05%		X twice daily
Watsky et al. (1992) [219]	Open-label bilateral-paired 4 weeks	Psoriasis (n=96)	Betamethasone dipropionate cream 0.05%	X	
Singh et al. (1995) [221]	Double-blind bilateral-paired 9 days	Psoriasis (n=36)	Betamethasone dipropionate ointment 0.05%	X	
Koopmans et al. (1995) [210]	Double-blind parallel multi-national 4 weeks	Atopic dermatitis (n=150)	Hydrocortisone 17-butyrate fatty cream 0.1%		X twice daily
Bleehen et al. (1995) [217]	Double-blind parallel multi-centered 4 weeks	Atopic dermatitis (n=270)	Fluticasone propionate cream 0.05%	X	
Tharp (1996) [213]	Double-blind parallel multi-centered 4 weeks	Atopic dermatitis (n=238)	Fluticasone propionate cream 0.05%	X	
Singh et al. (1998) [215]	Double-blind bilateral paired 2 weeks	Psoriasis (n=64)	Mometasone furoate ointment 0.1%	X	
Kanzler et al. (2001) [199]	Double-blind bilateral-paired 4 weeks	Psoriasis (n=24)	Triamcinolone acetonide cream 0.1%	X	

Pharmacokinetic aspect

Only few pharmacokinetic studies investigating multiple applications have been performed. Most of these studies draw their conclusions from data derived from drug concentration determination in plasma [222, 223] and urine [224, 225]. This means that percutaneous absorption and thus systemic, not topical bioavailability, is measured.

Melendres et al. investigated the effect of application frequency on the percutaneous absorption of ^{14}C-labeled hydrocortisone applied in acetone in human volunteers. The study compared a single low dose (1x13.3 µg/cm^2) to a threefold higher dose, applied either as a single dose (1x40 µg/cm^2) or as multiple doses (3x13.3 µg/cm^2). Percutaneous absorption was assessed by measurement of the urinary excretion over 7 days. A 3-fold increase in dose was supposed to yield a 3-fold increase in absorption. This turned out to be correct for application of the single high dose (1x40 µg/cm^2). Yet, the multiple application (3x13.3 µg/cm^2) resulted in a percutaneous absorption much higher than expected (Fig. 5-3), as well as in higher drug amounts within the stratum corneum [225].

A similar trend was obtained after hydrocortisone application in a cream vehicle. The application of the single high dose (1x40 µg/cm^2) resulted, as expected, in a percutaneous absorption 3 times higher compared to the single low dose (1x13.3 µg/cm^2). Again, the multiple application (3x13.3 µg/cm^2) resulted in an unexpected higher percutaneous absorption (Fig. 5-3) [226].

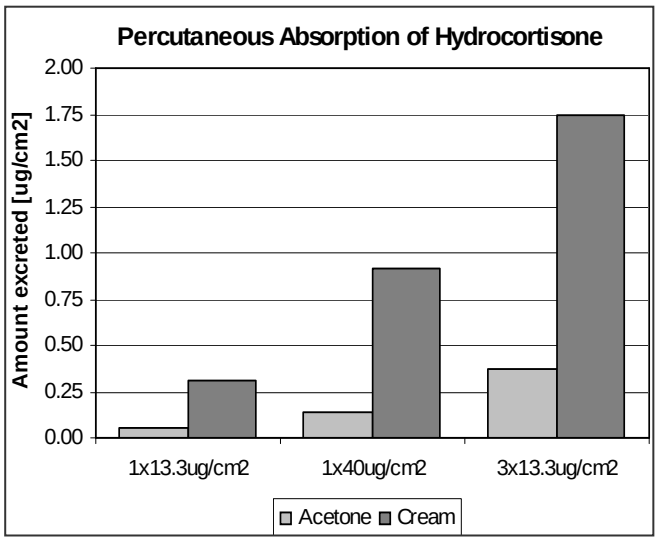


Fig. 5-3: Effect of application frequency on the percutaneous absorption of hydrocortisone applied on the forearm of human volunteers as an acetonic solution (data from [225]) and a cream (data from [226]. The percutaneous absorption was assessed as the total amount excreted in the urine over 7 days.

Investigations on the percutaneous absorption of hydrocortisone [224], testosterone, and estradiol [227] in the rhesus monkey were not consistent with human *in vivo* studies performed with the same design. Therefore, in some cases, the extrapolation of results obtained from animal studies to estimate percutaneous penetration in humans may be inappropriate.

The effect of application frequency on the *in vivo* penetration of TACA into stratum corneum was investigated in human volunteers in the experimental section, Project II: Effect of dose and application frequency.

5.4. Occlusion

Pharmacodynamic aspect

Occlusion is a simple method for enhancing the efficacy of topically applied drugs in clinical practice. The general effects of occlusion on the skin and on percutaneous absorption have already been described in chapter 2.4.4. An increased clinical efficacy of topical drugs under occlusion was first documented in 1960 by Garb with podophyllin (applied for the treatment of the naevus verrucosus) [228]. An enhanced efficacy of corticosteroids in the treatment of psoriasis was then observed for fluocinolone acetonide [229, 230] and hydrocortisone [231] applied under occlusion. The combination of topically applied calcipotriol plus occlusion for the treatment of psoriasis resulted as well in a significantly higher improvement of the lesion compared to calcipotriol or occlusion alone [232]. Interestingly, occlusion alone (without application of any topical formulation) also induced a significant improvement of psoriatic lesions which was comparable with the effect of fluocinolone acetonide [230].

The introduction of the McKenzie-Stoughton vasoconstriction test enabled the correlation of a pharmacodynamic endpoint (the vasoconstriction) with the efficacy of topical corticosteroids, and showed that the application of corticosteroids under occlusion clearly induced a higher vasoconstrictive response compared to non-occluded applications [153].

Pharmacokinetic aspect

The percutaneous absorption of different steroids (hydrocortisone, estradiol, testosterone, progesterone) after application under occluded and non-occluded conditions was investigated by Bucks et al. [233, 234]. The percutaneous absorption was significantly increased by 24 h-occlusion for estradiol, testosterone, and progesterone but not for hydrocortisone, the most hydrophilic steroid in this experiment. A linear trend of occlusion-induced absorption-enhancement with increasing compound lipophilicity was observed except for progesterone (the most lipophilic compound in the array). Similar results were obtained after application of different phenols: occlusion significantly increased the percutaneous absorption of some (the more lipophilic) but not of all phenols. The least effect of occlusion was encountered with the more hydrophilic phenols [52]. Treffel et al. investigated *in vitro* the compound-specific effect of occlusion. Occlusion enhanced the percutaneous absorption of citropten (a lipophilic compound) by a factor of 1.6, whereas the absorption of caffeine (an amphiphilic compound) remained unaffected [73]. Thus, occlusion seems to have a higher effect on percutaneous penetration and absorption for lipophilic compounds and less for hydrophilic compounds. The hydration of the normally lipophilic stratum corneum during occlusion alters the partitioning of the applied compound between stratum corneum and the hydrophilic viable epidermis.

The absorption enhancement encountered during occlusion appears to be dependent not only on the compound but also on the vehicle and on the material of the occlusive device. Cross and Roberts observed a clear penetration enhancement of parabens after application in volatile solvents (acetone, ethanol) under occlusion, whereas the occlusive application of an ointment resulted in a retardation of penetration. Depending on the vehicle, occlusion had significantly different effects on both partitioning and epidermal diffusivity. Moreover, occlusion of the volatile vehicles prevented their evaporation, and the solvents may have loosened the barrier of the stratum corneum by extracting intercellular lipids [235]. The material of the occlusive device seems to play a decisive role as well, since different materials display different water vapor permeability (Table 5-6) [68, 236].

Table 5-6: Water vapor permeability characteristics of different materials (data from [68]).

Material	Thickness [µm]	Water-vapor permeability [g/m^{2}h]	Manufacturer
polypropylene	15	0.9 ± 1.1	Tonen Chemical Co.
polyester	12	1.9 ± 0.0	Tonen Chemical Co.
nylon	25	12.0 ± 1.4	Unitika Ltd.
nylon	15	16.6 ± 0.4	Tonen Chemical Co.
polyurethane	80	24.9 ± 0.6	Toyo Kagaku Co. Ltd.
polyurethane	25	30.7 ± 0.2	Toyo Kagaku Co. Ltd.
polyurethane	60	32.1 ± 0.2	Toyo Kagaku Co. Ltd.
microporous polyethylene	38	80.9 ± 8.0	Sekisui Chemical Co. Ltd
polypropylene (non-woven)	18	145.6 ± 11.0	Tonen Chemical Co.

As described in chapter 4, the formation, the extent, and the duration of a reservoir are highly favorized by application of the compound under occlusion [148]. After removal of the occlusive wrap, the hydration level of the stratum corneum rapidly returns to normal levels, and so does the facilitated partitioning between stratum corneum (hydrated by occlusion) and epidermis (hydrophilic). Thus, topically applied compounds remain temporarily trapped within the stratum corneum, forming a reservoir. In the case of TACA, Carr and Wieland observed an accumulation of a double amount of ^{14}C-labeled triamcinolone acetonide within the stratum corneum after occlusion compared to non-occluded skin sites [152].

The effect of occlusion on the *in vivo* penetration of unlabeled TACA into human stratum corneum was investigated in the experimental section, Project III: Effect of occlusion.

EXPERIMENTAL SECTION

6. Project I: Validation of the methodology and proof of concept

Abstract

The aim of the investigations described in this chapter was to develope and validate a method set for the *in vivo* quantification of TACA in human stratum corneum by means of: 1) tape stripping as sampling method, 2) UV/VIS-spectroscopy for the quantification of corneocytes, and 3) HPLC for the quantification of TACA.

A standardized tape stripping protocol was developed and included the use of a template (ensures the removal of stratum corneum samples from the same skin site) and a hand roller (ensures a constant pressure on the tape before stripping), and, most importantly, the removal of the entire stratum corneum of one skin site to cope with inter- and intra-individual differences in stratum corneum thickness. The UV/VIS-spectroscopical method for quantification of the corneocytes had been previously validated and was adopted. The HPLC method for TACA quantification was developed and validated according to ICH Guidelines. The analytical challenge consisted in the development of a sensitive analytical method capable to quantify low amounts of TACA distributed on single tapes after extraction. The HPLC method was successfully validated and proved to have suitable specificity, linearity, accuracy, precision, and robustness in the working range. The LOQ of 0.1 µg/ml enabled the quantification of 27 ng/cm^2 TACA on single tapes.

The method set was then applied in a proof of concept performed with 6 healthy volunteers. TACA (100 µg/cm^2) was applied either as an acetonic solution or as an ethanolic gel. Stratum corneum samples were taken 0.5 h, 3h, and 24 h later by standardized tape stripping. Whereas the TACA penetration from the acetonic solution was high and TACA reached deeper layers of the stratum corneum, the TACA penetration from the ethanolic gel was only superficial, most TACA being retained within the cellulose matrix of the gel after evaporation of the solvent. The proof of concept proved the suitability of the methods to distinguish between different penetration patterns of TACA.

Keywords

HPLC
Standardization
Tape stripping
Triamcinolone acetonide
Validation

6.1. Introduction

Specific and sensitive analytical methods for the quantitative evaluation of drugs are decisive for the successful execution of preclinical and clinical trials. To demonstrate the suitability of a bioanalytical method for the intended use and to ensure reliable and reproducible results, validation of the methodology is required [237, 238].

Tape stripping is a sampling method which enables the removal of the stratum corneum layer by layer [93]. The amount of stratum corneum removed is influenced by several factors, e.g. the type of tape and the pressure applied on the tape before stripping [131], the topical formulation (vehicle effects) [93], and the skin site [6, 127, 239]. Thus, a standardized methodology and a critical evaluation of the results are essential. After stratum corneum sampling, the quantification of corneocytes and the quantification of drug adhering to the tapes are both required to assess the penetration of a drug into the stratum corneum.

For the quantification of corneocytes, different methods have been described [240-243]. The time consuming gravimetric method has been the conventional method over years. However, the mass of corneocytes is usually overestimated because of additional weight caused either by exogenous and endogenous substances like excess of formulation, sebum, and sweat [134] or by interferences caused by electrostatic charge. In addition, this method has to be combined with measurements reflecting the disruption status of the stratum corneum barrier, e.g. by TEWL, and thus indirectly reflecting the stratum corneum thickness. Recently, TEWL measurements have been shown not to be sensitive enough for the measurement of small changes in stratum corneum thickness [244]. Moreover, an estimation of the stratum corneum density is required and usually arbitrarily set at 1 g/cm^3 [131, 243]. Dreher et al. proposed a colorimetric assay (modified Lowry total protein assay) for quantification of the stratum corneum proteins extracted from the tapes, stained, and measured spectroscopically at 750 nm. The method was shown to be an accurate and reproducible alternative to the gravimetric method [240, 241]. Nevertheless, the quantification of the stratum corneum proteins directly on the tapes would be simpler and faster. Marttin et al. proposed a spectroscopic method based on the protein absorption at 278 nm. Unfortunately, this method turned out to be less reliable than the gravimetric method, since the light scattering effects caused by the corneocytes on the tape overshadowed the weak absorption of the proteins. Even an additional protein staining with Brilliant Blue did not improve the quantification [242].

Weigmann et al. proposed a novel method based on a spectroscopical measurement of the pseudo-absorption of corneocytes in the visible range. The pseudo-absorption was defined as the decrease in light transmission due to reflection, diffraction, and scattering by the corneocytes aggregates. The pseudo-absorption is nearly independent of the wavelength and was arbitrarily set at 430 nm, whereas all other components on the tapes usually absorb in the UV range [134]. The comparison of the novel spectroscopical method with established methods

has shown that UV/VIS-spectroscopy allows rapid, accurate, and reproducible quantification of corneocytes removed by tape stripping [245, 246].

For the quantification of drugs, HPLC represents a simple and common analytical method which, nevertheless, requires validation to prove specificity, accuracy, precision, and robustness in the working range. The analytical challenge concerns the development of a method capable of quantifying low drug amounts distributed on several tapes without the necessity of pooling the tapes.

The aim of the investigations described in this chapter is the validation of an HPLC method for the quantification of TACA, a moderately potent corticosteroid, on tape strips removed by tape stripping after topical application of a TACA formulation. The combination of: 1) the standardized tape stripping as sampling method, 2) the UV/VIS-spectroscopical method for quantification of corneocytes validated by Weigmann et al. [245], and 3) the new validated HPLC method for quantification of TACA, is then applied to a preliminary investigation (proof of concept). For this purpose, the TACA penetration into human stratum corneum was assessed *in vivo* after application of TACA in two different vehicles (acetonic solution, ethanolic gel).

6.2. Materials and methods

6.2.1. Material and formulations

Micronized TACA Ph.Eur. was purchased from Caesar & Loretz GmbH, Hilden, Germany. Hydroxypropylcellulose GF Ph.Eur. (Klucel GF®) was obtained from Synopharm GmbH, Barsbüttel, Germany and acetone Ph.Eur. from Hänseler AG, Herisau, Switzerland. HPLC grade methanol was obtained from Scharlau Chemie S.A., Barcelona, Spain. Water was purified by reverse osmosis (Osmostil LN 4/2 PH - Spetron 500 PH, Christ Aqua Ecolife AG, Aesch, Switzerland).

Because of the low solubility of TACA in methanol 60% (v/v), a stock solution of 2 mg/ml TACA in methanol 100% was prepared. TACA solutions of different concentrations were then diluted from the stock solution with methanol 60% (v/v).

For topical application, two TACA formulations were prepared: an acetonic solution (TACA 2 mg/ml) and an ethanolic gel consisting of 1% TACA, 4% hydroxypropylcellulose GF, and 95% ethanol 96% (v/v) (modified formulation from the “Neues Rezeptur-Formularium NRF”) [247].

6.2.2. Validation of the HPLC method

Chromatographic conditions

TACA was quantified by HPLC using a Waters Alliance HPLC System (2690 Separation Module, 996 Photodiode Array Detector, Millenium[32] Software), all Waters Corporation, Millford, Massachusetts, USA. An overview of the chromatographic conditions is given in Table 6-1.

Table 6-1: Chromatographic conditions of the HPLC method for TACA quantification.

Parameter	Setting
Column	Symmetry Shield™ RP18, 2.1 x 100 mm, 3.5 µm particle size
Mobile phase	methanol; water (60%;40%) (v/v)
Flow rate	0.3 ml/min
Running time	7 min
Temperature	35°C (column); 20°C (samples)
Injection volume	20 µl
DAD spectrum	210-300 nm
TACA quantification wavelength	240 nm

Validation

The validation of the HPLC method was performed according to the guidance of the "International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use" (ICH) [237]. Specificity, limit of detection and quantification, linearity and range, accuracy, precision (repeatability and intermediate precision), and robustness were assessed. The method of assessment of each validation parameter is presented together with the definition, results, and discussion in chapter 6.3.

The MVA® software (Method Validation in Analytics, Novia GmbH, Saarbrücken, Germany), specifically designed for the calculation of validation data and based on the ICH-guidelines, was used to conduct the statistical tests.

Tape spiking and tape extraction

Tape spiking was performed to determine specificity and accuracy. A solution volume of 50 µl was applied with a Hamilton® Syringe (250 µL, 22/2''/2, Supelco, Buchs, Switzerland) on the adhesive side of tape strips (approximate length of 3 cm). After evaporation of the solvent, each tape was individually placed in a scintillation vial (20 ml, glass, metal-foil sealing, polypropylene lock, VWR International AG, Dietikon, Switzerland) and extracted with 1.5 ml 60% methanol during 30 min on a horizontal shaker at 140 rpm (Heidolph Unimax 2010, Heidolph, Germany).

6.2.3. Proof of concept

Application of the formulations

The experiments were conducted on the volar aspect of the forearm of 6 healthy Caucasian volunteers of skin phototype II-III (4 female and 2 male, aged 22-38 years). The volunteers were divided into 2 groups (Table 6-2), and 3 skin sites per volunteer were treated with 100 µg/cm^2 TACA delivered either from an acetonic solution (group 1) or from an ethanolic gel (group 2). For the acetonic solution, a skin area of 10.5 cm^2 was delineated by a rectangular glass frame glued onto the skin (Sauer® skin glue, Manfred Sauer GmbH. Lobbach, Germany). A volume of 500 µl formulation was uniformly applied with a Hamilton® Syringe (Supelco, Buchs, Switzerland), and the solvent was allowed to evaporate. For the ethanolic gel, the application area was delineated by an adhesive template (HAWE® adhesive film, HAWE, Hugentobler AG, Bern, Switzerland) displaying a rectangular aperture of 10.5 cm^2. A mass of 100 mg gel was applied with a Gilson Microman® pipette (Gilson AG, Mettmenstetten, Switzerland) and homogeneously distributed with a glass rod. No skin washing was performed to remove excess of formulation.

Table 6-2: Overview of application and tape stripping procedure during the proof of concept experiments. The two TACA formulations (acetonic solution, ethanolic gel) were applied on 3 sites per arm (time 0 h, at 9 a.m.). Tape stripping followed at 3 different times (0.5 h, 3 h, 24 h). D = Dosing, T = Tape Stripping.

		0 h	0.5 h	3 h	24 h
Acetonic solution group (n=3)					
Site 1	TACA 100 µg/cm^2	D	T		
Site 2	TACA 100 µg/cm^2	D		T	
Site 3	TACA 100 µg/cm^2	D			T
Ethanolic gel group (n=3)					
Site 1	TACA 100 µg/cm^2	D	T		
Site 2	TACA 100 µg/cm^2	D		T	
Site 3	TACA 100 µg/cm^2	D			T
		9 a.m.	9.30 a.m.	12 a.m.	9 a.m.

Skin sampling by tape stripping

Stratum corneum removal occurred by tape stripping at 0.5 h, 3 h, and 24 h after application (Table 6-2). In order to remove the skin in a standardized way from the same skin area, a laminated paper template (protective film of HAWE® adhesive film, HAWE, Hugentobler AG, Bern, Switzerland) delineating a constant aperture of 3.3 x 1.7 cm (5.6 cm^2) was fixed onto the application area with adhesive tape. Tape stripping was carried out with Tesa® tape (Tesa Multi-Film Crystal-Clear® No. 57315, 19 mm width, Tesa, Beiersdorf, Hamburg, Germany). A hand roller supplying a pressure of 140 g/cm^2 was uniformly passed over the tapes 10 times for each stripping. Each tape was removed with a rapid, firm movement, and then fixed across a photographic slide frame, the adhering corneocytes being exposed through the frame aperture.

This procedure was repeated with new tapes, alternating the direction of tape removal (elbow-to-wrist, wrist-to-elbow).

The endpoint of tape stripping (total stratum corneum removal) was determined by UV/VIS-spectroscopy. When the amount of corneocytes adhering to the stripped tapes started to decrease macroscopically, the tapes were periodically measured as described below. As soon as transmission values ≥ 95% (calculated from the absorbance values and defined as complete removal of the stratum corneum) were attained, tape stripping was stopped.

Quantification of corneocytes by UV/VIS-spectroscopy

The amount of corneocytes adhering to each tape was quantified by measuring the pseudo-absorbance of the corneocytes at 430 nm as described in Weigmann et al. [245]. Each tape was directly measured against a blank tape in a Lambda 35 spectrophotometer (Perkin Elmer, Überlingen, Germany), custom-modified to obtain a rectangular light beam of 1 cm^2. Blank tapes were sequentially sampled from the stock roll of Tesa® tape after every fifth stripping, allowing a continuous correction for any irregularities in the optical properties of the tape from the start to the end of the roll. A solid-sample holder allowed a direct positioning of the photographic slide frame into the spectrophotometer. Continuous spectra of each tape were recorded between 400-500 nm, and absorbance values at 430 nm were extracted for quantification of the corneocytes.

Quantification of TACA by HPLC

After quantification of the corneocytes, each tape was individually extracted with 1.5 ml 60% methanol during 30 min on a horizontal shaker at 140 rpm (Heidolph Unimax 2010, Heidolph, Germany). TACA concentrations in the extract were quantified by the validated HPLC method described in chapter 6.2.2.

Data analysis

Qualitative TACA penetration into stratum corneum: penetration profiles

To localize the drug within the stratum corneum, TACA concentrations quantified on each tape were correlated to the tape number and to the depth of penetration into stratum corneum. Removal of the entire stratum corneum is a prerequisite for the profile calculation, as the total sum of corneocytes-(pseudo-)absorbance on one skin site (cumulative absorbance) represents 100% stratum corneum thickness. Thus, the relative amount of stratum corneum removed by each tape could be calculated from the individual corneocyte absorbance values as depicted in Fig. 6-1 [126]. To depict the penetration profiles, a Macro was programmed in Microsoft® Excel. Mean and standard deviation of the number of tapes required to remove the entire stratum corneum were calculated.

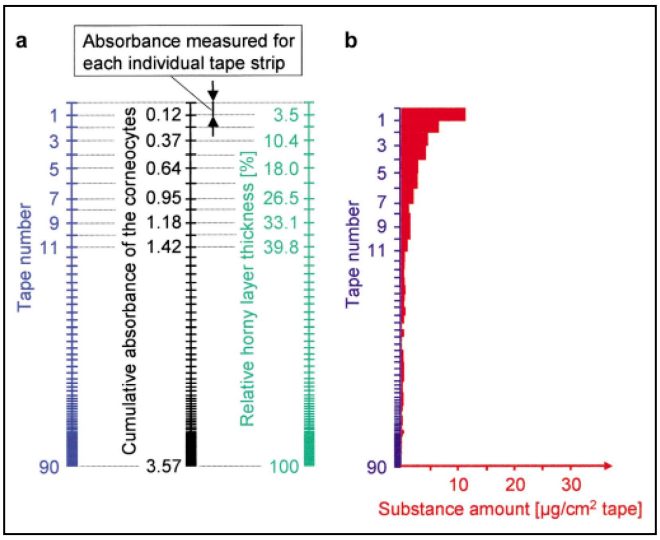


Fig. 6-1: Calculation of the penetration profiles. The pseudo-absorbance of the corneocytes on each tape is summed up to the cumulative absorbance values. The total cumulated absorbance (3.57 in this example) corresponds to 100% stratum corneum. From this value, the relative stratum corneum thickness can be deducted. After Jacobi et al. [126].

Quantitative TACA penetration into stratum corneum

The TACA amount on each tape of one skin site was added up to obtain the total TACA amount penetrated into stratum corneum. The total TACA amounts were then evaluated statistically. The difference between the treatment groups at each stripping time was tested in a 2-sided t-test ($\alpha = 0.05$). Two different types of evaluation were performed: the evaluation of a) the total TACA amount penetrated into the stratum corneum (sum of TACA amounts on all tapes), and b) the TACA amount without tapes 1-3 (on which formulation excess, e.g. TACA crystals and a thin gel film, could be located). Statgraphics® PLUS 5 software (Manugistic, Inc., Rockville, Maryland, USA) was used to conduct the statistical analysis.

Raster-electron-microscopic (REM) recording

Selected stripped tapes with adhering corneocytes and drug were observed with a Philips XL 30 ESEM scanning electron microscope. Sample preparation included fixation on a metal cylinder with a carbon adhesive tape and sputtering with gold (20 nm).

6.3. Validation of the HPLC method

6.3.1. Specificity

Definition

Specificity is the ability to unequivocally assess the analyte in the presence of components which may be expected to be present. Typically, this might include impurities, degradants, and matrix [237].

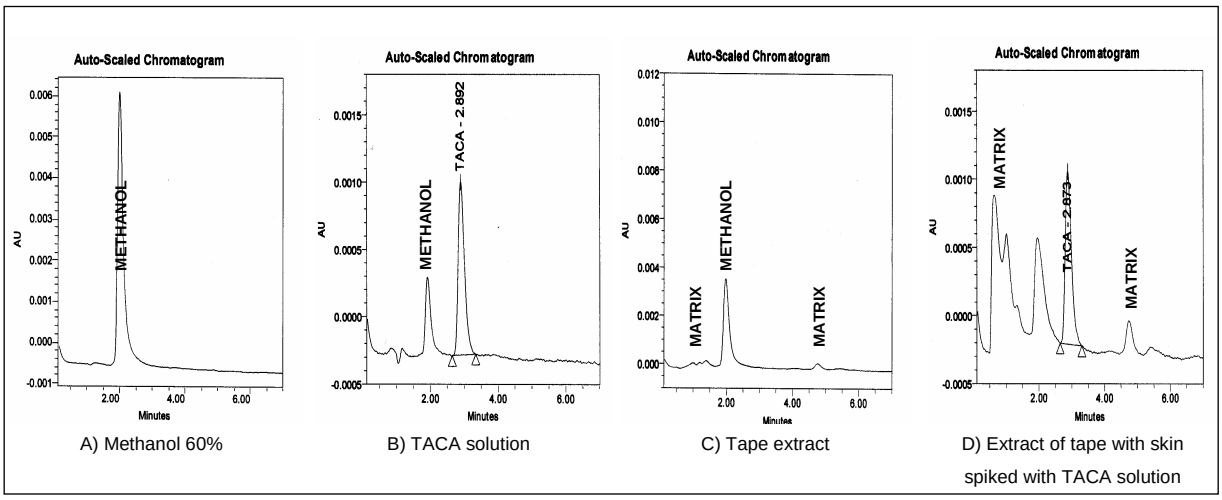
Method

To determine the retention time of TACA, a methanolic TACA solution (0.5 μg/ml) was assayed by HPLC (n = 3). Then, all the components which could possibly interact with the TACA peak, namely solvents (methanol 60%, acetone), tape matrix (e.g., polymer, adhesive), and stratum corneum constituents were individually assayed (each n = 3). The solvents were directly injected into the HPLC system. To obtain tape matrix samples, tape strips (n=3) were extracted by the standard extraction procedure described in chapter 6.2.2. To obtain stratum corneum constituents samples, tape strips (n=3) were placed on and peeled off the forearm skin of a healthy volunteer and were then extracted. The relative retention times were determined, and the chromatograms were visually searched for peak overlay of any component with the TACA peak.

In addition, tape strips of about 3 cm length were spiked with TACA solution following the standard spiking procedure described in chapter 6.2.2. A low (15 μg/ml) and a high (450 μg/ml) concentrated methanolic TACA solution was used as spiking solution (n=3 for each concentration). The spiking procedure was repeated with tape strips previously peeled off the forearm skin of a healthy volunteer (n=3 for each concentration). After evaporation of the solvent, the tapes were extracted, and the extracts were assayed by HPLC.

Results and discussion

Fig. 6-2 displays the chromatograms of methanol 60%, TACA solution (0.5 μg/ml), tape extract, and the extract of a spiked tape. The relative retention time (R_t) of TACA was 2.9 min, with the absorption maximum at 243.8 nm. The following additional peaks were recorded: methanol at 2.0 min and tape matrix at 1.0 min, 4.8 min, and 5.4 min. The relative R_t of acetone was 1.3 min and did not interfere with the TACA peak. Acetone is not expected to be present in the extracts, as it evaporates completely after application. The extract of tapes with and without adhering corneocytes resulted in identical chromatograms, indicating that stratum corneum constituents are not interfering in this wavelength range. No interference between the TACA peak and any other peak could be observed, assuring the specificity of the method for TACA.



A) Methanol 60% B) TACA solution C) Tape extract D) Extract of tape with skin spiked with TACA solution

Fig. 6-2: Chromatograms for specificity evaluation: A) Methanol 60% (R_t 2.0 min), B) TACA solution 0.5 µg/ml (TACA R_t 2.9 min), C) Tape extract (tape matrix R_t 1.0 min, 4.8 min, 5.4 min), D) extract of a tape with corneocytes spiked with TACA solution (15 µg/ml).

6.3.2. Limit of detection (LOD) and Limit of quantification (LOQ)

Definitions

The *limit of detection (LOD)* of an analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. *The limit of quantification (LOQ)* of an analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy [237].

Method

LOD and LOQ were assessed by calculating the signal-to-noise ratios from the chromatograms. The *signal* is defined as the height of the peak of interest (in this case the TACA peak), and the *noise* as the height of the baseline. The LOD was determined by diluting TACA solutions until a signal-to-noise ratio of ≥ 3-to-1 was obtained (n = 6), the LOQ until the signal-to-noise ratio was ≥ 10-to-1 (n = 6). The deviation of the LOQ signals should not exceed 20%, as described in chapter 6.3.3, Linearity and Range [238].

Results and discussion

The following signal-to-noise ratios were achieved for different TACA solutions: 2.8±0.2 to 1 for a TACA concentration of 0.030 µg/ml (n=3), 4.2±0.7 to 1 for 0.035 µg/ml (n=6), 5.2±0.4 to 1 for 0.040 µg/ml (n=3), and 11.1±0.7 to 1 for a TACA concentration of 0.1 µg/ml (n=6). Therefore, the LOD was set at 0.035 µg/ml (corresponding to a TACA amount of 9 ng/cm^2 on the tape) and the LOQ at 0.1 µg/ml (corresponding to 27 ng/cm^2). The deviation of the LOQ signals calculated with the linear weighted model was < 4%, thus not exceeding the maximal allowed value of 20% (compare chapter 6.3.3). The accuracy (chapter 6.3.4) and precision (chapter 6.3.5) achieved at LOQ were considered suitable.

6.3.3. Linearity and Range

Definitions

The *linearity* of an analytical procedure is its ability, within a given range, to obtain test results which are directly proportional to the concentration or the amount of an analyte in the sample. The *range* of an analytical procedure is the interval between the upper and lower concentration or amount of analyte in the sample, including those concentrations for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity [237].

Method

Standard TACA solutions of following concentrations were prepared: 20.0 μg/ml, 15.0 μg/ml, 10.0 μg/ml, 5.0 μg/ml, 1.0 μg/ml, 0.5 μg/ml, and 0.1 μg/ml. Each standard solution was assayed 5 times by HPLC. The measured signals (peak area) were plotted as a function of analyte concentration. The procedure was repeated on 3 different days with new standard solutions, yielding a total of 3 calibration curves.

To determine the model best describing the obtained calibration curves, the function of the 3 curves was calculated with following regression models: a) the linear unweighted model, b) the linear weighted model (weighting factor 1/x, where x = concentration), and c) the nonlinear (quadratic) model. The function, the correlation coefficient, the standard error of the slope (s_{x0}), the residual sum of square (SS), the residual standard deviation (SD), and the residual variance were calculated for each model [248].

Variance-homogeneity of the signals over the whole working range is a prerequisite for linear regression [249] and was tested in a Cochran test using peak-area data. If the signal variances displayed homogeneity, the residual variance of the linear regression models (both unweighted and weighted) could be tested against the non-linear model in a Mandel test [248, 250]. The best suitable model was chosen for TACA quantification. In addition, an analysis of the residuals was performed separately for each concentration level, with a maximal accepted deviation of 20% for the lowest concentration (LOQ) and 15% for the other concentrations [238].

Results and discussion

The key data of the 3 curves calculated with the different regression models are listed in Table 6-3. The correlation coefficient was good in all the models, but the evaluation of the simple correlation coefficient is a poor measure of the curve-fit quality of the data. The standard error of the slope (s_{x0}), representing the standard deviation of the procedure, displayed lower values with the linear unweighted model compared to the linear weighted model. However, the lowest residual variance was achieved with the linear weighted model.

Table 6-3: Key data of the calibration curves calculated with the linear unweighted, linear weighted (weighting factor 1/x, x = concentration), and nonlinear regression models. R = correlation coefficient, SD = standard deviation, SE = standard error, SS = sum of squares, ND = not determinable.

Calibration curve		Function	R^2	SE of slope (s_{x0})	Residual	
					SS	Variance
Linear unweighted	1	y = -731 + 35840 x	1.00000	0.20%	1.4 E+06	2.7 E+05
	2	y = -941 + 35780 x	0.99999	0.34%	4.0 E+06	7.9 E+05
	3	y = -1154 + 35510 x	0.99999	0.31%	3.3 E+06	6.7 E+05
Linear weighted	1	y = -545 + 35815 x	0.99997	0.94%	1.6 E+05	3.1 E+04
	2	y = -509 + 35718 x	0.99998	1.56%	4.2 E+05	8.4 E+04
	3	y = -660 + 35440 x	0.99997	1.88%	6.0 E+05	1.2 E+05
Non-linear	1	$y = -704 + 35820 x + 0.9 x^2$	1.00000	ND	1.3 E+06	3.4 E+05
	2	$y = -477 + 35500 x + 1.5 x^2$	1.00000	ND	1.8 E+06	4.4 E+05
	3	$y = -998 + 35410 x + 5.0 x^2$	0.99999	ND	3.1 E+06	7.7 E+05

According to the Cochran test, the variances were distributed homogeneously (test value 0.47 < critical value 0.75). Thus, the Mandel test could be performed, and the comparison of the linear models (unweighted/weighted) with the non-linear model resulted in a best fit achieved by the linear regression models.

To choose the best model between the linear unweighted and the linear weighted model, a residual analysis was performed for each concentration level of the curve. The values are summarized in Table 6-4. The residues displayed a normal distribution in all the models and randomly scattered around the zero line. The lowest concentration level (0.1 µg/m = LOQ) showed the highest residues with maximal values of 25% (linear unweighted model), 18% (non-linear model), and 4% (linear weighted model). The residues of the other concentration levels displayed maximal values of 1.2% (linear weighted model), 1.0% (linear unweighted model), and 0.8% (non-linear model).

Table 6-4: Mean percentage residual variance of the different concentration levels calculated with the linear unweighted, the linear weighted, and the non-linear model. The percentage residual variance is the percentage difference between experimental and calculated values divided by the calculated value.

Calibration curve		0.1µg/ml	0.5µg/ml	1.0µg/ml	5.0µg/ml	10.0µg/ml	15.0µg/ml	20.0µg/ml
Linear unweighted	1	7.9	0.8	-0.7	0.0	-0.2	0.2	-0.0
	2	20	0.8	-0.2	0.2	-0.5	-0.0	0.1
	3	25	1.0	0.2	-0.9	0.2	-0.0	0.0
Linear weighted	1	1.5	-0.2	-1.2	-0.0	-0.2	0.2	0.0
	2	3.2	-1.5	-1.3	0.1	-0.4	0.1	0.2
	3	3.9	-1.8	-1.0	-1.0	0.3	0.1	0.1
Non-linear	1	7.0	0.7	-0.8	0.0	-0.2	0.2	-0.0
	2	2.9	-1.1	-0.8	0.5	-0.2	0.1	0.0
	3	18	0.3	0.0	-0.8	0.3	-0.0	-0.0

According to the Mandel test and the residual analysis, the best results were achieved with the linear weighted regression model (curves displayed in the appendix, Fig. 11-1), which was chosen for data processing. The weighting of the calibration with the reciprocal of concentration (1/x) enables the curve adjustment to the lower concentration levels. The range for TACA quantification was set at 0.1-20.0 µg/ml.

6.3.4. Accuracy by recovery

Definitions

The *accuracy* of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value, and the experimental value [237].

Methods

The accuracy was evaluated in terms of recovery. For this purpose, tapes with adhering corneocytes were spiked with known amounts of TACA, extracted and assayed by HPLC. The measured TACA amount was then compared to the effective spiked TACA amount. To obtain tapes with adhering corneocytes, a skin area of 5.6 cm^2 on the forearm of a healthy volunteer was delineated by a template. A total of 18 tapes was subsequently applied and removed from this skin area. The tapes were then divided into 3 groups of 6 tapes each and spiked with 50 µl of the following methanolic TACA solutions: tape 1-6 with 450 µg/ml (corresponding to a high concentration level of 15 µg/ml on the calibration curve), tape 7-12 with 150 µg/ml (middle concentration level of 5 µg/ml) and tapes 13-18 with 15 µg/ml (low concentration level of 0.5 µg/ml). After evaporation of the solvent, each tape was extracted by the standard extraction procedure described in chapter 6.2.2. The extracts were assayed by HPLC and the data processed with the linear weighted (1/x) regression model to obtain recovery values. An accurate method should achieve recoveries of 80-120% [238].

Results and discussion

All the recovery values are displayed in the appendix (Table 11-1). The recovery was very good in the higher and middle calibration range (15 µg/ml and 5 µg/ml) with mean recovery values of 97 ± 2% (ranging from 94% to 99%) and 94 ± 2% (from 91% to 96%), respectively. In the lower calibration range (0.5 µg/ml), the mean recovery displayed values of 87 ± 4% (from 82% to 92%). The overall experimental recovery ranged from 82% to 99%, thus fulfilling the requirements for accuracy (80-120%).

6.3.5. Precision

Definitions

The *precision* of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision may be considered at 3 levels: repeatability, intermediate precision, and reproducibility. *Repeatability* expresses the precision under the same operating conditions over a short interval of time (intra-assay precision). *Intermediate precision* expresses within-laboratories variations (e.g., different days, different analysts, or different equipment). *Reproducibility* expresses the precision between laboratories. The precision of an analytical procedure is usually expressed as the variance, standard deviation and relative standard deviation (coefficient of variation) of a series of measurements. A minimum of 3 concentration levels and 3 replicates each are required to assess repeatability [237].

Methods

Repeatability was evaluated together with linearity, using the same data measured for the calculation of the 3 calibration curves. Absolute and relative standard deviations of the peak area were calculated for each concentration level of the calibration curve ($n = 5$ for each concentration level). A relative standard deviation of $\leq 15\%$ was required except for LOQ, for which a relative standard deviation of $\leq 20\%$ was accepted [238].

Repeatability and intermediate precision were calculated using the normalized peak areas (peak area divided by the respective concentration value) in the range with and without LOQ. The homogeneity of variance was tested in a Cochran test, and a variance analysis (ANOVA) was performed.

Repeatability was defined as:

$$CV_r = \frac{\sqrt{s_r^2}}{\bar{\bar{x}}} \cdot 100 \qquad \textbf{(Equation 6)}$$

s_r^2 being the intra-serial variance and $\bar{\bar{x}}$ being the overall mean.
Intermediate precision was defined as:

$$CV_R = \frac{\sqrt{s_R^2}}{\bar{\bar{x}}} \cdot 100 \qquad \textbf{(Equation 7)}$$

s_R^2 being the overall variance (sum of intra- and inter-serial variances).

Reproducibility was not assessed, since the method was designed for use within the same laboratory on the same HPLC apparatus.

Results and discussion

The peak area data for the different concentration levels are displayed in Table 6-5 (the complete data set is listed in the appendix, Table 11-2). The relative standard deviation of all the values excluding LOQ was ≤ 2% (≤ 15% required). At LOQ, a higher relative standard deviation (≤ 10%) was measured (≤ 20% required). Thus, repeatability was defined as good in the range of 0.1-20 µg/ml.

Table 6-5: Overview of the mean peak area, standard deviation (SD), and relative standard deviation (RSD) of the concentration levels for 3 different calibration curves (n=5 for each concentration level).

Calibration curve		0.1µg/ml	0.5µg/ml	1.0µg/ml	5.0µg/ml	10.0µg/ml	15.0µg/ml	20.0µg/ml
No. 1	mean	3078	17328	34855	178501	356972	537682	715799
	SD	300	351	450	327	1205	857	812
	RSD	**9.7**	**2.0**	**1.3**	**0.2**	**0.3**	**0.2**	**0.1**
No. 2	mean	3160	17086	34769	178324	355164	535626	715462
	SD	126	208	194	736	944	620	1280
	RSD	**4.0**	**1.2**	**0.6**	**0.4**	**0.3**	**0.1**	**0.2**
No. 3	mean	2998	16758	34420	174852	354667	531264	709124
	SD	148	169	177	628	619	774	1080
	RSD	**4.9**	**1.0**	**0.5**	**0.4**	**0.2**	**0.1**	**0.2**
Overall	mean	3079	17057	34681	177226	355601	534857	713462
	SD	203	338	339	1822	1353	2857	3330
	RSD	**6.6**	**2.0**	**1.0**	**1.0**	**0.4**	**0.5**	**0.5**

The mean values of the normalized peak areas are displayed in Table 6-6 for both the whole range and the range without LOQ. According to the Cochran test, the variances were homogenous. Repeatability (intra-serial precision) and intermediate precision (overall precision) calculated by ANOVA gave good precision values (1.88% and 1.89%, respectively) within the range excluding LOQ. If LOQ was included in the calculation, a value of 5.15% was obtained for both precision types, indicating that precision at LOQ was not optimal, but acceptable.

Table 6-6: Mean values and variance of the normalized peak areas (peak areas divided by the correspondent concentration) of 3 different calibration curves (whole range except LOQ).

	Range 0.5-20 µg/ml		Range 0.1-20 µg/ml	
Normalized peak area	Mean	Variance	Mean	Variance
Series 1	3.54 E+04	2.75 E+05	3.48 E+04	3.31 E+06
Series 2	3.52 E+04	4.24 E+05	3.47 E+04	2.27 E+06
Series 3	3.48 E+04	6.09 E+05	3.42 E+04	3.93 E+06
Overall	3.51 E+04	4.43 E+05	3.46 E+04	3.17 E+06
Cochran Test				
Test value	0.47		0.41	
Critical value	0.71		0.68	
	The variances are homogenous (test value < critical value)			
ANOVA				
Intra-serial variance	4.36 E+05		3.17 E+06	
Inter-serial variance	7.47 E+03		-3.42 E+05	
Overall mean $\bar{\bar{x}}$	3.51 E+04		3.46 E+04	
Overall variance	4.43 E+05		3.17 E+06	
	No significant difference of the means			
Repeatability CV_r	1.88%		5.15%	
Intermediate Precision CV_R	1.89%		5.15%	

6.3.6. Robustness

Definitions

The *robustness* of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters, and provides an indication of its reliability during normal usage [237].

Method

To evaluate method robustness, several method parameters were varied: ± 5% methanol in the mobile phase (methanol 55%, methanol 65%), ± 5% variation of the flow rate (0.285 ml/min, 0.315 ml/min), ± 5℃ variation of the column temper ature (30℃, 40℃) and ± 2 nm variation of the detection wavelength (238 nm, 242 nm). The extraction solutions used for accuracy evaluation were measured under these modified conditions (n = 6 per concentration). Recovery values of 80%-120% were aimed at [238].

Results and discussion

The variation of chromatographic and analytical conditions resulted in an overall mean recovery (± SD) of: 96.0 ± 1.2% in the higher concentration range of the calibration curve (15 µg/ml), 93.0 ± 2.2% in the middle concentration range (5 µg/ml), and 81.9 ± 9.7% in the lower concentration range (0.5 µg/ml). The mean recoveries obtained after variation of the different method parameters are depicted in Fig. 6-3. In particular, the variation in the mobile phase composition (methanol 65%) resulted in recoveries below 80% (70.2% to 82.2%) in the lower concentration range. Except for these unsatisfactory values, the recovery for all other parameters in the low range was 81-87%.

The robustness of the method was good in the mid and high concentration range but limited in the lower concentration range. The results were considered acceptable provided that the composition of the mobile phase is periodically checked.

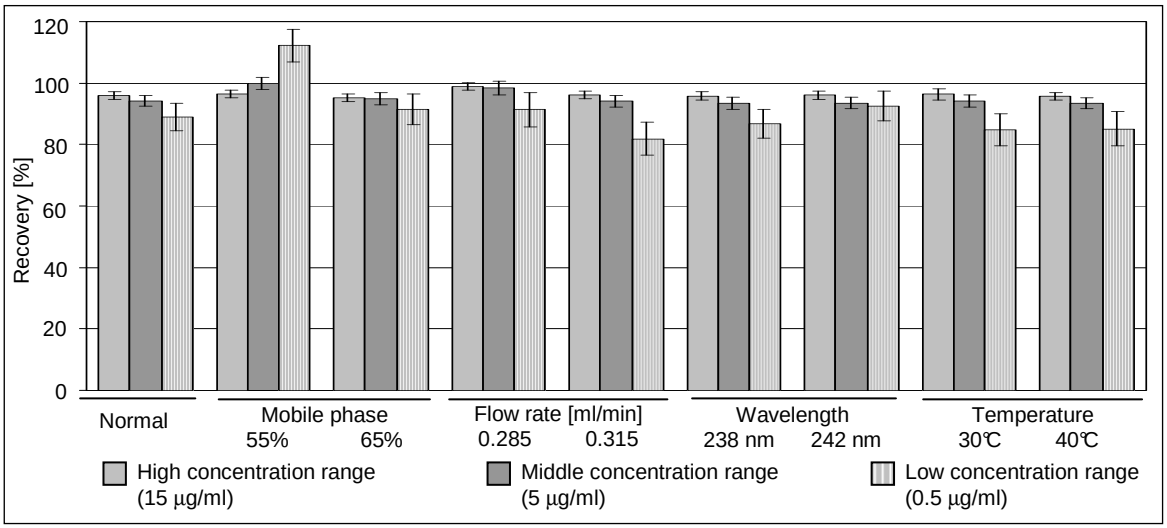


Fig. 6-3: Assessment of method robustness by variation of different chromatographic and analytical parameters such as mobile phase, flow rate, detection wavelength, and column temperature.

6.3.7. Overview of the validation

The HPLC method was successfully validated and proved to have suitable specificity, accuracy, precision, and robustness in the linear range of 0.1-20 µg/ml. The calibration curves were calculated with the linear weighted regression model, weighting factor 1/x (where x = concentration). The LOQ of 0.1 µg/ml enabled the quantification of corresponding concentrations of 27 ng/cm^2 TACA on each stripped tape, whereas the LOD of 0.035 µg/ml enabled the detection of 9 ng/cm^2 TACA. The validation results are summarized in Table 6-7.

Table 6-7: Overview of the validation parameters.

Specificity	n=3 (per component)	$R_{t\ (TACA)}$ 2.9 min, no peak overlay
LOD	n=6	35 ng/ml (corresponding to 9 ng/cm^2)
LOQ	n=6	100 ng/ml (corresponding to 27 ng/cm^2)
Linearity	3 calibration curves 7 conc. levels n = 5 (per concentration)	y = -573 + 35660 x R^2 = 0.99997 linear weighted (1/x, x = concentration)
Range	n=5 (per concentration)	0.1-20 µg/ml
Accuracy by recovery	n=6 (per concentration)	Overall: 93% ± 5% 0.5 µg/ml: 87% ± 4% 5.0 µg/ml: 94% ± 2% 15.0 µg/ml: 97% ± 2%
Repeatability	n=5 (per concentration)	1.9% without LOQ (range 0.5-20 µg/ml) 5.1% with LOQ (range 0.1-20 µg/ml)
Intermediate precision	n=5 (per concentration)	1.9% without LOQ (range 0.5-20 µg/ml) 5.1% with LOQ (range 0.1-20 µg/ml)
Robustness	n=6 (per concentration) 8 different conditions	0.5 µg/ml: 81.9 ± 9.7% 5.0 µg/ml: 93.0 ± 2.2% 15.0 µg/ml: 96.0 ± 1.2%

6.4. Proof of concept

6.4.1. Qualitative TACA penetration into stratum corneum: penetration profiles

Typical profiles of TACA penetration into stratum corneum over time are depicted in Fig. 6-4 (the entire set of profiles is displayed in the appendix, chapter 11.1.2). A clear vehicle effect can be observed: after application of the acetonic solution, TACA quickly penetrated and permeated the stratum corneum, reaching deeper skin layers. In contrast, the ethanolic gel formed a superficial film in which considerable TACA amounts remained trapped. This film was removed with the first stripped tape.

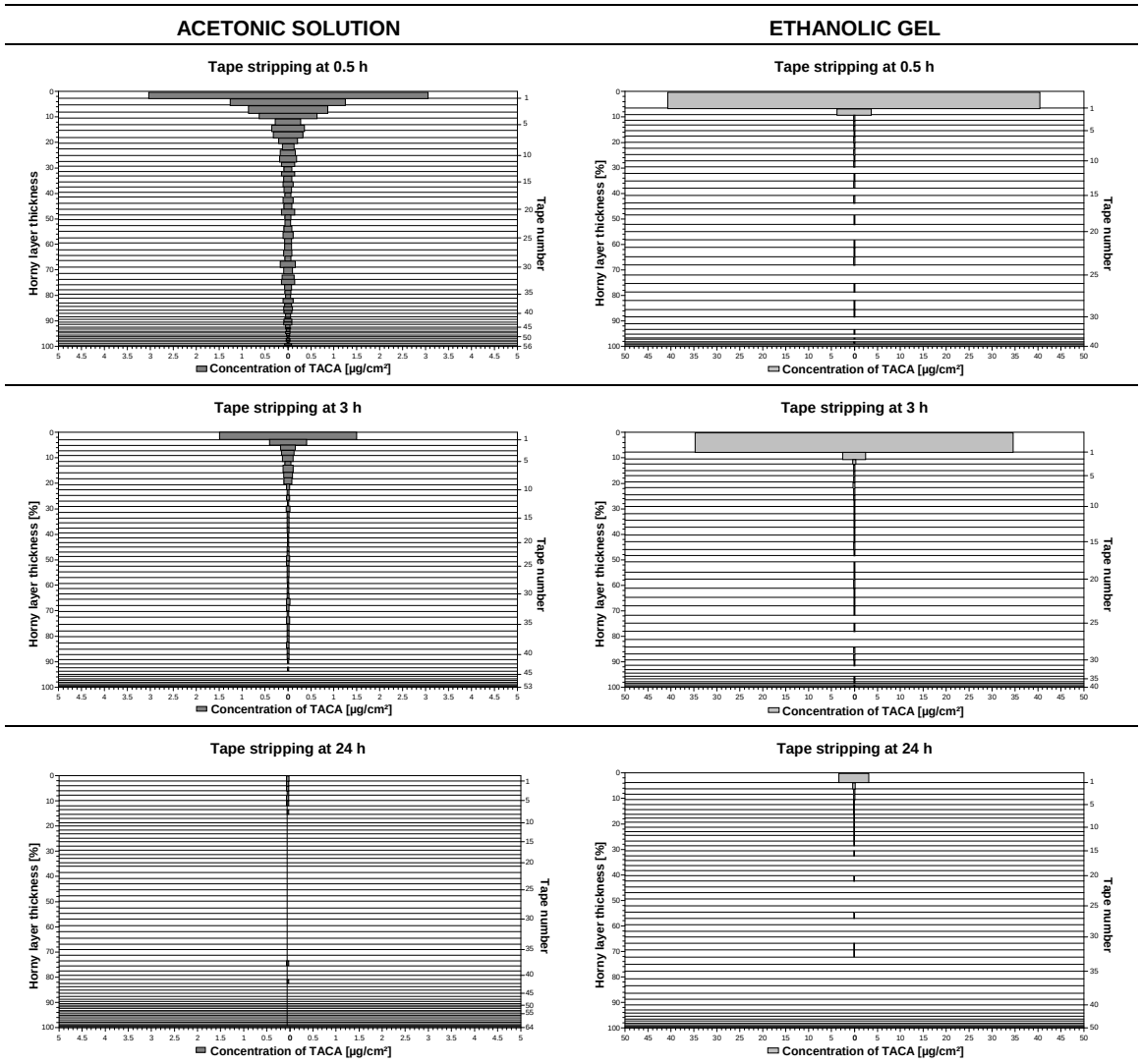


Fig. 6-4: Penetration profiles of TACA (100 µg/cm^2) into stratum corneum at 0.5 h, 3 h, and 24 h after application of the two different formulations acetonic solution (dark grey) and ethanolic gel (light grey). Note that the TACA concentration scale of the acetonic solution and ethanolic gel profiles differs by a factor of 10.

The mean tape number required to remove the entire stratum corneum was 56 ± 17 after application of the acetonic solution and 40 ± 5 after application of the alcoholic gel (Table 6-8). The intra-individual variability in the number of tapes used to remove the stratum corneum was low, but inter-individual differences were high. This is not unusual and has to be taken into account when drug penetration into stratum corneum is investigated.

Table 6-8: Number of tapes required to remove the entire stratum corneum of each skin site treated either with the acetonic solution or the ethanolic gel (TS = Tape Stripping; SD = Standard Deviation)

	Skin Site 1 (TS 0.5h)	Skin Site 2 (TS 3h)	Skin Site 3 (TS 24h)	Mean	SD
Acetonic solution group					
Volunteer 1	56	53	64	58	6
Volunteer 2	82	70	69	74	7
Volunteer 3	38	34	35	36	2
Overall acetonic solution group				56	17
Ethanolic gel group					
Volunteer 4	40	40	50	43	6
Volunteer 5	40	37	42	40	3
Volunteer 6	35	35	40	37	3
Overall ethanolic gel group				40	5
Overall (solution and gel group)					
Mean	49	45	50	48	4
SD	18	14	14	15	2

6.4.2. Quantitative TACA penetration into stratum corneum

A high amount of TACA was always recovered on the first stripped tapes, which also removed a large amount of corneocytes. On the skin surface not only corneocytes but also TACA crystals (in case of the acetonic solution) and a thin gel film (in case of the ethanolic gel) could be discerned.

The TACA amounts penetrated into stratum corneum after application of the two different formulations are displayed in Fig. 6-5, and the individual data are listed in the appendix (Table 11-3). If all tapes were considered, a significantly higher TACA amount was recovered within the stratum corneum after application of the ethanolic gel compared to the acetonic solution (94 µg/cm^2 vs. 24 µg/cm^2 at 0.5 h; $p < 0.01$). Over time, the amount recovered within the stratum corneum decreased with both formulations. At 3 h, mean TACA amounts of 84 µg/cm^2 vs. 13 µg/cm^2 ($p < 0.01$) were recovered after application of the ethanolic gel and the acetonic solution, and at 24 h still 7 µg/cm^2 vs. 0.8 µg/cm^2 ($p > 0.05$).

By excluding tapes 1-3, a statistically higher TACA amount was observed at 0.5 h after application of the acetonic solution (13 µg/cm^2 vs. 4 µg/cm^2; $p < 0.01$). This inversion of the results, i.e. higher penetration with the ethanolic gel (if all tapes were considered) vs. higher penetration with the acetonic solution (if tapes 1-3 were discarded), shows that a high TACA amount remained on tapes 1-3. Especially after application of the ethanolic gel, a high TACA amount remained bound to the gel matrix on the skin surface. Thus, the gel can be regarded as

a slow release formulation with a superficial drug reservoir. At 3 h, the penetrated TACA amount after application of the acetonic solution decreased, whereas the amount was unchanged after application of the ethanolic gel (6 µg/cm^2 vs. 4 µg/cm^2; $p > 0.05$). At 24 h, TACA amounts of 0.5 µg/cm^2 vs. 1.6 µg/cm^2 ($p > 0.05$) were measured after application of the acetonic solution and the ethanolic gel.

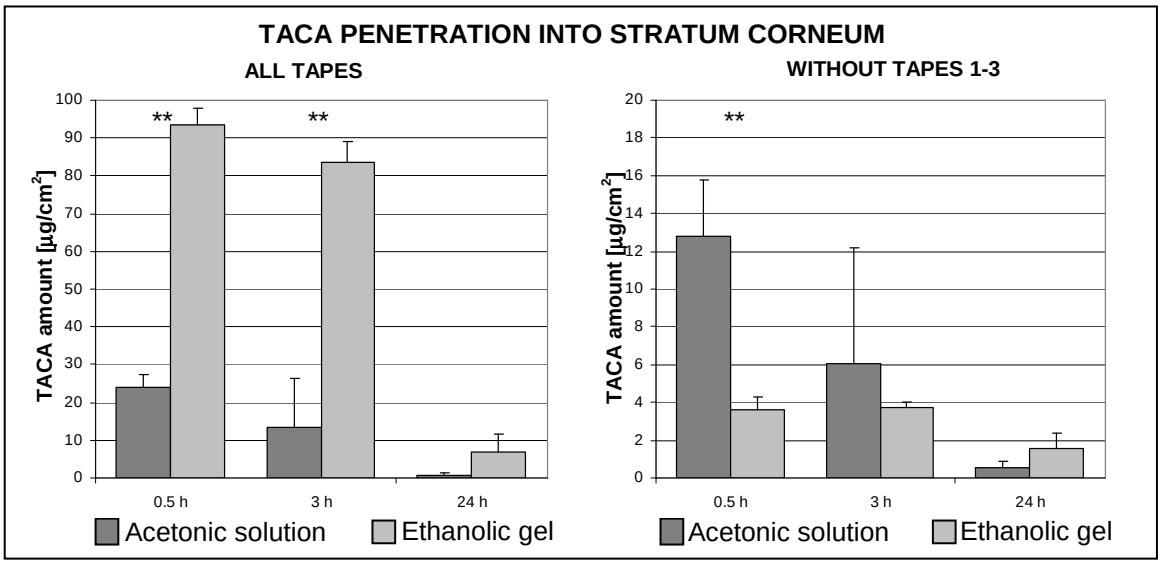
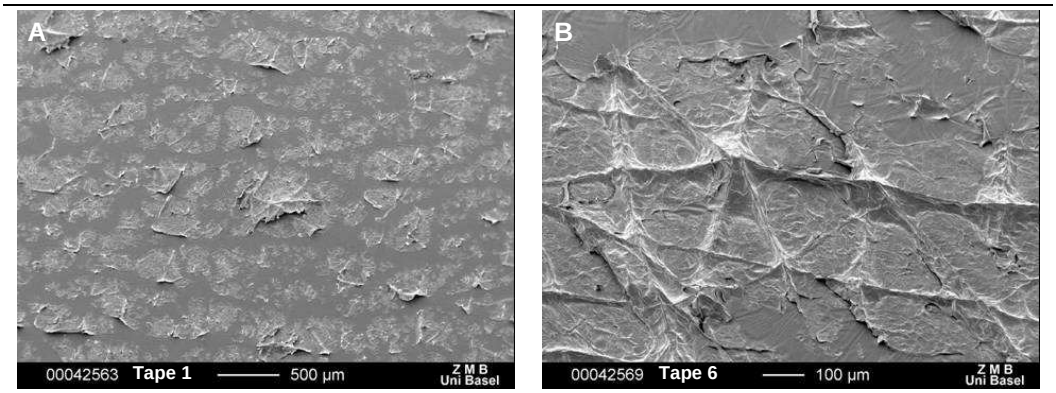


Fig. 6-5: TACA amounts penetrated into stratum corneum including all tapes (left diagram) and excluding tapes 1-3 (right diagram). Mean and standard deviation are displayed. The double asterisk (**) denotes a highly significant difference ($p < 0.01$, 2-sided t-test) between the formulations at the specified stripping time.

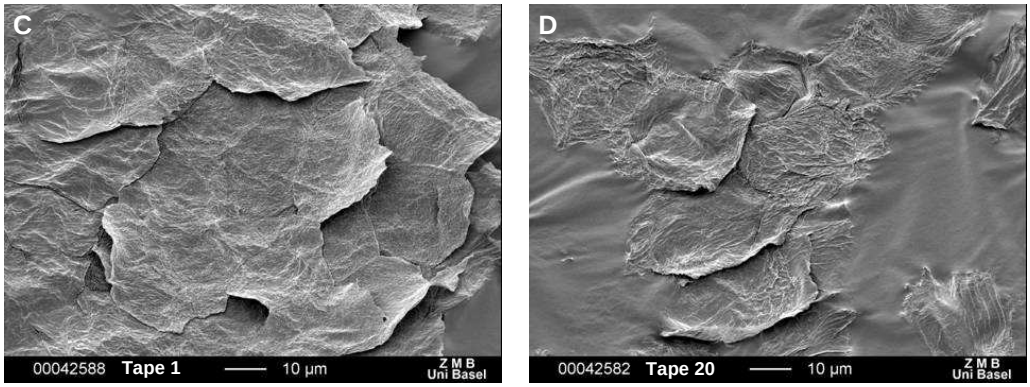
6.4.3. Raster-electron-microscopic (REM) recording

Tape stripping does not remove single, homogeneous layers of stratum corneum but rather skin frazzles. This is shown in Fig. 6-6 (A). The use of a hand roller to stretch the skin before stripping the tape from the skin also allowed removal of the stratum corneum of furrows (Fig. 6-6 B). Single, flat corneocytes can easily be discerned at higher magnifications, as well as their overlapping ("imbricated") arrangement (Fig. 6-6 C, D). TACA crystals precipitated on the skin surface after application of the acetonic solution could be observed on some tapes (Fig. 6-6 E, F).

STRATUM CORNEUM ON THE TAPE



CORNEOCYTES



TACA CRYSTALS

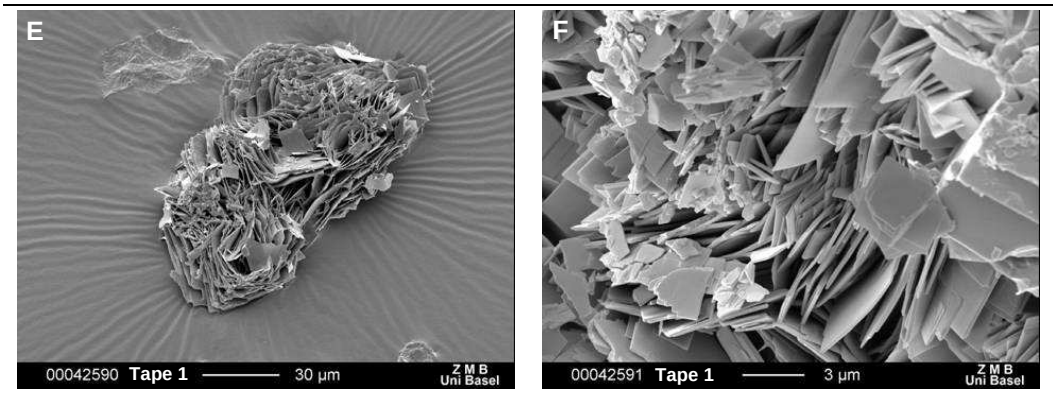


Fig. 6-6: Raster-electron-microscopic view of stratum corneum, corneocytes, and TACA crystals adhering to tapes removed from the skin by stripping. Tape stripping does not remove single layers of stratum corneum, but rather skin frazzles (A). Furrows can easily be discerned (B), as well as imbricatedly arranged corneocytes (C, D). TACA crystals could sometimes be observed on the first stripped tapes (E, F).

6.5. Discussion

The set of methods

In a first step, the HPLC method for the quantification of TACA on tapes removed by tape stripping was successfully validated. In a second step, the whole set of methods, i.e.: 1) the standardized tape stripping technique for stratum corneum sampling, 2) the UV/VIS-spectroscopical method for quantification of corneocytes on the tapes, and 3) the HPLC method for quantification of TACA on the tapes had to be tested. The preliminary tape stripping experiments described in this chapter should therefore be regarded as a proof of concept rather than an in-depth investigation of vehicle effects. The number of volunteers was small, and there was no intra-individual (contra-lateral) comparison of the 2 different formulations.

The amount of corneocytes removed by tape stripping is influenced by several factors (described *in extenso* in chapter 3.2). Therefore, a standardization of the tape stripping procedure is indispensable to obtain reproducible results. Our standardization included: a) the use of a template which ensured the removal of stratum corneum from the same skin area; b) the application of a constant pressure on the tape before stripping by the use of a hand roller; c) the removal of each tape with a rapid, firm movement; d) the alternation of tape removal (elbow-to-wrist, wrist-to-elbow); and e) the definition of a stripping endpoint (measured spectrosco-pically).

The quantification of corneocytes by UV/VIS-spectroscopy is based on the measurement of the pseudo-absorption of the corneocytes. In contrast to a real absorption (e.g. shown by sunscreen agents), the corneocytes aggregates, as particles, decrease the intensity of the transmitted light by reflection, scattering, and diffraction [134]. This happens nearly independently of the wavelength. Therefore, any wavelength which is undisturbed by other components, e.g. by the applied drug, could be used for quantification of the corneocytes. In our experiments, the corneocytes were quantified at 430 nm, as already described in the literature [134]. On the skin surface, the corneocyte cohesion is low, and large amounts of corneocytes were removed with the first tapes, displaying high pseudo-absorbance values. In the deeper stratum corneum, the measured pseudo-absorbance decreased, reflecting the decreasing amount of corneocytes.

The UV/VIS-spectroscopical method offers the advantage of a simultaneous quantification of both corneocytes and drug directly on the tape [251]. However, this is only possible if the drug has an absorption maximum which is not masked by the matrix of the tape, i.e. an absorption maximum > 300 nm in the case of Tesa Multi-Film Crystal-Clear® (compare Fig. 11-2 of the appendix for a UV/VIS-spectrum of a blank tape). Since the absorption maximum of corticosteroids is approximately at 240 nm, the simultaneous quantification of corneocytes and corticosteroids is not feasible. Thus, TACA has to be quantified with other methods, e.g. by the HPLC method successfully validated in this chapter.

The validated HPLC method was able to detect TACA on each stripped tape. Yet, on some of the last stripped tapes (deeper stratum corneum) and especially 24 h after application, TACA could be detected but not always quantified. In addition, the sampling of up to 82 tapes to remove the entire stratum corneum in some volunteers implied a higher TACA "dilution" compared to a lower tape number, and the probability to surpass the LOQ was higher.

Formulation and vehicle

The composition of the applied formulation plays a decisive role on the penetration of drugs into the stratum corneum. The application of a finite amount of drug was desired, and therefore 2 volatile formulations were compounded: a simple solution in acetone as a good solvent for TACA and a lesser volatile ethanolic gel. Acetone does not represent a vehicle normally used in dermatology. Yet, the investigation of the penetration of topical corticosteroids applied in acetone is known in the literature, since a volatile vehicle allows the application of a finite amount of drug [49, 59], and since organic solvents can play a relevant role in occupational skin exposure [61]. Comparisons with older investigations are possible.

Both acetone and ethanol are lipid solvents. The property of dissolving skin lipids, thus causing damage to the normal skin structure, has been described especially for acetone. In our study, skin damage is improbable, since the applied volume was low and the contact time short (about 30 seconds). At worst, only a superficial skin damage due to extraction of skin surface lipids is expected to occurr, whereas the stratum corneum structure would remain intact [252, 253].

Skin washing

Some authors are used to washing the skin after a definite application time to remove eventual excesses of formulation. Yet, different solvents wash the skin more or less successfully, and the washing procedure has been related to an enhanced penetration of the drug into deeper skin layers [224, 225] because of a remobilization of the drug through the solvent. Moreover, the washing procedure can affect the barrier function of the skin [254]. To minimalise any additional exogenous influence, no skin washing was performed in these experiments.

Tape evaluation

The number of tapes required to completely remove the stratum corneum is dependent on the cohesion of the corneocytes (depending on the volunteer and on the anatomical site), and on the vehicle, which can loosen the structure of the stratum corneum.

According to the withdrawn draft guidance "Topical dermatological drug products NDAs and ANDAs – *In vivo* bioavailability, bioequivalence, *in vitro* release, and associated studies" of the FDA (compare chapter 3.1) [1], some researchers are used to discarding the upper stripped tape from the evaluation, claiming that it contains excess formulation. Yet, discarding tapes is always a precarious and arbitrary procedure, and it is not well defined how many tapes are

sufficient to really eliminate potential formulation excesses. Furthermore, drug adhering to the skin can form a surface reservoir, from which the drug can be released at a later stage if re-dissolved.

Instead of discarding tapes, it was preferred for evaluation of the current experiments to present two different types of data. First, the total TACA amount calculated including all the tapes, which gives information about how much drug is in the entire stratum corneum, including the drug on the skin surface (e.g. as crystals or bound to the gel matrix). Second, the TACA amount calculated without the tapes 1-3 (usually up to 3 tapes were required to remove TACA crystals), which gives information about the TACA fraction penetrated into the stratum corneum that is not going to be removed by skin exfoliation and/or friction with clothes.

TACA penetration into stratum corneum

After application of the two different formulations, the vehicles rapidly changed their composition (metamorphosis of the vehicle) [255]. Acetone evaporated very quickly, and TACA partially precipitated on the skin as a solvent-deposited solid [49, 59]. After application of the ethanolic gel, the ethanol evaporated as well, but more slowly. A thin film of gel matrix (mainly cellulose) was macroscopically visible on the skin surface after evaporation of the ethanol. Since most TACA was bound within the cellulose matrix, the TACA penetration into stratum corneum after application of the ethanolic gel formulation was slow. The ethanolic gel resulted to be a slow release formulation, forming a superficial drug reservoir. Therefore, it was important to leave the cellulose film on the skin until tape stripping and not to remove any excess of formulation.

The existence of a surface TACA reservoir after application of the ethanolic gel is reflected by the high total TACA amount recovered at both 0.5 h (94%) and 3 h (84%). The lower TACA amount recovered at 24 h (7%) can be explained by the removal of the surface reservoir by textile friction and corneocyte desquamation. Nevertheless, after 24 h the total TACA amount recovered after application of the ethanolic gel is still higher than the TACA amount after application of the acetonic solution (1%).

The acetonic solution displayed a low total penetrated TACA amount (24%) already at 0.5 h, shortly after application. There are two possible explanations: either the acetonic vehicle enhanced the penetration of TACA to such an extent that the drug rapidly permeated through the stratum corneum into deeper, living skin layers (enhanced vertical diffusion), or TACA spread laterally (horizontal diffusion). A 5-fold enhanced penetration of cortisone after application in an acetonic solution compared to aqueous solutions was observed by Scheuplein and Ross [59], supporting the enhanced-vertical-penetration theory. Yet, horizontal diffusion has also been observed in experiments with clobetasol propionate applied in different vehicles [123], and the vehicle played a crucial role in inducing lateral diffusion. Thus, both explanations are possible, although the vertical diffusion was probably the predominant route of penetration.

6.6. Conclusions

An HPLC method for quantification of TACA on tapes obtained by tape stripping was validated. The combination of standardized tape stripping as sampling method and UV/VIS-spectroscopy and HPLC as validated analytical methods enabled the quantification of the penetration of TACA into human stratum corneum *in vivo*. This was successfully proven in a preliminary experiment investigating the vehicle effect on the TACA penetration into stratum corneum. The set of methods allowed a clear distinction of the penetration pattern of TACA after application in an acetonic solution and an ethanolic gel. The ethanolic gel resulted to be a slow-release formulation forming a surface TACA reservoir, whereas the acetonic solution displayed a good penetration into the stratum corneum.

7. Project II: Effect of dose and application frequency

The findings of this project have been accepted for publication: Pellanda C, Ottiker E, Strub C, Figueiredo V, Rufli T, Imanidis G, Surber C. Topical bioavailability of triamcinolone acetonide: effect of dose and application frequency. Arch Derm Res. The original publication is available at www.springerlink.com.

Abstract

The application frequency of topical corticosteroids is a recurrently debated topic. Multiple-daily applications are common, although a superior efficacy compared to a once-daily application is not unequivocally proven. Only few pharmacokinetic studies investigating application frequency exist. The aim of the study was to investigate the effect of dose (experiment 1) and application frequency (experiment 2) on the penetration of triamcinolone acetonide (TACA) into human stratum corneum *in vivo*. The experiments were conducted on the forearms of 15 healthy volunteers. In experiment 1, single TACA doses (300 μg/cm^2 and 100 μg/cm^2) were applied on 3 sites per arm. In experiment 2, single (1x300 μg/cm^2) and multiple (3x100 μg/cm^2) TACA doses were similarly applied. Stratum corneum samples were harvested by tape stripping after 0.5, 4, 24 h (experiment 1) and after 4, 8, 24 h (experiment 2). Corneocytes and TACA were quantified by UV/VIS-spectroscopy and HPLC, respectively. The amounts of TACA penetrated into stratum corneum were statistically evaluated by a paired-sample t-test. In experiment 1, TACA amounts within stratum corneum after application of 1x300 μg/cm^2 compared to 1x100 μg/cm^2 were only significantly different directly after application and similar at 4 and 24 h. In experiment 2, multiple applications of 3x100 μg/cm^2 yielded higher TACA amounts compared to a single application of 1x300 μg/cm^2 at 4 and 8 h. At 24 h, no difference was observed. In conclusion, considerable TACA amounts were retained within the stratum corneum independently of dose and application frequency. A low TACA dose applied once should be preferred to a high dose, which may promote higher systemic exposure.

Keywords

Application frequency
Reservoir
Triamcinolone acetonide
Tape stripping
Topical bioavailability

7.1. Introduction

In current dermato-pharmacotherapy, the application frequency of topical corticosteroids is a recurrently debated topic. Several studies have been performed to propose recommendations for optimal therapy. Once- or twice-daily applications are common [193], and many dermatologists usually follow twice-daily applications, although a superior efficacy of a multiple-daily application is not unequivocally proven. Recently, a systematic review reported a similar efficacy of once-daily versus multiple-daily applications of topical corticosteroids of the same potency in atopic dermatitis [208]. In addition to pharmacodynamic investigations, only few pharmacokinetic studies investigating application frequency exist (reviewed in [256]). Unfortunately, these studies make their statements on data derived from drug concentration determination in plasma [222, 223] and urine [224, 225]. This information is incomplete because these data document *systemic* bioavailability and not *topical* bioavailability.

Topical bioavailability can be estimated from the drug concentration within the stratum corneum, which is expected to be related to the drug concentration at the target site (i.e. viable epidermis or dermis) since the stratum corneum is the rate limiting barrier for percutaneous absorption. Similarly to the determination of the drug concentration in blood and/or urine as surrogate for the real concentration in the target tissue, the determination of the drug concentration in the stratum corneum is a surrogate for the concentration in the viable (epi-)dermis [257]. A useful dermato-pharmacokinetic technique for the assessment of drug amounts in stratum corneum as a function of time is tape stripping, which enables removal of the stratum corneum layer by layer [93].

Human stratum corneum has the property to store previously applied drugs depending on the drug, the formulation, the application procedure, and the state of the skin [146]. Drug accumulation in the skin forms a reservoir, from which minor amounts are released during a prolonged time period. The existence of a reservoir within the stratum corneum has been documented for several xenobiotics [146], particularly for topical corticosteroids [148]. This is a welcome phenomenon for topical corticosteroids and affects the choice of application frequency and dose.

Our investigation is focused on topical bioavailability. We determined the *in vivo* penetration of triamcinolone acetonide (TACA), a moderately potent corticosteroid often used in dermatology, into human stratum corneum after different application modes. The investigation was divided into two parts. In experiment 1, the influence of the dose was investigated by comparing the TACA penetration into stratum corneum after application of a high (1x300 $\mu g/cm^2$) and a low (1x100 $\mu g/cm^2$) TACA dose. In experiment 2, the influence of application frequency was investigated by comparing TACA penetration after a once-daily application of a high TACA dose (1x300 $\mu g/cm^2$) to TACA penetration after a thrice-daily application of a low TACA dose (3x100 $\mu g/cm^2$).

7.2. Subjects and methods

7.2.1. Material and formulations

Micronized TACA Ph.Eur. was purchased from Caesar&Loretz GmbH, Hilden, Germany and acetone Ph.Eur. from Hänseler AG, Herisau, Switzerland. Solutions of 4.2 mg/ml (for the application of 100 μg/cm^2) and 12.6 mg/ml (application of 300 μg/cm^2) TACA in acetone were prepared following current GMP guidelines.

7.2.2. Subjects and study design

A total of 15 healthy adult volunteers with skin phototype II-III (Caucasian) and with minor hairiness of the volar aspect of the forearm were recruited and underwent a preliminary dermatological examination one week prior to study-start. The experiments were conducted on the volar aspect of the forearms during 2 days as an open study with half-side intra-individual comparison. Experiment 1 (influence of dose) was regarded as explorative, whereas experiment 2 (influence of application frequency) was the main investigation. TACA in acetone was applied on selected skin sites, from which the stratum corneum was harvested afterwards by tape stripping at 3 different time points according to the protocol described below. No skin treatments were allowed during 24 h before study-start, and volunteers were not allowed to shower or practise sports during the 2-day experiment. Within 1 month after tape stripping, the wound healing was evaluated in a second dermatological examination[8]. Fig. 7-1 displays the flow chart of the study. The study was conducted according to the ethical rules stated in the Declaration of Helsinki and was approved by the local ethical committee and the national authorities (Swissmedic). The volunteers signed written consent for participation.

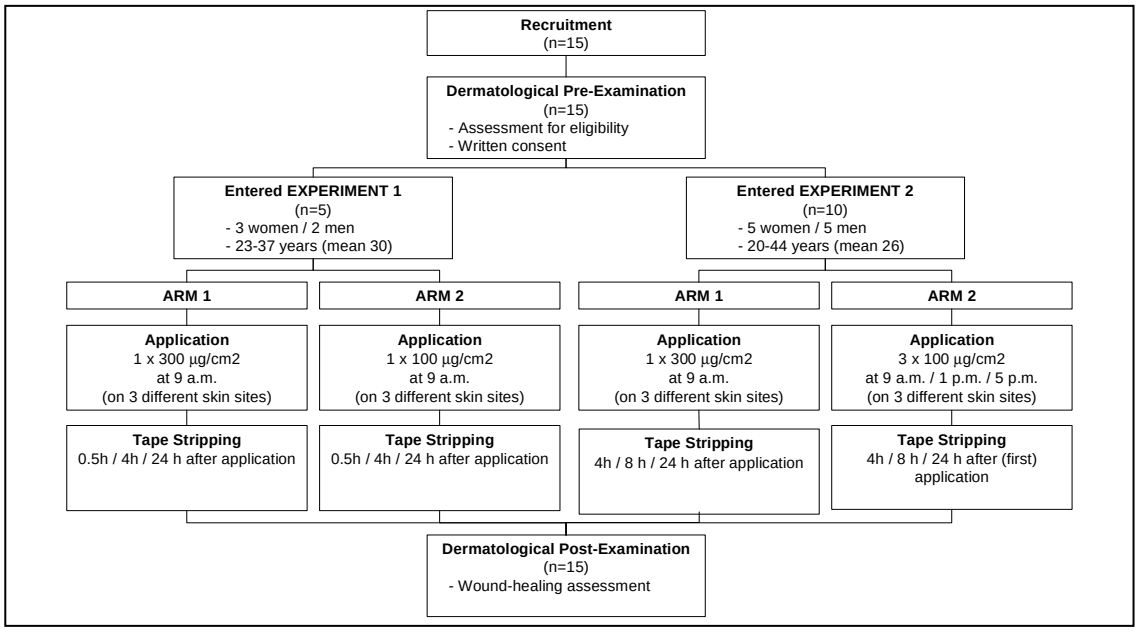


Fig. 7-1: Flow chart of the study: from recruitment to dismissal of the volunteers. The effect of dose (experiment 1) and application frequency (experiment 2) was investigated.

[8] The forms of the dermatological pre- and post-examination are displayed in the appendix (chapter 11.2.1 and 11.2.2).

7.2.3. Application of the formulations

Three skin sites per arm were treated (total of 6 skin sites per volunteer). The application area was delineated by a rectangular glass frame (10.5 cm^2) glued onto the skin (Sauer skin glue, Manfred Sauer GmbH, Lobbach, Germany). A volume of 250 µl formulation was uniformly applied with a Hamilton Syringe (Supelco, Buchs, Switzerland), and the vehicle was allowed to evaporate. In experiment 1, the high TACA dose (300 µg/cm^2) and the low TACA dose (100 µg/cm^2) were applied all at the same time (time 0 h, at 9 a.m.) on 3 different sites per arm. In experiment 2, the high TACA dose was applied at once on one arm (at 9 a.m.), whereas the low TACA dose was applied thrice on the other arm (at 9 a.m., 1 p.m., 5 p.m.) Details of application and tape stripping procedure are given in Table 7-1. Skin sites not stripped within 0.5 h after application were covered with non-occlusive cotton gauzes until tape stripping. No skin washing was performed to remove potential excess of formulation, because washing procedures have been correlated with an enhanced percutaneous absorption [258].

Table 7-1: Overview of application and tape stripping procedure.

				0 h	4 h	8 h	24 h
EXPERIMENT 1							
	ARM 1	Site 1	1 x 300 µg/cm^2	D T			
		Site 2	1 x 300 µg/cm^2	D	T		
		Site 3	1 x 300 µg/cm^2	D			T
	ARM 2	Site 4	1 x 100 µg/cm^2	D T			
		Site 5	1 x 100 µg/cm^2	D	T		
		Site 6	1 x 100 µg/cm^2	D			T
EXPERIMENT 2							
	ARM 1	Site 1	1 x 300 µg/cm^2	D	T		
		Site 2	1 x 300 µg/cm^2	D		T	
		Site 3	1 x 300 µg/cm^2	D			T
	ARM 2	Site 4	2 x 100 µg/$cm^{2\,(*)}$	D	D T		
		Site 5	3 x 100 µg/cm^2	D	D	D T	
		Site 6	3 x 100 µg/cm^2	D	D	D	T
				9 a.m.	1 p.m.	5 p.m.	9 a.m.

D = Dosing (application of the formulation), T = Tape Stripping (skin sampling). When tape stripping followed just after dosing (D T), a time span of 0.5 h was maintained between dosing and tape stripping to ensure an initial penetration of TACA into the stratum corneum. (*) Note that in experiment 2 just 2 doses were applied at 4 h (skin site 4); following the multiple application design, the total dose of 3 x 100 was not applied before 8 h.

7.2.4. Skin sampling by tape stripping

Stratum corneum tape stripping was performed after the following time intervals: in experiment 1 at 0.5 h, 4 h, and 24 h after application; in experiment 2 at 4 h, 8 h, and 24 h after (first) application (Table 7-1). To remove the stratum corneum in a standardized manner from the exact same skin area, a template delineating a constant aperture of 3.3 x 1.7 cm (5.6 cm^2) was fixed on the skin. An adhesive tape (Tesa Multi-Film Crystal-Clear® 57315, 19 mm width, Tesa, Beiersdorf, Hamburg, Germany) was placed on this skin site, and a hand roller supplying a pressure of 140 g/cm^2 was passed over the tape 10 times. The tape was removed with a rapid, firm movement and fixed across a photographic slide frame. This procedure was repeated with new tapes until total removal of the stratum corneum, which was defined as light transmission through the tape ≥ 95%, measured by UV/VIS-spectroscopy (see next section). Tape stripping of one skin site lasted about 15 min. No significant further drug diffusion into deeper skin layers is expected during the tape stripping time, since the highest amount of drug is removed with the first tapes [259].

7.2.5. Quantification of corneocytes

The amount of corneocytes adhering to each tape was quantified by measuring the pseudo-absorbance of the corneocytes at 430 nm as described by Weigmann et al. [245]. The slide frames on which the tapes had been fixed were inserted in the sample holder of a Lambda 35 spectrophotometer (Perkin Elmer, Überlingen, Germany, custom-modified to obtain a rectangular light beam of 1 cm^2), and each tape was directly measured against a blank tape.

7.2.6. Quantification of TACA

After quantification of the corneocytes, each tape was disassembled from the frame and extracted with 1.5 ml 60% methanol during 30 min on a horizontal shaker at 140 rpm (Heidolph Unimax 2010, Heidolph, Germany). To allow the calculation of a mass balance, the gauzes used to cover the treated skin sites were extracted similarly with 10 ml 60% methanol. TACA amounts in the extracts were quantified by an ICH-validated [237] HPLC method using a Symmetry Shield™ RP18 column (2.1 x 100 mm, 3.5 µm particle size) and a Waters Alliance HPLC System (2690 Separation Module, 996 Photodiode Array Detector), all Waters Corporation, Millford, Massachusetts, USA. The mobile phase consisted of methanol 60% in water (v/v) with a flow rate of 0.3 ml/min. Sample aliquots of 20 µl were injected, and quantification occurred at 240 nm. The limit of quantification (LOQ) was 100 ng/ml (corresponding to 27 ng/cm^2), the limit of detection (LOD) 35 ng/ml (9 ng/cm^2).

7.2.7. Data analysis

Sample size

The method deviation (intra-individual standard deviation) had been determined previously and was ± 40%. To provide a power of 80% in detecting a 50% difference between the 2 treatments groups at the 5% significance level, a total of 10 volunteers are needed according to the two-sided t-test nomogram for paired values after logarithmic transformation [260], and were thus enrolled in the main experiment 2. For the explorative experiment 1, a power of 50% was accepted, implicating the enrolment of 5 volunteers.

Qualitative TACA penetration into stratum corneum (penetration profiles)

To graphically visualize the drug distribution within the stratum corneum, TACA amounts quantified on each tape were correlated with tape number and depth of penetration into stratum corneum. Removal of the entire stratum corneum is a prerequisite for the profile calculation, since the sum of corneocytes (pseudo-)absorbance on one skin site represents 100% stratum corneum. Thus, the relative amount of stratum corneum removed by each tape can be calculated from the individual absorbance values as fully described in Jacobi et al. [126, 251].

Quantitative TACA penetration into stratum corneum

The TACA amounts on each tape (area 5.6 cm^2) of each skin site were added up to the total TACA amount penetrated into stratum corneum, which was evaluated statistically. The significance of differences between the treatment groups at each time was tested in a 2-sided paired-sample t-test after logarithmic transformation. Two different types of evaluation were performed: the evaluation of a) the total TACA amount within stratum corneum (sum of TACA amounts on all tapes) and of b) the TACA amount without tapes 1 to 3 (on which formulation excess, e.g. TACA crystals, could be located). Statgraphics® PLUS 5 software (Manugistic, Inc., Rockville, Maryland, USA) was used to conduct the analysis of the trial.

Mass balance

To gain further information on the fate of TACA, a mass balance was performed. The following TACA amounts were calculated: a) TACA in the gauzes used to protect the application sites until tape stripping; b) TACA in tapes 1-3; c) TACA in the stratum corneum (without tapes 1-3); and d) TACA not recovered and presumably penetrated into deeper skin layers or diffused laterally.

7.3. Results

7.3.1. Demographics of the subjects

A total of 15 healthy adult volunteers (7 male and 8 female) aged 20-44 years (mean 27) were recruited and finished the study. Five volunteers were assigned to experiment 1 and ten volunteers to experiment 2 (Fig. 7-1). The tape stripping experiments were conducted from March 2004 to July 2004 at the University Hospital Basel. The stripped skin sites displayed a good wound healing and no scarring at the final dermatological investigation. Slight hyperpigmentation was observed in some volunteers.

7.3.2. Qualitative TACA penetration into stratum corneum (penetration profiles)

The depiction of the results as penetration profiles visualizes the localization of TACA within the stratum corneum. The typical penetration profile displayed large TACA amounts in the upper stratum corneum and lower TACA amounts in the deeper stratum corneum, indicating TACA permeation through the stratum corneum and penetration into deeper tissues. In both experiments, similar penetration profiles of TACA over time were achieved, apart from a higher TACA amount usually located on the first stripped tape after application of the higher dose (300 µg/cm^2) in experiment 1 or after multiple application (3x100 µg/cm^2) in experiment 2. The penetration profiles obtained after the different applications of experiment 2 in one volunteer are displayed in Fig. 7-2[9].

The mean number of tapes required to remove the entire stratum corneum in both experiments was 55, with a minimum of 29 tapes and a maximum of 80 (independently of gender and age).

[9] The penetration profiles for each volunteer are displayed in the appendix (chapter 11.2.1 for experiment 1 and chapter 11.2.6 for experiment 2).

1 x 300 µg/cm^2	3 x 100 µg/cm^2

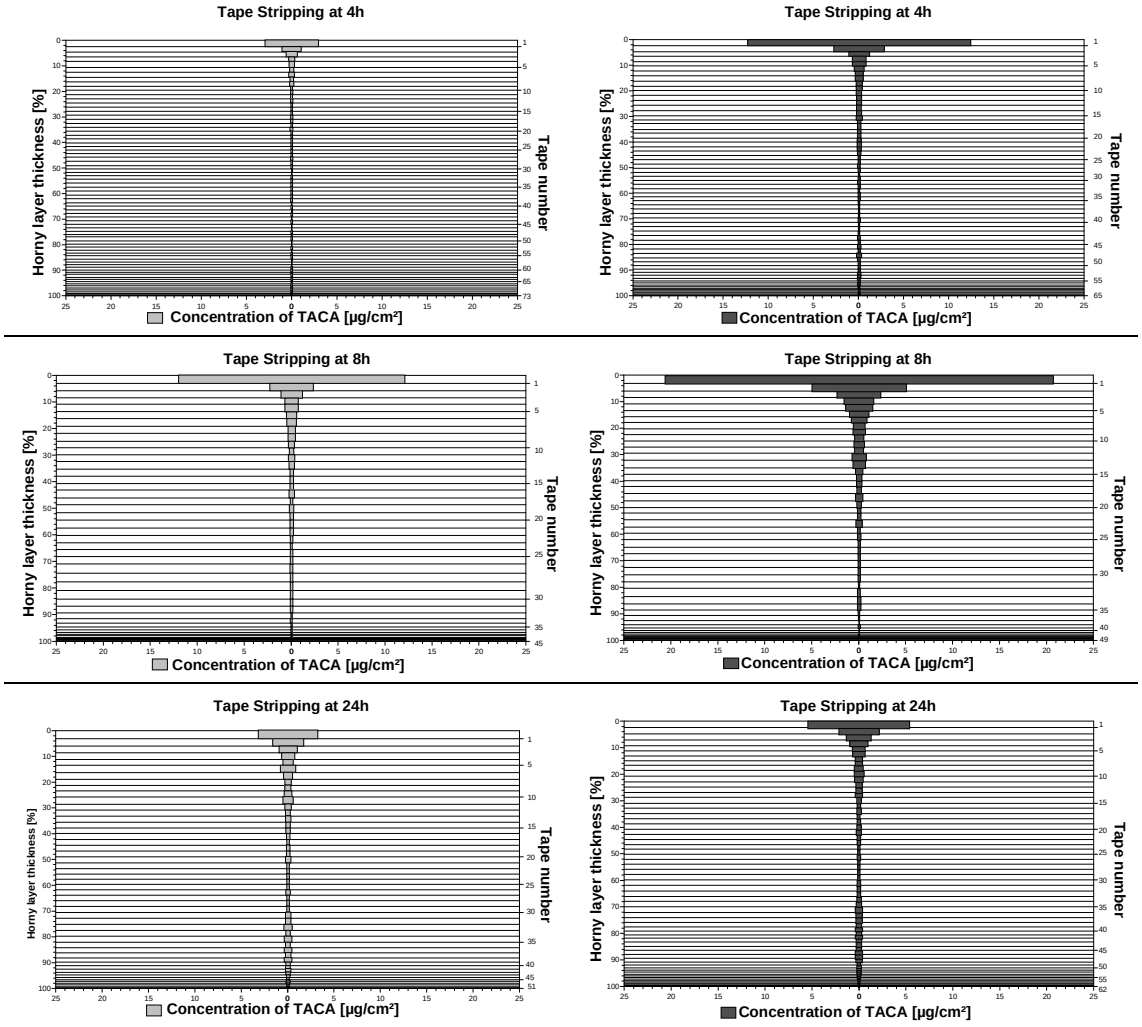


Fig. 7-2: Typical penetration profiles of TACA into stratum corneum over time (4 h, 8 h, 24 h) after application of 1x300 µg/cm^2 vs. 3x100 µg/cm^2 TACA in the same volunteer (experiment 2). TACA amounts on each tape (horizontal grey bars) are correlated to the tape number (right scale) and to the depth of penetration into stratum corneum, displayed as percentage of the total stratum corneum thickness (left scale). This correlation is enabled because the entire stratum corneum was stripped from each skin site, the total number of tapes thus representing 100% stratum corneum thickness.

7.3.3. Quantitative TACA penetration into stratum corneum

Experiment 1 - Effect of dose (1x300 µg/cm^2 vs. 1x100 µg/cm^2)

The total amounts of TACA penetrated into stratum corneum at the different time points are depicted in Fig. 7-3 (left side)[10]. At 0.5 h, almost the entire TACA dose applied was quantified within the stratum corneum: 245 ± 78 µg/cm^2 after application of 300 µg/cm^2 and 101 ± 17 µg/cm^2 after application of 100 µg/cm^2. At 4 h, lower TACA amounts of 80 ± 19 µg/cm^2 (after application of 300 µg/cm^2) and 52 ± 13 µg/cm^2 (after application of 100 µg/cm^2) were observed, and after 24 h still 46 ± 18 µg/cm^2 vs. 33 ± 19 µg/cm^2 were quantified.

By excluding tapes 1-3, a dramatically lower amount was measured at 0.5 h within the stratum corneum, with TACA amounts of 74 ± 12 µg/cm^2 (after application of 300 µg/cm^2) vs. 55 ± 14 µg/cm^2 (after application of 100 µg/cm^2). No difference in the TACA amounts recovered after application of the two different doses was observed at later time points: 31 ± 15 µg/cm^2 vs. 30 ± 16 µg/cm^2 at 4 h, and 27 ± 13 µg/cm^2 vs. 23 ± 14 µg/cm^2 at 24 h after application of 300 µg/cm^2 and 100 µg/cm^2, respectively. The extremely lower TACA amount obtained by exclusion of tapes 1-3 showed that considerable amounts are retained on the skin surface.

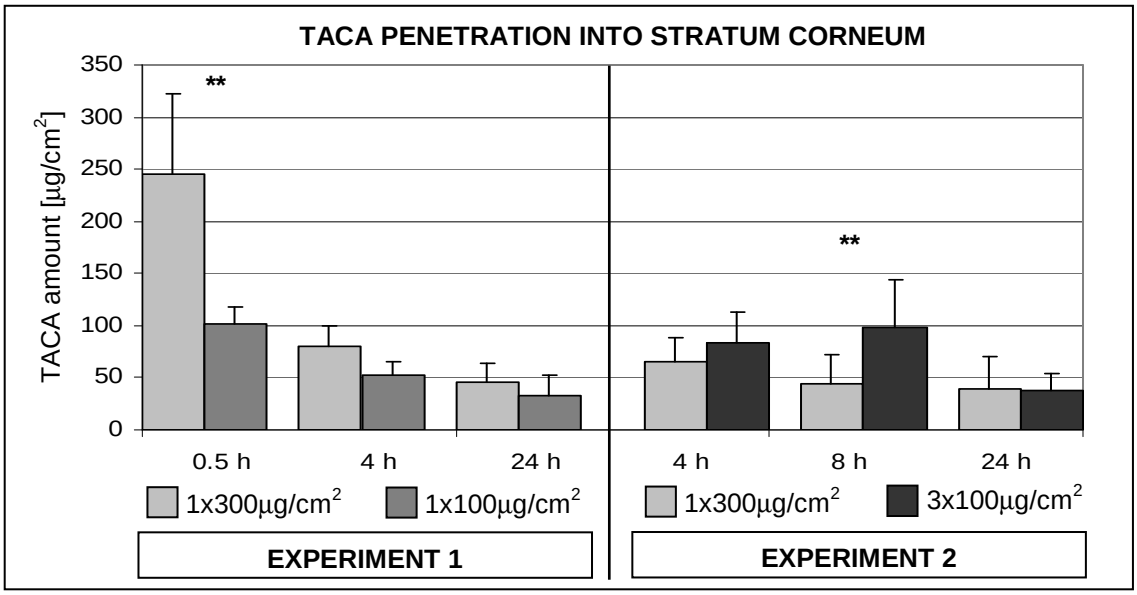


Fig. 7-3: Total TACA amounts penetrated into the stratum corneum in experiment 1 and experiment 2. Mean and standard deviation calculated with all tapes (including tapes 1-3) are displayed. The double asterisk (**) denotes a highly significant difference ($p < 0.01$, 2-sided paired-sample t-test) between the pair differences at the specified time.

Statistical evaluation of the corresponding pair differences yielded a highly significant difference of the TACA amounts quantified at 0.5 h when all tapes were considered ($p < 0.01$) but no significance when tapes 1-3 were excluded ($p > 0.05$). At 4 h and 24 h, no statistically significant difference was observed for both evaluations (all tapes / without tapes 1-3).

[10] The data table as well as the statistical evaluation are displayed in the appendix (chapter 11.2.4 and 11.2.5).

Experiment 2 - Effect of application frequency (1x300 μg/cm2 vs. 3x100 μg/cm2)

The total TACA amounts penetrated into stratum corneum at the different time points are depicted in Fig. 7-3 (right side)[11]. At 4 h, the total TACA amount quantified within the stratum corneum (all tapes) amounted to 65 ± 23 μg/cm^2 after application of 1x300 μg/cm^2 vs. 84 ± 29 μg/cm^2 after 2x100 μg/cm^2. This slight difference became inexistent after discarding the first 3 tapes (33 ± 12 μg/cm^2 and 35 ± 11 μg/cm^2, respectively). The pair difference was statistically not significant in both cases ($p > 0.05$). Note that this time point displays a different totally applied dose (300 μg/cm^2 vs. 200 μg/cm^2). Taking this into account, the amount observed after multiple application was quite high: despite the application of a lower total dose, a similar TACA amount was quantified within the stratum corneum.

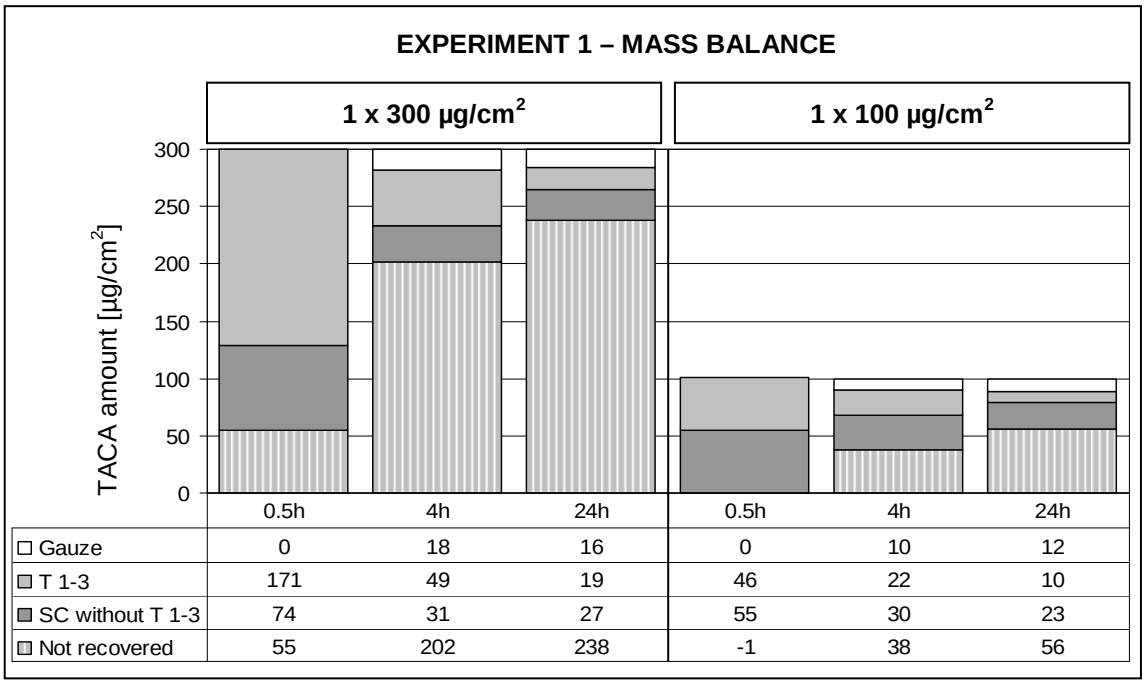
At 8 h, TACA within the stratum corneum amounted to 44 ± 29 μg/cm^2 (after application of 1x300 μg/cm^2) vs. 98 ± 45 μg/cm^2 (after application of 3x100 μg/cm^2). This difference was highly significant ($p < 0.01$) when all tapes were considered, but only a slight trend was seen after discarding tapes 1-3 (20 ± 8 μg/cm^2 and 29 ± 15 μg/cm^2 after application of 1x300 μg/cm^2 and 3x100 μg/cm^2, respectively, $p = 0.06$).

At 24 h, similar total TACA amounts were quantified within the stratum corneum: 39 ± 31 μg/cm^2 after application of 1x300 μg/cm^2 vs. 38 ± 16 μg/cm^2 after 3x100 μg/cm^2 (24 ± 14 μg/cm^2 and 23 ± 9 μg/cm^2, respectively, after discarding tapes 1-3). These values showed no statistically significant difference ($p > 0.05$).

7.3.4. Mass balance

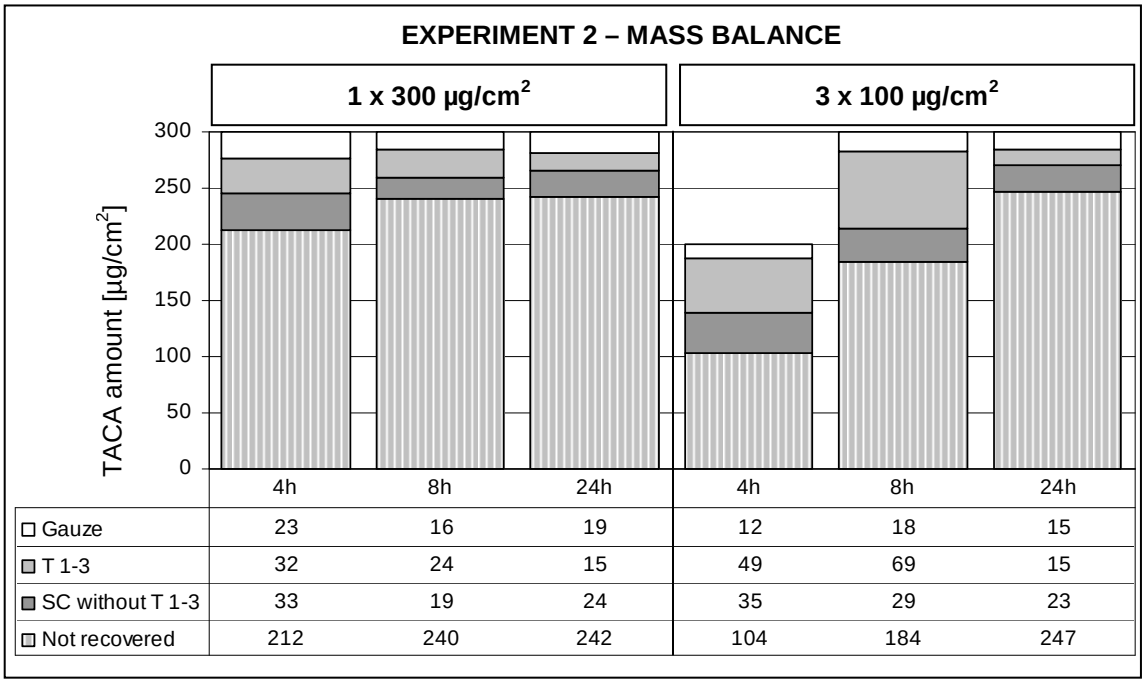
The application of a finite TACA dose permits the performance of a mass balance. For each treated skin site, 4 different values of recovered TACA amount were determined: 1) in the gauze; 2) in tapes 1-3; 3) in the stratum corneum without tapes 1-3 and; 4) the remnant amount not recovered and presumably penetrated into deeper tissues or diffused laterally. The results of the mass balance are presented in Fig. 7-4 (experiment 1) and Fig. 7-5 (experiment 2). The evaluation of both experiments showed that: a) TACA amounts of 10-23 μg/cm^2 did not penetrate into the stratum corneum and adhered to the gauze (corresponding to 5-12% of the applied TACA dose). b) About half the amount recovered in the entire stratum corneum persisted within the upper stratum corneum and was found in tapes 1-3 (up to 57% of the applied TACA dose). c) TACA amounts recovered in the stratum corneum without tapes 1-3 were not significantly different between the different application modes. d) TACA seemed to permeate the stratum corneum more rapidly after a single application of the high TACA dose (300 μg/cm^2), since the TACA amounts not recovered (and presumably penetrated into deeper tissues) were already observed after 0.5 h (experiment 1) and were high after 4 h (in both experiments 1 and 2).

[11] The data table as well as the statistical evaluation are displayed in the appendix (chapter 11.2.7 and 11.2.8).



	0.5h	4h	24h	0.5h	4h	24h
Gauze	0	18	16	0	10	12
T 1-3	171	49	19	46	22	10
SC without T 1-3	74	31	27	55	30	23
Not recovered	55	202	238	-1	38	56

Fig. 7-4: Mass balance of experiment 1. Skin sites are divided into 4 compartments displaying the mean TACA amounts [µg/cm²] recovered in gauze (white fields), in tapes (T) 1-3 (light grey), in stratum corneum (SC) without T 1-3 (dark grey), and the amounts of TACA not recovered and presumably penetrated into deeper or adjacent skin tissues (striped grey). At 0.5 h, no gauze was used, since tape stripping followed just after application of the formulation.



	4h	8h	24h	4h	8h	24h
Gauze	23	16	19	12	18	15
T 1-3	32	24	15	49	69	15
SC without T 1-3	33	19	24	35	29	23
Not recovered	212	240	242	104	184	247

Fig. 7-5: Mass balance of experiment 2. Each skin site is divided into 4 compartments displaying the mean TACA amounts [µg/cm²] recovered in gauze (white fields), in tapes (T) 1-3 (light grey), in stratum corneum (SC) without T 1-3 (dark grey), and the amounts of TACA not recovered and presumably penetrated in deeper or adjacent skin tissues (striped grey).

7.4. Discussion

A prerequisite for investigating topical products is the stringent differentiation between *topical* and *systemic* bioavailability determination. In previous investigations on percutaneous absorption of corticosteroids applied in acetone [224, 225], conclusions for topical therapy were drawn from data obtained by urinary excretion. Yet, data yielded from urinary excretion measure *systemic* bioavailability (of the topical application) and not *topical* bioavailability (of the topical application). Measurements of drug concentration in urine and/or blood are only a means for bioavailability evaluation when the pharmacological response is correlated to a systemic parameter or when the body burden is of interest. Furthermore, systemic drug concentrations after topical application do not represent drug concentrations at the target site in the skin but purely drug concentrations after permeation through the target site. Thus, the mere assessment of systemic bioavailability does not properly reflect topical bioavailability for the treatment of local skin diseases [34, 261].

This trial has been performed to assess the topical bioavailability of TACA in a simple vehicle after different application modes. Acetone does not represent a vehicle normally used in dermatology, but has often been used as a vehicle for investigational purposes [224, 225, 262]. TACA displays a very good solubility in acetone, and the volatile vehicle allows the application of a finite, solvent-deposited drug amount [49, 59]. Therefore, an acetonic vehicle is appropriate for the purpose of this study. Especially after application of the high TACA dose, some drug crystallized on the skin surface because of the rapid vehicle evaporation. On the one hand, the crystals can get lost due to friction with clothes or due to normal desquamation (this amount was retained and quantified in the protective gauze during our experiments). On the other hand, drug crystals can also become bioavailable at later stages if redissolved, either physiologically by humid micro environmental conditions on the skin surface or by fresh vehicle in case of multiple applications [61]. This was one reason for not washing the skin during our experiments, assuming that the protective gauze would retain the superficial, unbound TACA. Moreover, washing procedures have been shown to enhance percutaneous penetration of topically applied compounds [258].

A frequently debated question arising from tape stripping experiments is the inclusion or exclusion of the first tapes into the evaluation. The answer depends on the study design. In our case, the drug was solvent-deposited on the skin, and the mass balance required the consideration of all the tapes. Thus, a separate evaluation with and without tapes 1-3 was chosen.

In our experiments, the topical bioavailability of TACA was described by the TACA penetration into stratum corneum over time. This is possible because the stratum corneum is the rate limiting barrier to percutaneous absorption, and thus the amount of drug in the stratum corneum may be considered to reflect to the amount of drug at the target site [257]. Experiment 1 displayed higher TACA amounts within the stratum corneum after application of a high TACA

dose (300 μg/cm^2) compared to a lower TACA dose (100 μg/cm^2). However, this difference was only significant immediately after application, when almost the entire TACA dose applied was recovered within the stratum corneum, whereas similar TACA amounts were found after discarding tapes 1-3. Usually, increasing the applied drug dose leads to a higher absolute (but a lower relative) percutaneous penetration, provided that the drug is dissolved [203]. In our experiment, after application of the higher TACA dose, the immediate evaporation of acetone led to the precipitation of a high TACA amount on the skin surface and on the external layers of the stratum corneum.

Experiment 2 showed slightly higher TACA amounts within the stratum corneum after multiple application of a lower TACA dose (3x100 μg/cm^2) compared to the single application of the high TACA dose (1x300 μg/cm^2). As a result of multiple applications, the skin was periodically reloaded with new drug, thus achieving temporary higher amounts within the stratum corneum. In order to characterize this temporary trend, the stripping times after application of the second (at 4 h) and third (at 8 h) dose in case of multiple application were deliberately chosen. The highest drug amount was always localized within the upper stratum corneum layers and by excluding tapes 1-3 only insignificant differences between the TACA amounts recovered after the different application modes were observed (in both experiments).

At 24 h, still well quantifiable TACA amounts were retained within the stratum corneum independently of dose and application frequency. Yet, these TACA amounts were rather due to the slow diffusion through the stratum corneum barrier than to a “classical” reservoir formation. This was underlined by the slow but existent diffusion of TACA into deeper tissues.

A similar *topical* bioavailability within the stratum corneum does not necessarily imply a similar *systemic* bioavailability, not desired in topical therapy with corticosteroids. TACA amounts not quantified within the stratum corneum have presumably penetrated vertically into the viable epidermis and into the dermis, reaching the systemic blood circulation. In addition, a horizontal, lateral diffusion into adjacent stratum corneum has also been observed in the past [123]. Both penetration routes may have played a role in our experiments, but the vertical penetration usually represents the preferred route.

The mass balance was performed to estimate the extent of the systemic exposure. After application of a total TACA dose of 300 μg/cm^2 (either as a single dose or divided into multiple doses), a high TACA amount was not recovered and seemed to have penetrated into deeper tissues. At 24 h, the extent of percutaneous absorption and thus the extent of systemic bioavailability seemed independent of the application mode, but the release rate out of the stratum corneum into deeper tissues was lower after application of the multiple doses. On the contrary, after application of a total dose of 100 μg/cm^2, both extent and release of the drug out of the stratum corneum into deeper tissues was lower. Thus, a low TACA dose applied once may be preferable to a higher total TACA dose (applied once or thrice daily). This agrees with pharmacodynamic investigations [208], which showed that multiple-daily applications usually have no superiority.

7.5. Conclusions

In the present study, the effect of dose and application frequency on the topical (cutaneous) bioavailability of TACA was determined by standardized tape stripping in human volunteers. Actual drug amounts were measured directly within the stratum corneum, a layer preceding the target site.

Independently of the dose and the application frequency, considerable TACA amounts were retained for 24 h within the stratum corneum (reservoir formation). A difference between the TACA amounts penetrated into the stratum corneum after the different application modes was observed immediately after application and was mainly due to a different TACA amount in the first 3 tapes. No major differences were observed at later time points. Yet, a faster permeation through the stratum corneum and thus a higher systemic exposure, not welcome in topical therapy, may be promoted by application of a high TACA dose. Thus, a low TACA dose applied once may be preferable to a high TACA dose.

8. Project III: Effect of occlusion

The findings of this project have been accepted for publication: Pellanda C, Strub C, Figueiredo V, Rufli T, Imanidis G, Surber C. Topical bioavailability of triamcinolone acetonide: effect of occlusion. Skin Pharmacol Physiol. The original publication is available at www.karger.com/spp.

Abstract

Occlusion by covering the skin with an impermeable wrap enhances skin hydration, affects drug absorption, and can induce the formation of a drug reservoir within the stratum corneum. This is desired in local therapy with topical corticosteroids. The aim of the study was to investigate the effect of occlusion before (pre-occlusion, experiment 1) and after (post-occlusion, experiment 2) application on the penetration of triamcinolone acetonide (TACA) into the stratum corneum.

The experiments were conducted on the forearms of 10 healthy volunteers. In experiment 1, a dose of 100 $\mu g/cm^2$ TACA was applied on 3 sites per arm, one arm having been pre-occluded for 16 h. In experiment 2, the same dose was applied on 2 sites per arm, and one arm was occluded after application until skin sampling. Stratum corneum samples were removed by tape stripping at 0.5, 4, 24 h (experiment 1) and 4, 24 h (experiment 2) after application. Corneocytes and TACA were quantified by UV/VIS-spectroscopy and HPLC, respectively. The total TACA amount penetrated into stratum corneum was evaluated by multifactor ANOVA.

TACA penetration into stratum corneum with and without pre-occlusion (experiment 1) showed no significant difference and decreased with time. Occlusion after application (experiment 2) produced a marked TACA accumulation within the stratum corneum, which persisted for 24 h. In conclusion, pre-occlusion showed no effect on the topical bioavailability of TACA in stratum corneum. Contrariwise, post-occlusion enhanced the penetration by a factor of 2, favoring the development of a drug reservoir.

Keywords

Occlusion
Reservoir
Tape stripping
Topical bioavailability
Triamcinolone acetonide

8.1. Introduction

Occlusion by covering the skin with an impermeable wrap inhibits the physiological water loss through the skin and increases the skin temperature [67]. The enhanced skin hydration can induce morphological changes of the stratum corneum such as swelling of the corneocytes [69], water uptake into intercellular lipid domains [70], and deepening of skin furrows [68].

Such changes considerably affect the percutaneous absorption of topically applied drugs. Vickers observed in the early 60's that the application of corticosteroids under occlusion promoted the formation of a long-lasting drug reservoir within the stratum corneum [148]. This is a welcome effect in local therapy with topical corticosteroids. The longer the active drug is present within the skin, the longer a therapeutic effect can be expected.

Occlusion does not enhance the percutaneous absorption of all drugs [52, 233]. The enhancement appears to be compound and vehicle dependent [73]. Volatile solvents such as acetone and ethanol seem to induce a clear penetration enhancement after application under occlusion [235].

The aim of the study was to investigate the effect of occlusion on the *in vivo* penetration of triamcinolone acetonide (TACA), a moderately potent corticosteroid, into stratum corneum. In experiment 1, the effect of occlusion prior to topical application of TACA in acetone (pre-occlusion) was investigated. In experiment 2, the effect of occlusion after TACA application (post-occlusion) was investigated.

8.2. Subjects and methods

8.2.1. Material and formulation

A solution of 4.2 mg/ml micronized TACA Ph.Eur. (Caesar&Loretz GmbH, Hilden, Germany) in acetone Ph.Eur. (Hänseler AG, Herisau, Switzerland) was prepared following current GMP guidelines.

8.2.2. Subjects and study design

A total of 10 healthy adult volunteers with skin phototype II-III (Caucasian) without excessive hairiness of the inner forearm were recruited and underwent a dermatological examination one week prior to study-start. The experiments were conducted on the volar aspect of the forearms during 2 days as an open, explorative study with half-side intra-individual comparison. In experiment 1, one arm was pre-occluded prior to TACA application by wrapping it for 16 h in polypropylene film (Tangan® wrap). In experiment 2, occlusion of one arm followed TACA application, and the occlusive wrap was not removed until tape stripping. In both experiments, the contralateral arm served as control and was not occluded. Finite single doses (100 µg/cm^2) of TACA solution were applied on selected skin areas, which were afterwards sampled by tape stripping at different times following the protocol described below. Within 1 month after tape stripping, the wound healing was evaluated in a final dermatological examination[12]. Fig. 8-1 displays the flow chart of the study.

The study was conducted according to the ethical rules stated in the Declaration of Helsinki and was approved by the local ethical committee and the national authorities (Swissmedic). The volunteers signed written consent for participation.

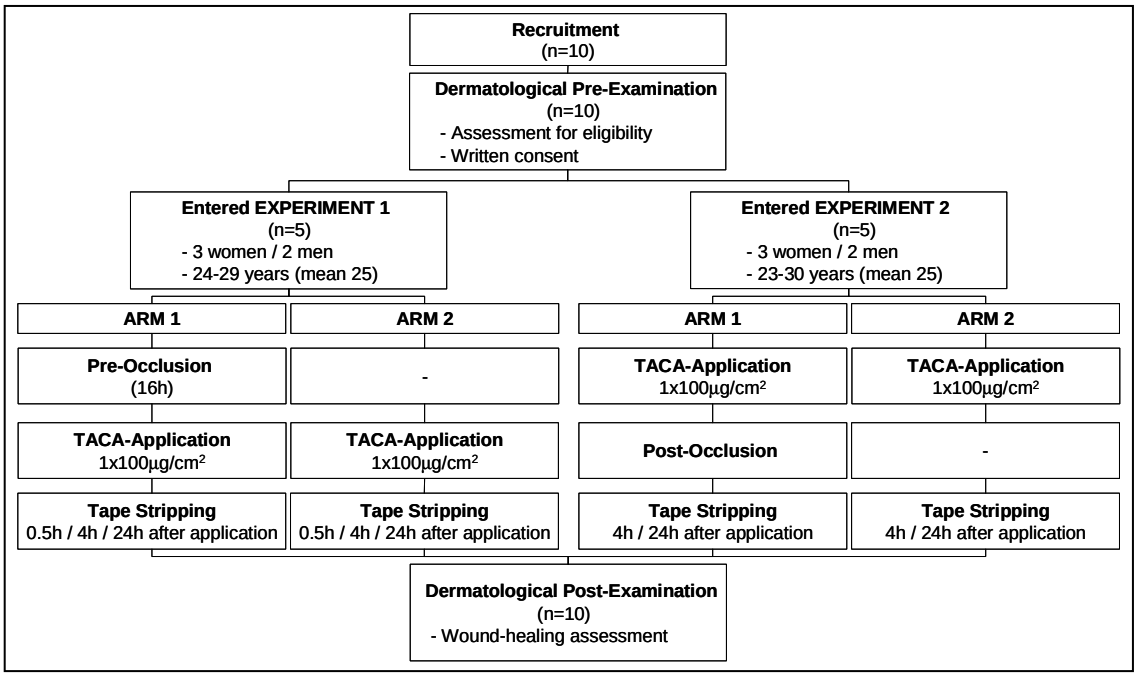


Fig. 8-1: Flow chart of the study: from recruitment to dismissal of the volunteers. The effect of pre-occlusion (experiment 1) and post-occlusion (experiment 2) on the TACA penetration into stratum corneum was investigated.

[12] The forms of the dermatological pre- and post-examination are displayed in the appendix (chapter 11.2.1 and 11.2.2).

8.2.3. Application of the formulation

In experiment 1, 3 skin sites per arm were treated (total of 6 skin sites per volunteer). In experiment 2, 2 skin sites per arm were treated (total of 4 skin sites per volunteer)[13]. The application area was delineated by a rectangular glass frame (10.5 cm^2) glued onto the skin (Sauer® skin glue, Manfred Sauer GmbH, Lobbach, Germany). A volume of 250 µl formulation, corresponding to a TACA dose of 100 µg/cm^2, was uniformly applied on the delineated area with a Hamilton Syringe (Supelco, Buchs, Switzerland). The vehicle was allowed to evaporate. Skin sites not stripped within 0.5 h after application were protected with non-occlusive cotton gauzes until tape stripping. No skin washing was performed to remove excess of formulation, and the volunteers were not allowed to shower during the 2-day experiment.

8.2.4. Skin sampling by tape stripping

Stratum corneum samples were removed by standardized tape stripping [93] with Tesa tape (Tesa Multi-Film Crystal-Clear® 57315, 19 mm width, Beiersdorf, Hamburg, Germany) at 0.5 h, 4 h, 24 h (experiment 1) and at 4 h, 24 h (experiment 2) after application (Fig. 8-1). A template delineating an area of 5.6 cm^2 was fixed onto the skin. Tapes were successively placed onto this area and stripped off with a rapid movement until the entire stratum corneum had been removed. A hand roller supplying a constant pressure of 140 g/cm^2 was passed 10 times over each tape before removal. Total stratum corneum removal was defined as light transmission through the tape ≥ 95%, measured by UV/VIS-spectroscopy as described below.

8.2.5. Analytics

The amount of corneocytes adhering to each tape was spectroscopically quantified directly on the tapes at 430 nm using a spectrophotometer (Lambda 35, Perkin Elmer, Überlingen, Germany), custom-modified to obtain a rectangular light beam of 1 cm^2, as described in Weigmann et al. [245]. The quantification of the corneocytes is required for the calculation of penetration profiles.

The amounts of TACA adhering to each tape were quantified by an ICH-validated [237] HPLC method after extraction with 1.5 ml 60% methanol on a horizontal shaker (30 min, 140 rpm, Heidolph Unimax 2010, Heidolph, Germany). The protective gauzes were similarly extracted with 10 ml 60% methanol. A Symmetry Shield™ RP18 column and a Waters Alliance HPLC System (2690 Separation Module, 996 Photodiode Array Detector), all Waters Corporation, Millford, Massachusetts, USA, were used. Methanol 60% (v/v) with a flow rate of 0.3 ml/min was the mobile phase, and the injection volume of the samples was 20 µl. Quantification of TACA occurred at 240 nm. The limit of quantification (LOQ) was 100 ng/ml (corresponding to 27 ng/cm^2), the limit of detection (LOD) 35 ng/ml (9 ng/cm^2).

[13] The detailed protocol of application, tape stripping, and occlusion is displayed in the appendix (chapter 11.3.1).

8.2.6. Data analysis

Sample size

The method deviation (intra-individual standard deviation) had been determined previously and was ± 40%. To provide a power of 50% in detecting a 50% difference between the 2 treatments groups at the 5% significance level, a total of 5 volunteers were needed according to the two-sided t-test nomogram for paired values after logarithmic transformation [260], and were thus enrolled in each experiment.

Quantitative TACA penetration into stratum corneum

The TACA amounts on each tape (area 5.6 cm^2) of each skin site were added up to the total TACA amount penetrated into stratum corneum, which was evaluated statistically. The significance of differences between the treatment groups at each time was tested after logarithmic transformation in a multifactor variance analysis (ANOVA) [30] with the following factors: volunteer, time, application and the interaction time/application. For the factors displaying a statistical significance in ANOVA ($p < 0.05$), a post-hoc comparison was performed with the least significance difference test (LSD test). Two different types of evaluation were performed: the evaluation of a) the total TACA amount within stratum corneum (sum of TACA amounts on all tapes) and of b) the TACA amount without tapes 1 to 3 (on which formulation excess, e.g. TACA crystals, could be located). Statgraphics® PLUS 5 software (Manugistic, Inc., Rockville, Maryland, USA) was used to conduct the analysis of the trial.

Qualitative TACA penetration into stratum corneum: penetration profiles and photographic recording

To visualize the drug distribution within the stratum corneum, TACA amounts quantified on each tape were correlated to the tape number and to the depth of penetration into the stratum corneum. Removal of the entire stratum corneum is a prerequisite for the profile calculation, since the sum of corneocyte (pseudo-)absorbance on one skin site represents 100% stratum corneum. Thus, the relative amount of stratum corneum removed by each tape can be calculated from the individual absorbance values as fully described in Jacobi et al. [126, 251].

The stratum corneum removal pattern was photographically documented. All photographs were taken at a standard camera-object distance.

8.3. Results

8.3.1. Demographics of the subjects

A total of 10 healthy adult volunteers (5 male and 5 female) aged 23-30 years (mean 25) were recruited and finished the study. Five volunteers were assigned to each experiment (Fig. 8-1). The tape stripping experiments were conducted from April 2004 to July 2004 in Basel. No skin damage was observed at the final dermatological investigation.

8.3.2. Quantitative TACA penetration into stratum corneum

Experiment 1: Effect of Pre-Occlusion

Pre-occlusion showed no effect on the TACA penetration into the stratum corneum (Fig. 8-2, left side)[14]. The penetrated TACA amount decreased significantly with time after both application modes, with and without pre-occlusion ($p < 0.001$). At 0.5 h, mean TACA amounts of 66 ± 28 μg/cm^2 and 67 ± 37 μg/cm^2 were quantified within the stratum corneum of normal and pre-occluded skin, respectively. At 4 h, mean values of 48 ± 17 μg/cm^2 and 43 ± 10 μg/cm^2 were recovered on normal and pre-occluded skin, and at 24 h values of 29 ± 12 μg/cm^2 and 18 ± 10 μg/cm^2, respectively.

By excluding tapes 1-3, the TACA amounts were approximately halved: at 0.5 h, mean values of 30 ± 12 μg/cm^2 and 35 ± 16 μg/cm^2 were obtained on normal and pre-occluded skin, respectively; at 4 h 25 ± 8 μg/cm^2 and 24 ± 8 μg/cm^2; and at 24 h 20 ± 9 μg/cm^2 and 12 ± 7 μg/cm^2.

Independently of the application mode (with/without pre-occlusion), 8-13% of the applied TACA amount was recovered in the gauzes used to protect the treated skin sites[15].

Statistical evaluation by ANOVA displayed a significant difference only for the factor time ($p < 0.001$), whereas the factors volunteer, application, and the interaction time/application showed no influence.

[14] The data table as well as the statistical evaluation are displayed in the appendix (chapter 11.3.3 and 11.3.4).

[15] The mass balance of experiment 1 is displayed in the appendix (chapter 11.3.8).

Experiment 2: Effect of Post-Occlusion

Post-occlusion induced a marked accumulation of TACA within the stratum corneum. The amount of TACA recovered in the stratum corneum in case of post-occlusion showed a highly significant difference ($p < 0.01$) compared to non-occluded skin (Fig. 8-2, right side)[16].

Over time, the TACA amount within the stratum corneum remained constant after both application modes: after normal application (without occlusion), the values amounted to 40 ± 28 µg/cm^2 and 42 ± 14 µg/cm^2 at 4 h and 24 h, respectively. In case of post-occlusion, a twofold higher TACA amount was quantified within the stratum corneum, with values amounting to 80 ± 32 µg/cm^2 and 79 ± 33 µg/cm^2 at 4 h and 24 h, respectively.

A similar trend was also seen after discarding tapes 1-3: after normal application, TACA amounts of 21 ± 12 µg/cm^2 and 24 ± 11 µg/cm^2 were quantified at 4 h and 24 h, vs. TACA amounts of 37 ± 13 µg/cm^2 and 42 ± 28 µg/cm^2 after application followed by post-occlusion.

The TACA amount extracted from the gauzes displayed values of 11% of the applied dose after normal application and 3-7% after application under occlusion[17].

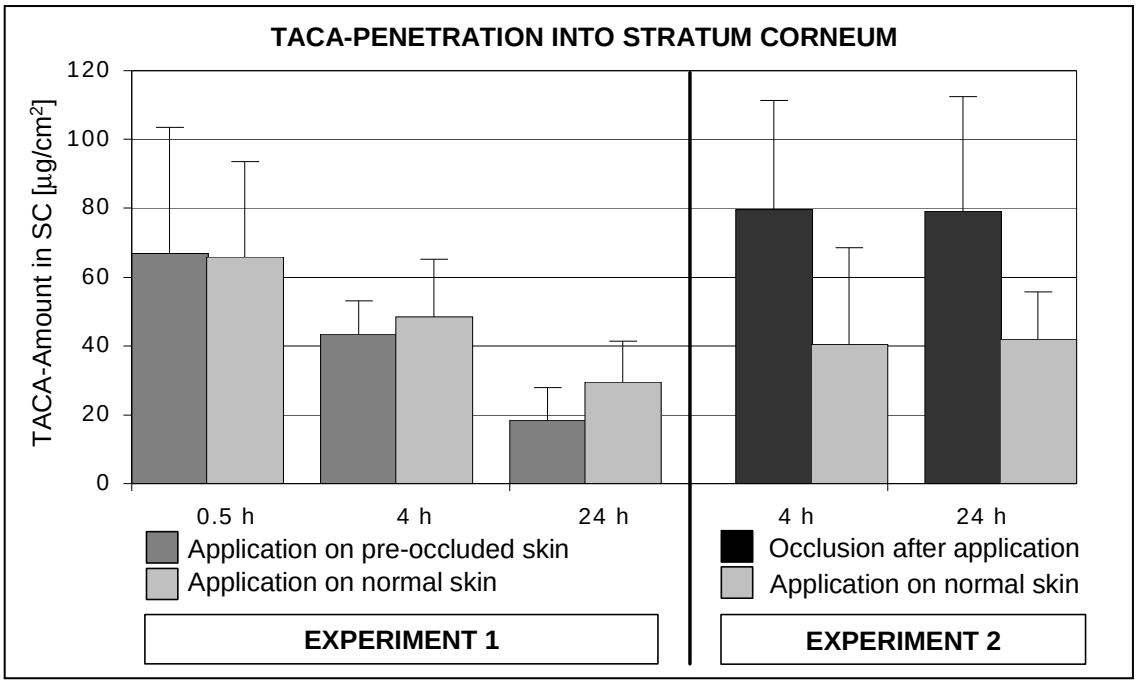


Fig. 8-2: Total TACA amount penetrated into the stratum corneum after application of 100 µg/cm^2 TACA in acetone under normal vs. pre-occluded conditions (experiment 1) and under normal vs. post-occluded conditions. Mean and standard deviation are displayed.

[16] The data table as well as the statistical evaluation are displayed in the appendix (chapter 11.3.6 and 11.3.7).

[17] The mass balance of experiment 2 is displayed in the appendix (chapter 11.3.9).

8.3.3. Qualitative TACA penetration into stratum corneum: penetration profiles and photographic recording

Two exemplar penetration profiles of TACA into the stratum corneum at 24 h after normal and post-occluded application are depicted in Fig. 8-3[18]. The profiles show that TACA permeated the stratum corneum and reached deeper tissues. The higher TACA amount retained within the stratum corneum after application under post-occlusion is clearly visible.

The increased hydration of the skin due to occlusion caused a decreased corneocyte cohesion. Fewer tapes were required to remove the entire stratum corneum of occluded skin sites, since larger amounts of corneocytes were removed on single tapes. This happened in both experiments 1 and 2 after long-time occlusion and tape stripping following within 0.5 h after removal of the occlusive wrap. The removal of larger amounts of corneocytes mostly occurred between tapes no. 5 and 10 (Fig. 8-4).

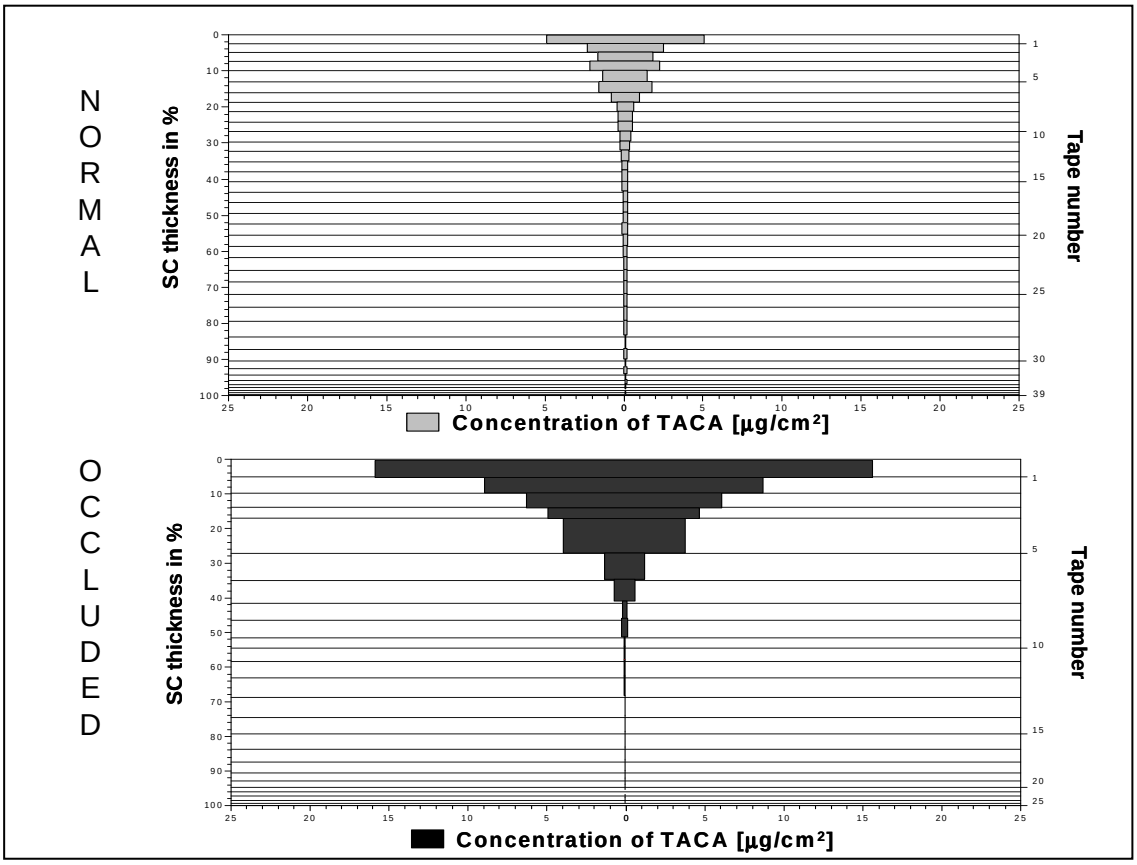


Fig. 8-3: Typical penetration profiles of TACA into the stratum corneum 24 h after normal application and after application followed by occlusion in the same volunteer. TACA amounts on each tape (horizontal grey or black bars) are correlated to the tape number (right scale) and to the depth of penetration into the stratum corneum, displayed as percentage of the total stratum corneum thickness (left scale).

[18] The penetration profiles for each volunteer are displayed in the appendix (chapter 11.3.2 for experiment 1 and chapter 11.3.5 for experiment 2).

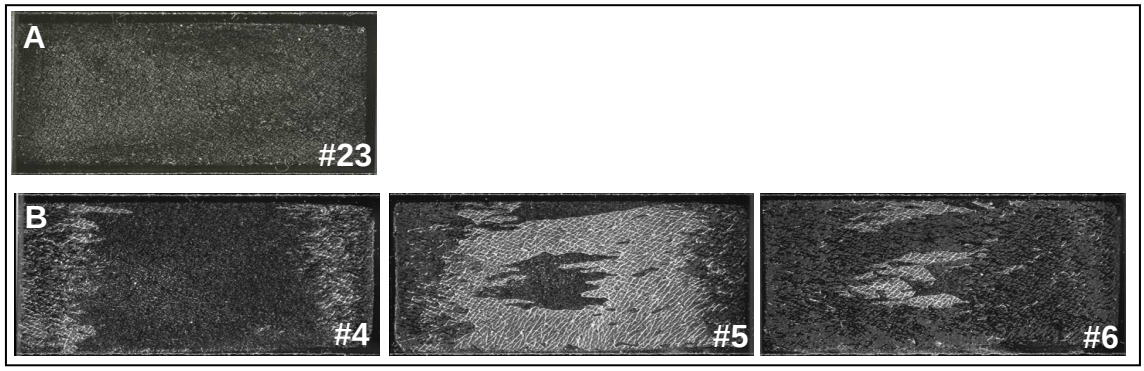


Fig. 8-4: Photographs of stripped tapes. Long-time occlusion induced a loosening of the stratum corneum structure. Larger amounts of corneocytes (skin sheets) were removed on single tapes. (A) 23rd tape stripped from a non-occluded skin site: a "normal", homogenous amount of skin is removed. (B) 4th to 6th tape stripped from a skin site occluded for 24 h: larger amounts of corneocytes are removed as sheets on single tapes. This artifact due to occlusion does not disturb the assessment of topical bioavailability provided that the entire stratum corneum of one skin site is sampled.

8.4. Discussion

In the present experiments, the topical bioavailability of TACA was described by the TACA penetration into stratum corneum over time. Because the stratum corneum is the rate limiting barrier of the skin, the TACA amount within the stratum corneum can be directly correlated to the amount of drug at the target site (viable epidermis, dermis). Two different modes of occlusion were investigated: pre-occlusion (occlusion before TACA application) and post-occlusion (occlusion after TACA application).

Experiment 1 (pre-occlusion) investigated the application on occluded and thus more hydrated skin, as it is the case after an extensive bath. Pre-occlusion did not show any effect on the TACA penetration into stratum corneum compared to normal application. Agner and Serup observed that the effect of occlusion on the stratum corneum hydration is transitory, transepidermal water loss (TEWL) returning to baseline values within 0.5-1 h after removal of the occlusive device [263]. Accordingly, in our experiment 1, larger amounts of corneocytes (skin sheets) due to the increased skin hydration and to the disruption of the stratum corneum were only observed at the first stripping time (0.5 h), when the occlusive wrap had just been removed. At later times (4 h and 24 h), the corneocyte amount removed by the tapes was homogeneous.

The disruptive effect of water on the stratum corneum structure was especially increased after about 5-10 tapes, whereas the outer and the innermost stratum corneum seemed to be less affected. Accordingly, swelling of corneocytes and formation of water pools between the cells in the middle stratum corneum could be observed microscopically by Bouwstra et al. after 24 h hydration of stratum corneum isolated from dermatomed skin [70].

Experiment 2 (post-occlusion) investigated the application followed by occlusion, which can be clinically useful to improve the effect of topical corticosteroids in severe forms of skin diseases. A “physiological” occlusion is possible as well in intertriginous skin areas, and also certain vehicles (e.g., ointments) can be occlusive. Post-occlusion enhanced the TACA retention into the stratum corneum by a factor of 2, favoring the development of a drug reservoir. The TACA amount retained by the stratum corneum remained constant between 4 h and 24 h and still amounted to 80% of the applied dose after 24 h post-occlusion. The amount of drug quantified in the gauze used to protect the treated sites until tape stripping was slightly lower after occlusion, showing that occlusion probably promoted the dissolution of drug crystals located on the skin surface and drug penetration into the skin.

The post-occlusion results obtained in our investigations agree with preliminary observations of Carr and Wieland [152]. They investigated the percutaneous penetration of ^{14}C-labeled TACA applied in 95% ethanol on a single male volunteer. After one day, 81% of the applied dose was found within the stratum corneum after occlusion vs. 38% without occlusion. Our trial performed with a larger number of volunteers and a different volatile vehicle yielded similar results. The extent of the topical bioavailability of TACA was significantly enhanced by post-occlusion, whereas the release rate from the stratum corneum was delayed because of the temporary accumulation of TACA within the hydrated stratum corneum (reservoir formation).

The standardized tape stripping technique in combination with the quantification of corneocytes by VIS-spectroscopy and the quantification of TACA by HPLC has shown to be adequate for the quantification of TACA within the stratum corneum. This technique is not influenced by artifacts due to occlusion, and does not require radiolabeling of the drug.

8.5. Conclusions

Pre-occlusion showed no effect on the topical bioavailability of TACA in stratum corneum. Contrariwise, occlusion after application enhanced the TACA penetration into stratum corneum by a factor of 2, favoring the development of a drug reservoir.

9. Project IV: Efficacy of low-dose corticosteroids in atopic dermatitis

An abbreviated version of the findings of this project has been published: Pellanda C, Weber M, Bircher A, Surber C. Low-dose triamcinolone acetonide in the phytocosmetic Lichtena® does reduce inflammation in mild to moderate atopic dermatitis. Dermatology 2005; 211(4): 338-340. The original publication is available at www.karger.com/drm.

Abstract

Background: Previously, we reported the efficacy of Lichtena® – a phytocosmetic cream product – in atopic dermatitis (AD). Later, fraudulent triamcinolone acetonide (TACA) was detected at low doses (16 to 40 μg/g) in Lichtena®. This suggested that TACA may be effective at much lower concentrations than used in commercial products (1000 μg/g).

Objectives: To investigate the efficacy in AD of low-dose TACA in Lichtena® compared to plain Lichtena®.

Methods: Fourteen patients presenting symmetrical lesions of AD were treated for one month with Lichtena® plus 25 μg/g TACA (=verum) and plain Lichtena® (=placebo). The severity of the lesions was assessed by SCORAD on day 0 (=baseline), 7, 14, and 28.

Results: Already after 1 week treatment, significant SCORAD differences to baseline were observed comparing verum and placebo treated areas. No improvement was observed using plain Lichtena®.

Conclusions: TACA displayed a significant improvement of AD at doses up to 40 times lower than in commercial products.

Keywords

Atopic dermatitis
Extemporaneous compounding
Lichtena®
Low dose corticosteroids
Phytocosmetic
SCORAD

9.1. Introduction

Atopic dermatitis is a chronic inflammatory pruritic skin disease that is often associated with elevated serum IgE levels and a personal or familial history of type I allergies, allergic rhinitis, and asthma. The relapsing course of atopic dermatitis requires both preventive and therapeutical measures. In the last years, new therapeutical approaches have been described, but topical corticosteroid therapy still remains the standard to which other treatments are compared [264, 265].

Concern and almost phobic fear about the side effect risk of corticosteroids, their chronic use, relapses after corticosteroid withdrawal, and lack of information leave more and more patients disappointed by steroid therapy [266, 267]. Alternative therapies such as phytotherapy [268, 269], traditional chinese herbal medicine [270], or complementary therapies [271] are often appreciated by patients because of their natural ingredients or the presumed absence of adverse effects. Yet, it has been repeatedly shown that some of these alternatives may also show potential adverse effects [272]. Cases are known where remedies marketed as “natural” were adulterated with non-declared corticosteroids, thus creating a more critical situation because of the unawareness of corticosteroid presence [273-279].

Previously, we have studied the apparent benefit of Lichtena®, a phytocosmetic cream marketed as a skin care product in some European countries and extensively used for its putative anti-inflammatory activity for different types of skin disorders including atopic dermatitis in children [280]. In a placebo controlled double-blind study including 21 patients (age 2 to 56 years) with symmetrical atopic eczema on the extremities, a marked reduction on the verum side was observed [281]. Later, triamcinolone acetonide (TACA), a moderately potent corticosteroid, was identified in Lichtena® at low concentrations ranging from 16 to 40 µg/g (mean 27) [282], whereas commercial TACA preparations typically contain 1000 µg/g. Meanwhile, measures have been established to control the correct composition of Lichtena®, which has been cleared of any corticosteroids (Table 9-1).

The reported efficacy of the adulterated Lichtena® cream leads to the presumption that commercially available corticosteroid formulations may be overdosed and would be effective at lower concentrations. We therefore performed a second study, described in this paper, with the aim to investigate the effect of a low-dose TACA addition in Lichtena® (verum) compared to plain, corticoid-free Lichtena® (placebo).

Table 9-1: Ingredients of Lichtena®, their function, and their allergenic potential. CA: contact allergen, PA: photo-contact allergen, IR: toxic irritative agent.

Ingredients	Function	Allergenic potential
Water	Vehicle	
Peg 8 Beeswax	Emollient, emulsifier, film former	
Caprylic-capric triglyceride	Emollient, solvent	
Isostearyl isostearate	Emollient, binding agent	
Octilmethoxycinnamate	Sunscreen agent (UVB)	PA
Buthylmethoxydibenzoilmethane	Sunscreen agent (UVA)	PA, CA
Butyrospermum parkii	Emollient	
Sorbityl furfural	Antioxidant	
Tocopherol	Antioxidant	CA
Glycerrhetinic acid	Adjuvant, moisturizer	
Bisabolol	Additive, penetration enhancer	
Chamomilla recutita	Anti-inflammatory agent, emollient	CA
Allantoin	Epithelialization and keratolysis enhancer	
Beta-sitosterol	Emulsion stabilizer	
Prunus armeniaca oil	Emollient	
Lactic acid	Moisturizer, pH-regulator	
Sodium (and) TEA lactate	Moisturizer, buffer	CA, IR
Urea	Moisturizer, penetration enhancer	
Serine	Additive	
Glycerine	Moisturizer, solvent	
Sorbitol	Moisturizer	
Laurylaminopropylglycine	Surface-active agent	
Lauryldiethylendiaminoglycine	Adjuvant	
Lecithin	Emollient, emulsifier	
Carbomer	Emulsion stabilizer, viscosity control agent	
Disodium EDTA	Preservative, viscosity control agent	
Methyl-ethyl-propyl-buthyl-paraben	Preservative	CA
Ascorbyl palmitate	Antioxidant	
Methylchloroisothiazolinone	Preservative	CA, IR
Magnesium chloride (and) nitrate	Antibacterial agent	
Methylisothiazolinone	Preservative	CA, IR
Triethanolamine	Buffer	CA, IR
Sodium dehydroacetate	Preservative	
Parfum	Additive	

9.2. Subjects and methods

9.2.1. Subjects

Patients were recruited at the Allergological and Dermatological outpatient clinic of the University Hospital of Basel, Switzerland. A total of 14 patients (9 females and 5 males) aged 3 to 41 years (median 20), who presented symmetrical lesions of subacute or chronic atopic dermatitis of the extremities were enrolled in the study. Atopic skin diathesis was diagnosed according to Hanifin and Rajka [283][19] and ranged from 7 to 23 (median 16). Exclusion criteria were allergy to Lichtena® or TACA, treatment with systemic corticosteroids, long-term treatment with topical steroids, immunosuppression, severe secondary diseases, pregnancy, or breast feeding.

9.2.2. Study design

A double-blind, vehicle-controlled, randomized explorative study with half-side comparison was performed. The study was approved by the local ethical committee and the patients signed a written consent for participation. The patients underwent a one-week run-in phase without specific therapy, and were then treated twice a day for one month at the corresponding symmetrical locations with a) Lichtena® to which 25 µg/g of TACA had been added (= verum), and b) Lichtena® without any supplement (= placebo). The creams were randomly allocated to the left or right side in a double-blinded manner. The randomization was performed at the Hospital Pharmacy, Basel, Switzerland using a random number generator, and the number sequence was concealed until the end of the study. The Lichtena® cream (placebo) was purchased from UCB Pharma, Italy. The Lichtena® cream with added TACA (verum) was prepared at the Hospital Pharmacy, Basel, Switzerland according to GMP-Guidelines. Both preparations were filled in identical tubes and blinded.

The patients were examined on day 0, 7, 14 and 28 (± 2 days) after beginning of the therapy. The severity of the lesions was assessed on each symmetrical body side separately by SCORAD [284] at each medical examination[20] and photographs of the lesions were taken. SCORAD (SCORing Atopic Dermatitis) is a score that combines A) the extent of the lesions; B) the intensity of six clinical signs: erythema, edema/papules, oozing/crusts, excoriation, lichenification, and dryness, ranging from 0 to 3 (0 = absent, 1 = mild, 2 = moderate, 3 = severe); and C) the subjective symptoms pruritus and sleep loss ranging from 0 to 10 on a visual analogue scale. The total score is defined as: SCORAD = (A/5) + (7 B/2) + C.

[19] The criteria for atopic skin diathesis assessment are displayed in the appendix (chapter 11.4.1).

[20] The form for SCORAD assessment is displayed in the appendix (chapter 11.4.2).

In addition, the patients had to record in a diary the daily use of cream (assessed as finger tip units FTU [285]), the efficacy of the creams (assessed as itching, erythema/papules, exudation), adverse effects (prickling/pricking, erythema after application, others), overall troubles (insomnia, well-being, subjective improvement of the skin), and the use of rescue medication. Rescue medication against itching consisted of cetirizine 10 mg (Zyrtec® drops or tablets). In case of aggravation of the skin lesions, a 0.25% prednicarbate cream (Prednitop® Cream) could be applied after medical consultation. To assess the allergenic potential of the cream, a patch test with the European series of contact allergens and a specific array of all Lichtena® ingredients was performed 2-4 weeks after the end of the study[21].

9.2.3. Comparison of the efficacy of the two different treatments (verum/placebo)

The assessment of the efficacy of the different treatments (verum, placebo) over time was defined as primary outcome of the study. SCORAD differences at day 7, 14, and 28 to the baseline (= day 0) were calculated for verum and placebo separately and evaluated statistically using the Wilcoxon matched pairs signed rank sum test at the 5% level of significance (2-sided analysis). In addition, a modified objective SCORAD was calculated omitting the subjective symptoms C: $SCORAD_{objective}$= (A/5) + (7 B/2), which was evaluated likewise with the Wilcoxon matched pairs signed rank sum test. Statgraphics® PLUS 5 software (Manugistic, Inc., Rockville, Maryland, USA) was used to conduct the analysis of the trial.

9.2.4. Efficacy within one treatment arm after one month therapy (verum/placebo separately)

As a secondary outcome, to assess the efficacy of the one-month treatment compared to the baseline for verum and placebo separately, the SCORAD of day 28 was compared to the SCORAD of day 0 in a Wilcoxon matched pairs signed rank sum test (5% level of significance). The same analysis was carried out with $SCORAD_{objective}$.

9.2.5. Intensity criteria and pruritus

The intensity of the six clinical signs erythema, edema/papules, oozing/crusts, excoriation, lichenification, and dryness as well as the subjective symptom pruritus were plotted in individual score-time curves. Significant influence of the one-month therapy on the different criteria was tested for verum and placebo separately in a Wilcoxon matched pairs signed rank sum test (5% level of significance) using the score pairs of day 28 / day 0.

[21] The patch-test ingredients are listed in the appendix (chapter 11.4.3).

9.3. Results

9.3.1. Demographics of the subjects

Of the 14 patients enrolled, 12 finished the study. Two drop outs (one male aged 3 years, one female aged 18) were recorded because of irregular attending the medical examinations and their data were excluded in the analysis. The medical examinations began in February and ended in July.

In addition to atopic dermatitis, 4 patients were suffering from asthma and 8 relatives were likewise atopic, suffering from atopic dermatitis, eczema, asthma, or allergic rhinitis. Clinical severity of atopic dermatitis, expressed by SCORAD, ranged from 5 to 69 on the verum side and from 12 to 77 on the placebo side during the whole study. The data are summarized in Table 9-2 [22].

Table 9-2: Summary of total SCORAD recorded during the investigation period, SCORAD differences of each treatment day to the baseline, $SCORAD_{objective}$, and $SCORAD_{objective}$-differences. To assess significant differences between the two treatments, the respective SCORAD differences to baseline were compared in a Wilcoxon matched pairs signed rank sum test at the 5% level of significance. The asterisk after the p-value denotes a statistically significant (*p < 0.05) or highly significant (p < 0.01) difference in treatment compared to the baseline.**

	Verum (Lichtena®+TACA)			Placebo (Lichtena®)				
	Mean (SD)	Median	Confidence Interval (95%)	Mean (SD)	Median	Confidence Interval (95%)	Mean Difference (SD)	P-value
SCORAD								
day 0	43.1 (12.9)	40.1	35.8 to 50.3	42.2 (12.7)	40.1	35.0 to 49.4	-0.9 (2.2)	
day 7	34.5 (13.2)	30.0	27.0 to 42.0	44.2 (11.3)	42.0	37.8 to 50.6	9.8 (12.5)	
day 14	29.8 (17.3)	25.8	20.0 to 39.6	44.1 (15.8)	41.5	35.2 to 53.0	14.3 (18.1)	
day 28	25.8 (18.5)	21.5	15.3 to 36.2	41.4 (20.5)	38.5	29.8 to 52.9	15.6 (15.0)	
SCORAD differences to baseline (day 0)								
day 0	baseline							
day 7	-8.6 (6.6)	-9.0	-12.3 to -4.8	-2.0 (11.7)	0.0	-4.6 to 8.6	10.6 (12.3)	0.04*
day 14	-13.3 (12.1)	-10.5	-20.1 to -6.5	1.9 (16.5)	-1.0	-7.4 to 11.3	15.2 (17.3)	0.02*
day 28	-17.3 (10.2)	-16.0	-23.1 to -11.5	-0.8 (19.4)	7.8	-11.8 to 10.1	16.5 (15.2)	0.01**
$SCORAD_{objective}$								
day 0	36.3 (10.9)	33.4	30.1 to 42.5	35.4 (10.8)	33.4	29.3 to 41.5	-0.9 (2.2)	
day 7	29.9 (11.3)	26.4	23.5 to 36.3	38.7 (10.7)	36.8	32.6 to 44.7	8.8 (11.1)	
day 14	24.9 (13.6)	23.8	17.2 to 32.7	37.2 (12.7)	35.1	30.0 to 44.4	12.3 (14.9)	
day 28	21.4 (15.6)	18.5	12.6 to 30.3	35.4 (17.9)	36.0	25.3 to 45.6	14.0 (13.1)	
$SCORAD_{objective}$-differences to baseline (day 0)								
day 0	baseline							
day 7	-6.4 (3.9)	-7.0	-8.6 to -4.2	3.2 (9.4)	5.3	-2.1 to 8.5	9.6 (10.9)	0.04*
day 14	-11.4 (9.3)	-10.5	-16.7 to -6.1	1.8 (12.7)	0.0	-5.4 to 8.9	13.1 (14.1)	0.02*
day 28	-14.9 (8.6)	-12.3	-19.7 to -10.0	0.0 (15.9)	5.3	-9.0 to 9.0	14.9 (13.2)	0.008**

[22] The data sheets of the single volunteers are displayed in the appendix (chapter 11.4.4).

9.3.2. Comparison of the efficacy of the two different treatments (verum/placebo)

SCORAD at day 0 (baseline) was comparable and ranging from 25 to 69 for the two treatment arms (verum, placebo). After beginning the therapy, the SCORAD values of the verum-treated side constantly decreased (median SCORAD at day 0 / 7 / 14 / 28 displaying values of 40 / 30 / 26 / 22 respectively). Contrariwise, the SCORAD values on the placebo-treated side persisted near the baseline and were slightly increased at the end of the therapy (median SCORAD ranging from 39 to 42 during the whole month). Statistical analyses of SCORAD differences to baseline resulted in a significant difference between the side treated with Lichtena® plus TACA (verum) compared to plain Lichtena® (placebo) already after 1 week treatment (median difference of 9, $p = 0.04$). This significant difference persisted at day 14 (median 11, $p = 0.02$) and became highly significant at the end of the therapy (median 16 at day 28, $p = 0.01$) (Fig. 9-1, left diagram).

By omitting the criterion "subjective symptoms" in the calculation of SCORAD (= $SCORAD_{objective}$), it is possible to evaluate the mere objective criteria. The evaluation of $SCORAD_{objective}$ yielded similar results: the difference between placebo and verum treatment became statistically different at day 7 ($p = 0.04$), stayed significant at day 14 ($p = 0.02$), and became highly significant at day 28 ($p < 0.01$). An additional slight impairment of the lesions was visible after one week on the placebo treated side (Fig. 9-1, right diagram).

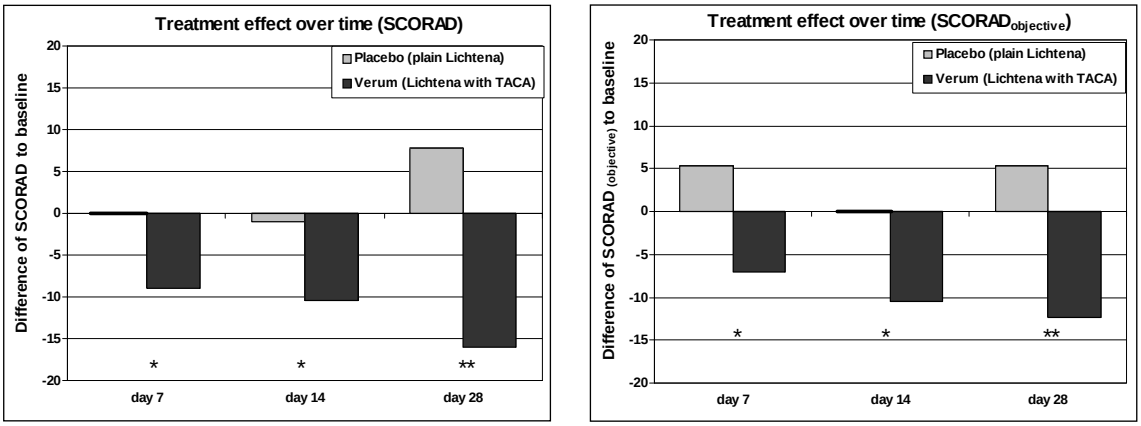


Fig. 9-1: Median SCORAD differences to baseline (day 0, beginning of therapy) after treatment during 7, 14 and 28 days with placebo (plain Lichtena®) and verum (Lichtena® with TACA) (diagram on the left) and, analogously, median $SCORAD_{objective}$-difference to baseline (diagram on the right). The verum-treated side shows an improvement of the lesion, whereas the placebo-treated side displays an impairment after one month therapy. The comparison of the two treatments (verum/placebo) shows a statistically significant difference on day 7 ($p = 0.04$*), day 14 ($p = 0.02$*), and a highly significant difference at the end of the therapy on day 28 ($p \leq 0.01$) for both SCORAD and $SCORAD_{objective}$.**

9.3.3. Efficacy within one treatment arm after one month therapy (verum/placebo separately)

After beginning the therapy, the SCORAD values of the verum-treated side decreased significantly ($p < 0.01$, day 28 vs. day 0). Median SCORAD at day 0, 7, 14, and 28 displayed values of 40, 30, 26, and 22, respectively (Fig. 9-2, left diagram). Contrariwise, the SCORAD values on the placebo-treated side persisted near baseline until the end of the therapy (median SCORAD ranging from 39 to 42 during the whole treatment period, $p > 0.05$). Similar results were recorded with $SCORAD_{objective}$-values (Fig. 9-2, right diagram).

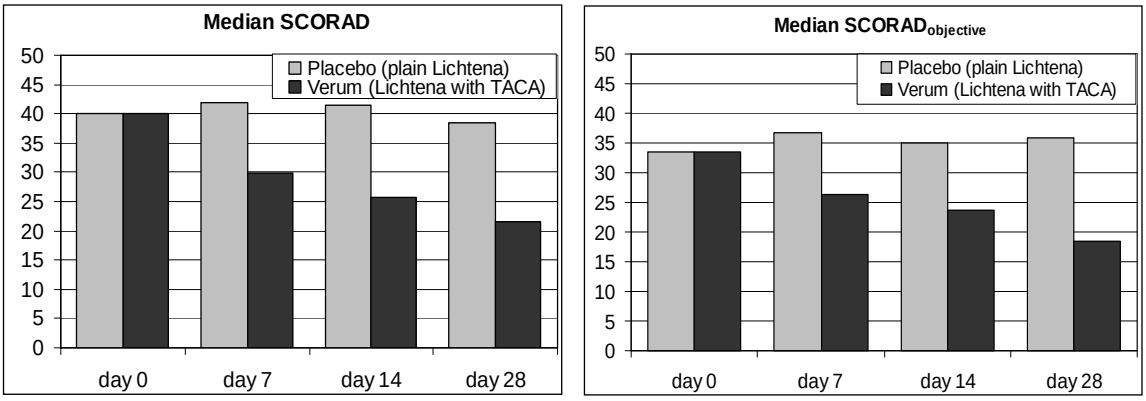


Fig. 9-2: Median SCORAD (left diagram) and median $SCORAD_{objective}$ (right diagram) during the one-month §treatment with placebo (plain Lichtena®) and verum (Lichtena® with TACA).

9.3.4. Intensity criteria and pruritus

The time response of the individual intensity factors erythema, edema/papules, oozing/crusts, excoriation, lichenification, and dryness as well as the subjective symptom pruritus are depicted in Fig. 9-3 and Fig. 9-4. The intensity factors scores range from 0 to 3 (0 = absent; 1 = mild; 2 = moderate; 3 = severe), and the pruritus score ranges from 0 to 10 (0 = absent; 10 = severe).

The analysis of the intensity factors separately showed as well a superiority of the verum treatment. On the verum-treated side, the one-month therapy resulted in a significant decrease of the score for the following criteria: erythema ($p = 0.02$), lichenification ($p = 0.02$), and dryness ($p < 0.01$), when day 28 was compared to day 0. The severity of the criterion edema/papules did not show any statistically significant decrease ($p > 0.1$), and the decrease in the severity of excoriation just showed a non-significant trend ($p = 0.06$). The criterion oozing/crusts was not present in our patients. No improvement was recorded on the placebo-treated side ($p > 0.9$). The criteria erythema and edema/papules even showed a temporary impairment during the therapy.

Similarly, a highly significant decrease of the pruritus intensity was recorded on the verum-treated side ($p = 0.008$), whereas no difference was reported on the placebo side ($p > 0.1$). The score showed an initial median value of 6 at day 0 and a lower median value of 1 (verum) and 4.5 (placebo) at day 28.

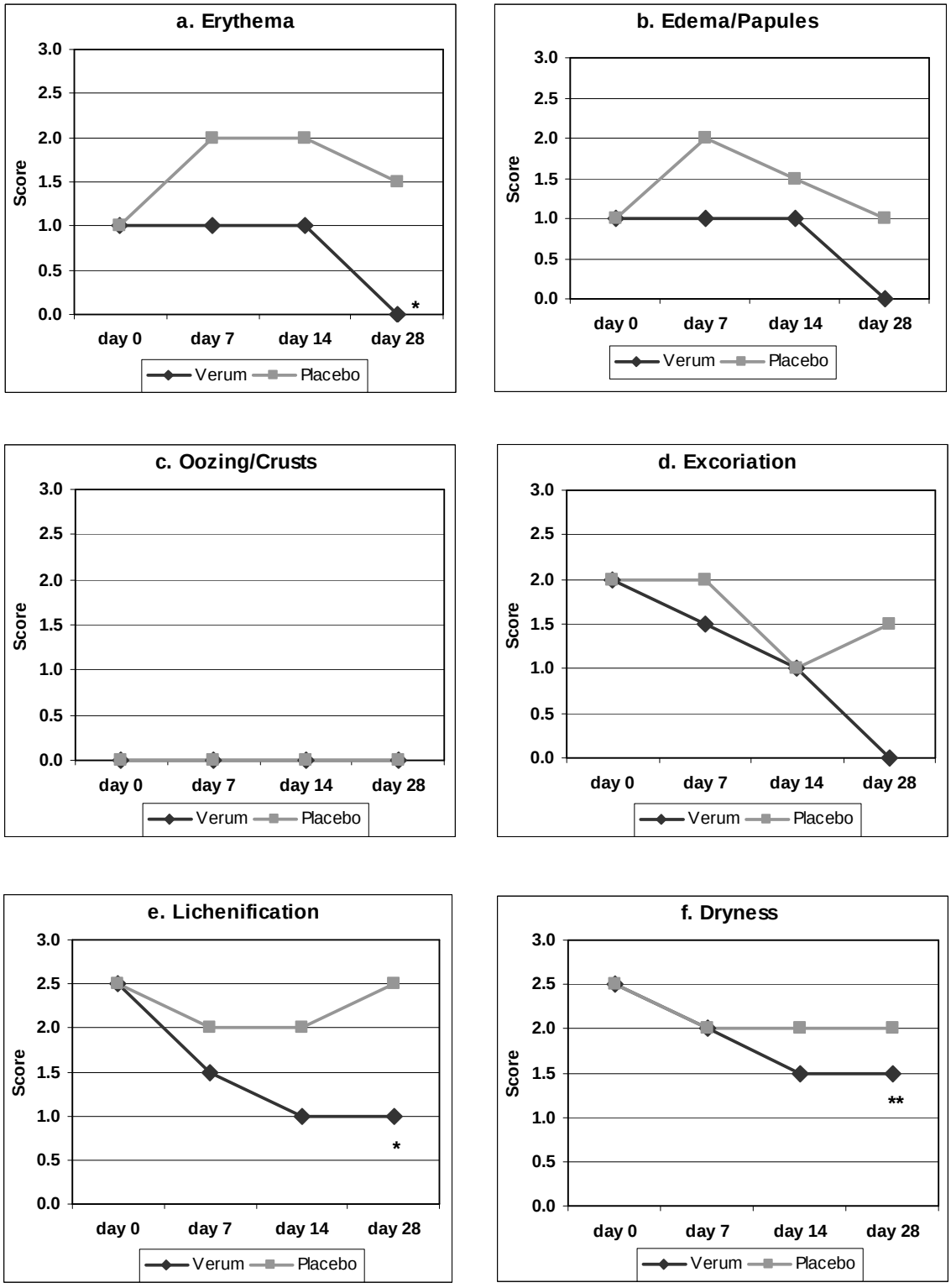


Fig. 9-3 : Progression of the intensity criteria a) erythema, b) edema/papules, c) oozing/crusts, d) excoriation, e) lichenification, f) dryness during the one-month therapy with Lichtena®+TACA (verum, black lines) and plain Lichtena® (placebo, grey lines). The scores range from 0 to 3 (0=absent; 1=mild; 2=moderate; 3=severe) and the median values are depicted. The asterisks near the curves on the verum side denote a statistically significant ($p < 0.05$*) respectively highly significant ($p < 0.01$) decrease of the intensity score between day 28 and day 0.**

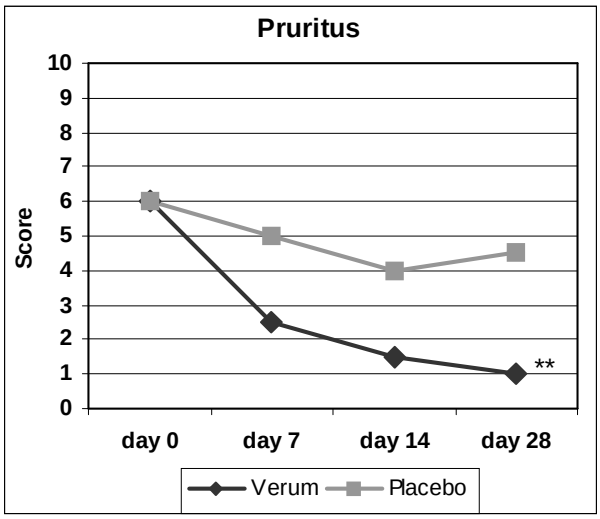


Fig. 9-4 : Progression of the criterion pruritus during the one-month therapy with Lichtena®+TACA (verum, black lines) and plain Lichtena® (placebo, grey lines). The scores range from 0 to 10 (0=absent; 10=severe) and the median values are depicted. The asterisks denote a statistically highly significant (p = 0.008) decrease of the pruritus score on the verum side between day 28 and day 0.**

9.3.5. Rescue medication

The use of additional topical corticosteroids (prednicarbate) in case of aggravation of the skin lesions was low: three patients applied prednicarbate 2-3 times and one patient 7 times during the whole month on both extremities. The use of cetirizine in case of itching was higher: two patients took 1 tablet once a day during the whole month, seven patients took it occasionally (a total of 3-15 tablets over the whole month), and three patients did not use cetirizine at all.

9.3.6. Adverse effects and subjective evaluation

Both formulations were well tolerated and no adverse effects were recorded. The qualities of quick skin penetration, good consistency, and smoothness of the creams were reported by the patients. Slight burning after application was recorded by 3 patients (for both placebo and verum). The duration of hydration and lipidization of the lesions was reported to be 1-8 h (mean 6 h). The amount of cream used was expressed as finger tip unit (FTU). The same amount of cream was applied on each side (1-2 FTU, depending on the extension of the lesions).

9.3.7. Patch test

Of the 12 patients, 10 accomplished the patch test. A total of 4 patients did not show any positive tests. Within the potential allergen ingredients of Lichtena®, the following appeared positive: Fudji perfume (2 patients), EDTA (1 patient) and Apifil (1 patient). The following standard allergens also gave positive reactions: Nickel(II)sulfate, Lyral, Thiuram-Mix (1 case each) and Sorbitan sesquioleate, Iodopropinylbutylcarbamate (2 cases each).

9.4. Discussion

Plain Lichtena® had no significant influence on the severity of atopic dermatitis measured by SCORAD. Contrariwise, the addition of TACA in a concentration which is 40 times lower than typical therapeutical corticosteroid concentrations (25 µg/g vs. 1000 µg/g) reduced the severity of the lesions.

Particularly, the signs indicating subacute or chronic inflammation displayed a marked improvement. These included erythema, lichenification, and dryness (Fig. 9-3a, e, f). Criteria representing acute lesions such as oozing and crusts were not present in our patients (Fig. 9-3c). While the decrease of the factors edema/papules and excoriation was not significant, complete remission indicated by a zero score at day 28 was recorded for their median score (Fig. 9-3b, d) as well as for the factor erythema (verum-treated side). For the factor edema/papules this may indicate that the initial score was already quite low (median score at day 0 = 1 = mild disorders), the following reduction being not large enough to result in a statistically significant decrease. Subjective symptoms like pruritus were as well successfully improved with the verum treatment (Fig. 9-4), thus contributing to an increased well-being of the patients.

The use of rescue medication as a marker of unsuccessful therapy was quite low, especially the use of the additional topical corticosteroid prednicarbate. The consequent intake of cetirizine once a day by two patients indicates that its relative high use may be due to additional troubles like allergic rhinitis, and not to pruritus alone.

The additional use of emollients and skin care products is considered to be standard therapy for the treatment of atopic dermatitis, but only few studies have tried to assess the effects of emollients alone on the severity of atopic dermatitis [286-288]. Interestingly, even the score “dryness” does not show a significant reduction after treatment with plain Lichtena®. During an acute episode, the simple use of an emollient does not seem to be sufficient to treat the lesions. A more aggressive therapy is required, emollients playing a decisive role later on in the skin care, particularly to prevent relapses.

The presence of several allergens in Lichtena® represents an additional exposure of the already affected skin to unnecessary irritations. In the patch test, two patients showed a positive reaction to Fudge perfume, EDTA, and Apifil, all ingredients of Lichtena®.

Our trial confirms that Lichtena® alone has no anti-inflammatory activity after topical application on atopic dermatitis lesions. However, the addition of TACA in a concentration which is 40 times lower than typical therapeutic corticosteroid concentrations reduces inflammation in mild to moderate eczema. These findings would justify the use of much lower corticosteroid concentrations than typically marketed and used today, particularly in subacute and chronic eczema and in children. This is sometimes overcome by dispensing brand corticosteroids which are “diluted” by the addition of vehicle or other agents. This extemporaneous dilution of topical corticosteroid formulations is intended to reduce the risk of adverse effects while maintaining

efficacy. However, improper dilution may compromise several factors such as stability (chemical and microbiological), rheology, and bioavailability, thus diminishing or even completely deactivating the therapeutical activity of the formulation (compare Table 9-3) [289-292].

Table 9-3: Consequences and risks due to improper dilution of drug formulations.

Factors that are affected by dilution of drug formulations	
Bioavailability	Concentration, state of solution/dispersion of the drug in the vehicle, diffusion coefficient, and partition coefficient all influence the optimum release of a drug out of the formulation. Change in any of these factors may lead to an ineffective formulation.
Chemical stability	Change of pH, as well as dilution of antioxidants and preservatives may lead to chemical instability and enhance chemical degradation.
Toxicology and safety	Drug degradation can lead to toxic degradation products.
Interactions	Drug-drug interactions but also drug-adjuvant interactions lead to chemical instability and can affect the efficacy of the formulation (both increase and decrease of the effect possible).
Microbiological quality	Dilution of preservatives below the effective concentration can lead to microbiological contamination, which leads to chemical degradation and iatrogenic contamination of the treated skin sides.
Rheology	Change of the composition of a formulation changes the rheology of the product and its cosmetic elegance.
Economic aspects	Extemporaneous compounding is generally expensive and leads to a higher price of the therapy.

Yet, the popularity of diluted corticosteroid formulations reveals the desire and the need for properly manufactured corticosteroid formulations to be available in much lower concentrations for some clinical situations, particularly for chronic skin disorders such as atopic dermatitis. Our trial shows that topical preparations of corticosteroids are possibly overdosed to achieve the desired therapeutic effect. Therefore, dose-finding studies should be an integral and important part of the clinical evaluation of topical therapeutics.

9.5. Conclusions

This study showed no anti-inflammatory properties of Lichtena®, a phytocosmetic cream marketed as a skin care product and extensively used for its putative anti-inflammatory activity for different types of skin disorders including atopic dermatitis in children. However, the addition of a low dose (40 times lower than in typical therapeutical concentrations) of the corticosteroid TACA to Lichtena® induced an improvement of the eczematous lesions.

These findings indicate that some corticosteroids may already be effective at much lower concentrations than usually used therapeutically, and that marketed formulations may contain a much higher concentration of corticosteroids than necessary.

10. Final conclusions and perspectives

The present thesis shows that the investigation of the topical bioavailability of TACA as model corticosteroid can successfully be performed using the DPK approach. The basic principle is the quantification of drug amounts penetrated into the stratum corneum over time. The analysis of the stratum corneum usually requires its removal, and tape stripping is a useful technique for this. The layer by layer removal and analysis of the stratum corneum enables the visualization of the penetration pattern of the topically applied drug. Even though the target site may not always be the stratum corneum, the drug must pass through the stratum corneum barrier to reach deeper sites of action. Moreover, the removal of this layer does not induce permanent skin damage.

The combination of following techniques was applied in this thesis for the investigation of topical bioavailability: 1) tape stripping for stratum corneum sampling, 2) UV/VIS-spectroscopy for quantification of corneocytes, and 3) HPLC for quantification of TACA as model corticosteroid. Since tape stripping is susceptible to numerous confounding factors, the technique requires an accurately standardized protocol, which was developed in **Project I**. The tape stripping protocol included a) the use of a template to ensure the removal of stratum corneum samples from the same skin site, b) the use of a hand roller to ensure a constant pressure on the tape before stripping, c) the removal of each tape with a rapid firm movement, d) the alternation of the tape removal (elbow-to-wrist, wrist-to-elbow) to ensure a homogenous removal and to minimize skin irritation, e) the removal of the entire stratum corneum of one skin site to cope with the inter- and intra-individual differences of stratum corneum thickness. The UV/VIS-spectroscopical method for quantification of the corneocytes had been validated by Weigmann et al. [245] and was adopted. The HPLC method for TACA quantification had to be developed and validated. The analytical challenge consisted in the development of a sensitive analytical method, capable to quantify low amounts of TACA distributed on single tapes. Therefore, a low limit of quantification (LOQ) was aimed at. The HPLC method was successfully validated and proved to have suitable specificity, linearity, accuracy, precision, and robustness in the working range. The LOQ of 0.1 µg/ml enables the quantification of 27 ng/cm^2 TACA on single tapes. The proof of concept proved that the set of method is suitable to distinguish between the different penetration pattern of TACA applied in different formulations (acetonic solution, ethanolic gel). Whereas the TACA penetration from the acetonic solution was high and TACA reached deeper layers of the stratum corneum, the TACA penetration from the ethanolic gel was only superficial, most TACA being retained within the cellulose-matrix of the gel after evaporation of the solvent. After the proof of concept, the method set was ready to be applied for the investigation of different factors determining the pharmacotherapy with topical corticosteroids.

Despite being a relative old drug class, topical corticosteroids are still the gold standard for the treatment of several dermatological affections, e.g. atopic dermatitis. A sufficient dose of steroid has to be provided in a suitable vehicle with an optimum application frequency to ensure an

adequate penetration into the affected skin areas and to finally ensure efficacy. Moreover, the accumulation of topically applied drug as a skin reservoir can considerably affect the therapy pattern in term of dose and application frequency. Particularly in case of corticosteroids, the formation of a stratum corneum reservoir and a slow drug release to deeper skin tissues over a longer time period is advantageous and desired to prevent systemic side effects.

To ensure an optimized pharmacotherapy, both pharmacokinetic and pharmacodynamic investigations are required and were performed in this thesis. **Project II** describes a pharmacokinetic clinical trial investigating the influence of dose and application frequency on the percutaneous penetration of TACA applied in an acetonic vehicle to 15 healthy human volunteers. In the dose experiment, higher TACA amounts were quantified within the stratum corneum after application of the high dose (300 $\mu g/cm^2$ vs. 100 $\mu g/cm^2$), as intuitively expected. However, this difference was only significant immediately after application. A high TACA amount crystallized on the skin surface after evaporation of the vehicle. The TACA crystals were mainly quantified on tapes 1-3, and are not supposed to be bioavailable unless additional solvent is applied or unless the hydration status of the skin is raised, e.g. by occlusion. The application frequency experiment showed slightly higher TACA amounts within the stratum corneum after multiple application (3x100 $\mu g/cm^2$) compared to the application of the total TACA dose at once (1x300 $\mu g/cm^2$). As a result of multiple applications, the skin was periodically reloaded with new drug, thus achieving temporary higher amounts within the stratum corneum, and re-solving eventual TACA crystals on the skin surface. The highest drug amount was always localized in the upper stratum corneum layers, and by excluding tapes 1-3, only minor differences between the TACA amounts after the different application modes were observed in both experiments. The performance of a mass balance showed that a high TACA dose may promote a faster permeation through the stratum corneum and thus a possibly higher systemic exposure, unwelcome in topical therapy. Thus, a low dose applied once daily may be preferable to a higher dose. These findings are in agreement with pharmacodynamic investigations, which have shown that usually multiple-daily applications have no superiority to a once-daily regimen.

The efficacy of low-dose TACA in the treatment of atopic dermatitis was proved by **Project IV**. In a pharmacodynamic clinical trial with 14 patients suffering from atopic dermatitis, the addition of low-dose TACA to a marketed skin care cream formulation (Lichtena®), applied twice-daily, induced a significant improvement of the eczematous lesions, whereas the application of the cream base alone did not. These findings indicate that some corticosteroids may already be effective at much lower concentrations than usually used therapeutically, and that marketed corticosteroid formulations may contain a much higher concentration than necessary.

A further factor enhancing the percutaneous penetration of topically applied compounds is occlusion, which increases skin hydration and temperature. We can distinguish between: 1) application on occluded skin (pre-occlusion), as it can be the case if a topical formulation is applied on thoroughly hydrated skin (e.g. after a bath), and 2) occlusion after application (post-occlusion), as is sometimes performed in clinical practice to improve the effect of topical

corticosteroids in severe forms of skin diseases. The effects of pre- and post-occlusion were investigated in a pharmacokinetic clinical trial with 10 healthy volunteers, described in **Project III**. Pre-occlusion did not show any effect on the percutaneous penetration of TACA into stratum corneum over time. On the contrary, post-occlusion showed a clear enhancement of the TACA penetration and an accumulation of a double TACA amount within the stratum corneum. The choice of TACA as relative lipophilic compound (logP 2.3) and acetone as volatile vehicle was advantageous for the investigation of the effect of occlusion, since the occlusion-induced penetration enhancement and reservoir formation appear to be compound and vehicle dependent. A higher effect is observed with lipophilic compounds and volatile vehicles.

During the pharmacokinetic investigations performed in this thesis, different types of reservoir could be discerned. Post-occlusion (Project III) induced a long-term TACA accumulation in the stratum corneum ("classical" reservoir). The TACA diffusion into deeper tissues was inhibited at least for 24 h. After application without occlusion (Project II), independently of dose and application frequency, considerable TACA amounts were still measured within the stratum corneum. Yet, these high TACA amounts were rather due to the slow diffusion through the stratum corneum barrier than to a "classical" reservoir formation. This was underlined by the slow but existent diffusion of TACA into deeper tissues. In Project I, not a stratum corneum reservoir, but a skin surface reservoir was induced after application of the gel formulation.

The DPK methodology described in this thesis can be applied for the bioavailability investigation of other compounds as well, provided that a specific analytical method for their quantification is developed and validated. Determinant for successful investigations using tape stripping is the awareness that a fixed number of tapes does never remove the same amount of stratum corneum. Endogenous (inter- and intra-individual variations) and exogenous factors (e.g., vehicle effects, occlusion) can change the stratum corneum amount removed by a single tape, as was demonstrated in Project III. The overhydration of the skin after occlusion induced a reduced cohesion of the corneocytes, and only half the number of tapes was required to remove the entire stratum corneum compared to non-occluded sites. The removal of a fixed tape number (e.g., 20 tapes) would have led to incorrect results. The most correct approach is without doubt the removal of the entire stratum corneum, as was performed in this thesis. Yet, the removal of up to 80 tapes for one test site is correlated to the time-consuming analytical analysis of each tape. In a recent investigation, Jacobi et al. developed a promising mathematical model for the prediction of the stratum corneum amount removed without the need of its complete removal. They observed that the pseudo-absorption, measured to quantify the corneocytes, correlates linearly to the cell layers removed, and that a pseudo-absorption of 0.25 reflects one single corneocyte layer [244].

The draft guidance "Topical dermatological drug products NDAs and ANDAs – *In vivo* bioavailability, bioequivalence, *in vitro* release, and associated studies" released in 1998 by the FDA already proposed tape stripping as DPK approach for the assessment of bioavailability and bioequivalence of topically applied compounds. The withdrawal of the draft guidance in 2002

showed that not the technique itself, but the proposed protocol (e.g., removal of a fixed number of 12 tapes, discarding of the first 2 tapes) was not yet mature for bioavailability and bioequivalence testing. The investigations described in this thesis show how tape stripping, correctly performed, asserts itself as a valuable technique for bioavailability assessment. Additional bioequivalence investigations with the proposed set of methods could lead to the reconsideration of tape stripping also on a regulatory level.

11. Appendix

11.1. Project I 113

11.1.1. Validation of the HPLC method 113

11.1.2. Proof of concept 116

11.2. Project II 124

11.2.1. Pre-Examination Form 124

11.2.2. Post-Examination Form 125

11.2.3. Experiment 1.1 (Dose) - Penetration profiles 126

11.2.4. Experiment 1.1 (Dose) - Data overview 131

11.2.5. Experiment 1.1 (Dose) - Data single volunteers 132

11.2.6. Experiment 1.2 (Application frequency) - Penetration profiles 133

11.2.7. Experiment 1.2 (Application frequency) - Data overview 143

11.2.8. Experiment 1.2 (Application frequency)- Data single volunteers 144

11.3. Project III 145

11.3.1. Overview of application and tape stripping procedure 145

11.3.2. Experiment 2.1 (Pre-occlusion) - Penetration profiles 146

11.3.3. Experiment 2.1 (Pre-occlusion) - Data overview 151

11.3.4. Experiment 2.1 (Pre-occlusion) - Data single volunteers 152

11.3.5. Experiment 2.2 (Post-occlusion) - Penetration profiles 153

11.3.6. Experiment 2.2 (Post-occlusion) - Data overview 156

11.3.7. Experiment 2.2 (Post-occlusion) - Data single volunteers 157

11.3.8. Mass balance experiment 2.1 (pre-occlusion) 158

11.3.9. Mass balance experiment 2.2 (post-occlusion) 158

11.4. Project IV 159

11.4.1. Atopic skin diathesis according to Hanifin and Rajka 159

11.4.2. Form for SCORAD assessment 160

11.4.3. Patch test 161

11.4.4. Data individual patients 163

11.1. Project I

11.1.1. Validation of the HPLC method

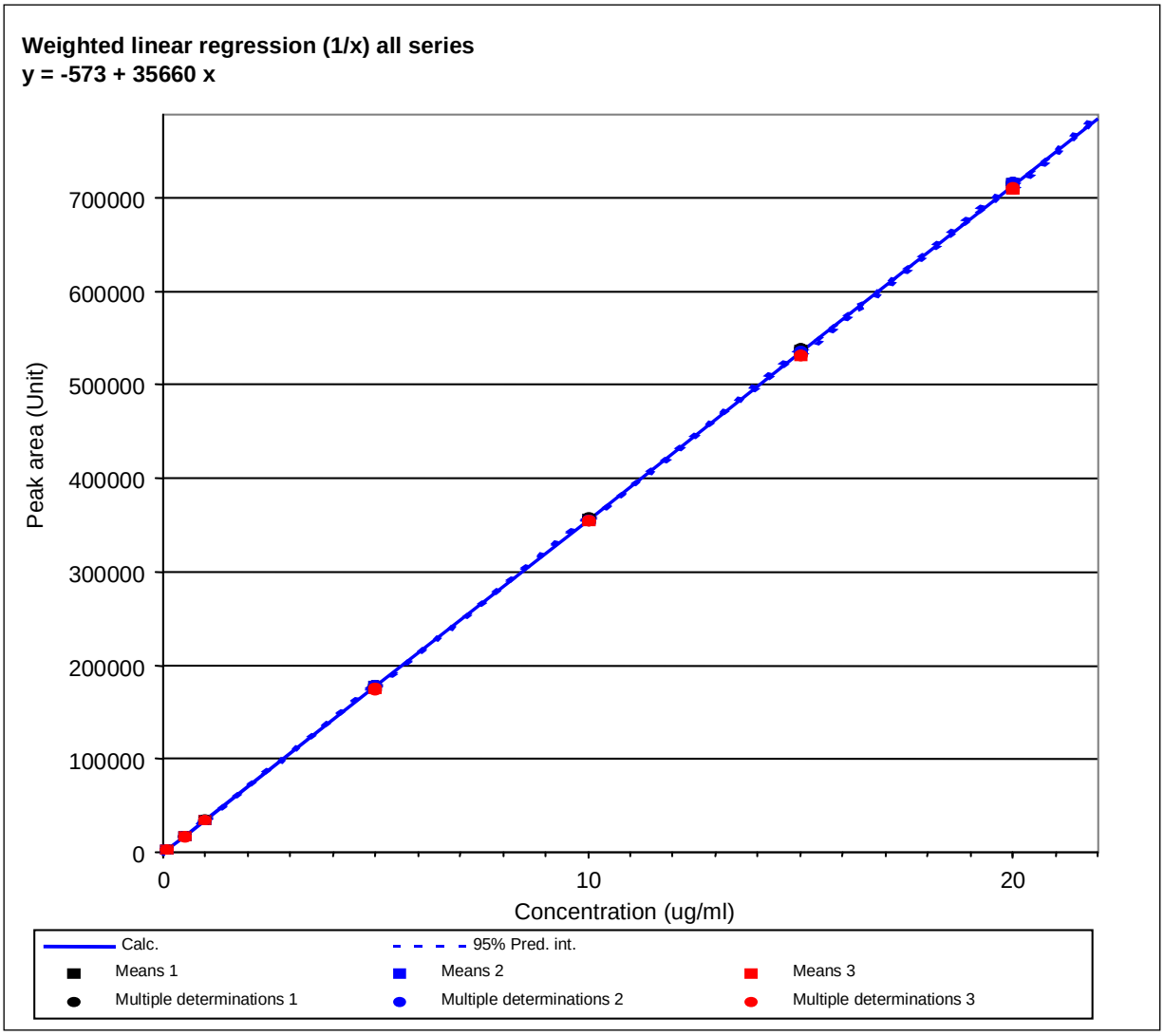


Fig. 11-1: Linearity assessment - Calibration curves (n=3) for the assessment of linearity; calculation with the weighted linear regression model (weighting factor 1/x, x = concentration)

Table 11-1: Accuracy assessement - Recovery data from tape spiking experiments for accuracy assessment.

Tape Number	Conc. level in calibration curve [µg/ml]	Amount on tape [ug] theoretical	Amount on tape [ug] measured	Difference [µg]	Recovery [%]
1	15	22.5	21.8	0.7	96.7
2*	15	22.5	22.4	0.1	99.4
3	15	22.5	21.9	0.6	97.3
4	15	22.5	21.1	1.4	93.8
5	15	22.5	21.7	0.8	96.4
6	15	22.5	21.5	1.0	95.4
mean		**22.5**	**21.7**	**0.8**	**96.5**
SD		**0.0**	**0.4**	**0.4**	**1.9**
7	5	7.5	7.2	0.3	96.0
8	5	7.5	7.0	0.5	93.2
9	5	7.5	7.1	0.4	95.0
10	5	7.5	7.1	0.4	95.0
11	5	7.5	6.8	0.7	91.0
12	5	7.5	7.0	0.5	92.8
mean		**7.5**	**7.0**	**0.5**	**93.8**
SD		**0.0**	**0.1**	**0.1**	**1.8**
13	0.5	0.8	0.7	0.1	88.0
14	0.5	0.8	0.7	0.1	90.0
15	0.5	0.8	0.7	0.1	92.0
16	0.5	0.8	0.7	0.1	88.0
17	0.5	0.8	0.6	0.1	82.0
18	0.5	0.8	0.6	0.1	82.0
mean		**0.8**	**0.7**	**0.1**	**87.0**
SD		**0.0**	**0.0**	**0.0**	**4.1**

* tape number 2 accidentally extracted with 1.8 ml instead of 1.5 ml

Table 11-2: Precision assessment - Peak areas of the concentration levels for 3 different calibration curves measured on 3 different days.

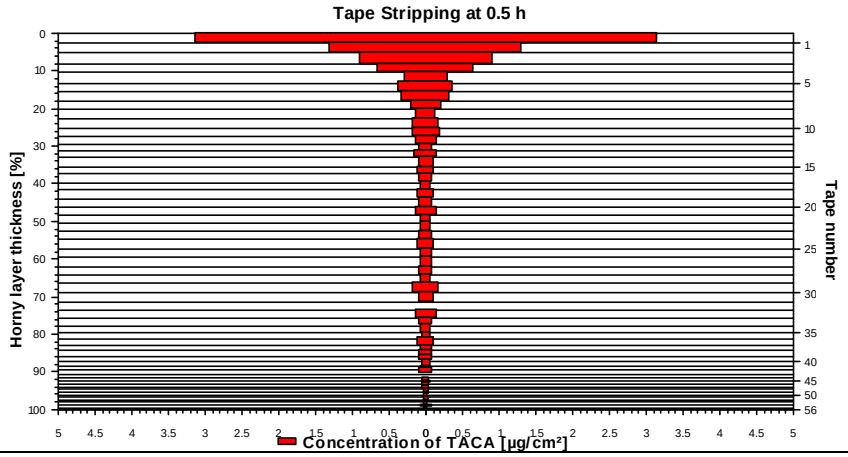
		0.1ug/ml	0.5ug/ml	1.0ug/ml	5.0ug/ml	10.0ug/ml	15.0ug/ml	20.0ug/ml
Day 1		3191	17337	34953	178630	358858	538634	716212
		3551	17152	35054	178168	356271	537021	715817
		2803	16969	35377	178987	357337	538516	715396
		2928	17280	34722	178248	355734	537485	714713
		2917	17904	34170	178472	356659	536756	716860
	mean	**3078**	**17328**	**34855**	**178501**	**356972**	**537682**	**715799**
	SD	**300**	**351**	**450**	**327**	**1205**	**857**	**812**
	rel. SD	**9.7**	**2.0**	**1.3**	**0.2**	**0.3**	**0.2**	**0.1**
Day 2		3025	17406	34514	178723	355394	534945	717020
		3170	17181	34924	178351	355859	535427	716478
		3049	16937	34938	178353	355296	536596	713874
		3328	16907	34613	179071	355745	535369	714757
		3231	16999	34858	177120	353528	535794	715182
	mean	**3160**	**17086**	**34769**	**178324**	**355164**	**535626**	**715462**
	SD	**126**	**208**	**194**	**736**	**944**	**620**	**1280**
	rel. SD	**4.0**	**1.2**	**0.6**	**0.4**	**0.3**	**0.1**	**0.2**
Day 3		2789	16727	34227	174586	354848	531455	707959
		2928	16840	34367	174166	354170	531061	709551
		3021	16932	34620	175806	354691	530039	708458
		3077	16806	34296	175104	355591	531719	708906
		3176	16487	34591	174599	354036	532045	710748
	mean	**2998**	**16758**	**34420**	**174852**	**354667**	**531264**	**709124**
	SD	**148**	**169**	**177**	**628**	**619**	**774**	**1080**
	rel. SD	**4.9**	**1.0**	**0.5**	**0.4**	**0.2**	**0.1**	**0.2**
	Overall mean	**3079**	**17057**	**34681**	**177226**	**355601**	**534857**	**713462**
	Overall SD	**203**	**338**	**339**	**1822**	**1353**	**2857**	**3330**
	Relative SD [%]	**6.6**	**2.0**	**1.0**	**1.0**	**0.4**	**0.5**	**0.5**

11.1.2. Proof of concept

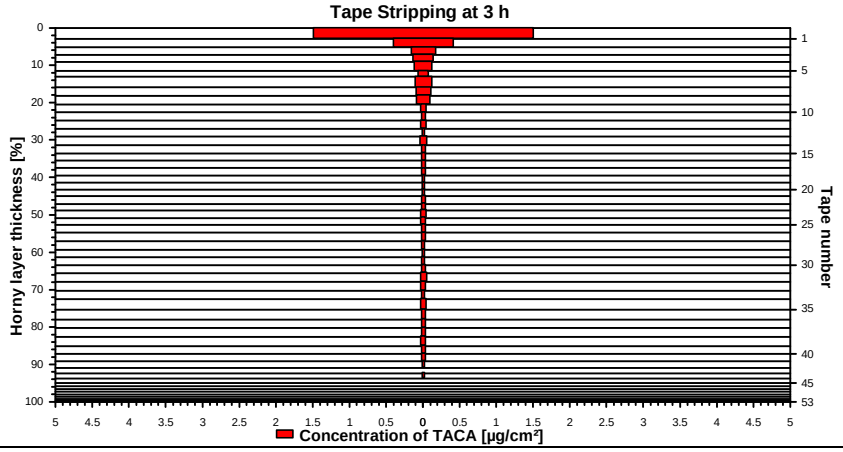
Qualitative TACA penetration into stratum corneum: penetration profiles

Volunteer 1: Acetonic solution

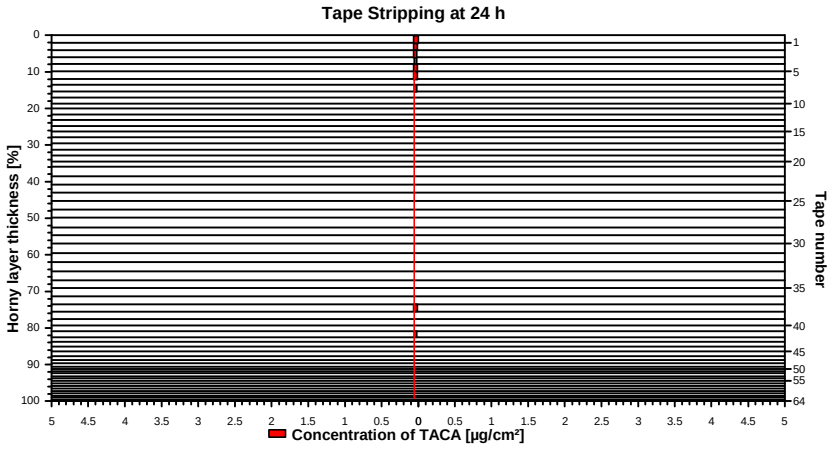
0.5 h



3 h

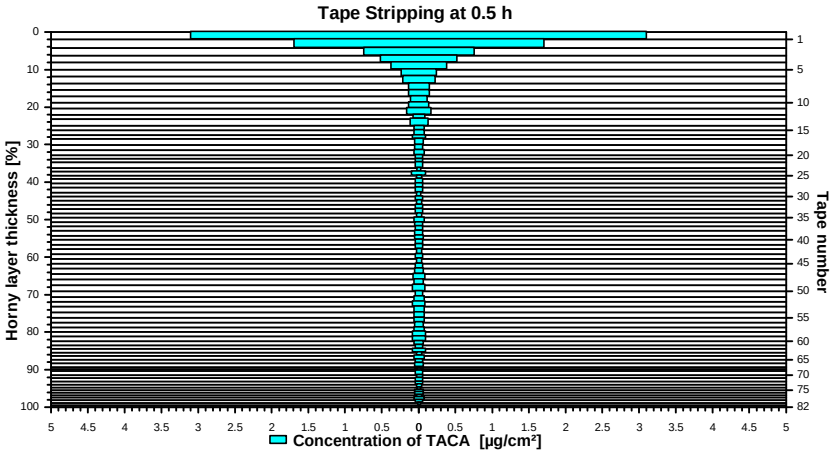


24 h

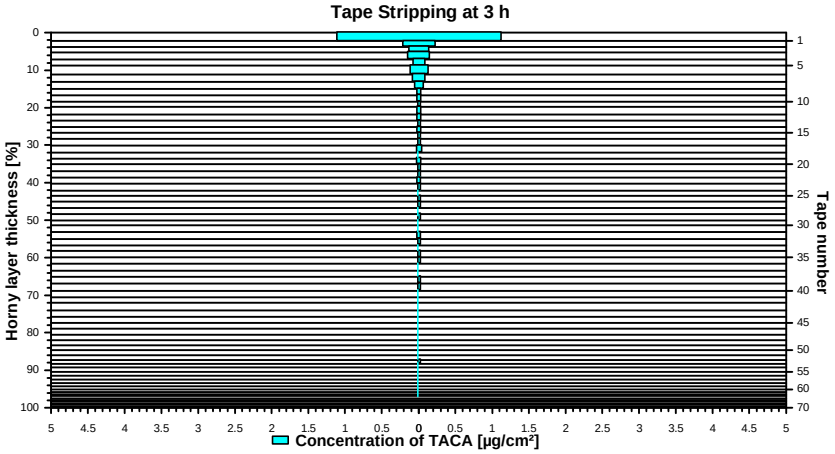


Volunteer 2: Acetonic solution

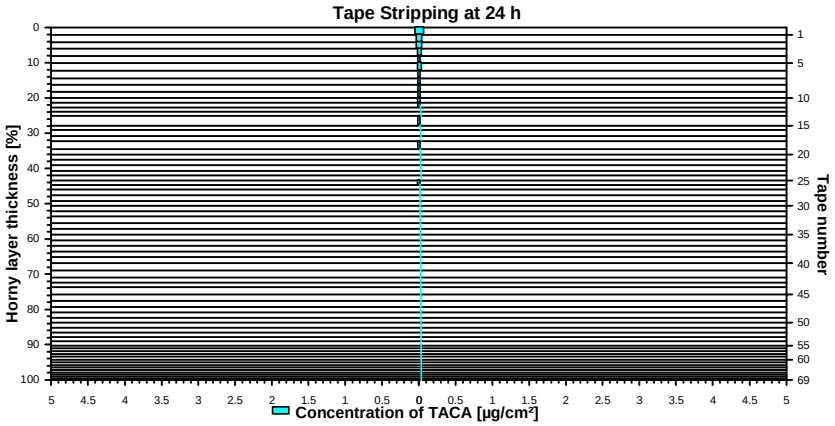
0.5 h



3 h

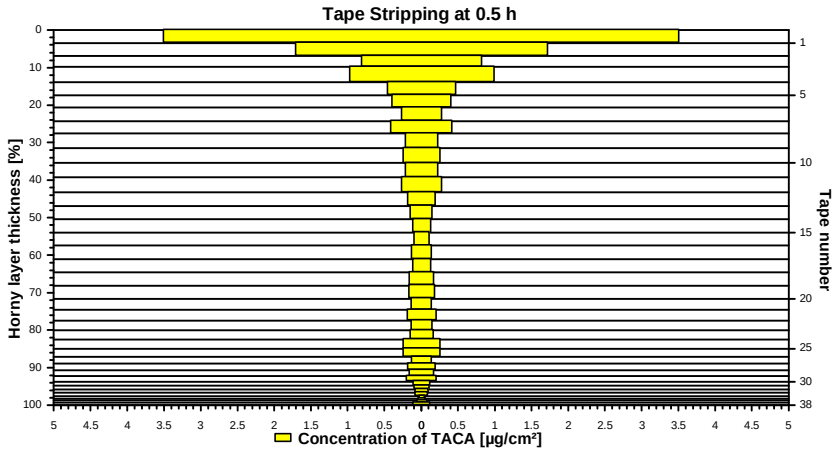


24 h

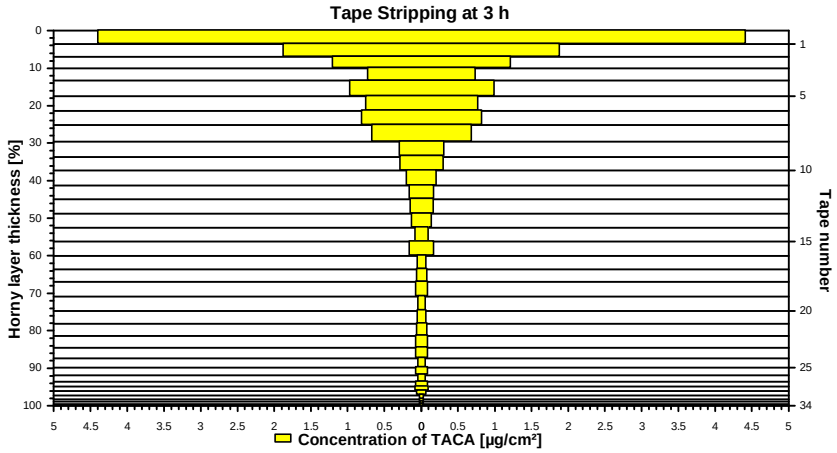


Volunteer 3: Acetonic solution

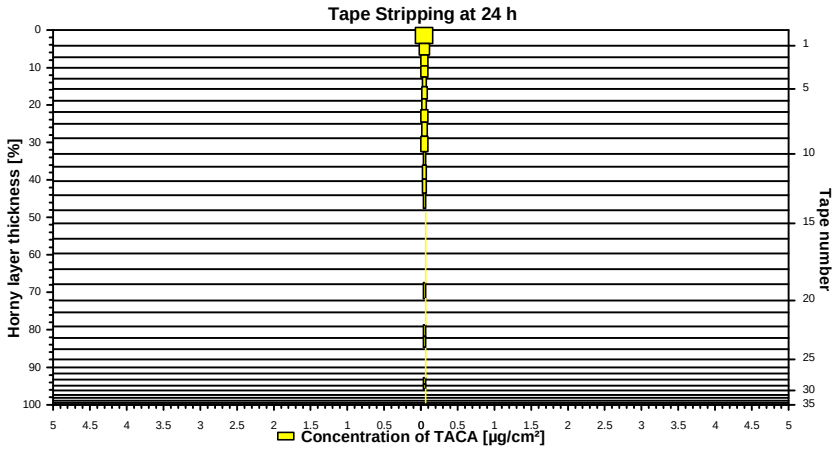
0.5 h



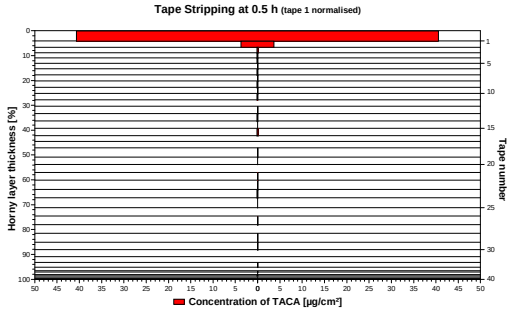
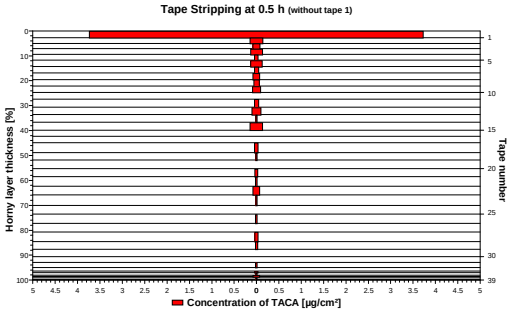
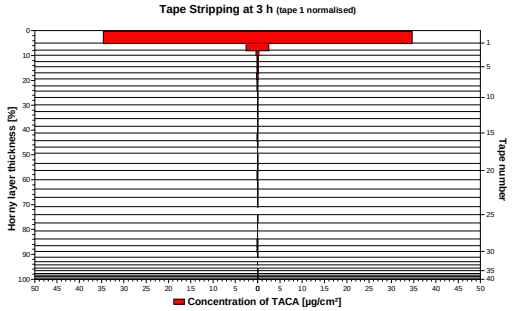
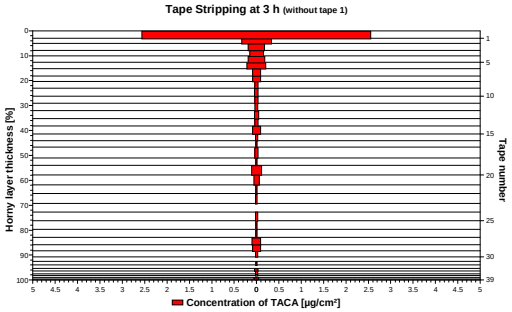
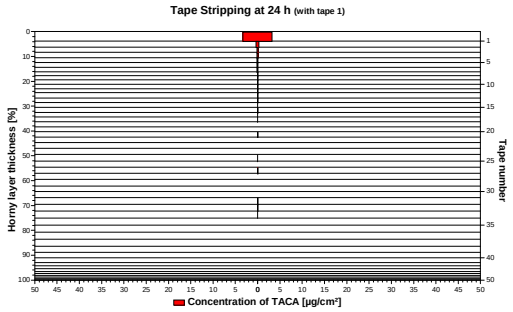
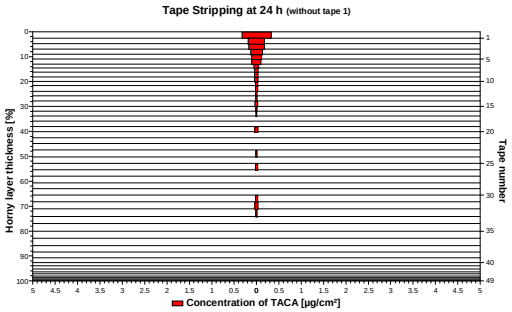
3 h



24 h



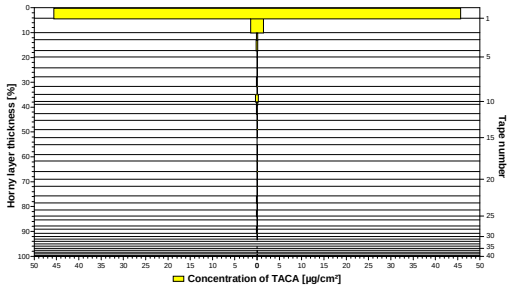
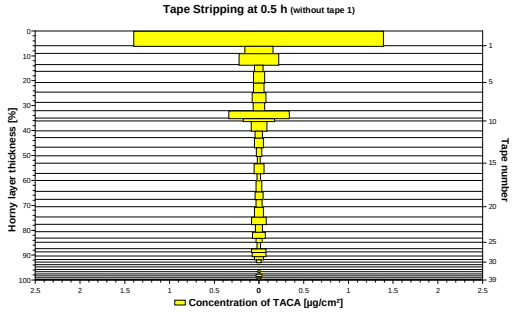
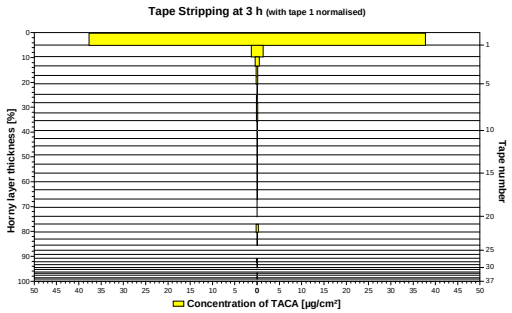
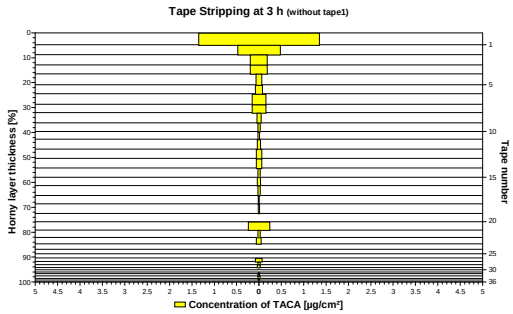
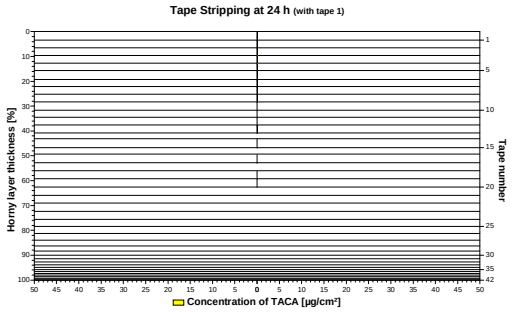
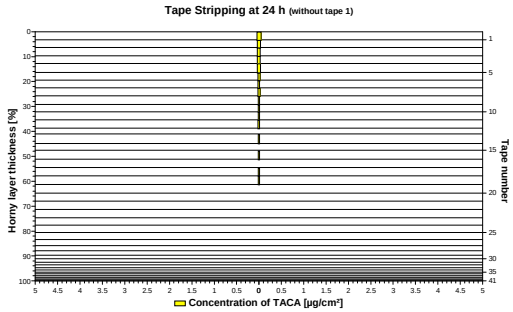
Volunteer 4: Ethanolic Gel

	ALL TAPES	WITHOUT TAPE 1
0.5h		
3h		
24h		

Notice:

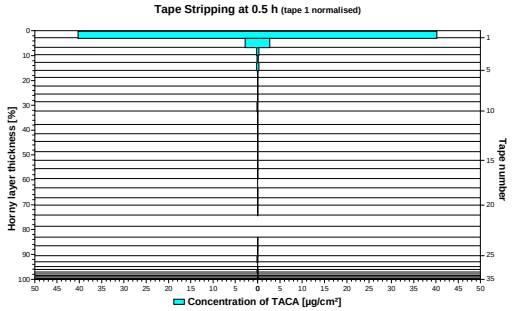
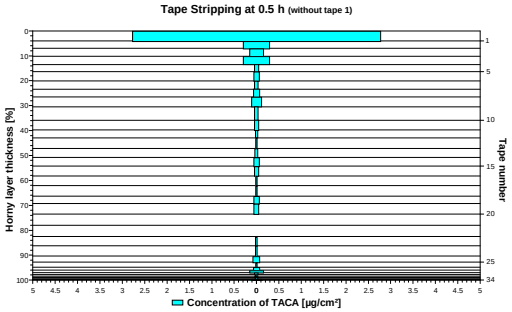
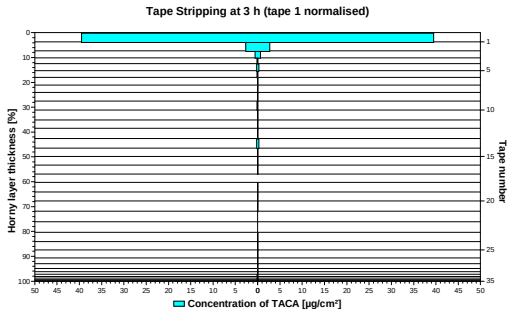
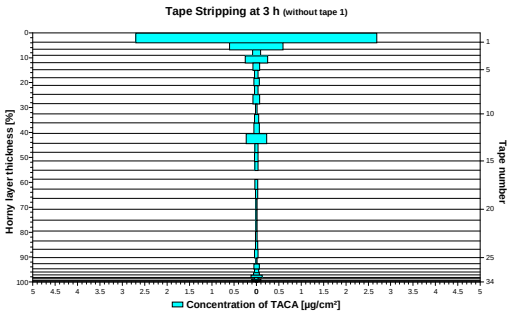
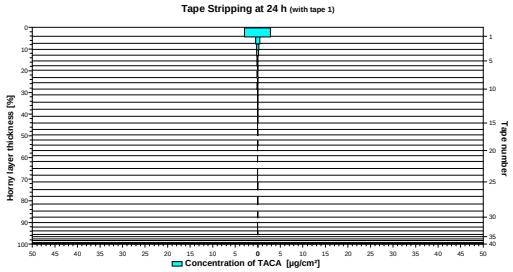
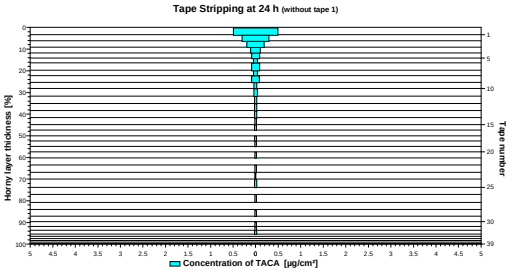
After application of the ethanolic gel and evaporation of the solvent, a thin film (consisting of cellulose and drug) remained on the skin surface. This film was removed with the first stripped tape and interfered with the spectroscopical quantification of the corneocytes. Therefore, a normalization of the corneocyte amount adhering on the first tape was necessary, and the profiles are depicted with normalized tape 1 (left side). Because the TACA amount on tape 1 was very large, the TACA concentration scale differs by a factor of 10 compared to the scale of the penetration profiles after application of the acetonic solution (50 µg/cm^2 vs. 5 µg/cm^2). Thus, to facilitate the comparison between the 2 formulations, the penetration profiles after application of the ethanolic gel are additionally depicted without tape 1 (right side, scale 5 µg/cm^2).

Volunteer 5: Ethanolic Gel

	ALL TAPES	WITHOUT TAPE 1
0.5h		
3h		
24h		

Please consider the notice on page 119.

Volunteer 6: Ethanolic Gel

	ALL TAPES	WITHOUT TAPE 1
0.5h		
3h		
24h		

Please consider the notice on page 119.

Quantitative TACA penetration into stratum corneum

Table 11-3: TACA amount penetrated into stratum corneum after application of an acetonic solution and an ethanolic gel, both delivering 100 µg/cm^2 TACA. Two types of evaluations were performed: total TACA amount penetrated (all tapes), and TACA amount calculated excluding tapes 1-3. The difference of the TACA amount penetrated after application of the 2 formulations was tested in a 2-sided t-test at the 5% level of significance. An asterisk (*) denotes a statistically significant difference (p<0.05), a double asterisk (**) a highly significant difference (p<0.01).

TOTAL TACA AMOUNT PENETRATED INTO STRATUM CORNEUM

	0.5 h		3 h		24 h	
No.	**Acetonic Sol.**	**Alcoholic Gel**	**Acetonic Sol.**	**Alcoholic Gel**	**Acetonic Sol.**	**Alcoholic Gel**
1	25.4	91.8	7.2	79.2	0.4	9.7
2	20.4	98.5	4.9	82.6	0.7	0.9
3	26.4	90.5	28.1	89.3	1.4	9.4
Mean±SD	24.1 ± 3.2	93.6 ± 4.3	13.4 ± 12.8	83.7 ± 5.2	0.8 ± 0.5	6.7 ± 5.0
95% CI	16.1 to 32.0	82.9 to 104.3	-18.3 to 45.1	70.9 to 96.5	-0.4 to 2.1	-5.8 to 19.1
p-value	< 0.01**		< 0.01**		0.1	

TACA AMOUNT PENETRATED INTO STRATUM CORNEUM WITHOUT TAPES 1-3

	0.5 h		3 h		24 h	
No.	**Acetonic Sol.**	**Alcoholic Gel**	**Acetonic Sol.**	**Alcoholic Gel**	**Acetonic Sol.**	**Alcoholic Gel**
1	14.7	2.8	3.1	4.0	0.2	2.0
2	9.3	4.1	2.0	3.5	0.4	0.6
3	14.3	3.8	13.1	3.8	0.9	2.1
Mean±SD	12.8 ± 3.0	3.6 ± 0.7	6.05 ± 6.1	3.8 ± 0.3	0.5 ± 0.3	1.6 ± 0.8
95% CI	5.3 to 20.3	1.9 to 5.3	-9.2 to 21.3	3.0 to 4.5	-0.3 to 1.4	-0.5 to 3.6
p-value	< 0.01**		0.6		0.1	

UV/VIS-Spectroscopy

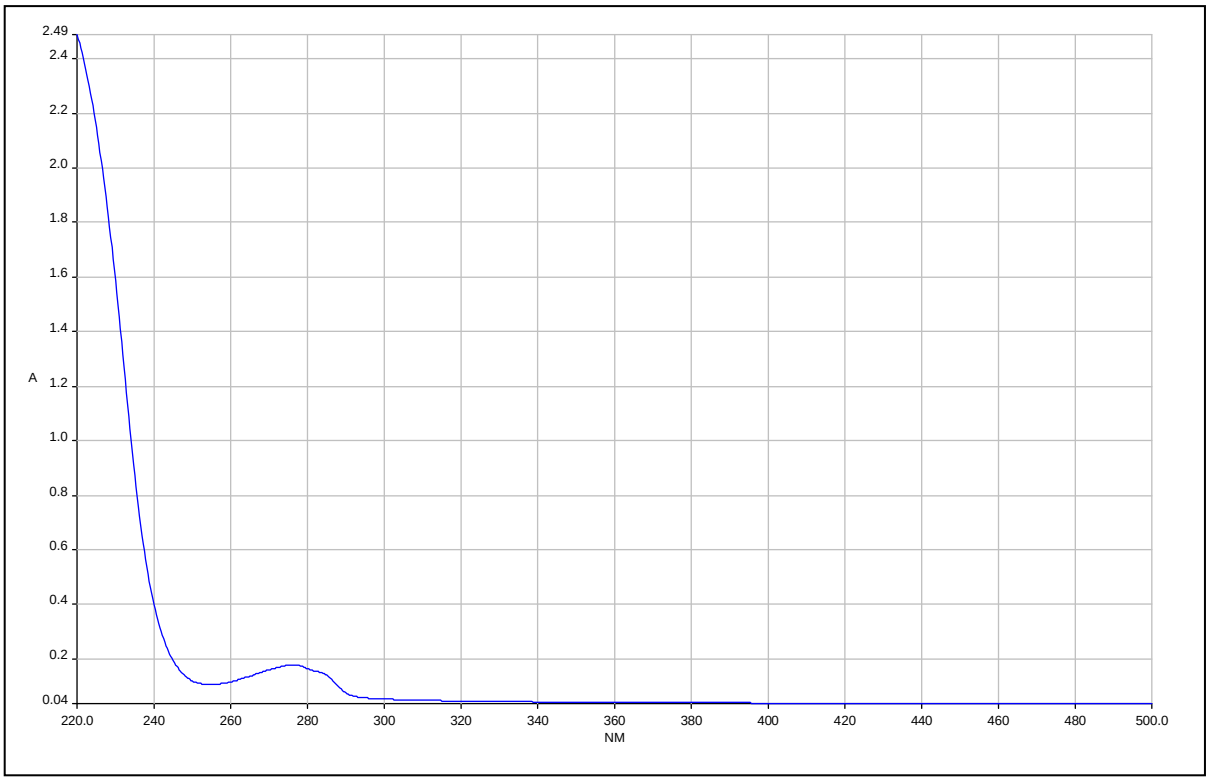


Fig. 11-2: UV/VIS-spectrum of a blank tape of Tesa Multi-Film Crystal-Clear®.

11.2. Project II

11.2.1. Pre-Examination Form

Voruntersuchung 02.2004

Voruntersuchung zur Aufnahme in die Studie:

„Explorative Pilotstudie zur Untersuchung der topischen Bioverfügbarkeit von Triamcinolonacetonid bei gesunden Probanden im Stratum corneum: in vivo Einfluss von mehrfacher Applikation und Okklusion"

Name ..... Vorname .....

Geburtsdatum ..... Geschlecht .....

Probandennummer .....

Einschlusskriterien:

Zur Aufnahme in dieser Studie müssen alle Fragen mit **Ja** beantwortet werden.	**Ja**	**Nein**
1. Ist der/die Proband/In mindestens 18 Jahre alt?	☐	☐
2. Ist der/die Proband/In von weisser Hautfarbe (Typ I-III)?	☐	☐
3. Liegt Aufklärung und schriftliche Einwilligung des/der Probanden/In durch den Prüfarzt vor?	☐	☐

Ausschlusskriterien

Zur Aufnahme in dieser Studie müssen alle Fragen mit **Nein** beantwortet werden.	**Ja**	**Nein**
1. Ist die Probandin schwanger oder stillt sie? (Bei männlichen Probanden „Nein" ankreuzen)	☐	☐
2. Leidet der/die Proband/In an einer akuten Entzündung der Haut?	☐	☐
3. Leidet der/die Proband/In an anderen Erkrankungen oder Allergien?	☐	☐
4. Sind bei dem/der Probanden/In beeinträchtigte Wahrnehmungs- und Beurteilungsfähigkeit (einschliesslich des Verdachtes auf Drogen und Alkoholmissbrauch) bekannt?	☐	☐
5. Leidet der/die Proband/In unter einer Abwehrschwäche (rezidivierende Infektionskrankheit)?	☐	☐
6. Nimmt der/die Proband/In andere Medikamente ein, insbesondere Glukokortikosteroide?	☐	☐
7. Nimmt der/die Proband/In zugleich an einer anderen Studie teil?	☐	☐

Kontrolle der Innenseite der Unterarme

Zur Aufnahme in dieser Studie müssen alle Fragen mit **Nein** beantwortet werden.	**Ja**	**Nein**
1. Sind bestehende, grossflächige Läsionen vorhanden?	☐	☐
2. Ist die Behaarung stark ausgeprägt?	☐	☐

Bemerkungen

.....

.....

Erfüllt der/die Proband/In **alle** Aufnahmekriterien?	**Ja**	**Nein**
Falls Nein darf der/die Proband/In nicht in die Studie aufgenommen werden.	☐	☐

Datum ..... Unterschrift des Prüfarztes .....

Visum / Datum Monitor.: ..... Seite 1 von 1

11.2.2. Post-Examination Form

Nachuntersuchung 02.2004

Nachuntersuchung – Beurteilung der Wundheilung

Im Rahmen der Studie: „Explorative Pilotstudie zur Untersuchung der topischen Bioverfügbarkeit von Triamcinolonacetonid bei gesunden Probanden im Stratum corneum: in vivo Einfluss von mehrfacher Applikation und Okklusion"

Name ..... Vorname .....

Geburtsdatum ..... Geschlecht .....

Probandennummer .....

Datum der Behandlung .....

Datum der Nachuntersuchung .....

Begutachtung des Verlaufs der Wundheilung durch den Prüfarzt:

- Ausgezeichnet ☐
- sehr gut ☐
- gut ☐
- befriedigend ☐
- schlecht ☐

Bemerkungen

.....

.....

.....

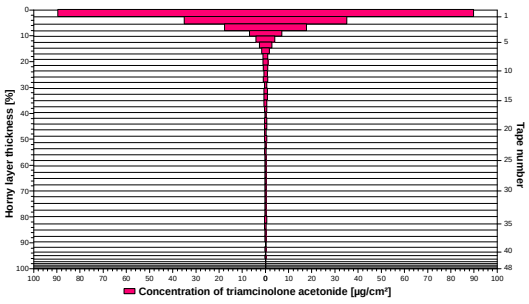
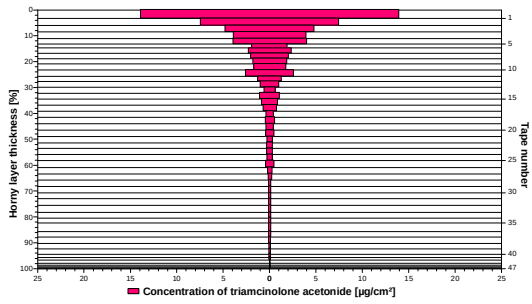
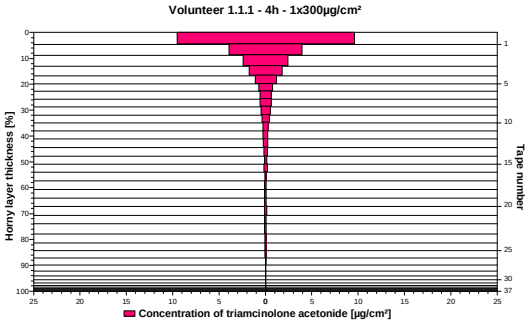
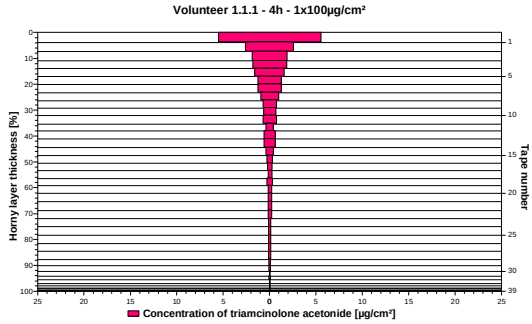
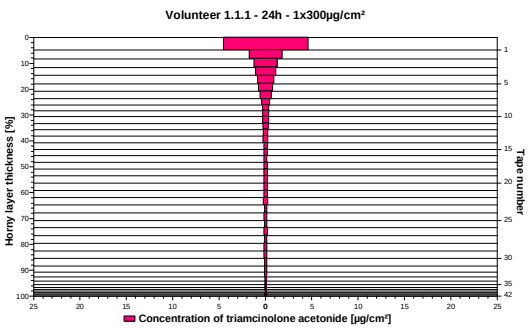
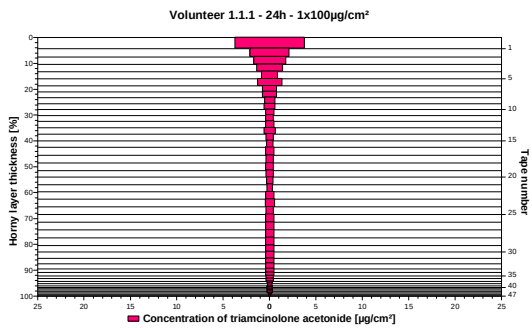
Datum Unterschrift des Prüfarztes

....

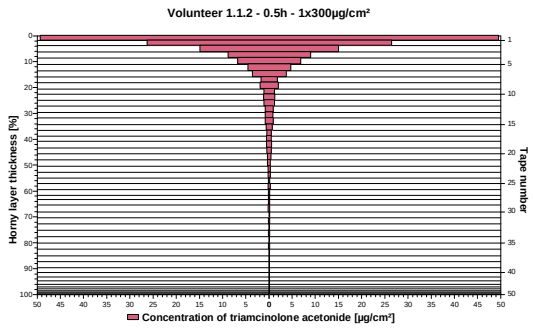
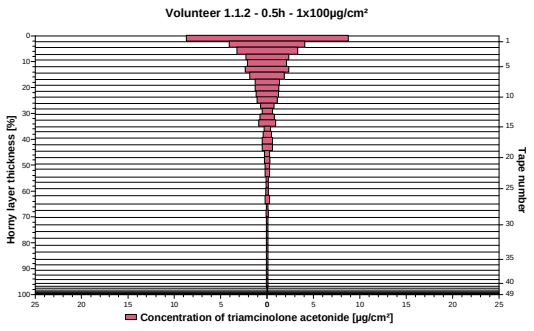
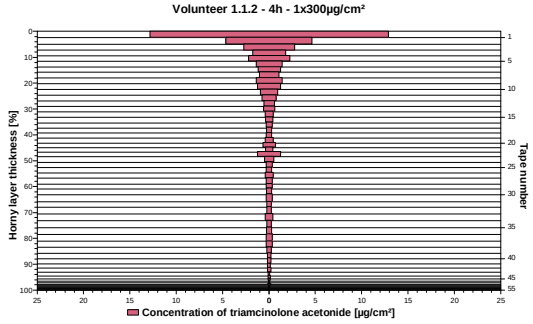
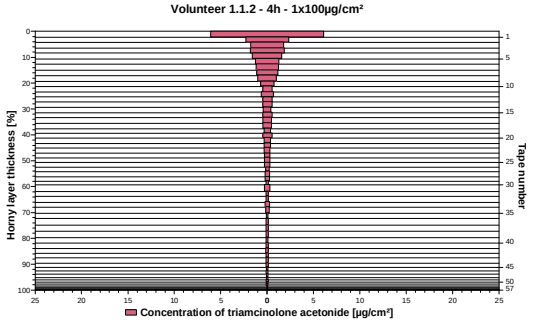
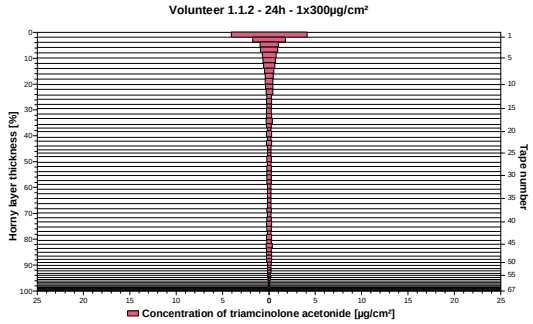
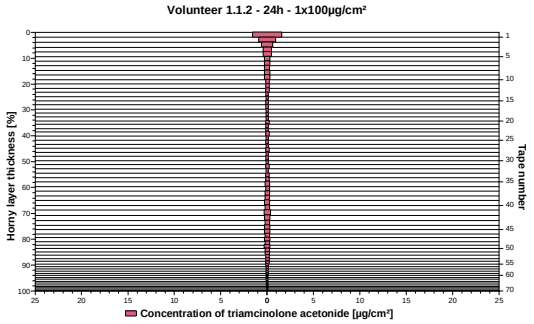
Visum / Datum Monitor.: ..... Seite 1 von 1

11.2.3. Experiment 1.1 (Dose) - Penetration profiles

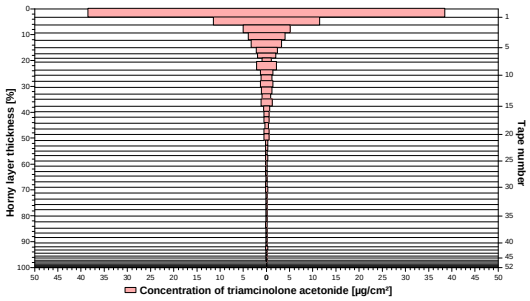
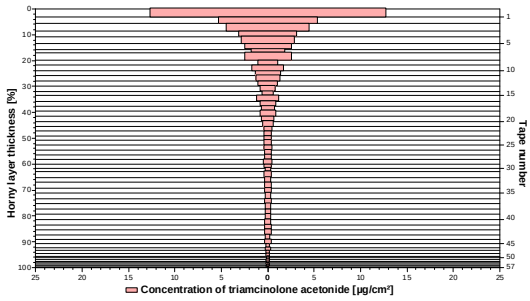
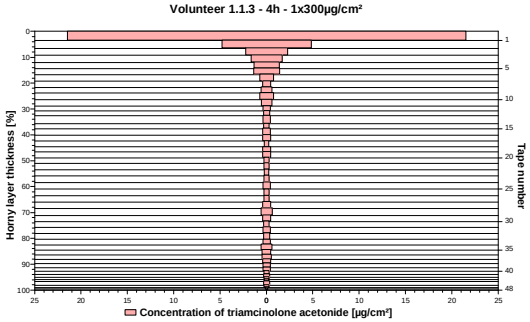
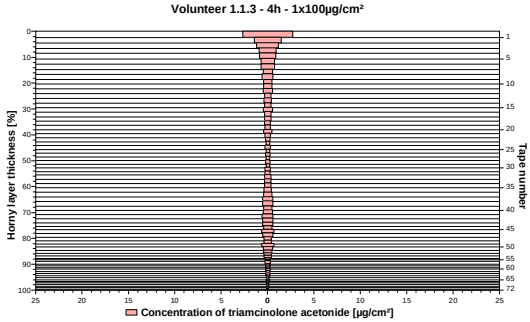
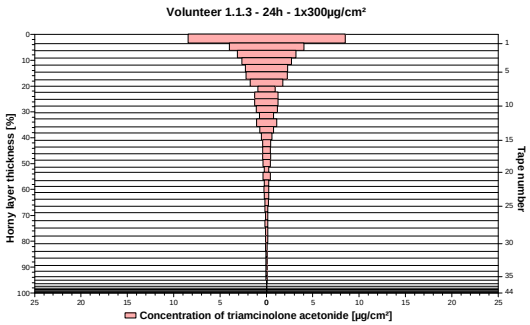
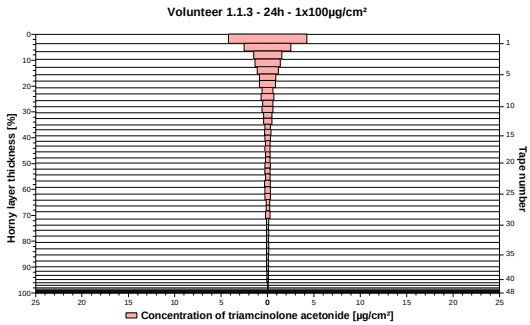
Volunteer 1.1.1

	1x300µg/cm²	1x100µg/cm²
0.5h		
4h		
24h		

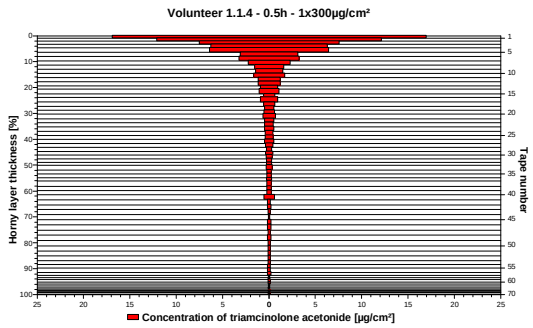
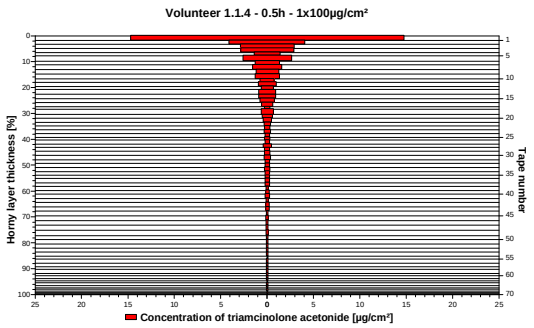
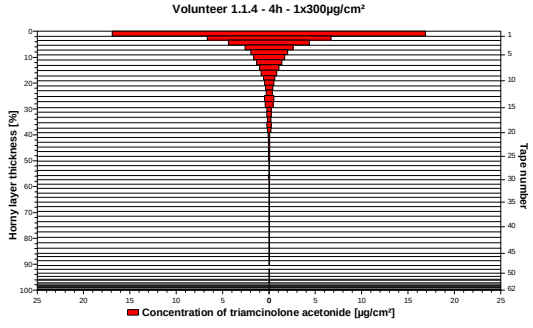
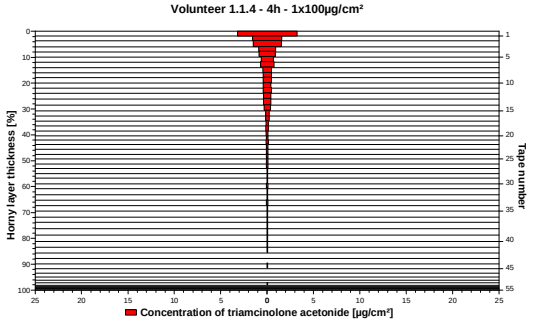
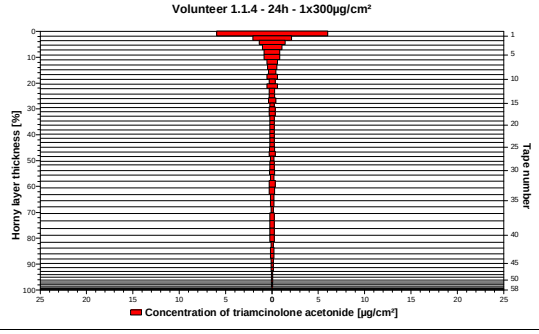
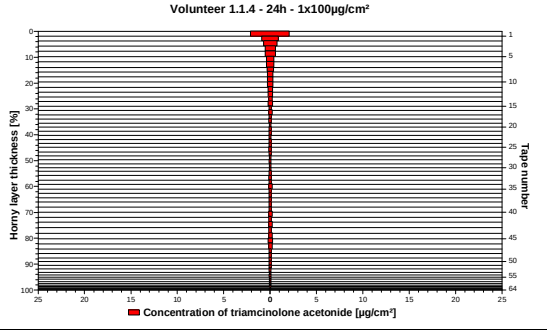
Volunteer 1.1.2

	1x300µg/cm²	1x100µg/cm²
0.5h		
4h		
24h		

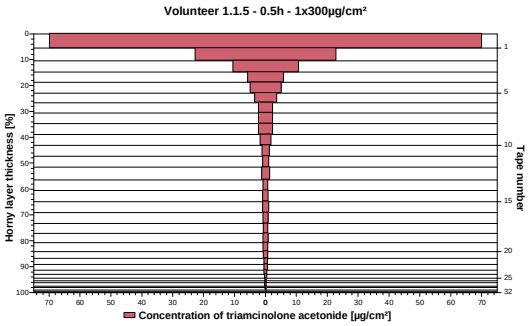
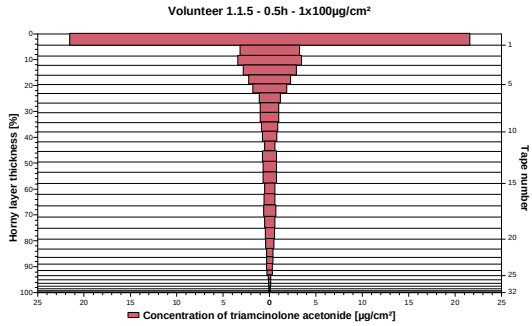
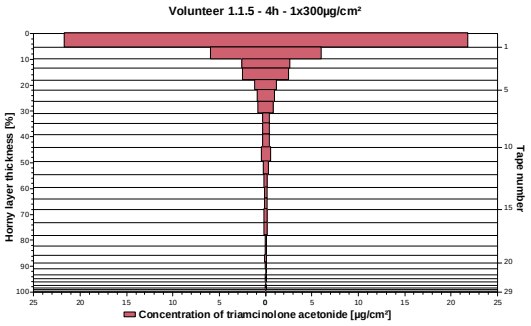
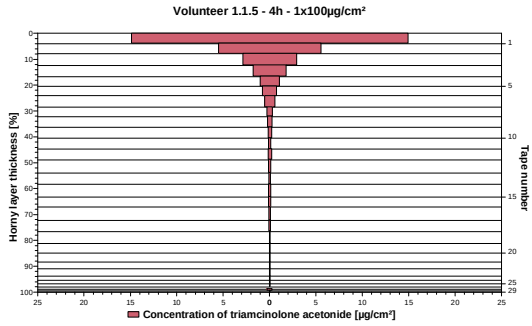
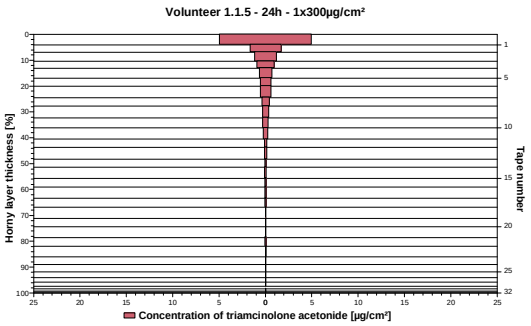
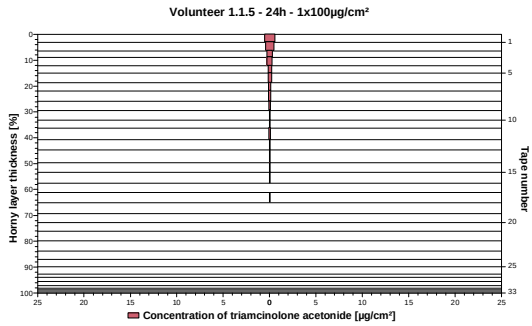
Volunteer 1.1.3

	1x300µg/cm²	1x100µg/cm²
0.5h		
4h		
24h		

Volunteer 1.1.4

	1x300µg/cm²	1x100µg/cm²
0.5h		
4h		
24h		

Volunteer 1.1.5

	1x300µg/cm²	1x100µg/cm²
0.5h		
4h		
24h		

11.2.4. Experiment 1.1 (Dose) - Data overview

Exp 1.1 (n=5)

Concentration	Tape Stripping	TACA-amount all tapes		TACA-amount without tape 1-3	
ORIGINAL VALUES		**Mean ± SD [µg/cm²]**	**[%]**	**Mean ± SD [µg/cm²]**	**[%]**
300 µg/cm²	0.5 h	245 ± 78	82 ± 26	74 ± 12	25 ± 4
300 µg/cm²	4 h	80 ± 19	27 ± 6	31 ± 15	10 ± 5
300 µg/cm²	24 h	46 ± 18	15 ± 6	27 ± 13	9 ± 4
100 µg/cm²	0.5 h	101 ± 17	101 ± 17	55 ± 14	55 ± 14
100 µg/cm²	4 h	52 ± 13	52 ± 13	30 ± 16	30 ± 16
100 µg/cm²	24 h	33 ± 19	33 ± 19	23 ± 14	23 ± 14
LN-VALUES		**Mean ± SD [µg/cm²]**	**[%]**	**Mean ± SD [µg/cm²]**	**[%]**
300 µg/cm²	0.5 h	5.5 ± 0.3	4.4 ± 0.3	4.3 ± 0.2	3.2 ± 0.2
300 µg/cm²	4 h	4.4 ± 0.3	3.3 ± 0.3	3.3 ± 0.5	2.2 ± 0.5
300 µg/cm²	24 h	3.8 ± 0.4	2.7 ± 0.4	3.2 ± 0.6	2.1 ± 0.6
100 µg/cm²	0.5 h	4.6 ± 0.2	4.6 ± 0.2	4.0 ± 0.3	4.0 ± 0.3
100 µg/cm²	4 h	3.9 ± 0.3	3.9 ± 0.3	3.3 ± 0.6	3.3 ± 0.6
100 µg/cm²	24 h	3.2 ± 0.9	3.2 ± 0.9	2.8 ± 1.1	2.8 ± 1.1
Pair Differences		**Confidence Interval 95%**	**p-value (2-sided)**	**Confidence Interval 95%**	**p-value (2-sided)**
	0.5 h	1.5 to 3.9	0.008 **	0.9 to 2.2	0.1
	4 h	1.0 to 2.5	0.06	0.6 to 1.8	0.7
	24 h	0.7 to 4.4	0.2	0.5 to 3.8	0.4

11.2.5. Experiment 1.1 (Dose) - Data single volunteers

Experiment 1.1 - TACA-amount penetrated - All Tapes

			1x300ug/cm²						1x100ug/cm²					
Volunteer	Gender	Age [years]	Tape Stripping at						Tape Stripping at					
			0.5h		4h		24h		0.5h		4h		24h	
			[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]
1.1.1	f	33	349.8	116.6	48.6	16.2	34.2	11.4	115.5	115.5	47.7	47.7	55.9	55.9
1.1.2	f	23	262.2	87.4	89.9	30.0	46.2	15.4	74.8	74.8	59.4	59.4	31.9	31.9
1.1.3	f	26	171.0	57.0	99.2	33.1	74.3	24.8	117.1	117.1	62.1	62.1	42.6	42.6
1.1.4	m	37	163.5	54.5	85.1	28.4	48.5	16.2	99.4	99.4	29.9	29.9	27.7	27.7
1.1.5	m	29	280.5	93.5	78.4	26.1	26.0	8.7	96.2	96.2	59.3	59.3	5.0	5.0
Mean		30	245.4	81.8	80.2	26.7	45.8	15.3	100.6	100.6	51.7	51.7	32.6	32.6
SD			78.5	26.2	19.2	6.4	18.3	6.1	17.2	17.2	13.4	13.4	18.9	18.9
RSD			32.0	32.0	24.0	24.0	40.0	40.0	17.1	17.1	25.9	25.9	58.0	58.0

Experiment 1.1 - TACA-amount penetrated - All Tapes, Logarithm (ln)

			1x300ug/cm²						1x100ug/cm²					
Volunteer	Gender	Age [years]	Tape Stripping at						Tape Stripping at					
			30min,		4h, 1x300ug/cm2		24h,		30min,		4h, 1x100ug/cm2		24h,	
			[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]
1.1.1	f	33	5.9	4.8	3.9	2.8	3.5	2.4	4.7	4.7	3.9	3.9	4.0	4.0
1.1.2	f	23	5.6	4.5	4.5	3.4	3.8	2.7	4.3	4.3	4.1	4.1	3.5	3.5
1.1.3	f	26	5.1	4.0	4.6	3.5	4.3	3.2	4.8	4.8	4.1	4.1	3.8	3.8
1.1.4	m	37	5.1	4.0	4.4	3.3	3.9	2.8	4.6	4.6	3.4	3.4	3.3	3.3
1.1.5	m	29	5.6	4.5	4.4	3.3	3.3	2.2	4.6	4.6	4.1	4.1	1.6	1.6
Mean		30	5.5	4.4	4.4	3.3	3.8	2.7	4.6	4.6	3.9	3.9	3.2	3.2
SD			0.3	0.3	0.3	0.3	0.4	0.4	0.2	0.2	0.3	0.3	0.9	0.9
RSD			6.0	7.6	6.4	8.5	10.5	14.8	3.9	3.9	7.8	7.8	29.4	29.4

Experiment 1.1 - TACA-amount penetrated - Without Tapes 1-3

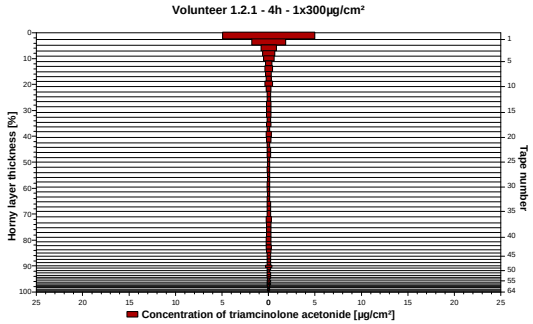
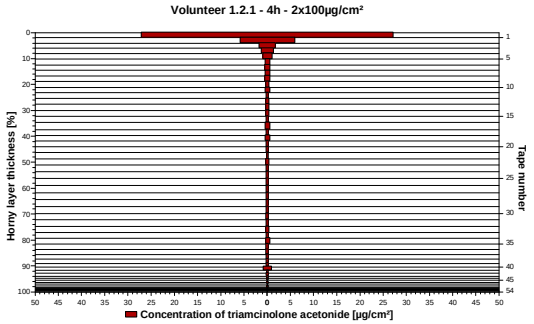
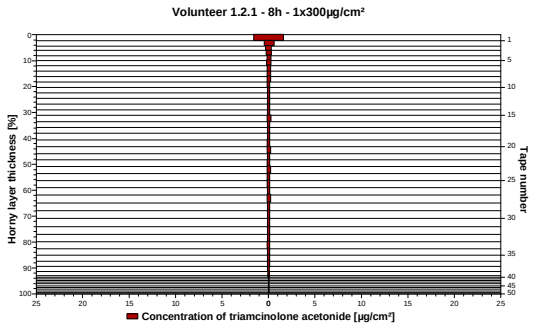
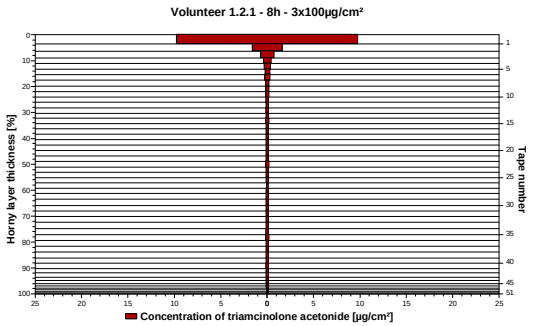
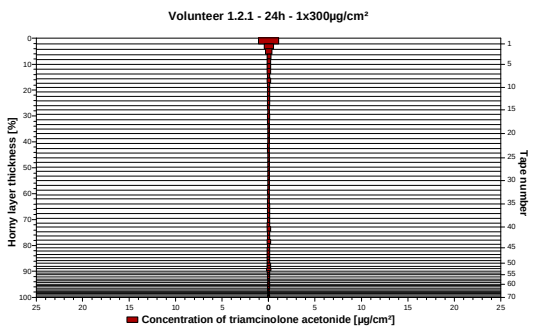
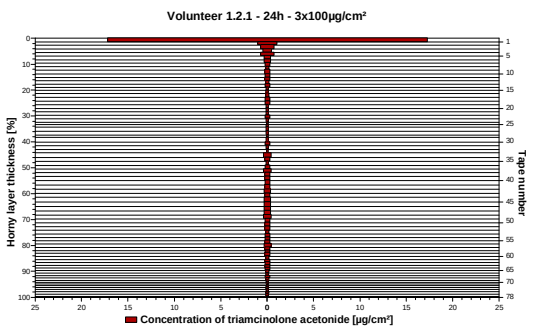
			1x300ug/cm²						1x100ug/cm²					
Volunteer	Gender	Age [years]	Tape Stripping at						Tape Stripping at					
			0.5h		4h		24h		0.5h		4h		24h	
			[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]
1.1.1	f	33	65.1	21.7	16.7	5.6	19.1	6.4	63.1	63.1	27.7	27.7	40.7	40.7
1.1.2	f	23	80.6	26.9	49.5	16.5	32.5	10.8	42.8	42.8	39.0	39.0	25.6	25.6
1.1.3	f	26	61.0	20.4	42.1	14.0	42.9	14.3	72.1	72.1	51.4	51.4	26.2	26.2
1.1.4	m	37	90.4	30.2	29.2	9.7	29.5	9.8	55.9	55.9	17.3	17.3	20.3	20.3
1.1.5	m	29	73.9	24.6	17.8	5.9	10.4	3.5	39.8	39.8	12.7	12.7	2.4	2.4
Mean		30	74.2	24.7	31.1	10.4	26.9	9.0	54.7	54.7	29.6	29.6	23.0	23.0
SD			11.8	3.9	14.5	4.8	12.5	4.2	13.6	13.6	15.9	15.9	13.8	13.8
RSD			16.0	16.0	46.8	46.8	46.6	46.6	24.8	24.8	53.5	53.5	60.0	60.0

Experiment 1.1 - TACA-amount penetrated - Without Tapes 1-3, Logarithm (ln)

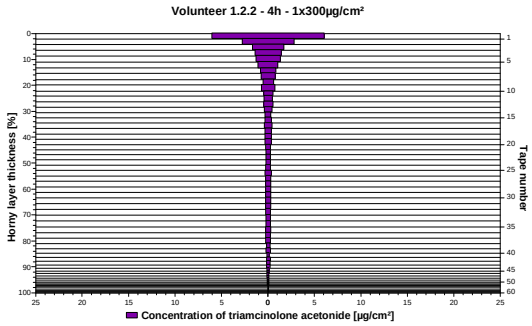
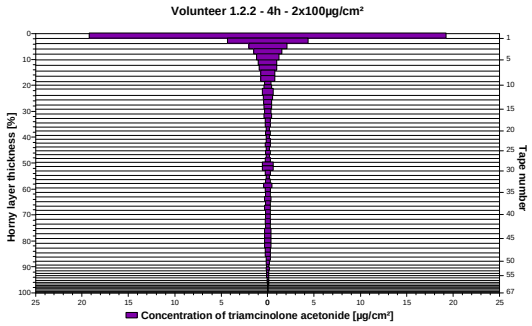
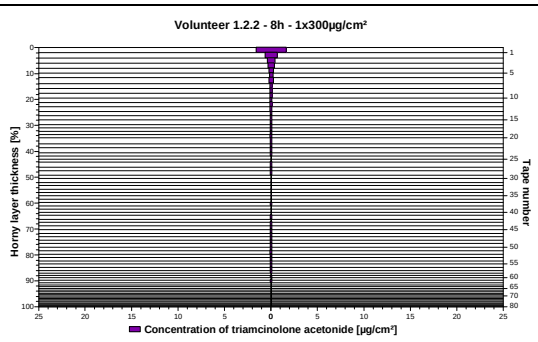
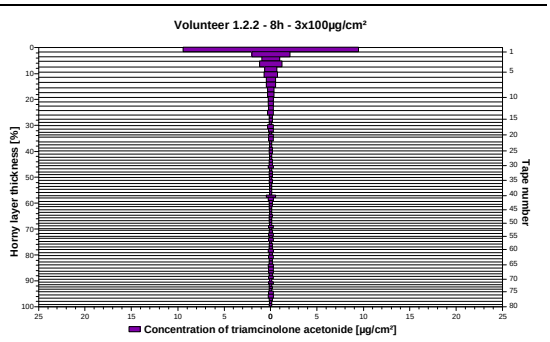
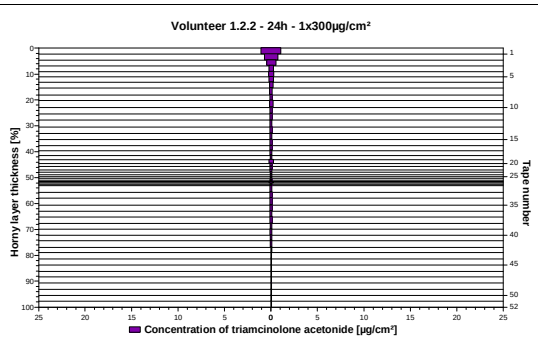
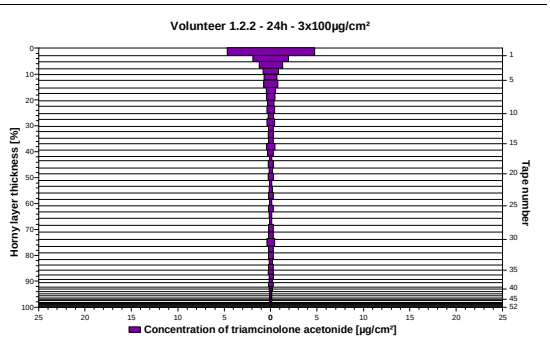
			1x300ug/cm²						1x100ug/cm²					
Volunteer	Gender	Age [years]	Tape Stripping at						Tape Stripping at					
			0.5h		4h		24h		0.5h		4h		24h	
			[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]
1.1.1	f	33	4.2	3.1	2.8	1.7	3.0	1.9	4.1	4.1	3.3	3.3	3.7	3.7
1.1.2	f	23	4.4	3.3	3.9	2.8	3.5	2.4	3.8	3.8	3.7	3.7	3.2	3.2
1.1.3	f	26	4.1	3.0	3.7	2.6	3.8	2.7	4.3	4.3	3.9	3.9	3.3	3.3
1.1.4	m	37	4.5	3.4	3.4	2.3	3.4	2.3	4.0	4.0	2.8	2.8	3.0	3.0
1.1.5	m	29	4.3	3.2	2.9	1.8	2.3	1.2	3.7	3.7	2.5	2.5	0.9	0.9
Mean		30	4.3	3.2	3.3	2.2	3.2	2.1	4.0	4.0	3.3	3.3	2.8	2.8
SD			0.2	0.2	0.5	0.5	0.6	0.6	0.3	0.3	0.6	0.6	1.1	1.1
RSD			3.7	5.0	14.7	21.8	17.4	26.6	6.4	6.4	17.6	17.6	39.6	39.6

11.2.6. Experiment 1.2 (Application frequency) - Penetration profiles

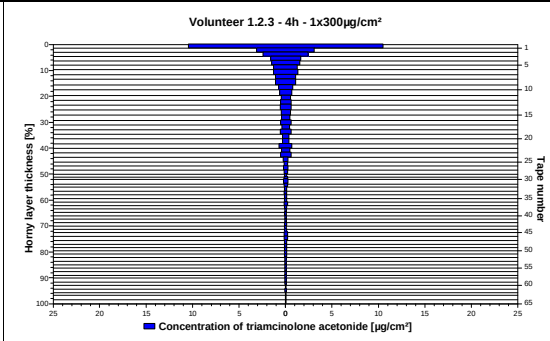
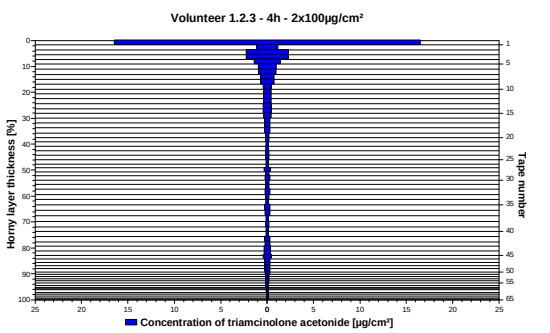
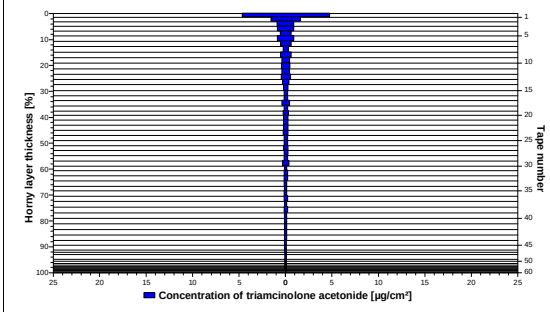
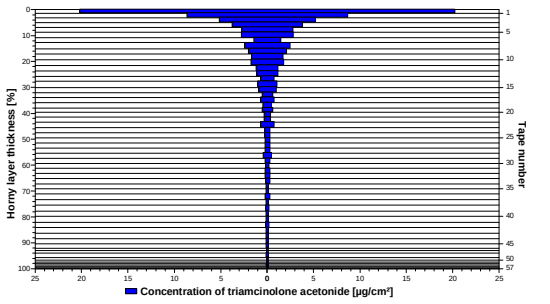
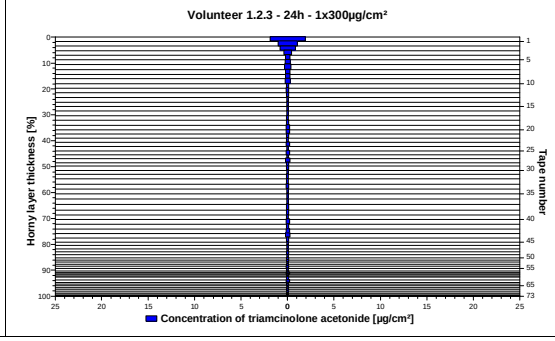
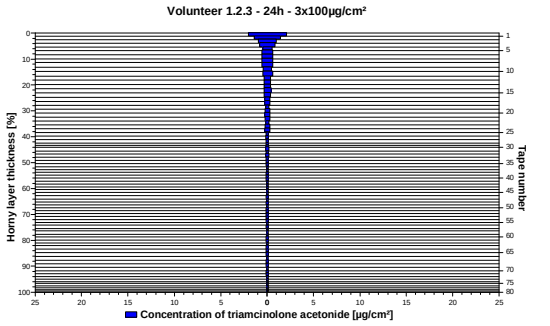
Volunteer 1.2.1

	1x300µg/cm²	3x100µg/cm²
4h		
8h		
24h		

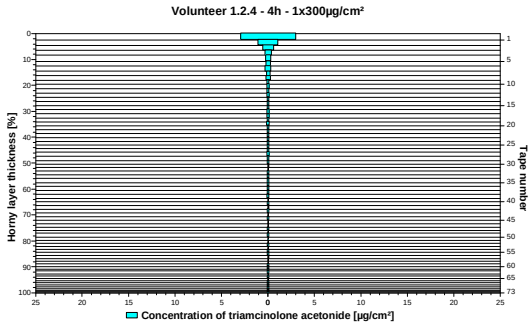
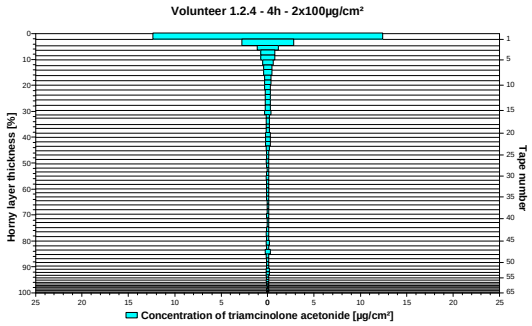
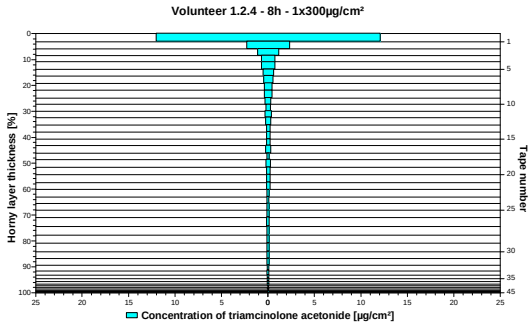
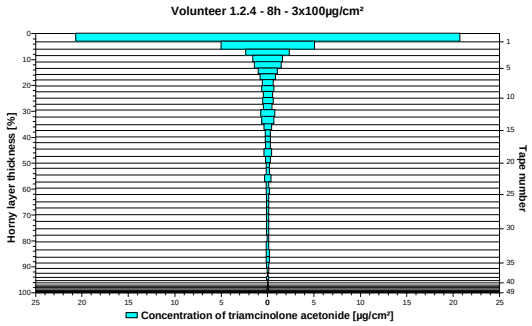
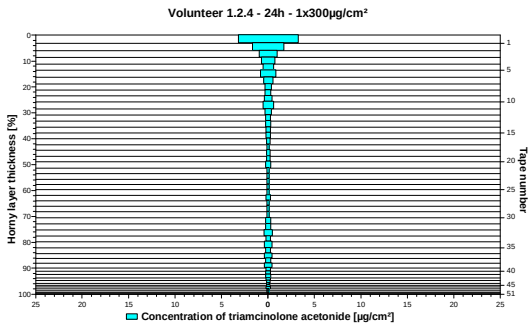
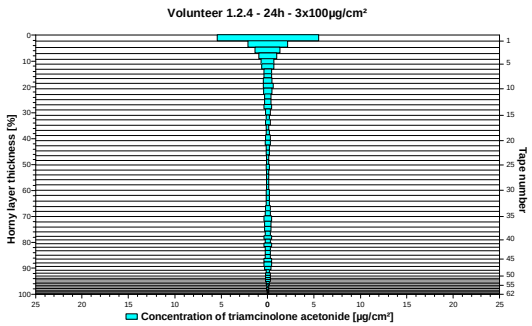
Volunteer 1.2.2

	1x300µg/cm²	3x100µg/cm²
4h		
8h		
24h		

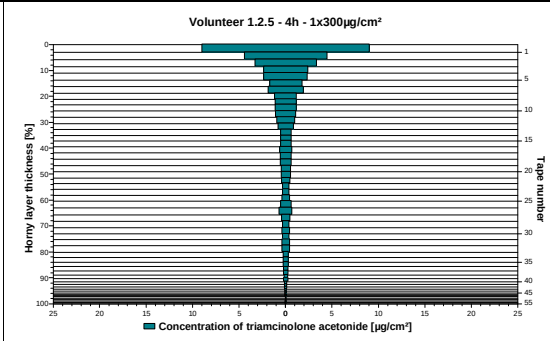
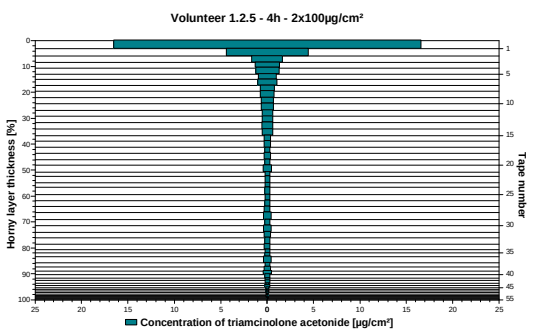
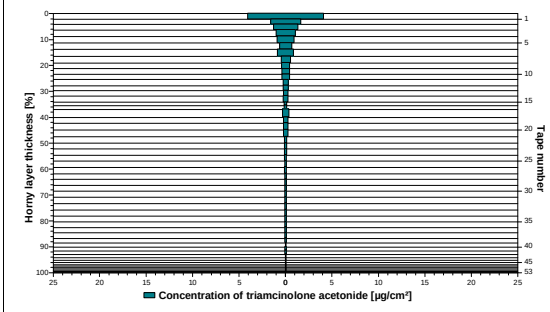
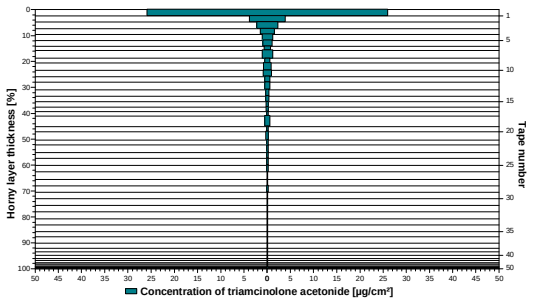
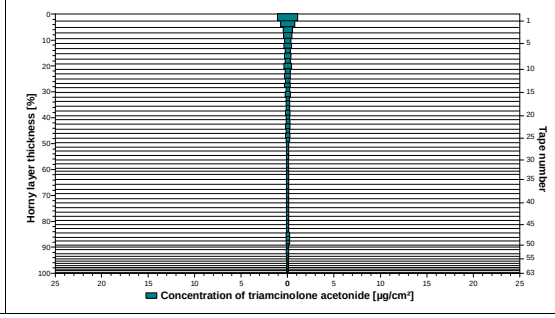
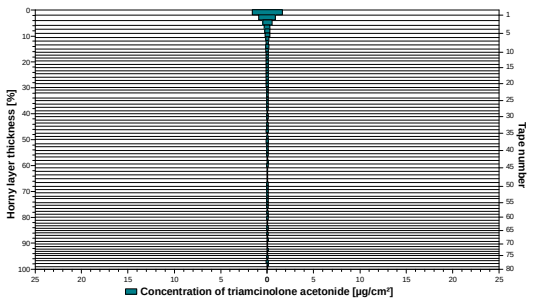
Volunteer 1.2.3

	1x300µg/cm²	3x100µg/cm²
4h		
8h		
24h		

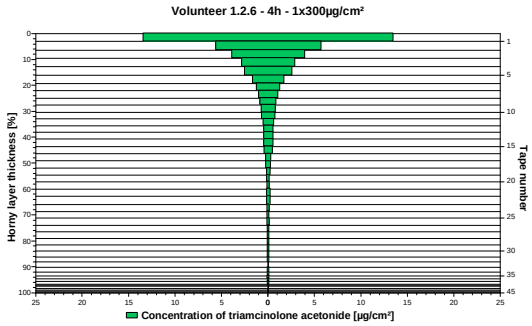
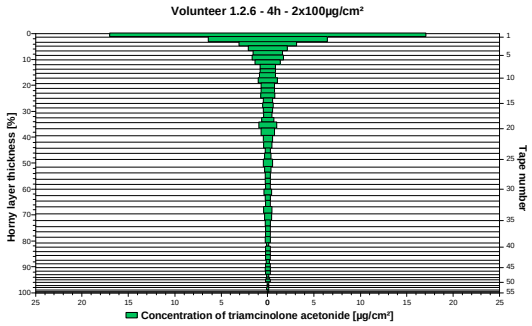
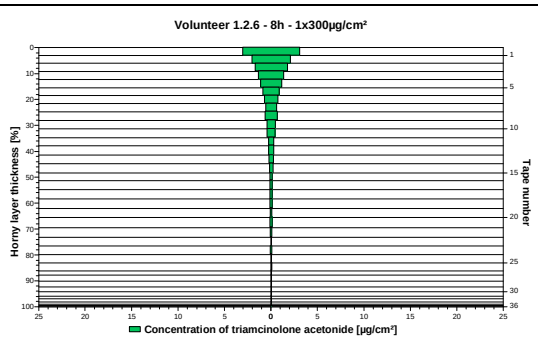
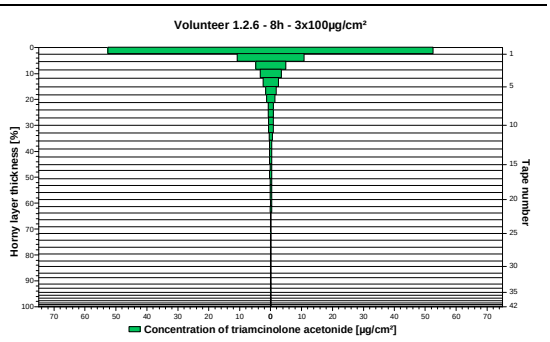
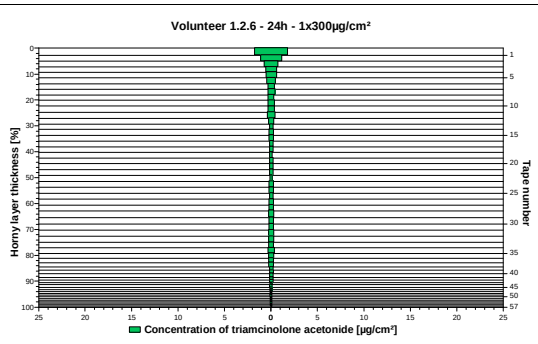
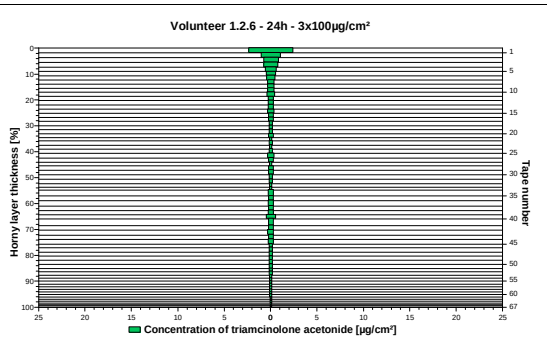
Volunteer 1.2.4

	1x300µg/cm²	3x100µg/cm²
4h		
8h		
24h		

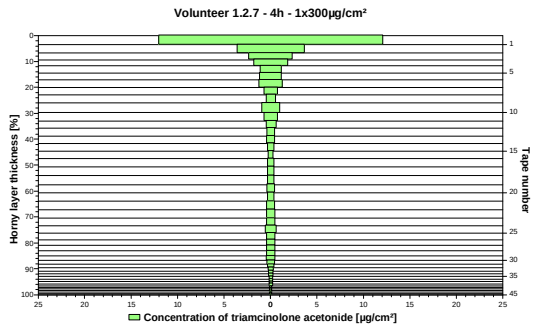
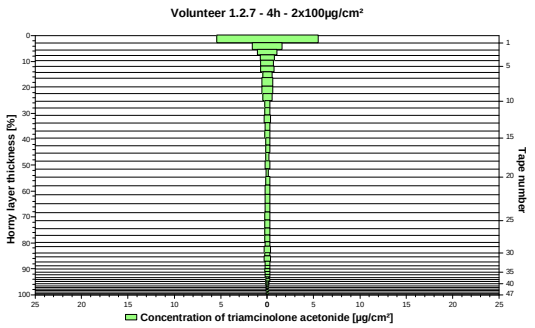
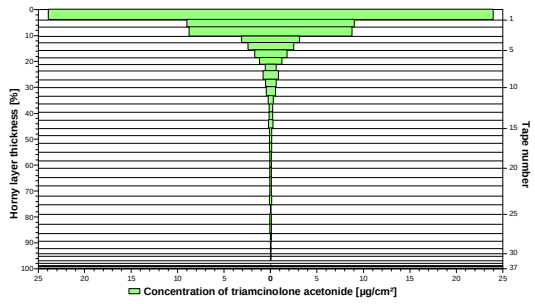
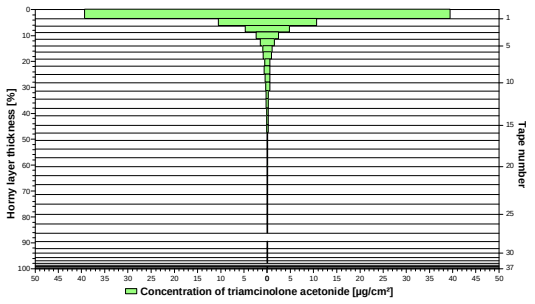
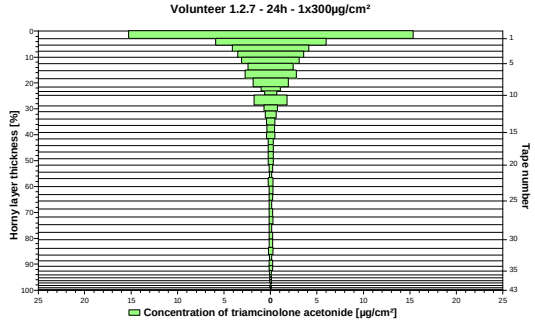
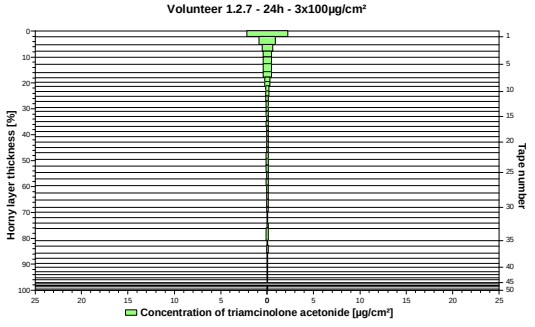
Volunteer 1.2.5

	1x300µg/cm²	3x100µg/cm²
4h		
8h		
24h		

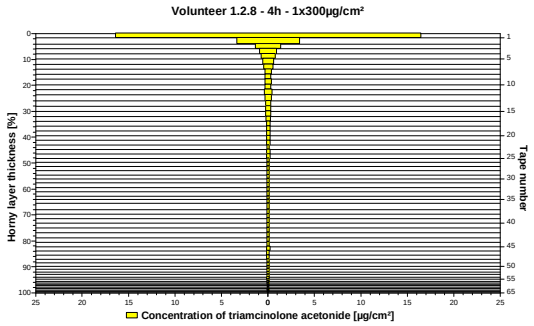
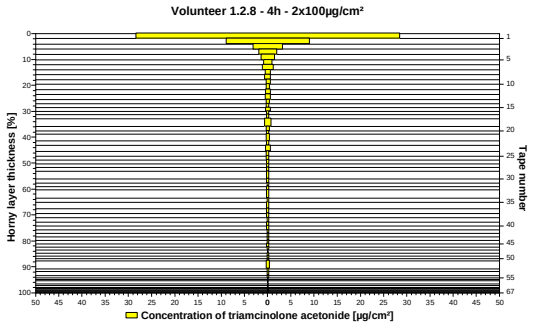
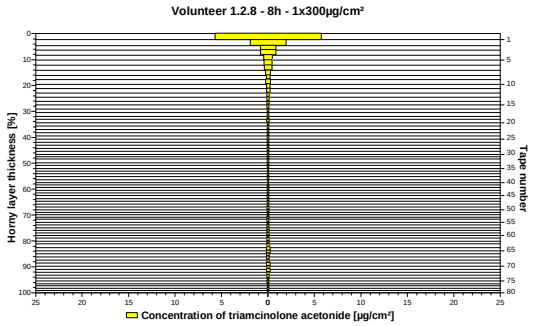
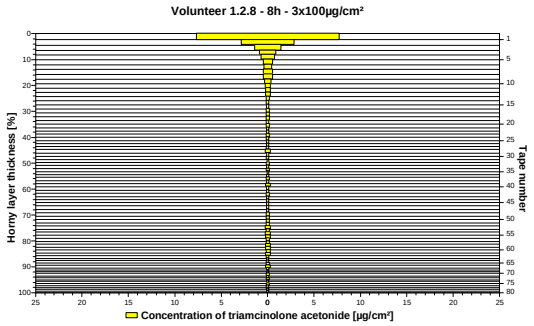
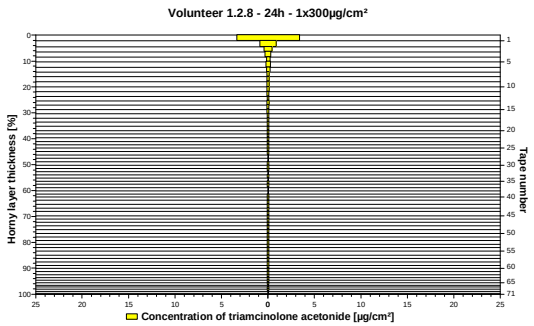
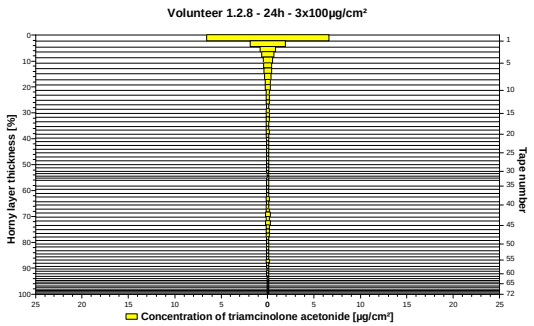
Volunteer 1.2.6

	1x300µg/cm²	3x100µg/cm²
4h		
8h		
24h		

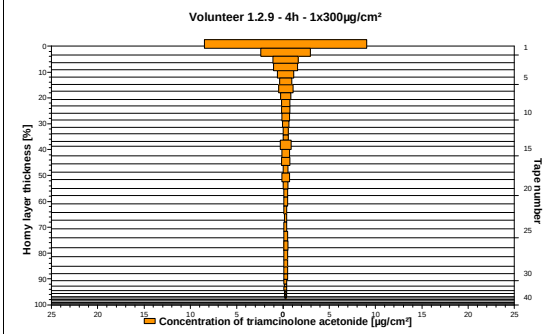
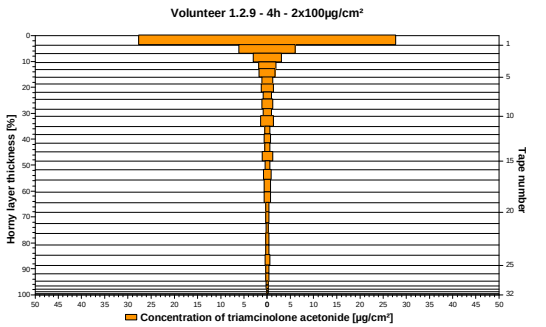
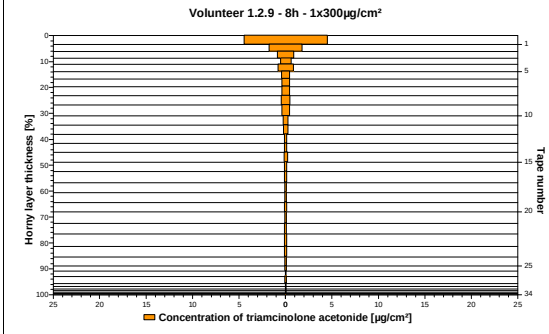
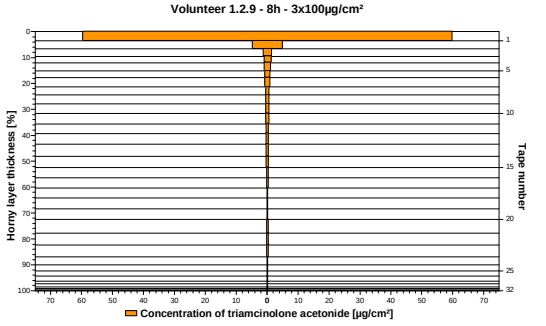
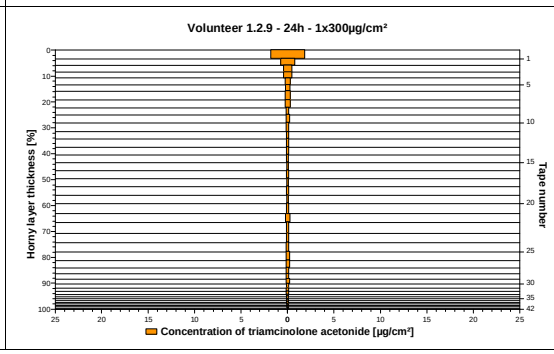
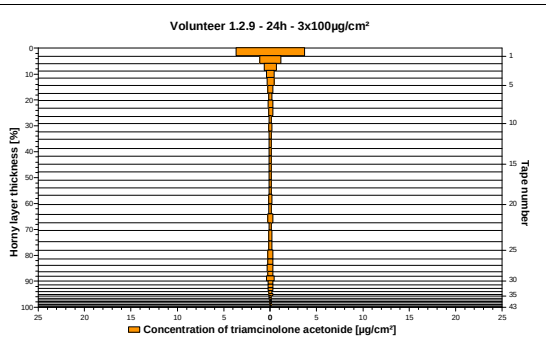
Volunteer 1.2.7

	1x300µg/cm²	3x100µg/cm²
4h		
8h		
24h		

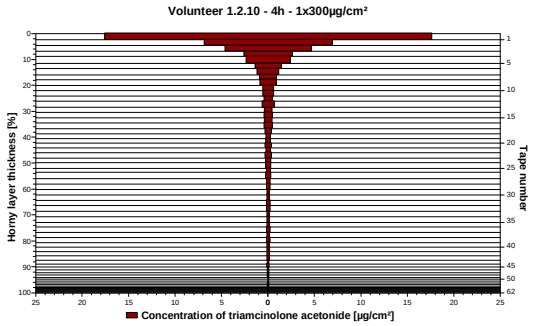
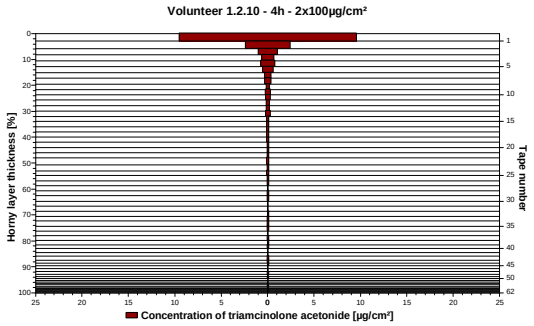
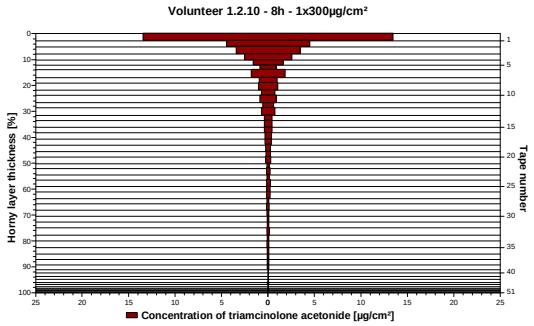
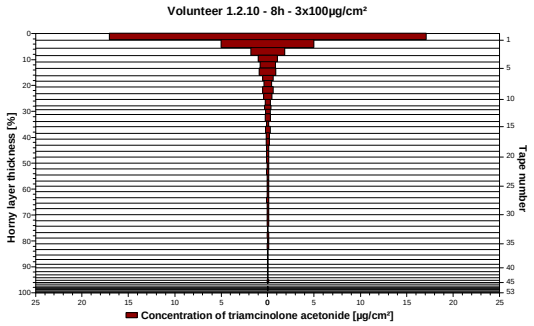
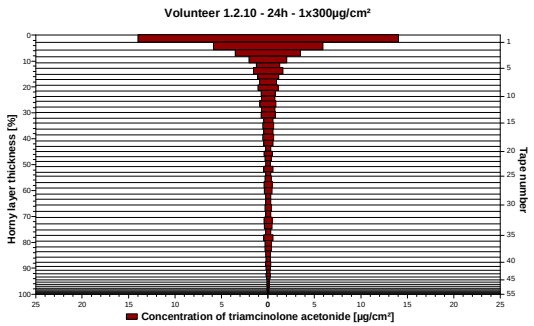
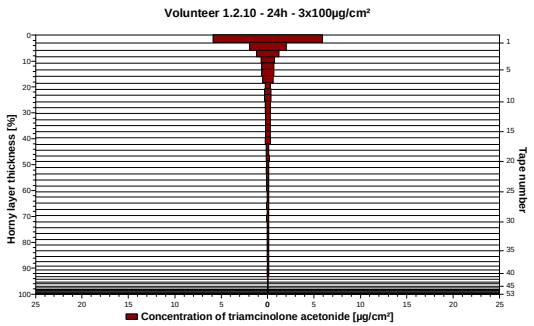
Volunteer 1.2.8

	1x300µg/cm²	3x100µg/cm²
4h		
8h		
24h		

Volunteer 1.2.9

	1x300µg/cm²	3x100µg/cm²
4h		
8h		
24h		

Volunteer 1.2.10

	1x300µg/cm²	3x100µg/cm²
4h		
8h		
24h		

11.2.7. Experiment 1.2 (Application frequency) – Data overview

Exp 1.2 (n=10)

Application pattern	Stripping time at	TACA-amount penetrated (all tapes)		TACA-amount penetrated (without tapes 1-3)	
ORIGINAL VALUES		**Mean ± SD** [µg/cm²]	[%]	**Mean ± SD** [µg/cm²]	[%]
1 x 300 µg/cm²	4 h	65.1 ± 23.0	21.7 ± 7.7	33.2 ± 11.7	11.1 ± 3.9
1 x 300 µg/cm²	8 h	43.6 ± 29.1	14.5 ± 9.7	19.5 ± 7.5	6.5 ± 2.5
1 x 300 µg/cm²	24 h	39.3 ± 31.3	13.1 ± 10.4	24.0 ± 14.0	8.0 ± 4.7
2 x 100 µg/cm²	4 h	84.0 ± 28.9	42.0 ± 14.5	35.1 ± 10.8	17.6 ± 5.4
3 x 100 µg/cm²	8 h	98.0 ± 45.2	32.7 ± 15.1	29.3 ± 15.1	9.8 ± 5.0
3 x 100 µg/cm²	24 h	37.9 ± 16.1	12.6 ± 5.4	22.9 ± 8.8	7.6 ± 2.9
LN-VALUES		**Mean ± SD** [µg/cm²]	[%]	**Mean ± SD** [µg/cm²]	[%]
1 x 300 µg/cm²	4 h	4.1 ± 0.5	3.0 ± 0.5	3.4 ± 0.4	2.3 ± 0.4
1 x 300 µg/cm²	8 h	3.6 ± 0.6	2.5 ± 0.6	2.9 ± 0.4	1.8 ± 0.4
1 x 300 µg/cm²	24 h	3.5 ± 0.7	2.4 ± 0.7	3.0 ± 0.5	1.9 ± 0.5
2 x 100 µg/cm²	4 h	4.4 ± 0.4	3.7 ± 0.4	3.5 ± 0.4	2.8 ± 0.4
3 x 100 µg/cm²	8 h	4.5 ± 0.5	3.4 ± 0.5	3.3 ± 0.5	2.2 ± 0.5
3 x 100 µg/cm²	24 h	3.6 ± 0.4	2.5 ± 0.4	3.1 ± 0.4	2.0 ± 0.4
Pair Differences		**Confidence Interval 95%**	**p-value (2-sided)**	**Confidence Interval 95%**	**p-value (2-sided)**
	4 h	0.5 to 1.2	0.2	0.6 to 1.4	0.7
	8 h	0.3 to 0.7	0.002 **	0.5 to 1.0	0.06
	24 h	0.5 to 1.7	0.7	0.6 to 1.7	0.9

11.2.8. Experiment 1.2 (Application frequency)- Data single volunteers

Experiment 1.2 - TACA-amount penetrated - All Tapes

Volunteer	Gender	Age [years]	1x300 ug/cm2 4h		1x300ug/cm2 8h		1x300 ug/cm2 24h		2x100 ug/cm2 4h		3x100 ug/cm2 8h		3x100 ug/cm2 24h	
			[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]
1.2.1	f	24	41.7	13.9	17.1	5.7	20.7	6.9	103.0	51.5	38.2	12.7	73.0	24.3
1.2.2	f	25	56.7	18.9	16.3	5.4	15.5	5.2	92.5	46.3	61.2	20.4	40.8	13.6
1.2.3	f	20	73.6	24.5	40.3	13.4	26.8	8.9	79.4	39.7	134.8	44.9	38.4	12.8
1.2.4	m	26	21.5	7.2	47.7	15.9	38.2	12.7	57.4	28.7	85.0	28.3	50.6	16.9
1.2.5	m	23	87.9	29.3	33.4	11.1	26.6	8.9	85.7	42.8	91.4	30.5	18.4	6.1
1.2.6	m	29	80.3	26.8	30.8	10.3	31.8	10.6	104.4	52.2	170.2	56.7	38.0	12.7
1.2.7	m	44	74.6	24.9	110.2	36.7	99.5	33.2	42.1	21.0	128.8	42.9	18.1	6.0
1.2.8	f	21	67.3	22.4	36.7	12.3	20.6	6.9	120.9	60.5	55.3	18.4	40.7	13.6
1.2.9	f	27	49.4	16.5	26.3	8.8	18.0	6.0	114.0	57.0	150.7	50.2	26.3	8.8
1.2.10	m	21	98.2	32.7	77.0	25.7	95.1	31.7	40.2	20.1	64.9	21.6	34.4	11.5
Mean		26	65.1	21.7	43.6	14.5	39.3	13.1	84.0	42.0	98.0	32.7	37.9	12.6
SD			23.0	7.7	29.1	9.7	31.3	10.4	28.9	14.5	45.2	15.1	16.1	5.4
RSD			35.4	35.4	66.8	66.8	79.7	79.7	34.5	34.5	46.1	46.1	42.5	42.5

Experiment 1.2 - TACA-amount penetrated - All Tapes, Logarithm (ln)

Volunteer	Gender	Age [years]	1x300 ug/cm2 4h		1x300ug/cm2 8h		1x300 ug/cm2 24h		2x100 ug/cm2 4h		3x100 ug/cm2 8h		3x100 ug/cm2 24h	
			[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]
1.2.1	f	24	3.7	2.6	2.8	1.7	3.0	1.9	4.6	3.9	3.6	2.5	4.3	3.2
1.2.2	f	25	4.0	2.9	2.8	1.7	2.7	1.6	4.5	3.8	4.1	3.0	3.7	2.6
1.2.3	f	20	4.3	3.2	3.7	2.6	3.3	2.2	4.4	3.7	4.9	3.8	3.6	2.6
1.2.4	m	26	3.1	2.0	3.9	2.8	3.6	2.5	4.0	3.4	4.4	3.3	3.9	2.8
1.2.5	m	23	4.5	3.4	3.5	2.4	3.3	2.2	4.5	3.8	4.5	3.4	2.9	1.8
1.2.6	m	29	4.4	3.3	3.4	2.3	3.5	2.4	4.6	4.0	5.1	4.0	3.6	2.5
1.2.7	m	44	4.3	3.2	4.7	3.6	4.6	3.5	3.7	3.0	4.9	3.8	2.9	1.8
1.2.8	f	21	4.2	3.1	3.6	2.5	3.0	1.9	4.8	4.1	4.0	2.9	3.7	2.6
1.2.9	f	27	3.9	2.8	3.3	2.2	2.9	1.8	4.7	4.0	5.0	3.9	3.3	2.2
1.2.10	m	21	4.6	3.5	4.3	3.2	4.6	3.5	3.7	3.0	4.2	3.1	3.5	2.4
Mean		26	4.1	3.0	3.6	2.5	3.5	2.4	4.4	3.7	4.5	3.4	3.6	2.5
SD			0.4	0.4	0.6	0.6	0.6	0.7	0.4	0.4	0.5	0.5	0.4	0.4
RSD			10.9	14.9	16.6	23.9	18.8	27.6	9.2	10.9	11.0	14.6	12.1	17.6

Experiment 1.2 - TACA-amount penetrated - Without Tape1-3

Volunteer	Gender	Age [years]	1x300 ug/cm2 4h		1x300ug/cm2 8h		1x300 ug/cm2 24h		2x100 ug/cm2 4h		3x100 ug/cm2 8h		3x100 ug/cm2 24h	
			[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]
1.2.1	f	24	26.4	8.8	12.2	4.1	16.7	5.6	33.4	16.7	13.9	4.6	35.0	11.7
1.2.2	f	25	35.7	11.9	11.0	3.7	11.0	3.7	41.3	20.7	36.2	12.1	25.1	8.3
1.2.3	f	20	41.7	13.9	25.9	8.6	19.3	6.4	39.7	19.8	66.6	22.2	29.6	9.9
1.2.4	m	26	12.2	4.1	16.6	5.5	26.4	8.8	24.7	12.4	28.8	9.6	32.7	10.9
1.2.5	m	23	54.3	18.1	19.4	6.5	21.8	7.3	40.4	20.2	27.0	9.0	12.3	4.1
1.2.6	m	29	34.2	11.4	17.2	5.7	24.4	8.1	51.4	25.7	33.8	11.3	29.5	9.8
1.2.7	m	44	38.5	12.8	26.7	8.9	48.7	16.2	25.8	12.9	19.3	6.4	10.9	3.6
1.2.8	f	21	24.9	8.3	19.7	6.6	11.3	3.8	39.9	19.9	31.4	10.5	22.1	7.4
1.2.9	f	27	23.8	7.9	11.9	4.0	11.9	4.0	40.5	20.2	18.9	6.3	15.3	5.1
1.2.10	m	21	39.9	13.3	34.3	11.4	48.2	16.1	14.2	7.1	17.1	5.7	16.3	5.4
Mean		26	33.2	11.1	19.5	6.5	24.0	8.0	35.1	17.6	29.3	9.8	22.9	7.6
SD			11.7	3.9	7.5	2.5	14.0	4.7	10.8	5.4	15.1	5.0	8.8	2.9
RSD			35.4	35.4	38.5	38.5	58.3	58.3	30.6	30.6	51.6	51.7	38.4	38.4

Experiment 1.2 - TACA-amount penetrated - Without Tape1-3, Logarithm (ln)

Volunteer	Gender	Age [years]	1x300 ug/cm2 4h		1x300ug/cm2 8h		1x300 ug/cm2 24h		2x100 ug/cm2 4h		3x100 ug/cm2 8h		3x100 ug/cm2 24h	
			[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]	[ug/cm2]	[%]
1.2.1	f	24	3.3	2.2	2.5	1.4	2.8	1.7	3.5	2.8	2.6	1.5	3.6	2.5
1.2.2	f	25	3.6	2.5	2.4	1.3	2.4	1.3	3.7	3.0	3.6	2.5	3.2	2.1
1.2.3	f	20	3.7	2.6	3.3	2.2	3.0	1.9	3.7	3.0	4.2	3.1	3.4	2.3
1.2.4	m	26	2.5	1.4	2.8	1.7	3.3	2.2	3.2	2.5	3.4	2.3	3.5	2.4
1.2.5	m	23	4.0	2.9	3.0	1.9	3.1	2.0	3.7	3.0	3.3	2.2	2.5	1.4
1.2.6	m	29	3.5	2.4	2.8	1.7	3.2	2.1	3.9	3.2	3.5	2.4	3.4	2.3
1.2.7	m	44	3.7	2.6	3.3	2.2	3.9	2.8	3.2	2.6	3.0	1.9	2.4	1.3
1.2.8	f	21	3.2	2.1	3.0	1.9	2.4	1.3	3.7	3.0	3.4	2.3	3.1	2.0
1.2.9	f	27	3.2	2.1	2.5	1.4	2.5	1.4	3.7	3.0	2.9	1.8	2.7	1.6
1.2.10	m	21	3.7	2.6	3.5	2.4	3.9	2.8	2.7	2.0	2.8	1.7	2.8	1.7
Mean		26	3.4	2.3	2.9	1.8	3.0	1.9	3.5	2.8	3.3	2.2	3.1	2.0
SD			0.4	0.4	0.4	0.4	0.5	0.5	0.4	0.4	0.5	0.5	0.4	0.4
RSD			12.1	17.8	13.0	20.9	17.9	28.0	10.7	13.3	13.9	20.9	13.8	21.6

11.3. Project III

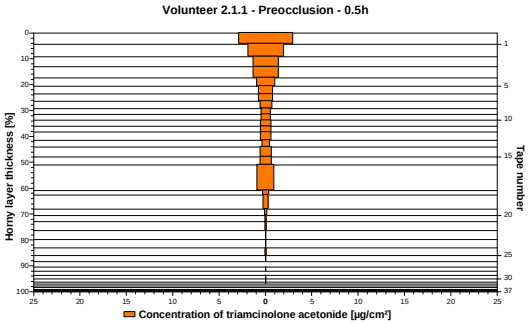
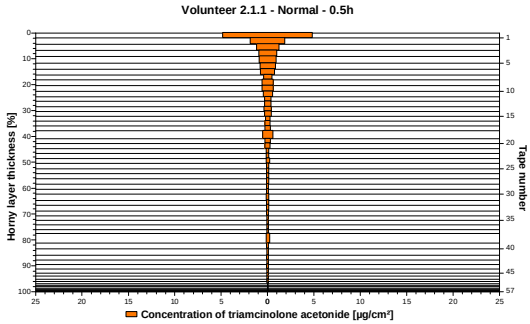
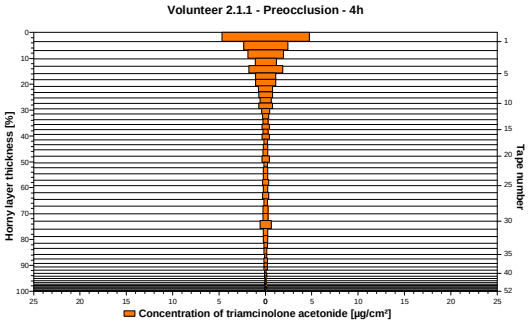
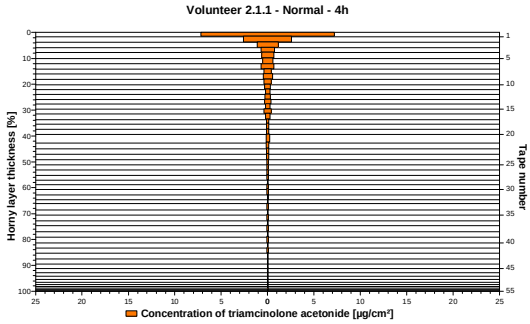
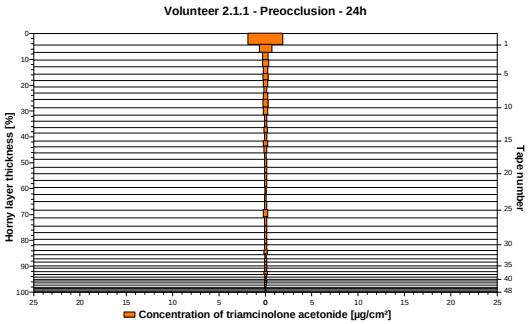
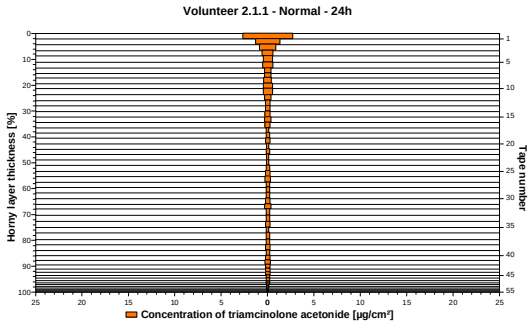
11.3.1. Overview of application and tape stripping procedure

						0 h	4 h	24 h
EXPERIMENT 1								
ARM 1	Site 1	Pre-Occlusion 16h	1 x 100 µg/cm^2	-		D T		
	Site 2	Pre-Occlusion 16h	1 x 100 µg/cm^2	-		D	T	
	Site 3	Pre-Occlusion 16h	1 x 100 µg/cm^2	-		D		T
ARM 2	Site 4	-	1 x 100 µg/cm^2	-		D T		
	Site 5	-	1 x 100 µg/cm^2	-		D	T	
	Site 6	-	1 x 100 µg/cm^2	-		D		T
EXPERIMENT 2								
ARM 1	Site 1	-	1 x 100 µg/cm^2	Post-Occlusion until T		D	T	
	Site 2	-	1 x 100 µg/cm^2	Post-Occlusion until T		D		T
ARM 2	Site 3	-	1 x 100 µg/cm^2	-		D	T	
	Site 4	-	1 x 100 µg/cm^2	-		D		T
						9 a.m	1 p.m	9 a.m.

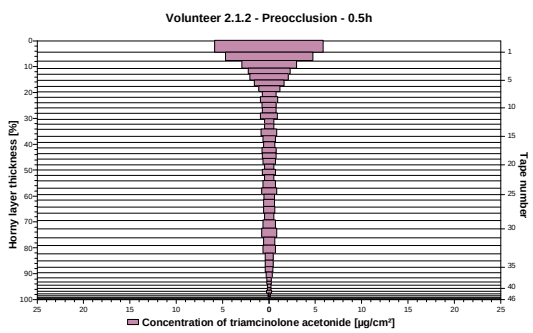
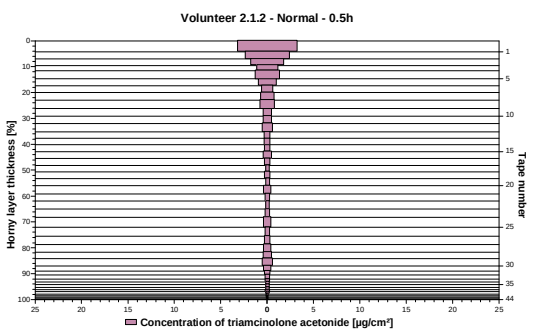
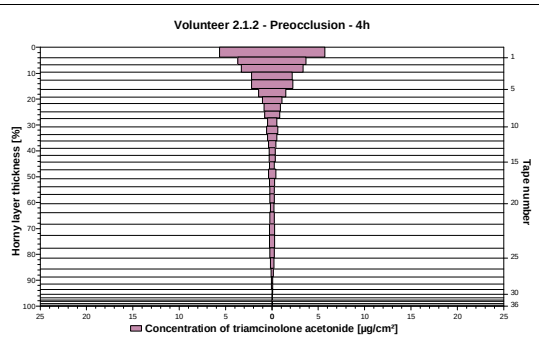
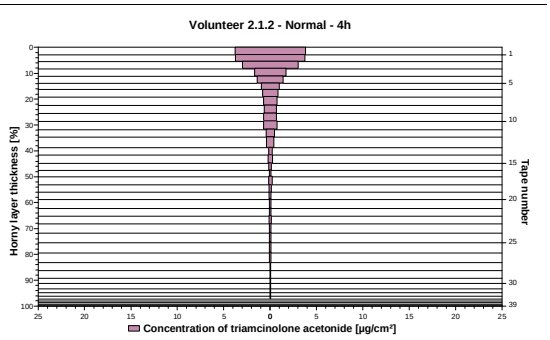
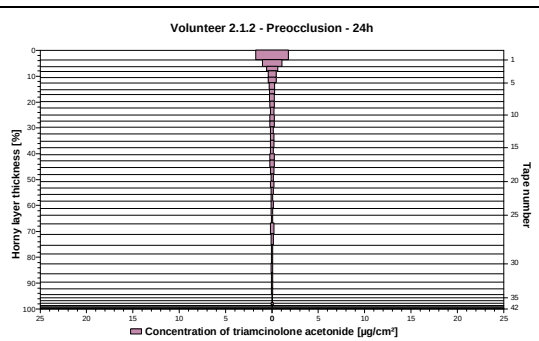
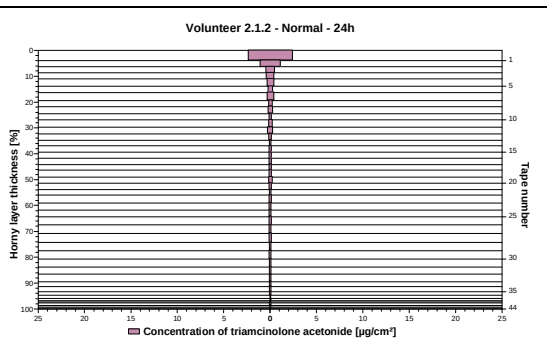
D = Dosing (application of the formulation), T = Tape Stripping (skin sampling). When tape stripping followed just after dosing (D T), a time span of 0.5 h was maintained between dosing and tape stripping to ensure an initial penetration of TACA into the stratum corneum.

11.3.2. Experiment 2.1 (Pre-occlusion) - Penetration profiles

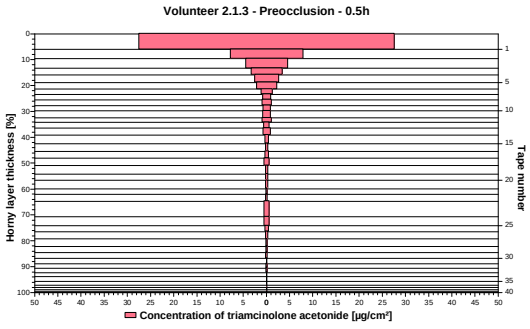
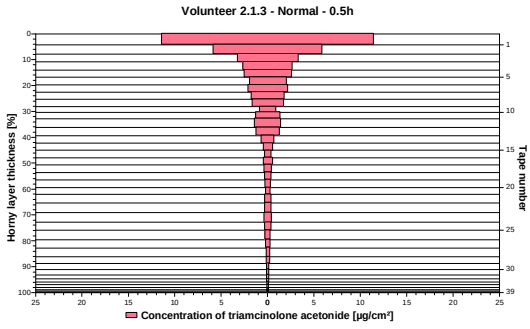
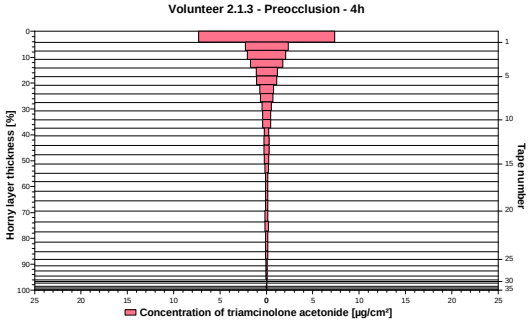
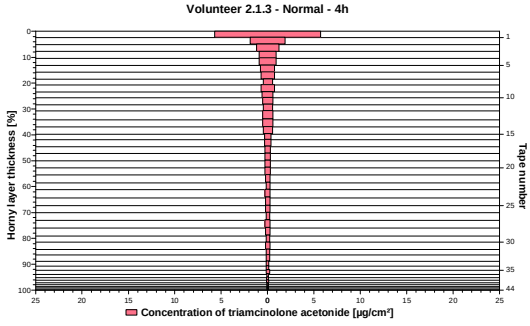
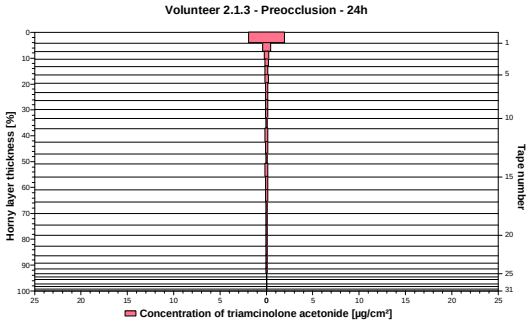
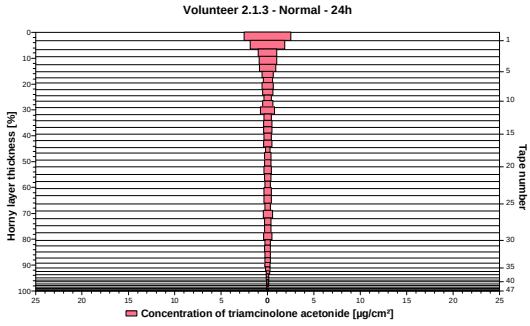
Volunteer 2.1.1

	Pre-occlusion 1x100µg/cm^2	Normal 1x100µg/cm^2
0.5h		
4h		
24h		

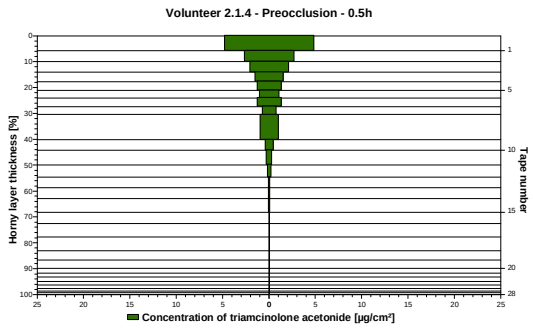
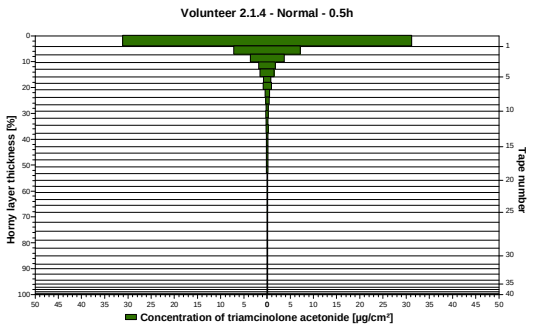
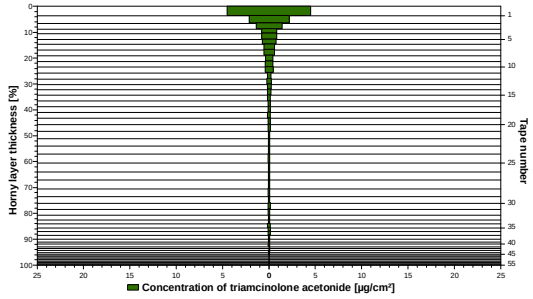
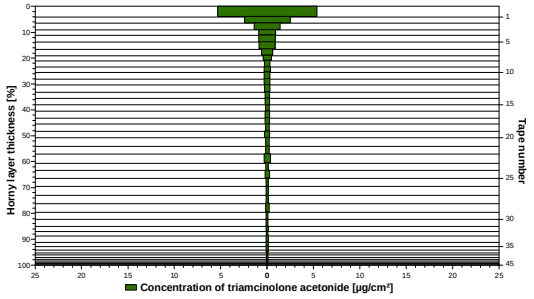
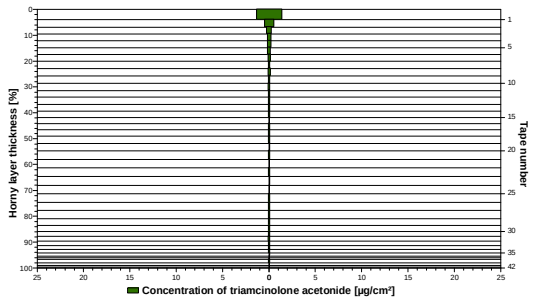
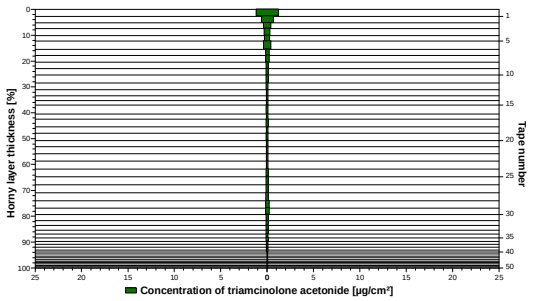
Volunteer 2.1.2

	Pre-occlusion 1x100µg/cm^2	Normal 1x100µg/cm^2
0.5h		
4h		
24h		

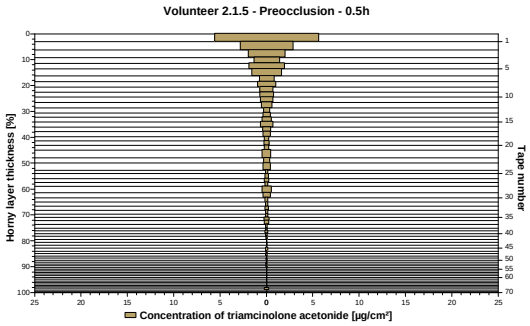
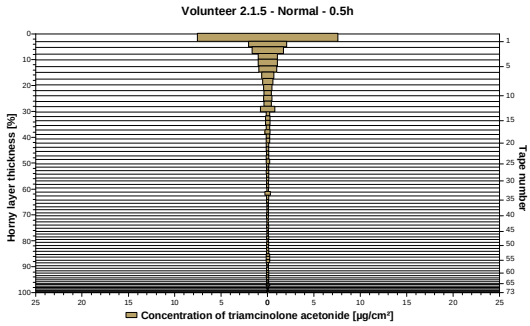
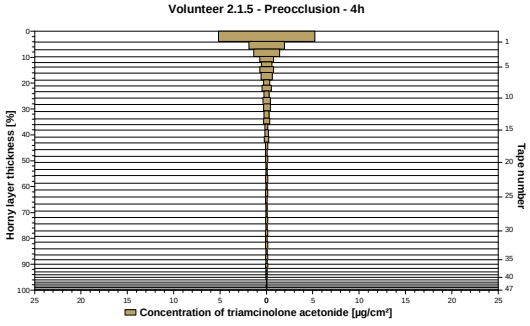
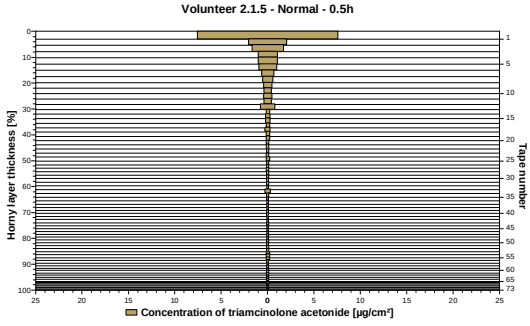
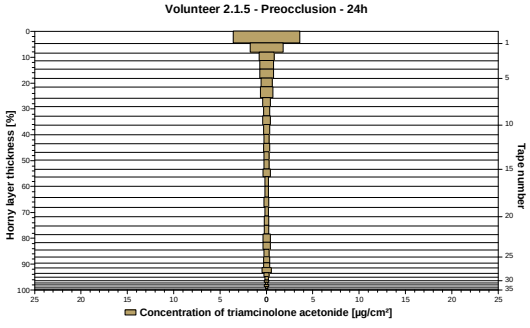
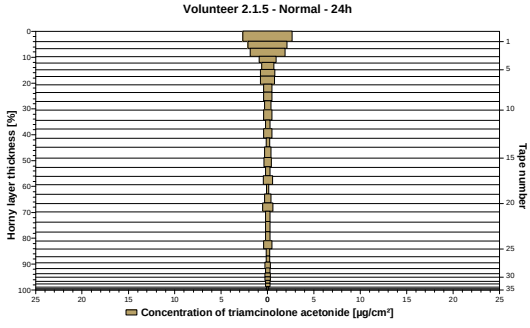
Volunteer 2.1.3

	Pre-occlusion 1x100µg/cm^2	Normal 1x100µg/cm^2
0.5h		
4h		
24h		

Volunteer 2.1.4

	Pre-occlusion 1x100µg/cm^2	Normal 1x100µg/cm^2
0.5h		
4h		
24h		

Volunteer 2.1.5

	Pre-occlusion 1x100µg/cm^2	Normal 1x100µg/cm^2
0.5h		
4h		
24h		

11.3.3. Experiment 2.1 (Pre-occlusion) - Data overview

Exp 2.1 (n=5), Application of 1x100µg/cm^2

Concentration	Tape Stripping	TACA-amount all tapes	TACA-amount without tape 1-3
ORIGINAL VALUES		**Mean ± SD**	**Mean ± SD**
Preocclusion	0.5 h	67 ± 37	35 ± 16
Preocclusion	4 h	43 ± 10	24 ± 7.6
Preocclusion	24 h	18 ± 10	12 ± 6.6
Normal	0.5 h	66 ± 28	30 ± 12
Normal	4 h	48 ± 17	25 ± 8.1
Normal	24 h	29 ± 12	20 ± 9.3
LN-VALUES		**Mean ± SD**	**Mean ± SD**
Preocclusion	0.5 h	4.1 ± 0.6	3.5 ± 0.5
Preocclusion	4 h	3.8 ± 0.2	3.1 ± 0.3
Preocclusion	24 h	2.8 ± 0.5	2.3 ± 0.6
Normal	0.5 h	4.1 ± 0.4	3.4 ± 0.4
Normal	4 h	3.8 ± 0.3	3.2 ± 0.3
Normal	24 h	3.3 ± 0.5	2.9 ± 0.5
ANOVA (ln-values)		**All tapes**	**Without Tapes 1-3**
Factor:			
- volunteer		0.3	0.08
- time		0.0001 **	0.0008 **
- application		0.2	0.2
- Interaction time/application		0.4	0.1

11.3.4. Experiment 2.1 (Pre-occlusion) - Data single volunteers

Experiment 2.1 - TACA-amount penetrated - All Tapes [ug/cm^2]

			Preocclusion - 1x100ug/cm^2			Normal - 1x100ug/cm^2		
Volunteer	Gender	Age [years]	Tape Stripping at 0.5h	4h	24h	Tape Stripping at 0.5h	4h	24h
2.1.1	f	26	34.02	51.89	19.19	40.57	38.95	35.22
2.1.2	f	24	82.83	54.51	18.51	45.96	41.90	18.60
2.1.3	f	24	122.16	43.07	10.50	89.70	44.86	41.97
2.1.4	m	29	36.09	34.11	10.12	101.27	38.49	14.94
2.1.5	m	24	58.99	33.71	33.64	51.79	78.03	36.54
Mean		25	66.82	43.46	18.39	65.86	48.45	29.46
SD			36.74	9.69	9.54	27.64	16.74	11.92
RSD			54.99	22.30	51.85	41.97	34.54	40.47

Experiment 2.1 - TACA-amount penetrated - All Tapes [ug/cm^2], Logarithm (ln)

			Preocclusion - 1x100ug/cm^2			Normal - 1x100ug/cm^2		
Volunteer	Gender	Age [years]	Tape Stripping at 0.5h	4h	24h	Tape Stripping at 0.5h	4h	24h
2.1.1	f	26	3.53	3.95	2.95	3.70	3.66	3.56
2.1.2	f	24	4.42	4.00	2.92	3.83	3.74	2.92
2.1.3	f	24	4.81	3.76	2.35	4.50	3.80	3.74
2.1.4	m	29	3.59	3.53	2.31	4.62	3.65	2.70
2.1.5	m	24	4.08	3.52	3.52	3.95	4.36	3.60
Mean		25	4.08	3.75	2.81	4.12	3.84	3.30
SD			0.55	0.23	0.50	0.41	0.29	0.46
RSD			13.36	6.02	17.67	10.00	7.67	13.91

Experiment 2.1 - TACA-amount penetrated - Without Tape 1-3 [ug/cm^2]

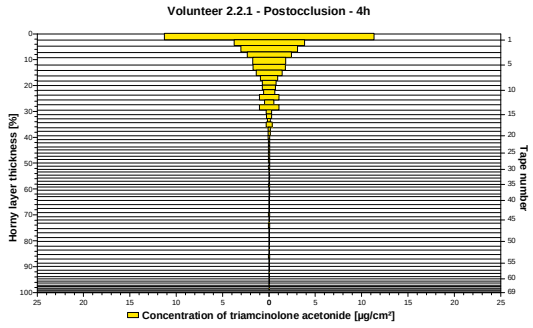
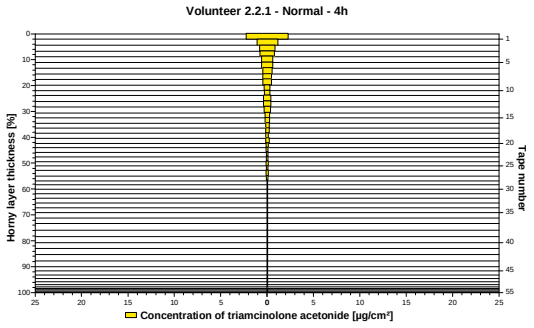
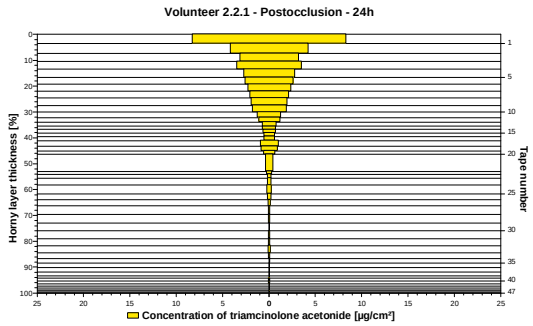
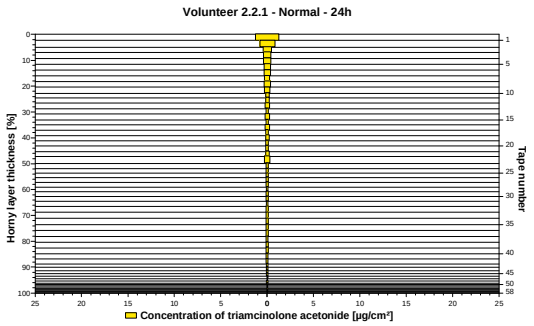
			Preocclusion - 1x100ug/cm^2			Normal - 1x100ug/cm^2		
Volunteer	Gender	Age [years]	Tape Stripping at 0.5h	4h	24h	Tape Stripping at 0.5h	4h	24h
2.1.1	f	26	21.75	33.96	13.53	24.70	17.13	25.52
2.1.2	f	24	55.79	29.22	11.71	31.19	20.69	10.71
2.1.3	f	24	42.44	19.67	5.17	48.63	27.27	31.21
2.1.4	m	29	16.91	17.92	5.85	17.42	20.04	10.49
2.1.5	m	24	38.09	16.82	21.34	29.17	37.40	23.26
Mean		25	35.00	23.52	11.52	30.22	24.51	20.24
SD			15.81	7.62	6.58	11.57	8.11	9.27
RSD			45.18	32.41	57.09	38.28	33.08	45.78

Experiment 2.1 - TACA-amount penetrated - Without Tape 1-3 [ug/cm^2], Logarithm (ln)

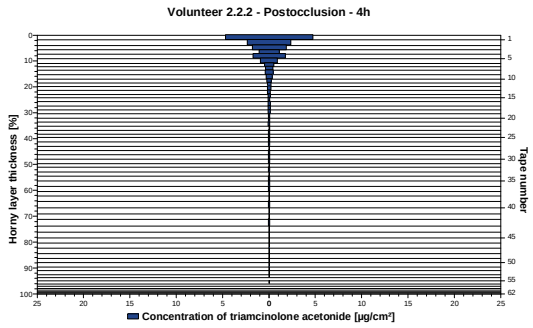
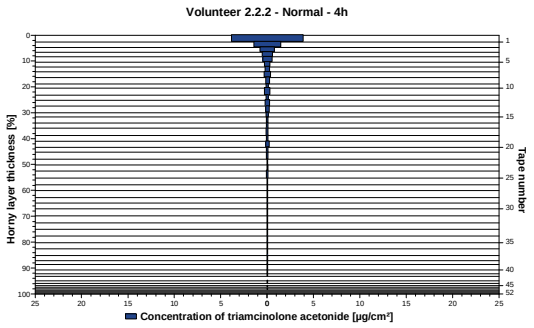
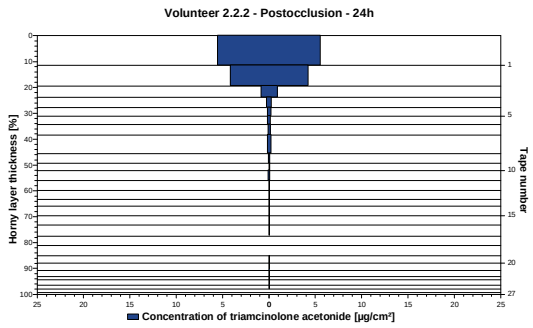
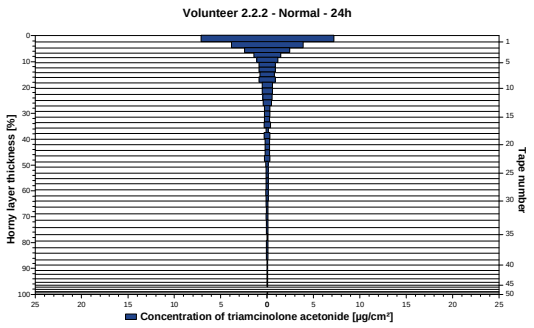
			Preocclusion - 1x100ug/cm^2			Normal - 1x100ug/cm^2		
Volunteer	Gender	Age [years]	Tape Stripping at 0.5h	4h	24h	Tape Stripping at 0.5h	4h	24h
2.1.1	f	26	3.08	3.53	2.60	3.21	2.84	3.24
2.1.2	f	24	4.02	3.37	2.46	3.44	3.03	2.37
2.1.3	f	24	3.75	2.98	1.64	3.88	3.31	3.44
2.1.4	m	29	2.83	2.89	1.77	2.86	3.00	2.35
2.1.5	m	24	3.64	2.82	3.06	3.37	3.62	3.15
Mean		25	3.46	3.12	2.31	3.35	3.16	2.91
SD			0.49	0.31	0.59	0.37	0.31	0.51
RSD			14.25	10.04	25.77	11.13	9.75	17.61

11.3.5. Experiment 2.2 (Post-occlusion) – Penetration profiles

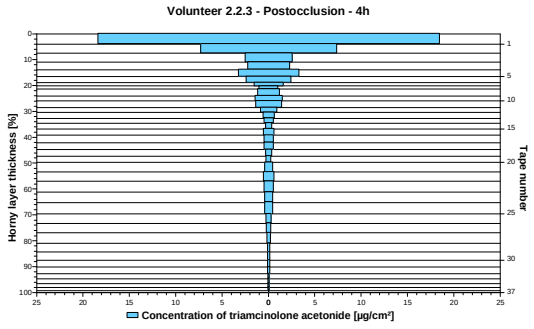
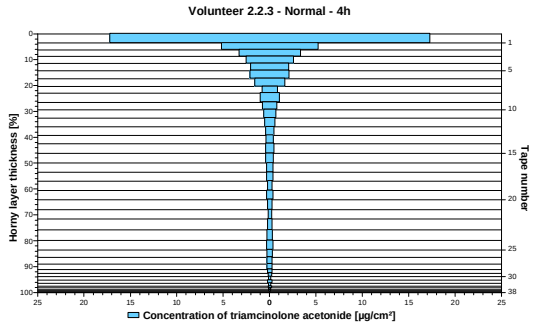
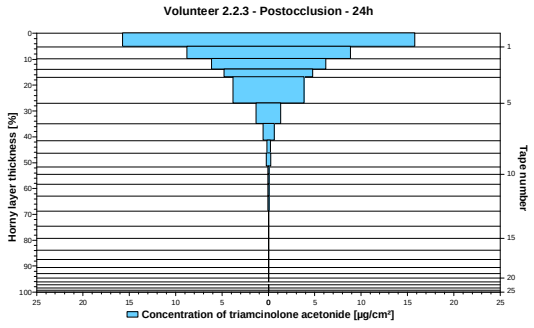
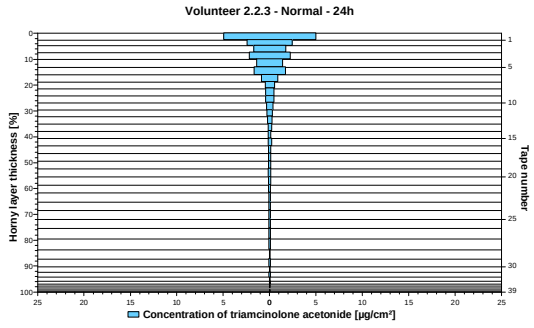
Volunteer 2.2.1

	Post-occlusion 1x100µg/cm²	Normal 1x100µg/cm²
4h		
24h		

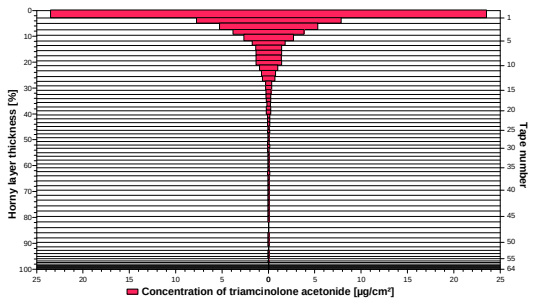
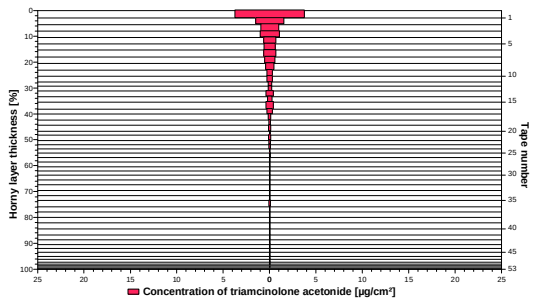
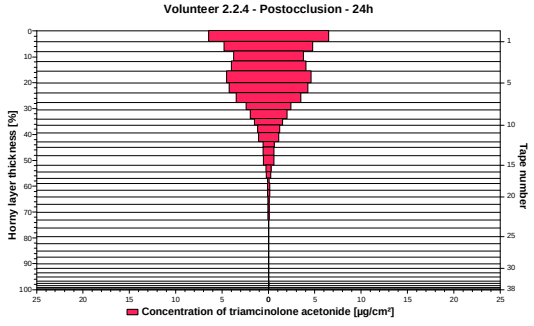
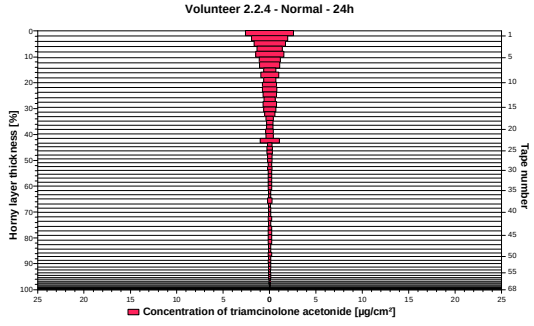
Volunteer 2.2.2

	Post-occlusion 1x100µg/cm²	Normal 1x100µg/cm²
4h		
24h		

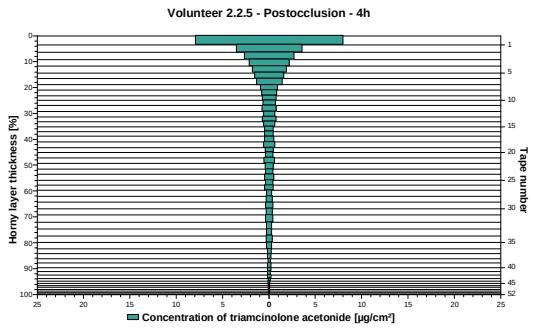
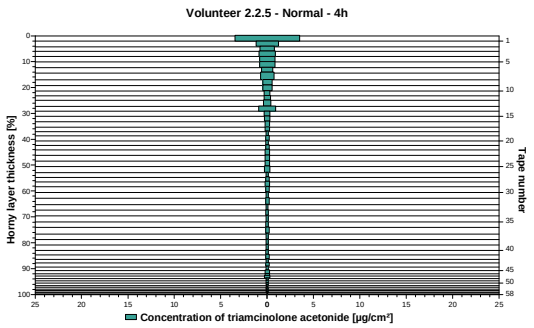
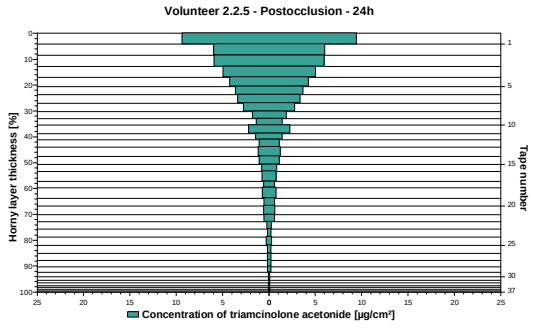
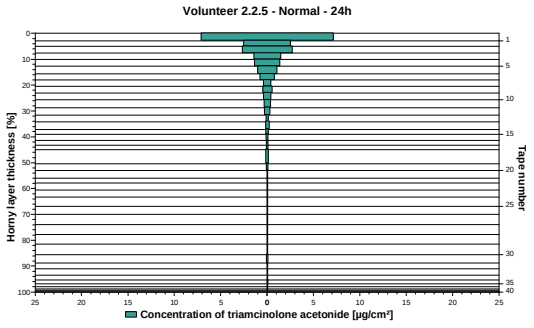
Volunteer 2.2.3

	Post-occlusion 1x100µg/cm^2	Normal 1x100µg/cm^2
4h		
24h		

Volunteer 2.2.4

	Post-occlusion 1x100µg/cm^2	Normal 1x100µg/cm^2
4h		
24h		

Volunteer 2.2.5

	Post-occlusion 1x100µg/cm^2	Normal 1x100µg/cm^2
4h		
24h		

11.3.6. Experiment 2.2 (Post-occlusion) - Data overview

Exp 2.2 (n=5), Application of 1x100µg/cm^2

Concentration	Tape Stripping	TACA-amount all tapes	TACA-amount without tape 1-3
ORIGINAL VALUES		**Mean ± SD**	**Mean ± SD**
Post-occlusion	4 h	80 ± 32	37 ± 13
Post-occlusion	24 h	79 ± 33	42 ± 28
Normal	4 h	40 ± 28	21 ± 12
Normal	24 h	42 ± 14	24 ± 11
LN-VALUES		**Mean ± SD**	**Mean ± SD**
Post-occlusion	4 h	4.3 ± 0.5	3.6 ± 0.4
Post-occlusion	24 h	4.3 ± 0.6	3.3 ± 1.3
Normal	4 h	3.6 ± 0.6	2.9 ± 0.6
Normal	24 h	3.7 ± 0.4	3.1 ± 0.4
ANOVA (ln-values)		**All tapes**	**Without Tapes 1-3**
Factor:			
- volunteer		0.08	0.1
- time		0.8	0.9
- application		0.005 **	0.2
- Interaction time/application		0.7	0.5

11.3.7. Experiment 2.2 (Post-occlusion) - Data single volunteers

Experiment 2.2 - TACA-amount penetrated [ug/cm2] - All Tapes

Volunteer	Gender	Age [years]	Post-Occlusion Tape Stripping at 4h	Post-Occlusion Tape Stripping at 24h	Normal Tape Stripping at 4h	Normal Tape Stripping at 24h
2.2.1	f	25	68.6	86.8	24.1	21.0
2.2.2	m	30	34.8	24.1	21.1	52.3
2.2.3	f	23	102.5	84.6	89.0	39.6
2.2.4	f	24	116.3	85.8	28.3	55.8
2.2.5	m	24	75.6	114.5	39.7	41.0
Mean		25	79.6	79.2	40.4	41.9
SD			31.7	33.2	28.1	13.7
RSD			39.8	42.0	69.4	32.6

Experiment 2.2 - TACA-amount penetrated [ug/cm2] - All Tapes - Logarithm (ln)

Volunteer	Gender	Age [years]	Post-Occlusion Tape Stripping at 4h	Post-Occlusion Tape Stripping at 24h	Normal Tape Stripping at 4h	Normal Tape Stripping at 24h
2.2.1	f	25	4.2	4.5	3.2	3.0
2.2.2	m	30	3.5	3.2	3.1	4.0
2.2.3	f	23	4.6	4.4	4.5	3.7
2.2.4	f	24	4.8	4.5	3.3	4.0
2.2.5	m	24	4.3	4.7	3.7	3.7
Mean		25	4.3	4.3	3.5	3.7
SD			0.5	0.6	0.6	0.4
RSD			11.0	14.4	16.2	10.5

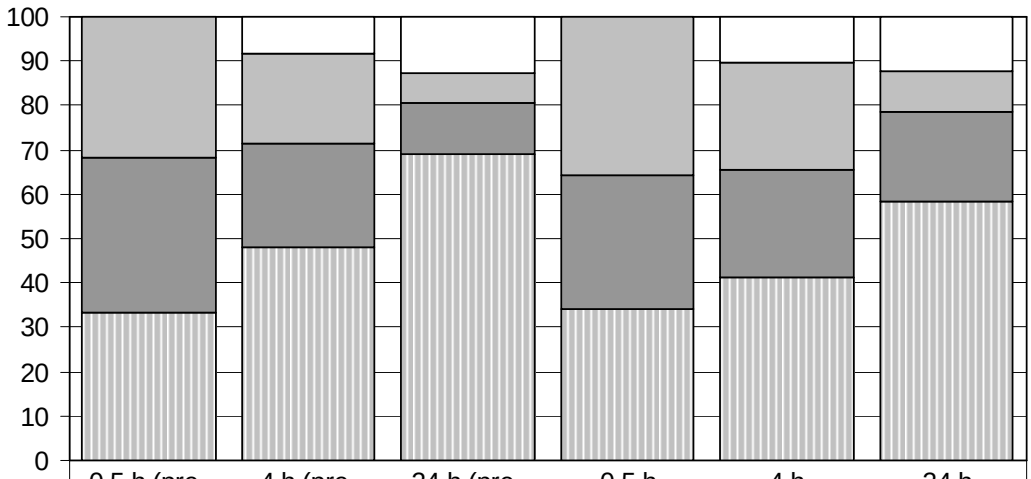
Experiment 2.2 - TACA-amount penetrated [ug/cm2] - Without Tapes 1-3

Volunteer	Gender	Age [years]	Post-Occlusion Tape Stripping at 4h	Post-Occlusion Tape Stripping at 24h	Normal Tape Stripping at 4h	Normal Tape Stripping at 24h
2.2.1	f	25	32.3	55.6	15.7	16.0
2.2.2	m	30	17.0	2.9	9.0	25.4
2.2.3	f	23	46.0	23.1	37.6	21.3
2.2.4	f	24	43.0	55.7	15.8	43.4
2.2.5	m	24	47.3	71.9	28.9	16.4
Mean		25	37.1	41.8	21.4	24.5
SD			12.7	28.1	11.6	11.3
RSD			34.2	67.1	54.2	46.0

Experiment 2.2 - TACA-amount penetrated [ug/cm2] - Without Tape 1-3 - Logarithm (ln)

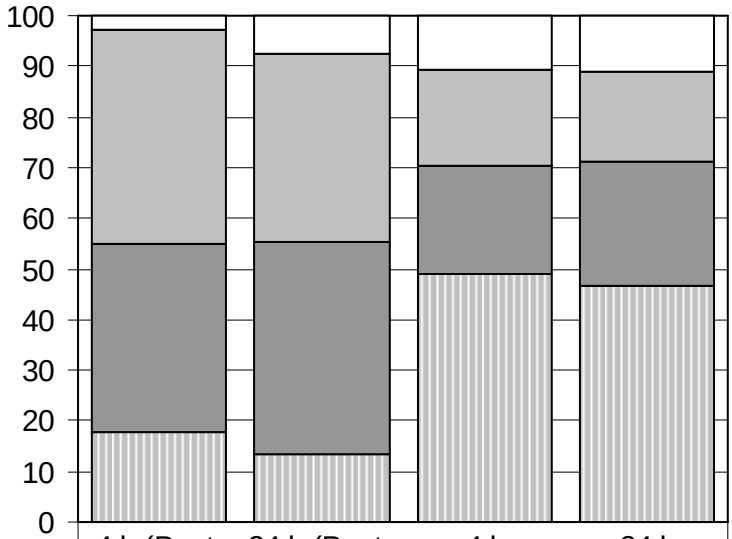
Volunteer	Gender	Age [years]	Post-Occlusion Tape Stripping at 4h	Post-Occlusion Tape Stripping at 24h	Normal Tape Stripping at 4h	Normal Tape Stripping at 24h
2.2.1	f	25	3.5	4.0	2.8	2.8
2.2.2	m	30	2.8	1.0	2.2	3.2
2.2.3	f	23	3.8	3.1	3.6	3.1
2.2.4	f	24	3.8	4.0	2.8	3.8
2.2.5	m	24	3.9	4.3	3.4	2.8
Mean		25	3.6	3.3	2.9	3.1
SD			0.4	1.3	0.6	0.4
RSD			12.0	40.3	19.2	13.1

11.3.8. Mass balance experiment 2.1 (pre-occlusion)



	0.5 h (pre-occlusion)	4 h (pre-occlusion)	24 h (pre-occlusion)	0.5 h (normal)	4 h (normal)	24 h (normal)
Gauze	0	8	13	0	10	12
T 1-3	32	20	7	36	24	9
SC without T 1-3	35	24	12	30	25	20
Not recovered	33	48	69	34	41	58

11.3.9. Mass balance experiment 2.2 (post-occlusion)



	4 h (Post-occlusion)	24 h (Post-occlusion)	4 h (normal)	24 h (normal)
Gauze	3	7	11	11
Tape 1-3	42	37	19	17
SC without T1-3	37	42	21	24
Not recovered	18	14	49	47

11.4. Project IV

11.4.1. Atopic skin diathesis according to Hanifin and Rajka

Erlangter Atopiescore

Nr.	Substanz	Punkte	Patient	Bemerkungen
1	Juckreiz beim Schwitzen	3		
2	Wollunverträglichkeit	3		ev andere Textilien
3	Xerosis	3		≠ Ichtyosis vulgaris
4	Weisser Dermographismus	3		Stirne, Rücken
5	Herthoge Zeichen	2		laterale Augenbrauen
6	Ohrläppchenrhagaden	2		ev retroaurikulär
7	Perlèche, Cheilitis	2		
8	Hyperlineare Palmae	2		Thenar/Hypothenar
9	Pityriasis alba	2		Gesicht/Oberarme
10	Atopische Familienanamnese	1		Ekzema flexuarum
11	Milchschorf	1		exsudativ-ekzematös
12	Allergische Rhinokonjunktivitis	1		Eigenanamnese
13	Allergisches Athma	1		Eigenanamnese
14	Nahrunsgmittelintoleranz	1		Exacerbation des Ekzemes
15	Metallunverträglichkeit	1		Kontaktallergie (Nickel)
16	Lichtempfindlichkeit/scheu	1		Photophobie
17	Zentrofaciale Blässe/Wangenerythem	1		
18	Dennei Morgan Falte	1		Unterlid, ev verdoppelt
19	Keratosis pilaris	1		Extremitäten
20	Gesamt-IgE > 150 kU/l	1		> 400 kU/l = 2 Punkte
21	Phadiatop (SX-1) positiv	1		
Total Patientenscore / Beurteilung				

Bei nicht eindeutigen Kriterien halbe Punktzahl, Laborteste (Punkt 20/21) nur bei Bedarf bestimmen.
Hauptkriterien: Pruritus, typische Morphe und Lokalisation, chronisch(-rezidivieren), atopische EA/FA.
Seltene Nebenkriterien (=1 Punkt): Dishydrosis, „atopic foot“, Mamillenekzem, „dirty neck“, Orbita-verschattung.

Punkte	Beureteilung	Patient
0-6:	keine atopische Hautdiathese	☐
7-9:	Grenzbefund (atopische Hautdiathese nicht auszuschliessen)	☐
10-14:	atopische Hautdiathese	☐
15-19:	deutliche atopische Hautdiathese	☐
> 19:	ausgeprägte atopische Hautdiathese	☐

Nach Th. Diepgen et al. 1991/1992 Bircher 1/93

11.4.2. Form for SCORAD assessment

SCORAD Europäische Experten-Gruppe für Atopische Dermatitis

Patient: Name/Vorname

Geburtsdatum

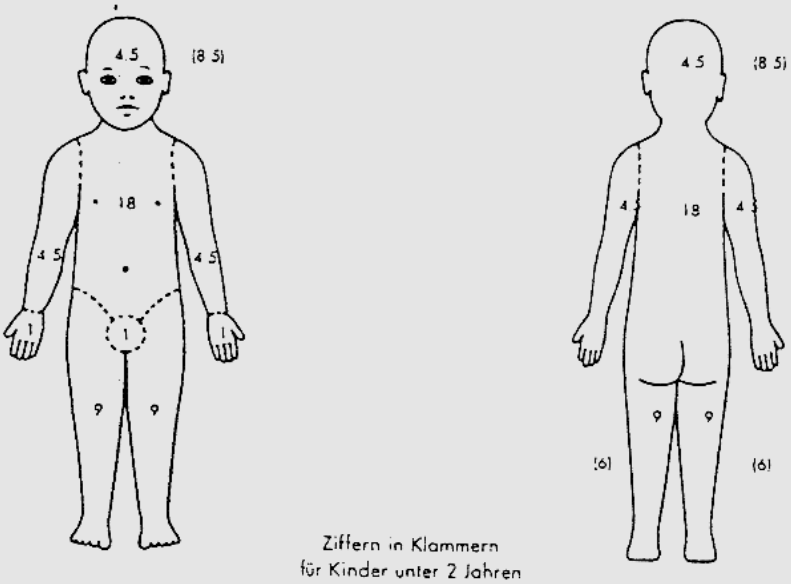
Besuchsdatum

Eingesetztes topisches Steroid

Wirkstoff (Handelsname, Konzentration)

Menge/Monat

Anzahl der Erytheme/Monat



Ziffern in Klammern
für Kinder unter 2 Jahren

A: Ausmaß

Bitte geben Sie die Summe der betroffenen Hautareale an

B: Intensität

Bemessungswerte

Angaben zur Intensität (üblicherweise typische Stellen)

0 = keine 1 = leicht 2 = mäßig 3 = stark

Kriterien	*Intensität*	*Kriterien*	*Intensität*
Erythem		Exkoriation	
Ödem/Papelbildung		Lichenifikation	
Nässen/Krustenbildung		Trockenheit	

Die Hauttrockenheit wird an nicht betroffenen Stellen bewertet

C: Subjektive Symptome

Pruritus und Schlaflosigkeit

SCORAD A/5+7B/2+C

Visuelle Analog-Skala (Durchschnitt für die letzten drei Tage oder Nächte)

Pruritus (0–10)

0 10

Schlaflosigkeit (0–10)

0 10

Behandlung

Anmerkungen

11.4.3. Patch test

Standard Patch Test (True-Test)

No	Ingredient	Conc. [µg/cm^2]	Vehicle	48h	72h	…h	Relevance
1	Nickel(II)-sulfat	200	Aqua				
2	Wollwachsalkohole	1000	Ethanol				
3	Neomycinsulfat	230	Aceton				
4	Kaliumdichromat	23	MethylenChlorid				
5	Cain-Mix	630	NaBicarbonat				
6	Parfum-Mix	430	NaCarbonat				
7	Kolophonium	850	Polyvidon				
8	Epoxidharz	50	Propylcellulose				
9	Quinolin-Mix	190	Methylcellulose				
10	Perubalsam	800	β-Cyclodextrin				
11	Ethylendiamindihydrochlorid	50	β-Cyclodextrin				
12	Kobalt(II)-chlorid	20	β-Cyclodextrin				
13	p-Butylphenol / Formaldehydharz	40	β-Cyclodextrin				
14	Paraben-Mix	1000	β-Cyclodextrin				
15	Gummi-Mix	250	β-Cyclodextrin				
16	Schwarzgummi-Mix	75	β-Cyclodextrin				
17	Chlormethylisothiazolon Kathon	4	β-Cyclodextrin				
18	Quaternium 15	100	β-Cyclodextrin				
19	Mercaptobenzotriazol	75	β-Cyclodextrin				
20	p-Phenyldiamin	90	β-Cyclodextrin				
21	Formaldehyd	180	β-Cyclodextrin				
22	Mercapto-Mix	75	β-Cyclodextrin				
23	Thiomersal	8	β-Cyclodextrin				
24	Thiuram-Mix	25	β-Cyclodextrin				

Standard Additives / M1

No	Ingredient	Conc. [%]	Vehicle	48h	72h	…h	Relevance
1	Euxyl K 400	0.1	Vaseline				
2	Terpentin	10	Vaseline				
3	Cetylstearylalkohol	20	Vaseline				
4	Bufexamac	5.0	Vaseline				
5	Kompositen-Mix	6.0	Vaseline				
6	Lyral	5.0	Vaseline				
7	Propolis	10	Vaseline				
8	Natriumdisulfit (A)	5.0	Vaseline				
9	Tixocortolpivalat (CT)	1.0	Vaseline				
10	Budesonid	0.1	Vaseline				

Standard M1							
1	Sorbitansesquioleat	20	Vaseline				
2	Iodpropinylbutylcarbamat	0.3	Vaseline				
3	Dispersionsmix Blau 124/106	1.0	Vaseline				

Patch Test Lichtena Ingredients

No	Ingredient	Conc. [%]	Vehicle	48h	72h	…h	Relevance
1	AC Glycyrrhetic Fitosoma	1	petrolatum				
2	Glycerrhetinic acid	1	petrolatum				
3	Alpha Bisabolol nat.	5	petrolatum				
4	Allantoin	5	petrolatum				
5	Allantoin	0.5	water				
6	Apifil	10	petrolatum				
7	AR-GB 11	1	petrolatum				
8	Ascofluile Palmitate	1	petrolatum				
9	Beta-sitosterol	1	petrolatum				
10	Shea butter	1	petrolatum				
11	Carbopol Ultrez 10	10	petrolatum				
12	Delta-Tocopherol	1	petrolatum				
13	EDTA 2Na	1	petrolatum				
14	Fudji Perfume	1	petrolatum				
15	Hydroviton	1	petrolatum				
16	Hydroviton	5	petrolatum				
17	Isostearyl isostearat	1	water				
18	Labrafac CC	1	petrolatum				
19	Neo Heliopan 357	2	petrolatum				
20	Neo Heliopan AV	1	petrolatum				
21	Chamomile oil	2.5	petrolatum				
22	Paracombin (methyl-,buthyl-, ethyl-, propyl-paraben-mix)	3	petrolatum				
23	Sodium dehydroacetate	3	water				
24	Triethanolamine	2.5	petrolatum				

11.4.4. Data individual patients

PATIENTENBOGEN KLINISCHE STUDIE LICHTENA A.I.

Verum links appliziert

Patient Nr 1

Geschlecht w

Geburtsdatum 1961

Lokalisation Ellenbeugen

Atopiescore 18

	Untersuchung Nr **Tag** **Datum**	**1** **Voruntersuchung** 05.02.2002		**2** **Tag 0** 12.02.2002		**3** **Tag 7** 19.02.2002		**4** **Tag 14** 26.02.2002		**5** **Tag 28** 12.03.2002		**6** **Tag** ___ _____	
A	**Ausmass**	9	9	9	9	9	9	9	9	9	9		
B	**Intensität**	8	8	9	9	6	13	4	10	4	15		
	Seite	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
	Erythem	1	1	2	2	1	3	0	2	0	3		
	Ödem/Papel	1	1	1	1	1	3	1	3	1	3		
	Nässen/Kruste	0	0	0	0	0	0	0	0	0	1		
	Exkoriation	1	1	1	1	1	2	1	1	0	2		
	Lichenifikation	2	2	2	2	1	2	0	1	1	3		
	Trockenheit	3	3	3	3	2	3	2	3	2	3		
C	**Subjektive Symptome**	3	3	4	4	1	5	1	4	6	12		
	Pruritus (VAS)	3	3	4	4	1	5	1	4	1	7		
	Schlaflosigkeit (VAS)	0	0	0	0	0	0	0	0	5	5		
SCORAD	(A/5) + (7B/2) + C	32.8	32.8	37.3	37.3	23.8	52.3	16.8	40.8	21.8	66.3		
EPI-Teste	Positive												
SCORAD ohne Subjektive Symptome		29.8	29.8	33.3	33.3	22.8	47.3	15.8	36.8	15.8	54.3		

PATIENTENBOGEN KLINISCHE STUDIE LICHTENA A.I.

Verum rechts appliziert

Patient Nr 2

Geschlecht w

Geburtsdatum 1997

Lokalisation Handgelenke

Atopiescore 12

	Untersuchung Nr **Tag** **Datum**	**1** **Voruntersuchung** 06.02.2002		**2** **Tag 0** 13.02.2002		**3** **Tag 7** 20.02.2002		**4** **Tag 14** 27.02.2002		**5** **Tag 28** 13.03.2002		**6** **Tag ___** _____	
A	**Ausmass**	33.5	33.5	51.5	51.5	51.5	51.5	51.5	51.5	51.5	51.5		
B	**Intensität**	9	9	12	12	10	10	12	7	15	9		
	Seite	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
	Erythem	1	1	2	2	2	2	2	1	3	2		
	Ödem/Papel	0	0	1	1	0	0	2	1	2	1		
	Nässen/Kruste	1	1	1	1	0	0	0	0	1	0		
	Exkoriation	1	1	2	2	2	2	2	1	3	1		
	Lichenifikation	3	3	3	3	3	3	3	2	3	2		
	Trockenheit	3	3	3	3	3	3	3	2	3	3		
C	**Subjektive Symptome**	5	5	7	7	5	5	4	3	5	4		
	Pruritus (VAS)	5	5	7	7	5	5	4	3	5	4		
	Schlaflosigkeit (VAS)	0	0	0	0	0	0	0	0	0	0		
SCORAD	(A/5) + (7B/2) + C	43.2	43.2	59.3	59.3	50.3	50.3	56.3	37.8	67.8	45.8		
EPI-Teste	Positive	keine Positive											
SCORAD ohne Subjektive Symptome		38.2	38.2	52.3	52.3	45.3	45.3	52.3	34.8	62.8	41.8		

PATIENTENBOGEN KLINISCHE STUDIE LICHTENA A.I.

Verum rechts appliziert

Patient Nr 3

Geschlecht m

Geburtsdatum 2002

Lokalisation Handgelenke

Atopiescore 21

	Untersuchung Nr **Tag** **Datum**	**1** **Voruntersuchung** 11.02.2002		**2** **Tag 0** 19.02.2002		**3** **Tag 7** 26.02.2002		**4** **Tag 14** 05.03.2002		**5** **Tag 28** 19.03.2002		**6** **Tag ___** _____	
A	**Ausmass**	9	9	9	9	9	9	9	9	9	9		
B	**Intensität**	8	8	10	10	9	9	7	7	6	3		
	Seite	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
	Erythem	0	0	1	1	1	1	1	1	1	0		
	Ödem/Papel	0	0	0	0	0	0	0	0	1	0		
	Nässen/Kruste	0	0	0	0	0	0	0	0	0	0		
	Exkoriation	2	2	3	3	2	2	1	1	1	0		
	Lichenifikation	3	3	3	3	3	3	2	2	1	1		
	Trockenheit	3	3	3	3	3	3	3	3	2	2		
C	**Subjektive Symptome**	2	2	2	2	2	2	2	2	0	0		
	Pruritus (VAS)	2	2	2	2	2	2	2	2	0	0		
	Schlaflosigkeit (VAS)	0	0	0	0	0	0	0	0	0	0		
SCORAD	(A/5) + (7B/2) + C	31.8	31.8	38.8	38.8	35.3	35.3	28.3	28.3	22.8	12.3		
EPI-Teste	Positive	keine Positive											
SCORAD ohne Subjektive Symptome		29.8	29.8	36.8	36.8	33.3	33.3	26.3	26.3	22.8	12.3		

PATIENTENBOGEN KLINISCHE STUDIE LICHTENA A.I.

Verum links appliziert

Patient Nr 4

Geschlecht w

Geburtsdatum 1965

Lokalisation Unterschenkel

Atopiescore 14

	Untersuchung Nr **Tag** **Datum**	**1** **Voruntersuchung** 14.02.2002		**2** **Tag 0** 21.02.2002		**3** **Tag 7** 27.02.2002		**4** **Tag 14** 06.03.2002		**5** **Tag 28** 20.03.2002		**6** **Tag ___** _____	
A	**Ausmass**	27	27	27	27	27	27	27	27	27	27		
B	**Intensität**	10	10	8	8	8	6	7	4	5	2		
	Seite	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
	Erythem	2	2	2	2	2	1	1	0	0	0		
	Ödem/Papel	2	2	1	1	1	1	2	1	1	0		
	Nässen/Kruste	1	1	0	0	0	0	0	0	0	0		
	Exkoriation	2	2	2	2	2	1	2	1	2	1		
	Lichenifikation	1	1	1	1	1	1	0	0	0	0		
	Trockenheit	2	2	2	2	2	2	2	2	2	1		
C	**Subjektive Symptome**	14	14	13	13	12	10	12	10	7	5		
	Pruritus (VAS)	8	8	8	8	8	6	6	4	4	2		
	Schlaflosigkeit (VAS)	6	6	5	5	4	4	6	6	3	3		
SCORAD	(A/5) + (7B/2) + C	54.4	54.4	46.4	46.4	45.4	36.4	41.9	29.4	29.9	17.4		
EPI-Teste	Positive	Nickel(II)sulfat (48h/72h ++)											
SCORAD ohne Subjektive Symptome		40.4	40.4	33.4	33.4	33.4	26.4	29.9	19.4	22.9	12.4		

PATIENTENBOGEN KLINISCHE STUDIE LICHTENA A.I.

Verum links appliziert

Patient Nr 5

Geschlecht w

Geburtsdatum 1988

Lokalisation Unterarme/Ellenbeugen

Atopiescore 20

	Untersuchung Nr **Tag** **Datum**	**1** **Voruntersuchung** 27.02.2002		**2** **Tag 0** 06.03.2002		**3** **Tag 7** 13.03.2002		**4** **Tag 14** 20.03.2002		**5** **Tag 28** 03.04.2002		**6** **Tag ___** _____	
A	**Ausmass**	9	9	9	9	9	9	9	9	9	9		
B	**Intensität**	11	10	13	11	13	12	13	9	13	15		
	Seite	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
	Erythem	2	2	2	1	2	2	2	1	2	3		
	Ödem/Papel	1	1	2	2	2	2	2	1	1	1		
	Nässen/Kruste	0	0	0	0	0	0	1	0	2	2		
	Exkoriation	2	1	3	2	3	2	2	1	3	3		
	Lichenifikation	3	3	3	3	3	3	3	3	3	3		
	Trockenheit	3	3	3	3	3	3	3	3	2	3		
C	**Subjektive Symptome**	9	9	9	9	10	11	13	11	13	15		
	Pruritus (VAS)	4	4	6	6	7	8	9	7	6	8		
	Schlaflosigkeit (VAS)	5	5	3	3	3	3	4	4	7	7		
SCORAD	(A/5) + (7B/2) + C	49.3	45.8	56.3	49.3	57.3	54.8	60.3	44.3	60.3	69.3		
EPI-Teste	Positive	fehlen!											
SCORAD ohne Subjektive Symptome		40.3	36.8	47.3	40.3	47.3	43.8	47.3	33.3	47.3	54.3		

PATIENTENBOGEN KLINISCHE STUDIE LICHTENA A.I.

Verum rechts appliziert

Patient Nr 6

Geschlecht m

Geburtsdatum 1977

Lokalisation Ellenbeugen

Atopiescore 16

	Untersuchung Nr **Tag** **Datum**	**1** **Voruntersuchung** 27.02.2002		**2** **Tag 0** 08.03.2002		**3** **Tag 7** 15.03.2002		**4** **Tag 14** 22.03.2002		**5** **Tag 28** 05.04.2002		**6** **Tag ___** _____	
A	**Ausmass**	9	9	9	9	9	9	9	9	9	9		
B	**Intensität**	8	7	6	7	10	5	11	5	9	2		
	Seite	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
	Erythem	1	1	1	1	2	1	2	1	1	0		
	Ödem/Papel	1	0	1	1	2	1	2	1	1	0		
	Nässen/Kruste	0	0	0	0	0	0	0	0	0	0		
	Exkoriation	1	1	1	2	2	1	2	1	2	0		
	Lichenifikation	2	2	1	1	2	1	3	1	3	1		
	Trockenheit	3	3	2	2	2	1	2	1	2	1		
C	**Subjektive Symptome**	9	9	5	5	6	3	7	2	5	1		
	Pruritus (VAS)	7	7	5	5	6	3	7	2	5	1		
	Schlaflosigkeit (VAS)	2	2	0	0	0	0	0	0	0	0		
SCORAD	(A/5) + (7B/2) + C	38.8	35.3	27.8	31.3	42.8	22.3	47.3	21.3	38.3	9.8		
EPI-Teste	Positive	(EDTA 72h +) (Fudji Parfum 72h +)											
SCORAD ohne Subjektive Symptome		29.8	26.3	22.8	26.3	36.8	19.3	40.3	19.3	33.3	8.8		

PATIENTENBOGEN KLINISCHE STUDIE LICHTENA A.I.

Verum rechts appliziert

Patient Nr 7

Geschlecht w

Geburtsdatum 1974

Lokalisation Ellenbeugen/Unterarme

Atopiescore 23

	Untersuchung Nr **Tag** **Datum**	**1** **Voruntersuchung** 06.03.2002		**2** **Tag 0** 06.03.2002		**3** **Tag 7** 14.03.2002		**4** **Tag 14** 21.03.2002		**5** **Tag 28** 04.04.2002		**6** **Tag** ___ _____	
A	**Ausmass**			40.5	40.5	40.5	40.5	40.5	40.5	40.5	40.5		
B	**Intensität**			14	14	16	11	16	11	11	11		
	Seite	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
	Erythem			3	3	3	2	2	1	2	2		
	Ödem/Papel			2	2	3	2	2	1	1	1		
	Nässen/Kruste			1	1	1	1	3	1	0	0		
	Exkoriation			2	2	3	2	3	2	2	2		
	Lichenifikation			3	3	3	2	3	3	3	3		
	Trockenheit			3	3	3	2	3	3	3	3		
C	**Subjektive Symptome**			12	12	5	5	13	12	9	9		
	Pruritus (VAS)			6	6	5	5	8	7	7	7		
	Schlaflosigkeit (VAS)			6	6	0	0	5	5	2	2		
SCORAD	(A/5) + (7B/2) + C			69.1	69.1	69.1	51.6	77.1	58.6	55.6	55.6		
EPI-Teste	Positive	Lyral 5% (48h +) Fudji Parfum (48h +)		Apifil (48h +)									
SCORAD ohne Subjektive Symptome				57.1	57.1	64.1	46.6	64.1	46.6	46.6	46.6		

PATIENTENBOGEN KLINISCHE STUDIE LICHTENA A.I.

Verum links appliziert

Patient Nr 8

Geschlecht w

Geburtsdatum 1991

Lokalisation Kniekehlen

Atopiescore 7

	Untersuchung Nr **Tag** **Datum**	**1** **Voruntersuchung** 02.04.2002		**2** **Tag 0** 02.04.2002		**3** **Tag 7** 09.04.2002		**4** **Tag 14** 16.04.2002		**5** **Tag 28** 02.05.2002		**6** **Tag ___** _____	
A	**Ausmass**			36	36	36	36	36	36	36	36		
B	**Intensität**			7	7	4	6	4	7	4	12		
	Seite	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
	Erythem			1	1	1	1	1	2	1	3		
	Ödem/Papel			0	0	0	0	0	0	0	1		
	Nässen/Kruste			0	0	0	0	0	0	0	1		
	Exkoriation			1	1	0	0	0	0	0	1		
	Lichenifikation			3	3	2	3	2	3	2	3		
	Trockenheit			2	2	1	2	1	2	1	3		
C	**Subjektive Symptome**			8	8	2	2	2	3	1	3		
	Pruritus (VAS)			6	6	1	1	1	2	1	3		
	Schlaflosigkeit (VAS)			2	2	1	1	1	1	0	0		
SCORAD	(A/5) + (7B/2) + C			39.7	39.7	23.2	30.2	23.2	34.7	22.2	52.2		
EPI-Teste	Positive	Thiuram-Mix (72h +)											
SCORAD ohne Subjektive Symptome				31.7	31.7	21.2	28.2	21.2	31.7	21.2	49.2		

PATIENTENBOGEN KLINISCHE STUDIE LICHTENA A.I.

Verum rechts appliziert

Patient Nr 9

Geschlecht m

Geburtsdatum 1996

Lokalisation Unterarme/Ellenbeugen

Atopiescore 11

	Untersuchung Nr **Tag** **Datum**	**1** **Voruntersuchung** _____		**2** **Tag 0** 09.04.2002		**3** **Tag 7** 16.04.2002		**4** **Tag 14** 23.04.2002		**5** **Tag 28** 07.05.2002		**6** **Tag ___** _____	
A	**Ausmass**			9	9	9	9	9	9	9	9		
B	**Intensität**			5	5	10	4	13	2	5	1		
	Seite	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
	Erythem			1	1	2	1	2	0	0	0		
	Ödem/Papel			0	0	2	0	3	0	0	0		
	Nässen/Kruste			0	0	0	0	1	0	1	0		
	Exkoriation			1	1	2	1	3	0	1	0		
	Lichenifikation			1	1	2	1	2	1	1	0		
	Trockenheit			2	2	2	1	2	1	2	1		
C	**Subjektive Symptome**			6	6	11	6	18	9	13	7		
	Pruritus (VAS)			?	?	5	0	9	0	6	0		
	Schlaflosigkeit (VAS)			6	6	6	6	9	9	7	7		
SCORAD	(A/5) + (7B/2) + C			25.3	25.3	47.8	21.8	65.3	17.8	32.3	12.3		
EPI-Teste	Positive	Keine Positive											
SCORAD ohne Subjektive Symptome		0	0	19.3	19.3	36.8	15.8	47.3	8.8	19.3	5.3		

PATIENTENBOGEN KLINISCHE STUDIE LICHTENA A.I.

Verum links appliziert

Patient Nr 10

Geschlecht w

Geburtsdatum 1976

Lokalisation Unterschenkel

Atopiescore 21

	Untersuchung Nr **Tag** **Datum**	**1** **Voruntersuchung** 12.04.2002		**2** **Tag 0** 12.04.2002		**3** **Tag 7** 19.04.2002		**4** **Tag 14** 26.04.2002		**5** **Tag 28** 17.05.2002		**6** **Tag ___** _____	
A	**Ausmass**			18	18	18	18	18	18	18	18		
B	**Intensität**			7	7	5	9	8	10	5	10		
	Seite	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
	Erythem			1	1	1	2	1	2	1	2		
	Ödem/Papel			1	1	1	2	2	2	0	1		
	Nässen/Kruste			0	0	0	0	1	1	0	0		
	Exkoriation			2	2	1	2	2	2	2	3		
	Lichenifikation			1	1	1	1	1	1	1	2		
	Trockenheit			2	2	1	2	1	2	1	2		
C	**Subjektive Symptome**			2	2	6	6	2	3	0	0		
	Pruritus (VAS)			2	2	2	2	1	2	0	0		
	Schlaflosigkeit (VAS)			0	0	4	4	1	1	0	0		
SCORAD	(A/5) + (7B/2) + C			30.1	30.1	27.1	41.1	33.6	41.6	21.1	38.6		
EPI-Teste	Positive	Sorbitansesquioleat 20% in Vaseline (48h/72h) (Standard M1) Iodpropinbutylcarbamat 0.3% in Vaseline (48h/72h) (Standard M1)											
SCORAD ohne Subjektive Symptome				28.1	28.1	21.1	35.1	31.6	38.6	21.1	38.6		

PATIENTENBOGEN KLINISCHE STUDIE LICHTENA A.I.

Verum rechts appliziert

Patient Nr 12

Geschlecht w

Geburtsdatum 1987

Lokalisation Ellenbeugen

Atopiescore 14

	Untersuchung Nr **Tag** **Datum**	**1** **Voruntersuchung** 24.05.2002		**2** **Tag 0** 24.05.2002		**3** **Tag 7** 31.05.2002		**4** **Tag 14** 10.06.2002		**5** **Tag 28** 21.06.2002		**6** **Tag ___** _____	
A	**Ausmass**			9	9	9	9	9	9	9	9		
B	**Intensität**			10	10	7	8	6	2	5	2		
	Seite	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
	Erythem			1	1	1	1	1	0	0	0		
	Ödem/Papel			1	1	1	1	0	0	1	0		
	Nässen/Kruste			0	0	0	0	0	0	0	0		
	Exkoriation			2	2	1	2	1	0	0	0		
	Lichenifikation			3	3	2	2	2	1	2	1		
	Trockenheit			3	3	2	2	2	1	2	1		
C	**Subjektive Symptome**			6	6	4	3	0	0	4	4		
	Pruritus (VAS)			6	6	4	3	0	0	4	4		
	Schlaflosigkeit (VAS)			0	0	0	0	0	0	0	0		
SCORAD	(A/5) + (7B/2) + C			42.8	42.8	30.3	32.8	22.8	8.8	23.3	12.8		
EPI-Teste	Positive	Sorbitansesquioleat 20% in Vaseline (48h) (Standard M1) Iodpropinbutylcarbamat 0.3% in Vaseline (48h) (Standard M1)											
SCORAD ohne Subjektive Symptome				36.8	36.8	26.3	29.8	22.8	8.8	19.3	8.8		

PATIENTENBOGEN KLINISCHE STUDIE LICHTENA A.I.

Verum rechts appliziert

Patient Nr 13

Geschlecht m

Geburtsdatum 1996

Lokalisation Ellenbogen

Atopiescore 12

	Untersuchung Nr **Tag** **Datum**	**1** **Voruntersuchung** 07.02.2002		**2** **Tag 0** 31.05.2002		**3** **Tag 7** 07.06.2002		**4** **Tag 14** 14.06.2002		**5** **Tag 28** 02.07.2002		**6** **Tag** ___ _____	
A	**Ausmass**			27	27	27	27	27	27	27	27		
B	**Intensität**			8	8	10	5	8	1	2	0		
	Seite	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**	**L**	**R**
	Erythem			1	1	2	1	3	0	0	0		
	Ödem/Papel			2	2	2	1	0	0	0	0		
	Nässen/Kruste			1	1	0	0	2	0	0	0		
	Exkoriation			0	0	2	1	0	0	0	0		
	Lichenifikation			2	2	2	1	1	0	1	0		
	Trockenheit			2	2	2	1	2	1	1	0		
C	**Subjektive Symptome**			7	7	0	0	8	0	0	0		
	Pruritus (VAS)			7	7	0	0	8	0	0	0		
	Schlaflosigkeit (VAS)			0	0	0	0	0	0	0	0		
SCORAD	(A/5) + (7B/2) + C			40.4	40.4	40.4	22.9	41.4	8.9	12.4	5.4		
EPI-Teste	Positive	Keine Positive											
SCORAD ohne Subjektive Symptome				33.4	33.4	40.4	22.9	33.4	8.9	12.4	5.4		

DROP OUTS: Patient Number 13 and 14

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