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A COMPARISON *IN VITRO* STUDY OF PERMEATION OF SELECTED DRUGS FROM LIPOPHILIC SOLUTIONS TROUGH HUMAN SKIN

SROVNÁVACÍ *IN VITRO* STUDIE PERMEACE VYBRANÝCH LÉČIV Z LIPOFILNÍCH ROZTOKŮ PŘES LIDSKOU KŮŽI

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ABSTRACT

The aim of the study was to compare the permeation of testosterone (TST), caffeine (CAF), flufenamic acid (FA) and benzoic acid (BA) from highly liquid paraffin through human heat-separated epidermis (HSE).

For evaluation of saturation concentration of the drugs in the vehicle(c_{sat}), an excess of BA, CAF, FA or TST was suspended in 5 ml of highly liquid paraffin in a screw top scintillation vial (500 rpm, 32 °C; 24 h). After sedimentation of non-dissolved substance, samples were drawn from the supernatant, diluted 1:10 with dichloromethane and analyzed via UV/VIS-spectroscopy: (Lambda 35; Perkin Elmer, BA 228 nm; CAF 277 nm; FA 287 nm; TST 238 nm).

Permeation experiments over human HSE of two donors (6 pieces of each) were performed in Franz diffusion cells at 32 °C. The donor was composed of TST, FA, BA (0.4 mg/ml), CAF (0.1 mg/ml) dissolved in paraffin. Samples of 0.4 ml were collected over the time, replaced by fresh acceptor solution (Soerensen phosphate buffer pH 7.4) and quantified by validated HPLC methods.

The steady state flux (J) was evaluated from the linear part of cumulative amount versus time plots and dividing it by donor concentration (c_{don}), the apparent permeability coefficient (k_P) was obtained**.** The k_P, were calculated by multiplying diffusion coefficient (D) and the stratum corneum-donor partition coefficient ($K_{sc/don}$); dividing this by the thickness of HSE (h), maximum flux (Jmax) was also calculated

Based on $K_{sc/don}$ is established the partitioning of drug between highly liquid paraffin and SC lipids. $K_{sc/don}$ was determined from the decrease of c_{don} after 24 h incubation of SC with BA, CAF, FFA, or TST in highly liquid paraffin at 32 °C
(3 different c_{don} each, skin 1 and skin 2). The remaining amount of drug in paraffin (c_{Rest}) was analyzed using UV-spectrophotometer by wavelength determined from UV spectrum of drug. °The results obtained are summarized in Table 1.

TABLE 1	**TST**	**CAF**	**FA**	**BA**
$C_{sat.}$ ± SD (µg/ml)	498.15 ± 55.3	134.1± 3.3	1176.7 ± 5.2	9852.4±826
Log $K_{oct/water}$	3.47	-0.08	4.80	1.90
K_P (cm/s) ×10-7	2.50 ± 0.56	11.43 ± 5.2	7.11 ± 0.8	29.68 ± 9.9
J_{MAX} (µg/cm²/h)	0.45	0.53	3.02	105.25
$K_{SC/don}$	168.3 ± 47.9	103.74 ± 39.1	52.5 ± 23.4	61.7 ± 34.4

The potential of vehicle to influence the permeation of model drugs is present in my work. Normally, the permeability is higher for hydrophilic drug using the lipophilic donor in comparison to the aqueous. However, for testosterone the permeation was very similar. This may lead to the conclusion, that skin absorption of testosterone is not dependent on the vehicle used. Generally, we can assert that hydrophilic drugs show high permeabilities out of lipophilic vehicle and vice versa.

The maximum flux **(**J_{MAX}) is primarily dependent on solubility of drug in vehicle **(**C_{sat}**)**, which corresponds well to lipophilicity of drugs expressed as the octanol/water partition coefficient (log $K_{oct/water}$).

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SOUHRN

Cílem práce bylo porovnat permeaci testosteronu (TST), kofeinu (KOF), flufenamové kyseliny (FK) a benzoové kyseliny(BK) z nízkoviskózního parafínu skrz teplem separovanou lidskou epidermis (TSE)

Pro zjištění saturační koncentrace léčivých látech v nosiči (c_{sat}) bylo rozpuštěno větší množství BK, KOF, FK nebo TST v 5 mililitrech nízkoviskòzního parafínu uvnitř scintilačních nádobek (500 rpm, 32°C, 24 h). Po sedimentaci nerozpuštěné části léčiva byly odebrány vzorky roztoku, naředěny v poměru 1:10 dichlormetanem a analyzovány pomocí UV/ VIS spektroskopu (Lambda 35, Perkin Elmer, BK 228 nm, KOF 277 nm, FK 287 nm, TST 238 nm).

Permeační pokusy na lidské TSE od 2 dárců (6 kusů od každého) byly provedeny ve Franzových difúzních celách (FDC) při 32°C. Do donorové části cely byly aplikovány léčivé látky rozpuštěné v parafínu (TST, FK, BK o koncentraci 0,4mg/ml, KOF 0,1 mg/ml). Vzorky o objemu 0,4 ml byly odebírány z akceptorové části FDC v určitých časových intervalech a nahrazeny čerstvým pufrem (Soerensenův fosforečnanový pufr pH 7,4). Odebrané vzorky byly kvantifikovány pomocí validovaných HPLC metod.Steady state flux (J) byl zjištěn z linární části grafu permeovaného množství látky v závislosti na čase. Zdánlivý permeační koeficient (k_P) se vypočítá jako podíl J a koncentrace léčivé látky v donoru (c_{don}), nebo jako násobek difúzního koeficientu (D) a stratum corneum(SC)- donor rozdělovacího koeficientu($K_{sc/don}$) dělených tloušťkou TSE (h), také byl vypočten maximální flux (J_{max}).

$K_{sc/don}$ sloužící ke zjistištění rozdělení léčivých látek mezi nízkoviskózní parafín a SC lipidy(rovnice 3) se určilo z poklesu c_{don} po 24h inkubaci SC s parafínem obsahujícím BK, KOF, FK nebo TST při 32°C (3 různé c_{don} pro obě kůže). Zbylé množství léčivé látky v parafínu (c_{Rest}) bylo analyzováno pomocí UV spektrometru při vlnové délce zjištěné z UV spektra látek.

Zjištěné výsledky pro všechny látky s kůží shrnuje tabulka 1.

Tab. 1	**TST**	**KOF**	**FK**	**BK**
C_{sat} ±SD (µg/ml)	498 ± 55.3	134 ± 3.3	1176.65±5.2	9852.4 ± 826
Log $K_{okt/voda}$	3.47	-0.08	4.80	1.90
K_P (cm/s) ×10-7	2.50 ± 0.56	11.43 ± 5.2	7.11 ± 0.8	29.7 ± 9.9
J_{MAX} (µg/cm²/h)	0.45	0.53	3.02	105.25
$K_{SC/don}$	168.3 ± 47.9	103.7 ± 39.1	52.5±23.4	61.7 ± 34.4

V práci bylo ověřeno, že vehikulum ovlivňuje permeaci modelových léčiv. Permeace z lipofilního nosiče byla vyšší v porovnání s hydrofilním nosičem. TST měl velmi podobnou permeaci z obou vehikul, což může vést k závěru, že permeace TST není závislá na použitém nosiči. Obecně lze tvrdit, že hydrofilní látky vykazují vysokou permeaci z lipofilního nosiče a naopak

Maximální flux (J_{max}) je primárně závislý na rozpustnosti léčiva ve vehikulu (c_{sat}), která je přímo úměrná lipofilitě léčiva vyjádřené jako oktanol/ voda rozdělovací koeficient (Log Kokt/voda).

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1. INTRODUCTION

Transdermal and dermal drug administration represent an important way of drug delivery to human body, not only for its high acceptance by the patients, but mainly for minimization of first pass metabolism in liver, for evasion or lowering of gastrointestinal bleeding or irritation due to drugs such as NSAIDs, and for ability to cease absorption in overdosing.

Topical application could remain superficial or be delivered to the site of action by various penetration pathways. To deliver the drug to the site of action, it has to overcome the main skin barrier the stratum corneum. According to Fick`s first law the permeations parameter of a drug, the apparent permeability coefficient (Kp) is calculated, which is a significant criteria for comparison of drug permeation. Kp is influenced by physical and chemical properties of a drug, by the character of the donor and by the kind of skin. This means that change of the donor could influence the permeation of a drug.

Although lipophilic excipients are part of many ointments, creams and other topical dosage forms in everyday use, majority of permeation experiments are performed with hydrophilic donors. To get a deeper insight in the influence of a lipophilic donor on skin permeation, the experiments with liquid paraffin were carried out in this thesis performed using the following drugs: testosterone, caffeine, flufenamic acid, benzoic acid. These drugs were chosen, because they cover wide range of physical and chemical properties especially concerning lipophilicity and hydrophilicity. Moreover, these substances have been already tested in permeation studies with a hydrophilic donor. Based on published results found in literature, we could compare the permeation of the same drugs in a lipophilic and a hydrophilic donor.

The experiments were performed using Franz diffusion cell (FDC) on human heat separated epidermis (HSE) and drug quantification was done by using previously validated HPLC methods.

HSE was chosen, because the main skin barrier and rate-limiting step of transdermal absorption resides normally in the stratum corneum. However, for very lipophilic substances the rate limiting step could be partition between stratum corneum and viable epidermis and therefore the use of full thickness skin should be avoided due to extremely long experimental time periods.

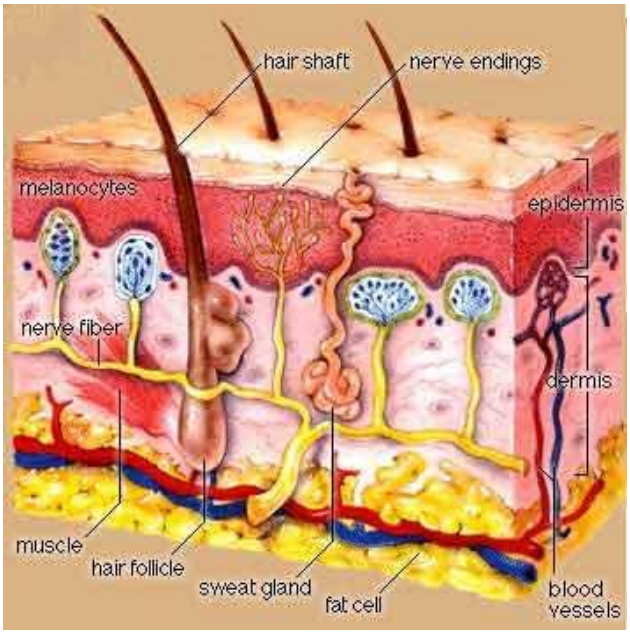
2. THEORY

2.1. Structure and function of human skin

The skin is the largest organ of human body with a size of about 1.8 m^2. The skin evolved as an organ to separate us from the environment. It stops the ingress of bacteria, allergens, and wide range of additional proteins and compounds and it controls the loss of water, electrolytes and other body constituents. To a certain degree, skin protects us from the sun and of course, the skin keeps the body fluids and our tissues. Furthermore, it is responsible for maintaining the body temperature at 37°C.

All these functions can only be filled up due to a specialized anatomical structure. It is composed of stratified epidermis, dermis, subcutaneous fat and a range of structure called appendages.

Figure 1: Structure of human skin[1]



[1] http://images.google.com/imgres?imgurl=http://www.thisistruth.org/images/AboutHumanBody_01s.jpg&imgrefurl=http://www.thisistruth.org/truth.php%3Ff%3DAboutHumanBody&h=167&w=143&sz=5&hl=cs&start=82&tbnid=HJTbyLfjczEAjM:&tbnh=99&tbnw=85&prev=/images%3Fq%3Dhuman%2Bskin%26start%3D80%26gbv%3D2%26ndsp%3D20%26svnum%3D10%26hl%3Dcs%26sa%3DN

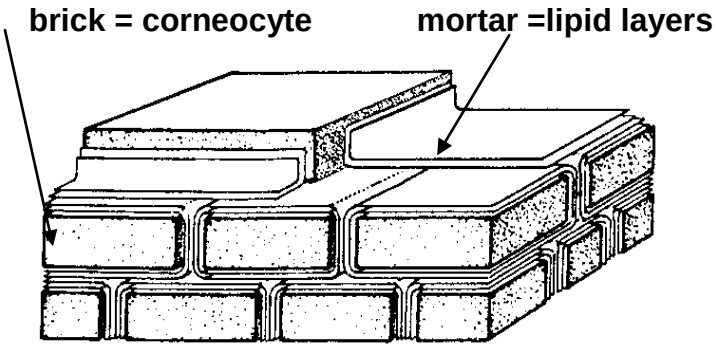
2.1.1. The epidermis

The epidermis is a layer with a thickness of 80 µm to 160 µm and it is divided in the viable epidermis and the stratum corneum. The stratum corneum or horny layer is the outermost layer, which provides most of the skin protective function. It is composed of flattened, anuclear, metabolically inactive, cornified cells (corneocytes) of hexagonal shape, arranged in overlapping stacks. The keratin-rich corneocytes are embedded in an intercellular lipid-rich matrix. This two-compartment arrangement is usually called brick and mortar model (Fig. 2).

The intercellular lipids have an organized bilayer structure.[1] The lipids are packed into lamellae, with the hydrocarbon chains mirroring each other and the polar groups dissolving in an aqueous layer.[2] (Fig.6)

In the intercellular region are desmosomes, which act as rivets to hold the layers of the stratum corneum together, and which are keys for cohesion. In the upper layers, where the desmosomes are not present, the stratum corneum undergoes a continuous process of desquamation. The normal period of turnover is four weeks.

Figure 2: The brick and mortar model[2]



The stratum corneum has a thickness of 10 to 25 µm. It can be up to ten times thicker at the corns, calluses, soles, and palms. In general, as the density of external hair or fur increases, the stratum corneum thins, because hair provides an additional protection from the environment. As the consequence, humans have

[2]Landmann, L.(1991), Die Permeabilitaetsberriere der Haut, Pharmazie in unserer Zeit 4, 155-163, modification according Schaefer, Ulrich F.

thicker stratum corneum than fur bearing animals [3]. After hydration the SC swells and its thickness may reach values up to 40 μm.[2]

The viable epidermis consists of four layers: stratum germinativum (basal layer), stratum spinosum, stratum granulosum, stratum lucidum, which is present only on the palms and soles.

2.1.2. The dermis

The dermis lies between the epidermis and the subcutaneous fatty tissue. Its thickness ranges from 1 mm to 5 mm. The major function of the dermis is to provide nutrition to other layers of the skin. From an anatomical perspective, the dermis is composed of two layers, a papillary and reticular layer. Cells within the dermis include collagen fibers and elastin, which are part of the epidermis's support mechanism. The dermis contains nerve fibers, such as Pacinian fibers, which sense and measure pain, Meissner's corpuscles, which sense pressure and vibration, Langerhans cell, which are major antigen presenting immune cells and melanocytes, which are important for pigmentation. Lymphatic and blood vessels innervate also the dermis. Blood underlay the epidermal gradient by keeping lower concentration in the dermis, which is important for transdermal absorption.

2.1.3. The subcutaneous tissue

The subcutaneous fat layers acts as a heat insulator, mechanical cushion and stores readily available high energy chemicals.

2.1.4. The skin appendages

Hair follicles are found over all the skin except for the lips, palms and soles. Associated sebaceous glands produce sebum, which is made up of glycerides, free fatty acids and cholesterol. Sebum provides protection to the skin, lubrication and enables it to maintain a pH of about 5[4].

Other structures of potential importance include the eccrine, apocrine and apoeccrine sweat ducts. The most important of these in terms of thermal regulation is the eccrine gland, which helps to maintain body temperature at the

desired 37°C. On the other hand, apocrine sweat glands are found at philosebaceous follicles, which secrete a milky or oily odourless liquid. When metabolized by surface bacteria, it induces characteristic body smell.
The nails, as well as the hair and stratum corneum contain keratin proteins. The difference between SC and nails is the higher permeability of nails for hydrophilic substances.

2.1.5. Skin metabolism

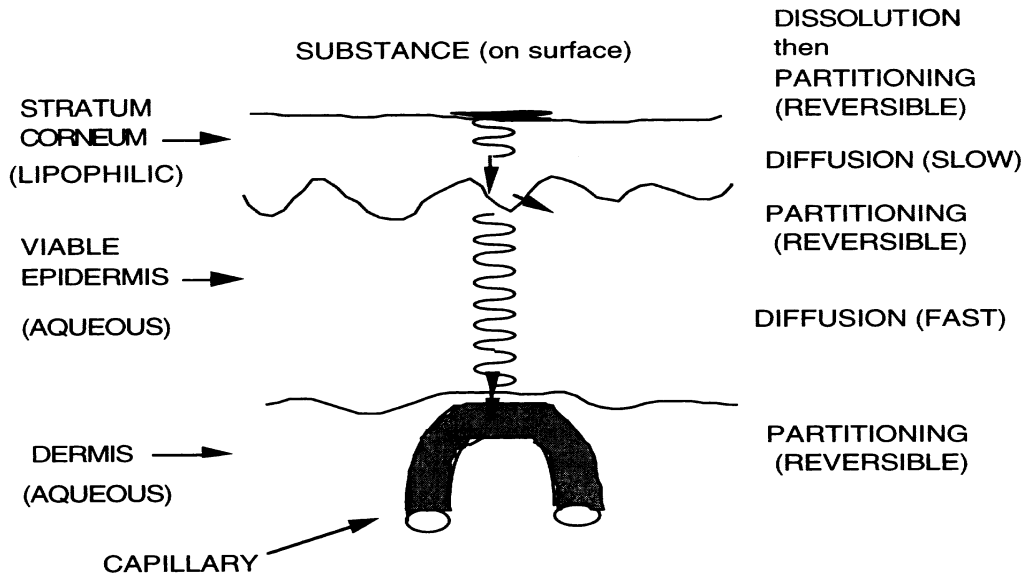
One of many reasons for drug delivery trough the skin is to avoid the first pass effect that happens after oral drug administration. However, liver enzymes such as the esterases and cytokine P450 are also present in the skin. Some investigators have published data suggesting that the metabolizing capacity of the skin is about 2 % of the liver, while others have found that skin esterase metabolism can actually be up to 50 % of the liver[3]. A full understanding of skin metabolism would be very important for an optimized transdermal delivery of drugs, but there are many difficulties, e.g. our limited knowledge of exactly where and to what concentration enzymes are located within the skin.

2.2. Transdermal absorption

The process of transdermal absorption involves several individual transport processes, some of which occurs in series and other in parallel. The two key determinants for a drug crossing a membrane are solubility and diffusivity.
The relative solubility of a drug in two phases determines its partition coefficient and, therefore, the likelihood of the drug being taken up into the SC from a vehicle. In addition, solubility determines the permeation of drug into deeper layers.
The diffusivity is a measure of the speed at which a drug crosses a given barrier and is affected by binding, viscosity of environment, and tortuosity of the path.
When a formulation is applied to the skin, the first limiting step is the release of the drug from formulation before absorption can take place. The second step is the partitioning of drug between formulation and SC. The formulation’s penetrative

ability depends on the affinity of the drug to the skin components, and the diffusion of the drug into and trough the cell layers. After drug penetration into SC partitioning of drug between the more lipophilic SC and hydrophilic stratum granulosum (the outer layer of viable epidermis) occurs. If no partitioning takes place at this level, the molecule will stay in the stratum corneum and will not penetrate more deeply. This is typically for very hydrophobic substance. In the viable epidermis the drug penetrates mostly faster than in SC. If the drug reaches the dermis, than it partitions into the dermis, where the absorption into circulating blood could occur. If there is no systemic uptake, we cannot talk in terms of the pharmacokinetics of transdermal absorption (Fig.3).

Figure 3: Steps of absorption[3]



[3] Robert L. Bronaugh, Howard I. Mailbach, Topical Absorption of Dermatological Products, 2002 New York

2.2.1. Partition coefficients

We first considered the partitioning between the SC and vehicle. The partitioning of drug between the SC and vehicle is defined by the chemical potential difference between the drug in the SC μ_{SC} and that in the vehicle μ_v. At equilibrium, the chemical potential of the drug in two phases is equal (i.e., $\mu_{SC} = \mu_v$). However, we could also define an SC-vehicle partition coefficient based on concentration

eq.1 $$K_{V\text{-}SC} = \frac{C_{SC}}{C_V}$$

C_{SC}= concentration of the drug in SC (mg/cm^3)

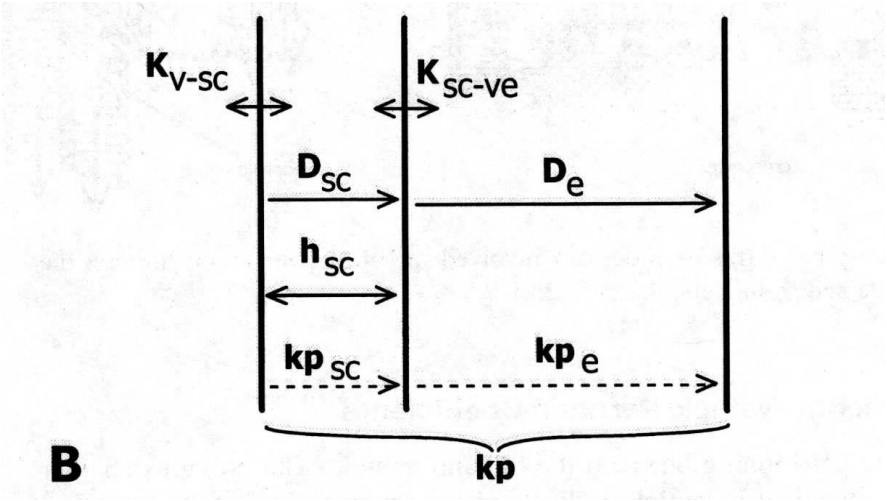
C_V= concentration of drug in vehicle (mg/cm^3)

Generally, it is accepted that the higher the value of K is the faster occurs the penetration of the drug through skin. However, an optimum is supposed for partition coefficients between 10 and 1000 (log K 1 to 3).

To explain partitioning of drug between stratum corneum and vehicle so called “push effects“ and “pull effects“ are assumed. Push effects occurs, if interaction between drug and vehicle decreases and that is why increases the escaping tendency of drug from vehicle into stratum corneum. Pull effects is increasing of the interaction between SC lipids and drug, thus increasing of affinity to the stratum corneum. For both cases, the partition is higher to the stratum corneum.

For lipophilic substances in lipophile vehicle, the partition coefficient between SC and vehicle decreases, because the drug has a significant afinity to the vehicle. On the other hand, hydrophilic substances in lipophilic vehicle are very low soluble and that‘s why the partition coefficient between SC and vehicle increases. We can see the tendency, that substances which are difficult soluble in the vehicle show higher permeation, because they have more suitable Kv-sc [5]

Figure 4: Diagram of partitioning and diffusion in the skin [4]



Kv-sc = partition coefficient vehicle/stratum corneum, responsible for stratum corneum invasion

Ksc-ve = partition coefficient stratum corneum/ viable epidermis, responsible for viable epidermis invasion

Dsc = diffusion coefficient stratum corneum

De = diffusion coefficient viable epidermis

hsc = thickness of stratum corneum

kpsc = permeability coefficient stratum corneum

kpe = permeability coefficient viable epidermis

kp = permeability coefficient epidermis

Partition coefficient between the SC and vehicle can be interpreted using “dual sorption model”. This explanation postulates the existence of “bound” and “freely diffusible” molecules within the SC. As the number of molecules available for partitioning into the SC increases, the immobilized fraction becomes saturated while the unbound compound can continue to rise. Hence, the measured partition coefficient may not be constant with increasing concentration until the “binding” sites in the SC are saturated.[6]

Partition coefficient between lipophile stratum corneum and hydrophile viable epidermis (K_{SC-VE}) is the critical step by permeation of extrem lipophile substances.

[4] Rougier A,Goldberg A. Maibach Hi, eds. In vitro skin toxicology: Irritation, Phototoxicity and Sensitization, New York: Mary Ann Liebert, 1994

2.2.2. Mathematical model in transdermal absorption

Diffusion is a passive process that leads to equilibration of concentration between the high concentrated donor and less concentrated receptor side (skin). When the drug is first applied onto the skin, the system is not in equilibrium. Diffusion is slow and nonlinear until the system reaches equilibrium. Flux is generated by the concentration of drug in the formulation applied to the donor compartment. The period between application and beginning of linear permeation is called the “lag time”. This “ lag time“ symbolises the time of delay which described the first contact of the drug with the skin’s surface. After this point, diffusion is strictly proportional to the concentration and physical chemical properties of the applied drug. These are the steady state conditions.

Fick’s diffusion laws describe the passive diffusion of a drug in equilibrium through skin.

Fick’s first law

According to Fick`s first law flux (J) could be stated from linear part of a diagram of the cumulative amount permeated per area versus time. J is the slope of the streight line (Fig. 5).

eq.2 $$J=\frac{M_A}{A*dt}$$

J = flux (mg/cm²/s)

A = area (cm²)

M_A= amount of drug in acceptor (mg)

t= time point (h)

Flux is directly related to the drug concentration in the vehicle.

eq.3 $$J=\frac{D*Kv-sc*(C_D-C_R)}{h}=\frac{D*Kv-sc*C_D}{h}=C_D*Kp$$

In most circumstances $C_R << C_D$, and equation (3) can be simplified.

Kp = apparent permeability coefficient (cm/s)

C_D = drug concentration in the vehicle (mg/cm^3)

C_R = drug concentration in the acceptor (mg/cm^3)

D= diffusion coefficient of drug (cm^2/s)

h= thickness of SC (cm)

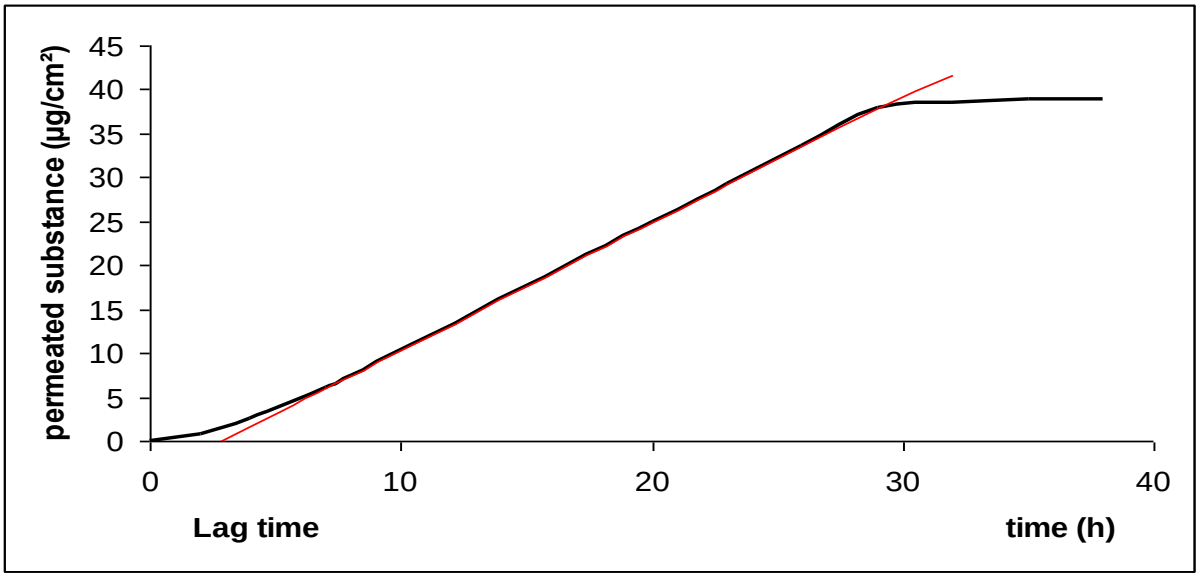
From equation 2 and 3, we could calculate the apparent permeability coefficient Kp (eq. 4), which is independent on donor concentration. That is very important for the comparison of drugs, if different donor concentrations of the drugs are used.

eq.4 $$Kp = \frac{dM_A}{A * dt * C_D} = \frac{J}{C_D}$$

The next parameter for calculation is maximum flux, Jmax (eq.5). This Jmax described the maximal amount of drug, which is possible to penetrate through the skin. The flux is maximal for the saturation concentration of the drug in the formulation.

eq. 5 $$J_{MAX} = Kp * C_{Sat.} = \frac{D * C_{SC}}{h}$$ (mg/cm²/s)

Figure 5: Example of Flux-diagram



2.2.3. Penetrations pathway through the stratum corneum

The routes to the viable epidermis are through the skin appendages, like hair follicles and sweat ducts and across the intact stratum corneum. Across the intact stratum corneum two ways are possible: either the intercellular route or the transcellular route. It would be a great simplification to assume that one route prevails under all conditions (Fig.6).

Transappendageal route of penetration

Hair follicles may also play a major role in transdermal penetration, especially for larger polar molecules and ions. However, this route is not the most important for reaching a steady state flux.

The relative difference in depth between the hair follicle and sebaceous glands is not significant for drug permeation because, unlike the hair shaft, the sebaceous duct has no stratum corneum. Only one layer of epidermal cells separates it from the dermis. Partitioning is affected because this epidermal cell layers separates a very lipophilic environment from a very hydrophilic medium. Therefore, if a molecule has the required solubility to partition between lipids and water, it will cross the epidermal layer via the sebaceous duct.

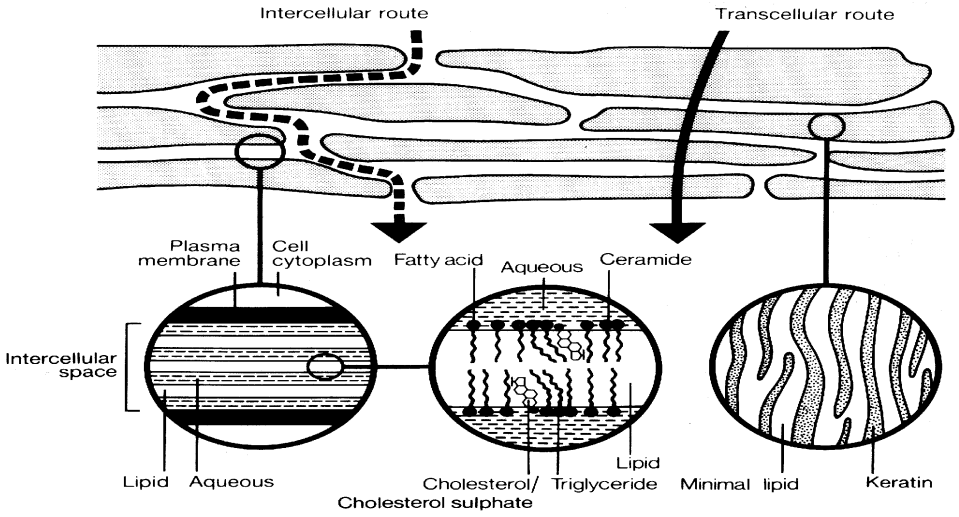
Intercellular route of penetration

The major route is via the tortuous but continuous intercellular region. Because the intercellular region of the stratum corneum is structured as bilayers with lipophilic and hydrophilic regions, it is stated out that lipophilic substances penetrate predominantly in the lipophilic and hydrophilic in the more hydrophilic part of the bilayer. Some studies show that the intercellular lipid and not the corneocyte is the main epidermal permeability barrier.

Transcellular route of penetration

The transcellular route presents a very complicated pathway of drug penetration due to unavoidable repeated partitioning of the drug between the hydrophilic corneocytes and the lipophilic intercellular matrix. In addition, keratin and keratohyalin being content of the corneocytes are very low permeable for drugs. Therefore, this pathway does not contribute to drug permeation significantly.

Figure 6: Absorption via the stratum corneum and structure of intracellular bilayer[5]



2.2.4. Parameters for diffusion

It counts that these parameters influence diffusion of drug through skin:

Physical and chemical properties of drug

Character of donor

Variability of skin

Physical and chemical properties of drug

Quantitative structure permeability relationships indicate that molecular weight and hydrophobicity are the main determinants of transdermal penetration[7].

Molecular weight

When the size of the molecule increases, such as when the number of carbon atoms increases, diffusion normally decreases[3].

Log P

Hydrophobicity of substance is expressed as the logarithm of the octanol – water partition coefficient, Log P.

[5] Elias, P.M. ,1983, Epidermal lipids, barrier function, and desquamation. J. Invest. Dermatol. 80,44S-49S

Character of donor

Multiple vehicle factors can influence the penetration of the drug. In addition to drug solubility, these include the thermodynamic activity of the drug, penetration enhacement due to barrier function modification, and disruption of skin lipids' structure or protein denaturation.

The thermodynamic activity and solubility

In context with diffusion experiments, the thermodynamic activity is calculated as a quotient of donor concentration of the drug and the saturation solubility of drug in the vehicle.

If the donor concentration of drug is changed, changing of the thermodynamic activity occurs and that is why permeation flux would be different.

The maximal thermodynamic activity (a=1) dominates in saturation preparations and it induces the maximal flux of drug through the skin[8].

If the drug is not enough soluble in the vehicle the danger of early "bleeding" of drug exists.

Modification of barrier function and penetration enhancement

It is proved, that the vehicle could influence drug permeation by modification of the skin barrier function. Wagner`s experiments show significantly different diffusion values trough the skin by use of various semisolid preparations with the same concentration of drug. Enhancer effects could often take place by the use of complex preparations or vehicle with emulsifiers, e.g. creams [9].

pH value of donor

For drugs, which could be ionized, for hydrophilic vehicles it is very important to know the pH value of vehicle as well as the pKa value of the drug. Because the saturation concentration depends on the pH value pH changes influence consequently the thermodynamic activity of drug.

Viscosity of vehicle

In the case of semisolid preparations, the diffusion coefficient of drug inside the vehicle has to be considered. For systems with high viscosity, drug release may be reduced and therefore the diffusion through the skin may be influenced.

Variability of skin

Although the skin covers the total external human body surface, there are big differences between skins from various parts of the human body concerning the

permeation of drugs. The reason is a local difference in the structure of skin. Experiments with hydrocortisone have shown that these local differences could evocate higher absorption. Dosages of Hydrocortisone were applied on various parts of human body and evocated difference of absorption 50 times greater[10]. Extremely high absorption occurs in parts of head, neck, shrug and scrotum.

In summary, the specific skin condition (disease), blood flow, hydration due to occlusion, and differences in species, sex and age are some of the factors that can affect skin penetration. In a in vitro situation even larger variations have been reported, as excision, storage and experimental manipulations may cause additional modifications [11].

Individual skin metabolism can also affect skin penetration due to stratum corneum enzymes alone and in interaction with the bacterial flora on top of the skin.

2.3. Test systems

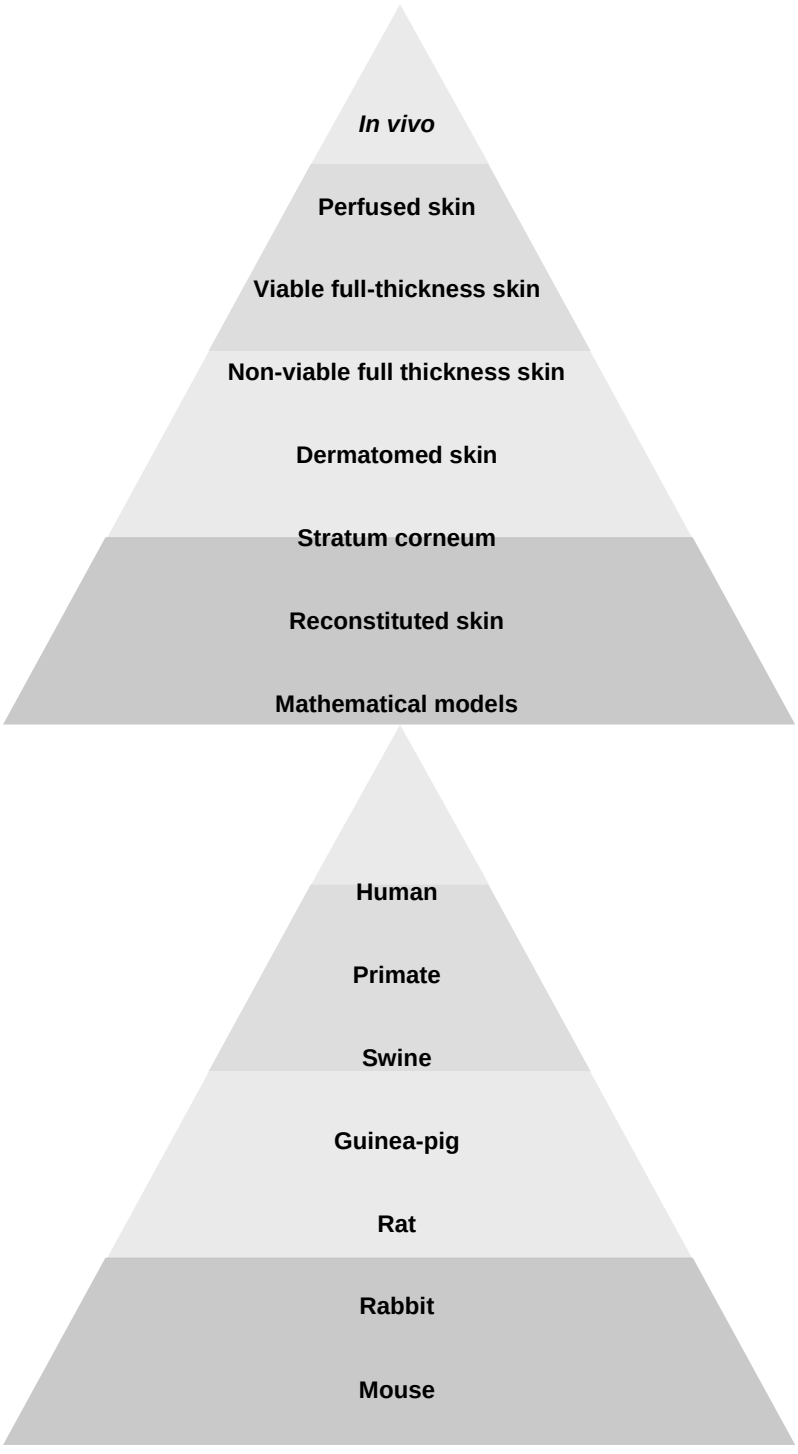
Several official documents, provided by the European authorities and the FDA, are at the disposal of researchers in the field of skin research[12-16]. Where ambiguities of interpretation remain, advice, on how to practically apply this guidance to protocols in current use, is at hand [17].

In vivo skin absorption measurements are “standard” to investigate the behaviours of drugs in the local dermal therapy. However, considering ethical, economical and analytical aspects, in vitro alternative methods had to be found [18-21].

A comprehensive compilation of literature data, comparing the permeability of chemicals across animal and human skin *in vivo*, as well as *in vitro*, has been published by European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) [15].

Howes *et al.* introduced a hierarchy of frequently applied *in vitro* methods for measuring transdermal absorption according to their resemblance of the *in vivo* situation (Fig. 7) [22].

Figure 7: Hierarchy of frequently applied methods for measuring transdermal absorption according to resemblance of the *in vivo* situation.[6]



[6] Howes, D., et al., *Methods for assessing transdermal absorption. The art and recommendations of ECVAM Workshop 13.* Alternatives Lab. Anim., 1996. **24**: p. 81-106.

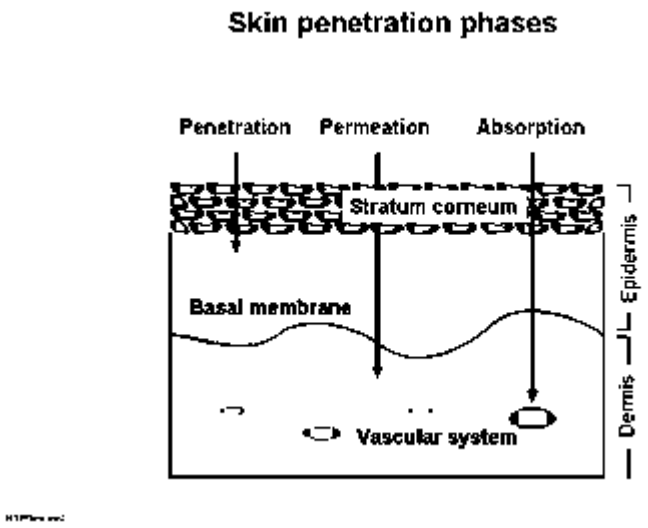
2.3.1. Penetration× Permeation

Currently, two different experimental designs are established: permeation and penetration studies.

Permeation: transdermal transport through the skin into an acceptor compartment

Penetration: transport into various skin layers, which act as individual acceptor compartments.

Figure 8:[7]



In vitro penetration

Penetration studies involve determining the concentration of a topically applied substance in the various skin layers. For this purpose is used the so called "Saarbrücken model". The model uses the tape stripping technique to remove the various horny layers and afterwards the skin is horizontally segmented by means of a cryo-microtome.

In vitro permeation

The goal of permeation study is to obtain pharmacokinetics profile of drug, which reflects how the concentration of drug changes within the time in a receptor compartment.

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[7] http://images.google.com/imgres?imgurl=http://www.gd-online.de/english/originals_e/imagesoriginals2000/Fig%25201%2520b%2520skin%2520penetration%2520phases%2520Pitterman%2520webcopy.gif&imgrefurl=http://www.gd-online.de/english/originals_e/Pittermann2000.htm&h=275&w=397&sz=17&hl=cs&start=2&tbnid=KzyDsF-_kOJk0M:&tbnh=86&tbnw=124&prev=/images%3Fq%3Dpenetration%2Bskin%26gbv%3D2%26svnum%3D10%26hl%3Dcs%26sa%3DG

Currently, the most widely used permeation test system is the Franz diffusion cell model (FDC).

Franz diffusion cell

The FDC enables to obtain data on the drug penetration through the skin in the course of time, for example this kind of measurements allow to determine steady state flux, apparent permeability coefficient, diffusion constant and lag time.

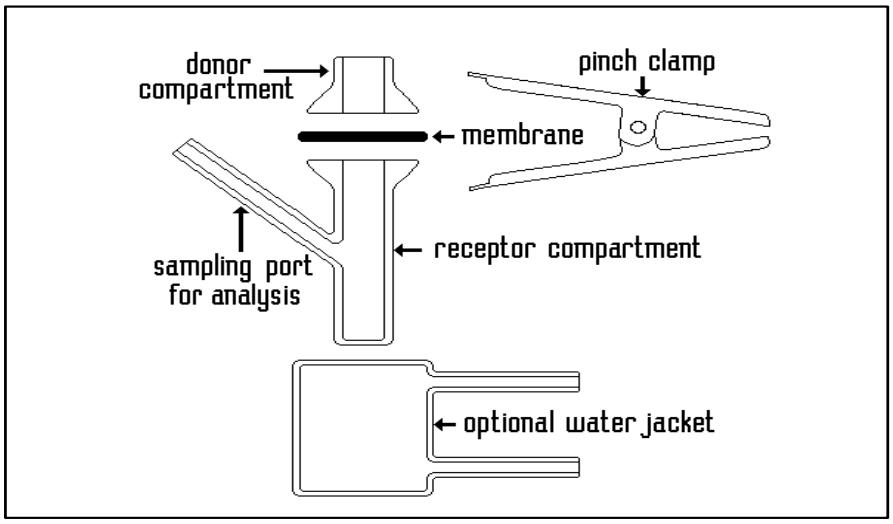
The FDC consists of two compartments: a donor compartment and an acceptor compartment. The used barrier (animal or human skin, skin equivalent, and artificial membrane) is placed between these two compartments. The donor compartment is filled with drug preparation (ointment, solution or patch) and the acceptor compartment contains an aqueous or alcoholic medium with or without solubilizers. The composition of the acceptor fluid is determined by the solubility of the drug in this medium, whereas the concentration of the drug should not exceed 10% of saturation solubility during experiment.

Stirring of the acceptor medium guarantees the uniform distribution of the dissolved drug during the experiment.

Samples of acceptor medium are collected through the sampling port over the time and replaced by fresh buffer solution. The entire system can be either jacketed or put into a water bath to maintain a membrane surface temperature of 32°C, the normal temperature at the surface of human skin.

The amount of applied dose varies with type of study; for finite dose study, the applied dose should mimic the “in use“ conditions which is to be considered up to 10 mg/ cm^2 or 10µl/ cm^2. Infinite dose experiments, with typical doses of 100µl/ cm^2 (or >10 mg/ cm^2) and more, may be appropriate to obtain steady state conditions from which steady state flux or absorption rate and Kp value can be calculated[12].

Figure 9: FDC



Human skin is most favourably used in FD-C experiments. Usually the supply is provided from plastic surgery, amputations or cadavers. It can be differentiated between investigations with full thickness skin, dermatomed skin, separated epidermis, isolated stratum corneum and stripped skin. All these barriers originate from full thickness skin. Especially dermatomed skin and separated epidermas were established because the permeations experiment with full thickness skin lead to several hours of incubation until drug reaches the acceptor medium due to its high thickness and high barrier properties.

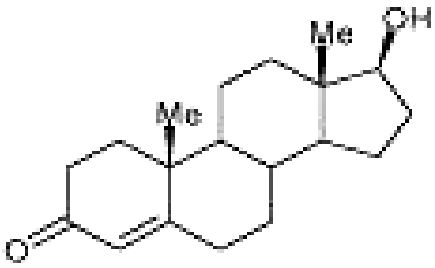
2.4. Drugs

2.4.1. Testosterone

Testosterone is a physiological steroid hormone with dominant androgen effect and minor anabolic effect. It is built primarily in testes, lower amount in ovary and in adrenal cortex.

Structure:

Figure 10: structure of testosterone



Indication:

Androgens are used at men for substitution at androgen deficit (primary and secondary hypogonadismus, impotence, testes retention, oligospermie, fertility diseases, psycho vegetative disorders, activity decreasing). Testosterone could be used also in high doses for man sterilization. At women, it cures mamma and endometrial carcinomas. Its anabolic effect improves protein deficiency, renal and aplastic anemia, osteoporosis and cirrhosis of the liver.

Pharmacokinetic:

Testosterone is not oral available due to its fast deactivation in the liver. It is effective as implant or as the ester form.

Testosterone propionate is usable in injections, sublingual or transdermal form. Big patches application seems to have the advantage producing constant blood levels.

The next esters are testosterone enanthate and testosterone cyclopentyl propionate.

Majority of testosterone (97%) binds to plasma proteins.

Testosterone is excreted as glucuronide and sulfate into urine.

Dosage:

The dosage is depended on way of administration.

Physical-chemical properties:

Testosterone is a white or light yellow, crystalline, odourless powder.

Solubility: insoluble in water, very soluble in ether, soluble in vegetable oils

Ultraviolet spectrum: The UV maximum is at 240 nm (ethanol, methanol) and at 249 nm (0.1 N-HCl, 0.1 N- NaOH)

Log $P_{oct/wat}$= 3.47

Molecular weight: 288.40[23, 24]

2.4.2. Caffeine

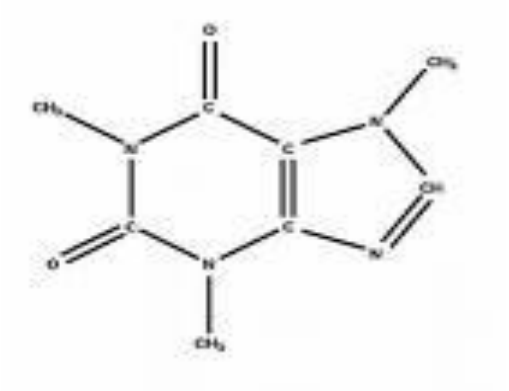
Caffeine belongs to psychostimulancia. Its effect depends on the applied dose. It can be used as an antagonist of adenosine receptors and an inhibitor of phosphodiesterase.

Adenosine acts on two receptor subtypes: A1 and A2 receptor, and changes activity of adenylat cyclase in cells trough G-protein. Through binding with A1, adenylat cyclase can be inhibited. On the other hand, association with A2 receptors stimulates adenylat cyclase.

Therefore, caffeine affects as on A1 as on A2 receptors like competitive antagonist.

Structure:

Figure 11: Structure of caffeine



Indication:

Caffeine can be used by fatigues. It avoids longer apnoe at premature infants. Because of vasoconstriction effect on brain vessels, it is applied by headaches.

In many formulations, it amplifies the effect of analgetics.

Pharmacokinetic:

Caffeine is fully and quickly absorbed by oral administration.

30-40% of caffeine binds to plasma proteins.

It overcomes blood-brain barrier and placenta barrier.

Caffeine and its metabolites are excreted mainly renal way.

Dosage:

Adult dosage moves along 100-200 mg 3 times a day. Intoxication occurs by dosage of 1g. Lethal dosage lies by 3-10g.

Physical-chemical properties:

Caffeine is a white powder or white, glistening needles, usually matted together. It is odourless and has a bitter taste.

Solubility: 1 mg of caffeine dissolves in about 50 ml water; 6 ml water at 80°C; 75 ml alcohol, about 25 ml alcohol at 60°C, about 6 ml chloroform, 600 ml ether, 50 ml acetone, 100 ml benzene and 22 ml boiling benzene. Freely soluble in pyrrole, in tetrahydrofuran containing 4% water, also soluble in ethyl acetate and slightly in petroleum ether.

The solubility of caffeine in water is increased by the presence of organic acids or their alkali salts.

Ultraviolet spectrum: Reported UV spectral data are as follows: 272 nm (methanol, 0.1 N-HCl); 273 nm (ethanol); 275 nm (0.1 N-NaOH); 278 nm (trichloroethylene).

Log $P_{oct/wat}$= -0.08

pKa= 1.39

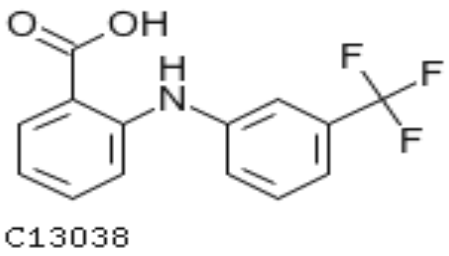
Molecular weight: 194.20[23, 24]

2.4.3. Flufenamic acid

Flufenamic acid is a derivate of anthranilic acid. It belongs in the group of the no steroidal anti-inflammatory drugs. The basic principle of NSAIDs effect is an inhibitory action on the synthesis of prostaglandins and other eicosanoids responsible for induction of pain, inflammation and fever by inhibiting of cyclooxygenases. In addition, flufenamic acid influences the synthesis of leucotriens by inhibition of lipooxygenase activity.

Structure:

Figure 12: structure of flufenamic acid



Indication:

Flufenamic acid as NSAID is used for relief of various painful states including musculoskeletal disorders, injuries, surgery, and menstruation troubles. Its anti–inflammatory effect is widely used in treatment of rheumatoid arthritis and osteoarthritis.

Pharmacokinetic:

Anti-inflammatory drugs with fenamate group are absorbed very slowly by oral administration.

CF_3 group contributes significantly to the binding affinity, which is 90% for plasma proteins.

Flufenamic acid is metabolized in the liver and it is excreted in conjugated form by urine.

Dosage:

Flufenamic acid can be administrated per os in doses of 200 to 600 mg per day.

Physical-chemical properties:

Flufenamic acid builds pale yellow needles, practically odourless with a slight bitter taste.

Solubility: It is very readily soluble in dimethylformamid, alkali hydroxide solvents, diethyl ether, ethanol 96%, chloroform and dichloromethane. The solubility of this lipophilic substance in water is very low, but can be increased by using alkaline solutions.

Ultraviolet spectrum: UV- maxima of absorption are 288 nm and 340.5 nm in methanol and 288 nm in 0.1 N- NaOH.

Log $P_{oct/wat}$= 4.88

pKa= 3.90

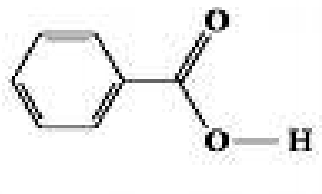
Molecular weight: 281.23[23-25]

2.4.4. Benzoic acid

As ointment, benzoic acid is used for treatment of fungal infections. It can be used as corrosion inhibitor in emulsion and paints, as well as an anti-freeze formulation, plugging agent, and modifier in oil well applications. It has also found use as a textile dye carrier.

Structure:

Figure 13: structure of benzoic acid



Indication:

Benzoic acid possesses anti-bacterial and anti fungal properties. It can be clinically used to treat hyperammonemia caused by defect in urea metabolism.[26]

Pharmacokinetics:

It is metabolized in the liver by conjugation with glycine, and rapidly excreted in the urine as hippuric acid in most of animal species.

Rougier et al. reported that transdermal penetration of benzoic acid in human skin depended on the anatomical location of the skin. The rank order of skin permeability of benzoic acid appears to be: arm< abdomen< post auricular< forehead.[27]

Downard et al. showed that benzoic acid could induce cutaneous vasodilatation after topical application caused by local formation of prostaglandin D_2 in the skin.[28]

Dosage:

At concentration of 0.1%, benzoic acid is a moderately effective preservative providing that the pH of the formulation does not exceed 5.0.

Physical-chemical properties:

Benzoic acid is a colourless or white powder, which can be odourless or with a slight characteristic odour. It is obtained as a crystalline material.

Solubility: Benzoic acid is soluble in definite proportion of acetone, benzene, carbon tetrachloride, chloroform, ethanol ethyl ether, hexane, methanol, and toluene at ambient temperature. The aqueous solubility was found to exhibit strong pH dependence, with the ionized form of the substance being more soluble than the free acid. The pH of a saturated solution of benzoic acid in water at 25°C was found to be 2.8.

Ultraviolet spectrum: An absorbance maximum of 227 nm was noted for the 96% ethanol solution, while a maximum at 229 nm was found in the 0.01 N- HCl as solvent.

Log $P_{oct/wat}$= 1.90

pKa= 4.20

Molecular weight: 122.10[23]

3. EXPERIMENTAL PART

3.1. Materials and Instruments

Chemicals

Testosterone T-1500, SIGMA Aldrich, Steinheim, Germany

Caffeine anhydrous C-0750, SIGMA Aldrich, Steinheim, Germany

Flufenamic acid F9005, SIGMA Aldrich, Steinheim, Germany

Benzoic acid, ACS Reagent B7521, SIGMA Aldrich, Steinheim, Germany

Paraffin highly liquid, MERCK, Darmstadt, Germany

Methanol for HPLC, VWR, Darmstadt, Germany

Dichloromethane for HPLC, VWR, Darmstadt, Germany

Acetonitrile for HPLC, Acros Organics Geel, Belgium

Aqua destilata (Pharm. Eur.), Dep.Biopharm.Pharm.Techn.Saarbruec.,Germany

Trypsin T-8003, SIGMA Aldrich, Steinheim, Germany

Buffers

Soerensen phosphate buffer pH 7.4

PBS buffer pH 7.4

McIlvaine buffer pH 2.2

Phosphate-buffer pH 2.6

Phosphate-buffer pH 6.5

Instruments

UV/ VIS spectrophotometer Lambda 35, Perkin Elmer, Germany

Glass cuvettes Suprasil, HELLMA

Freeze dryer ALPHA 2-4 LSC, CHRIST, Osterode am Harz

HPLC system

UV detector UVD 170S, Dionex GmbH, Idstein, Germany

Pump, Dionex P580

Autosampler, Dionex ASJ 100 automated sample injector

Software Chromeleon 6.5 SP2 build 9, 68

Column oven Dionex STH 585

HPLC cartridge LiChroCART 125-4, MERCK, Darmstadt, Germany

HPLC vials, CS-Chromatographie- Service GmbH, Langerwehe, Germany
HPLC micro insert G30 and cap

Electronic, analytic balance, Sartorius Gem^{plus}, Goettingen, Germany
Filters: OPTI-flow PTFE 0, 2µm, Wicom GmbH, Heppenheim, Germany
Diffusion experiments devices
Franz diffusion cells type 4G-01-00-20-15 (area= 1.767 cm^2) Perme Gear, Riegelsville, USA
Tripod Clamp, Fischer, Frankfurt, Germany
Drying chamber, Memmert, Schwabach, Germany
Water bath, Koettermann, Heidelberg, Germany
Punch Ø= 25 mm
Magnetic stirrer, Janke & Kunkel, GmbH, Staufen, Germany
Magnetic stiring bar, 7 x 1.5 mm, Brand, Mainz, Germany
Parafilm, American Nation Can^{TM}, Menasha, USA
Disposable syringe, sterile, 1.0 ml, Becton Dickinson S.A., Madrid, Spain
Disposable needle, sterile, 0, 8×80 mm, Rose GmbH, Trier, Germany
Eppendorf pipette, Research 20/50/100/250/1000µl Eppendorf AG, Hamburg, Germany
Cellulose membrane cut off 10000 Da, Medicell Int., LTD, London, Great Britain
Petri dishes, Fischer Frankfurt, Germany
pH-meter; Knick, Berlin, Germany

Preparation Aids
Aluminum foil
Scissors, Forceps, Aesculap, Tuttlingen, Germany
Freezer bag Cotton, Hartmann AG, Heidenheim, Germany
Teflon foil, J. Mettler & Co, Saarbruecken, Germany

3.2. Buffer preparation

Buffer solution was prepared in accordance to:

Soerensen phosphate buffer pH 7.4		
Na_2HPO_4 ×2 H_20	9.2 g	Gruessing, Filsum, Germany
KH_2PO_4	2.0 g	Merck, Darmstadt, Germany
Aqua dest.	1000.0 ml	
PBS buffer pH 7.4		
Na_2HPO_4 ×2 H_20	1.75 g	Gruessing, Filsum, Germany
KH_2PO_4	0.20 g	Merck, Darmstadt, Germany
NaCl	8.0 g	Merck, Darmstadt, Germany
KCl	0.2 g	Merck, Darmstadt, Germany
Aqua dest.	1000.0 ml	
McIlvaine buffer pH 2. 2		
Citric acid	20.8 g	Merck, Darmstadt, Germany
Na_2HPO_4 ×2 H_20	0.4 g	Gruessing, Filsum, Germany
Aqua dest.	1000.0 ml	
Buffer pH 2.6		
Orthophosphoric acid	1.16 ml	Riedel de Haen, Seelze, Germany
KH_2PO_4	2.04 g	Merck, Darmstadt, Germany
Aqua dest.	1000.0 ml	
Buffer pH 6. 5		
Na_2HPO_4 × H_20	2.94 g	Merck, Darmstadt, Germany
Na_2HPO_4 × $7H_20$	2.33 g	Riedel de Haen, Seelze, Germany
Aqua dest.	1000.0 ml	

3.3. Skin samples preparation

Skin was obtained from three Caucasian patients, who had undergone abdominal plastic surgery in hospital Lebach, Germany (approved by the Etical Committee of the Caritas Krankenhaus, Lebach, Germany).

Donor 1: female, 38 years old

Donor 2: female, 61 years old

Donor 3: female, 25 years old

Donor skin did not show any abnormality and that is why it could be used for diffusion experiments.

3.4. Choice of vehicle

By the selection of the vehicle possible enhancement effects had to be considered. To have well defined experimental conditions during our experiments we decided to avoid enhancement effects provided by the vehicle. As Bunge et al. showed that paraffin does not change the skin properties [29] and liquid paraffin is part of many ointments, creams and other topical applications we selected liquid paraffin as appropriate vehicle..

3.5. Preparation of biological material

Human skin from three donors was used. Immediately after excision, the subcutaneous fatty tissue was removed using a scalpel and forceps. The skin was cut into 10×10 cm pieces, wrapped in aluminum foil and stored in freezer bags in a freezer at - 26°C until use for a maximum time of 6 months.

At time of use, the skin was thawed and used for preparing heat-separated epidermis as well as isolated stratum corneum.

3.5.1. Heat-separated epidermis (HSE)

For isolation of epidermis, many ways are described in literature[30-35].

We used the heat-separation technique in a water bath of 60°C. Skin disks with a diameter of 25 mm were punched out from full thickness skin. They were transferred into a water bath and incubated for 90 seconds. After this time, the skin disks were placed on a filter paper and epidermis was pelt off using forceps. In this way, prepared epidermis sheets could be used for diffusion experiments after 30 min. of floating in water.

3.5.2. Trypsin- isolated stratum corneum (SC)

The skin was cleaned and divided in small pieces using a pair of scissors. The pieces were transferred, dermal side down, into a Petri dish, which contained an 0.15% trypsin solution in PBS buffer, and incubated for 24 h in drying chamber at 32°C. At the following days , this procedure was repeated with fresh trypsin solution until CS was fully isolated.[33, 36, 37]

The SC was washed three times with distilled water, dried up with a freeze dryer and stored in an exsiccator at room temperature until use.

3.6. Determination of saturation solubility of drugs in the vehicle

100 mg of drug was added to 10 ml of highly liquid paraffin in a glass vials and stirred for 24h in a drying chamber set to 32 °C. Then the excess solid was removed by filtering through a 0.2 µm pore size filter (OPTI-flow PTFE). The clear filtrate was diluted appropriately maintaining a final proportion of paraffin: dichloromethane 1: 10. Dichloromethane reduced absorption of paraffin, what is especially important for UV spectroscopy. The dilution was analyzed using UV-spectroscopy by wavelength of 238 nm for testosterone, 277 nm for caffeine, 287 nm for flufenamic acid and 274 nm for benzoic acid.

Using calibration curve, saturation solubility of drug in the vehicle was established.

3.7. Determination of partition coefficient (Kp-sc)

To establish partitioning of drug between highly liquid paraffin and SC lipids, Kp-sc was calculated.

At first, dried SC from donors 2 and 3 was placed into glass vials with 5ml paraffin containing 3 different concentration of each drug (2 repetitions for skin 2; 3 repetitions for skin 3). After 24 h in a drying chamber set to 32°C, the SC was taken out and the 2.5 ml of supernatant was diluted with dichlormethane to final

volume of 25ml. The remaining amount of drug in paraffin was analyzed using UV-spectrophotometer by wavelenght determined from spectrum of drug.
The difference between initiation amount of drug in paraffin and remain amount of drug in paraffin is amount of drug, which SC absorbed .
According to equation 1. Kv-sc was calculated.

3.8. Determination of partition coefficient (K_{Soe-SC})

$K_{Soe\ -SC}$ is the partition coefficient between Soerensen phosphate buffer and stratum corneum. For this determination, SC of donors 2 and 3 was placed into glass vials with 5 ml of Soerensen phosphate buffer and 3 different concentrations of benzoic acid (2 replicates for skin 2, 3 replicates for skin 3). The vials were placed into a drying chamber at 32°C and left to incubate for 24 h. After this time, the SC was removed and the fluids were diluted with fresh Soerensen phosphate buffer to the required concentration. This dilution was quantificated by a HPLC method.
$K_{Soe\ -SC}$ was calculated only for BA, because $K_{Soe\ -SC}$ for testosterone, caffeine and flufenamic acid were obtained from literature.

In liquid- liquid partitioning processes, it is often advisable to allow the equilibration time to be as long as possible. When one phase is a biological membrane, one must be cautious about the use of very long incubation periods because changes in the membrane may occur.[38]

3.9. Permeation experiments ("Infinite dose")

For all permeation experiments, the skin from donors 1 and 2 was used (n= 6, for each skin and for each drug).
Heat-separeted epidermis (HSE) was prepared from full thickness skin in the time of use according Chap., par. 3.2. Because the HSE is very thin and fragile, a support membrane from cellulose was used for its stabilization and better

handling. The membrane was pre-hydrated with Soerensen phosphate buffer. The proof of inert properties of this membrane was adduced (Medicell Int., London).

In the FDCs with diffusion area of 1.767 cm^2, the HSEs with support membranes were carefully attached between the donor and acceptor compartments. The donor compartment was filled with 500µl liquid paraffin containing the drug in following concentrations: 400µg/ml in case of testosterone, flufenamic acid and benzoic acid and 100µg/ml for caffeine. Prepared FDCs were stabilized by clamps and positioned into a water bath set to 32° C. In the acceptor compartment, Soerensen phosphate buffer pH 7.4 was continually stirred by a magnetic bar at 500 rpm. The samples of 0.4 ml were drawn from the middle of the acceptor area at different time intervals (depending on the used drug). The samples were collected and immediately replaced with 0.4 ml of fresh buffer solution. Experimental time was between 10 and 29 h depending on drug. During the experiment, the diffusion cells had to be checked for air bubbles in the acceptor fluids. These bubbles reduce the diffusion area and therefore could change the results of the diffusion experiments. Preservative was not added in the acceptor fluid, because of its influence on drug permeation.

At the end, collected samples were analyzed by validated HPLC methods.

N.B.

All experiments with benzoic acid were performed in glasses, which were washed with isopropanol and left to dry out during the night before our experiments. These processes should avoid microbial contamination of benzoic acid samples.

3.10. HPLC determination

The chromatographic separation and quantification occurred in accordance to below described conditions:

3.10.1. Testosterone

Detection wavelength: 250 nm

Mobile phase: methanol / water 70/ 30 (V/V)

Flow rate: 1.2 ml/ min

Analyze time: 5 min.

Retention time: 4.8 ± 0.2 min.

Injection volume: 50 µl

3.10.2. Caffeine:

Detection wavelength: 262 nm

Mobile phase: buffer pH 2.6 / acetonitrile 90/ 10 (V/V)

Flow rate: 1.2 ml/ min

Analyze time: 6 min.

Retention time: 5.08 ± 0.2 min.

Injection volume: 50 µl

3.10.3. Flufenamic acid:

Detection wavelength: 284 nm

Mobile phase: methanol / McIlvaine buffer pH 2.2 80/ 20 (V/V)

Flow rate: 1.2 ml/ min

Analyze time: 5 min.

Retention time: 3.1 ± 0.2 min.

Injection volume: 50 µl

3.10.4. Benzoic acid:

Detection wavelength: 228 nm

Mobile phase: buffer pH 6.5 / methanol 95/ 5 (V/V)

Flow rate: 1.2 ml/ min

Analyze time: 5 min.

Retention time: 3.5 ± 0.03 min.

Injection volume: 50 µl

The above-described HPLC analytical methods could be used for the concentration interval of drug between 50 to 15 000 ng/ ml. Before quantification, the samples of known drug concentration were measured (extern standard method). Quantification of drug was done by comparison of the peak area of standards with the peak area of samples.

3.11. Calculations

3.11.1. Permeation experiments

The removed 0.4 ml samples of drug were collected over the time and quantified by HPLC. From the drug concentration in acceptor medium the cumulative amount of drug permeated per area was calculated. We drew a diagram of this cumulative amount permeated per area versus time for each drug. From a certain time point, the course of curve showed linear correlation between cumulative amount permeated per area and time. The point, where the curve cuts the X- axis, was so called ‘lag time’. After this point, a constant concentration gradient in skin occured and steady state conditions could be postulated. The steady state flux was determined from the slope of the linear portion of the plots. The apparent permeability coefficient (Kp) was calculated by dividing steady state flux by donor concentration, according eq.4.

The next parameter for calculation was Jmax. This could be calculated, when Kp was multiplied by Csat according to eq. 5.

These calculations were performed using Microsoft Excel 2003 and Sigma Plot 10.0.

4. RESULTS

4.1. Saturation solubility

At first, scans of all substances in mix of highly liquid paraffin and dichlormethane (1:10) was performed using UV-spectroscopy.

Figure 14: Scan of testosterone

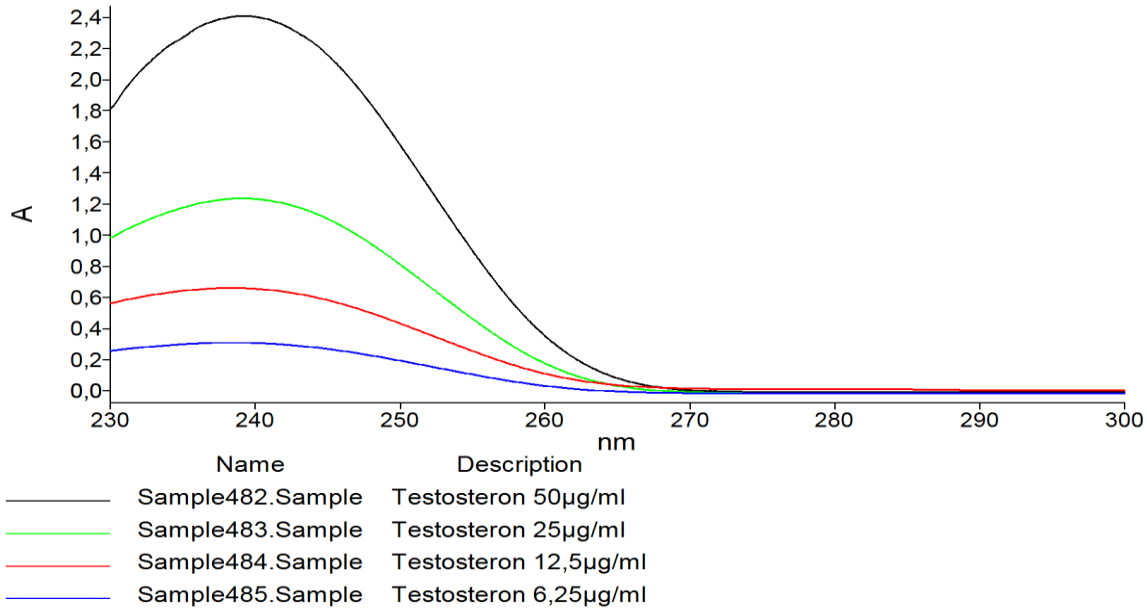


Figure 15: Scan of caffeine

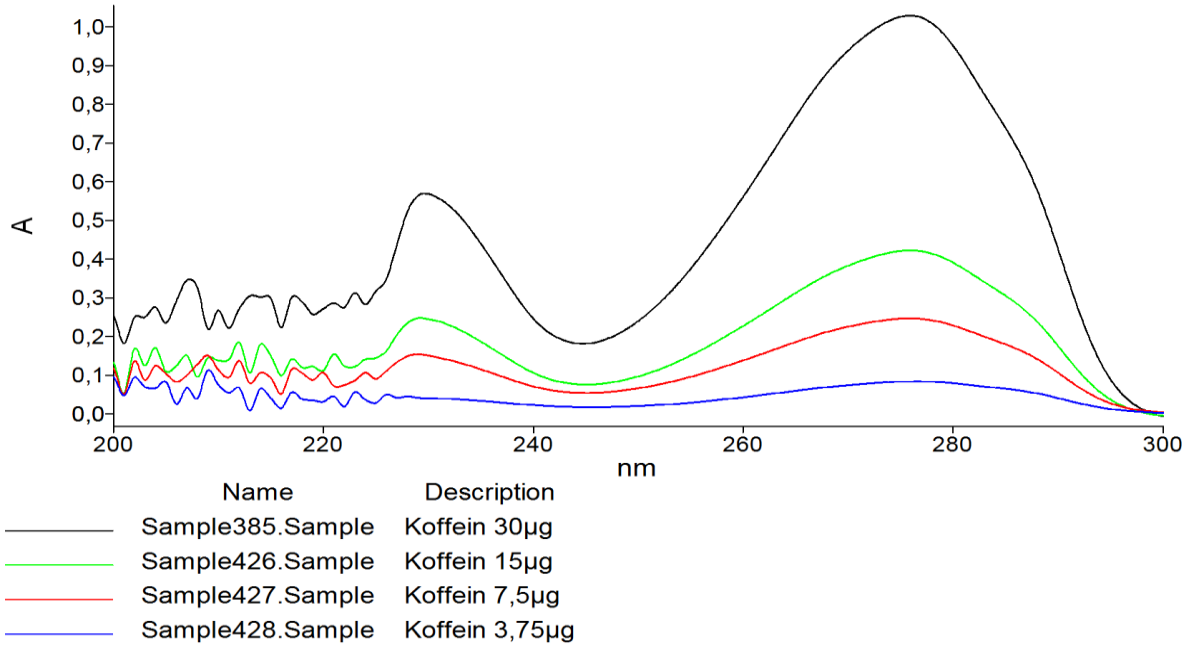


Figure 16: Scan of flufenamic acid

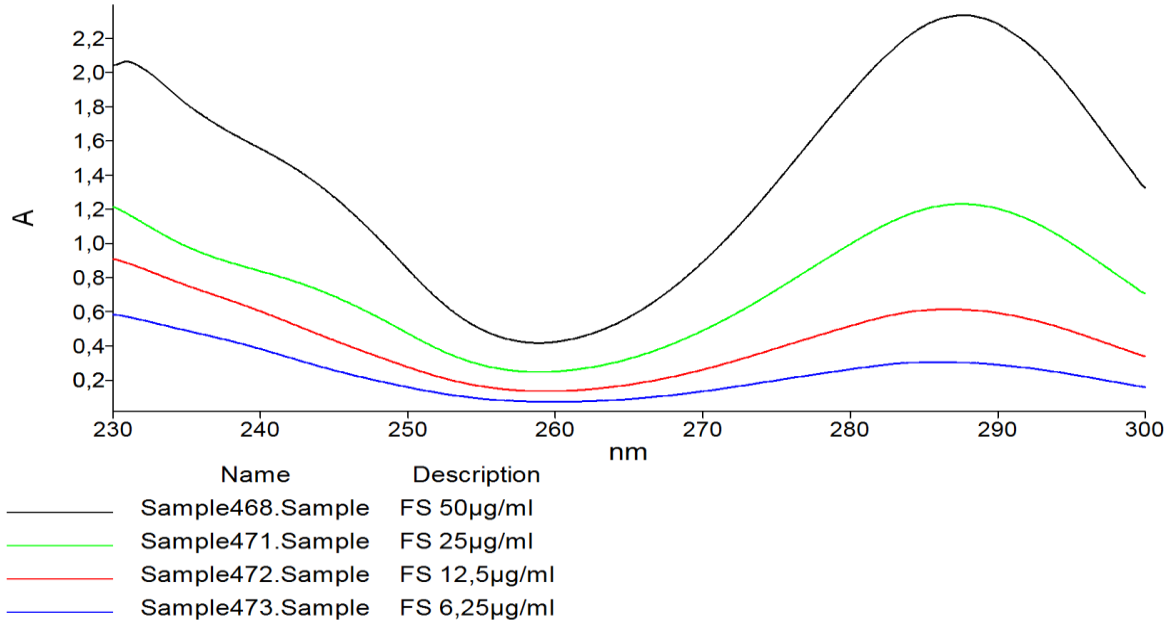
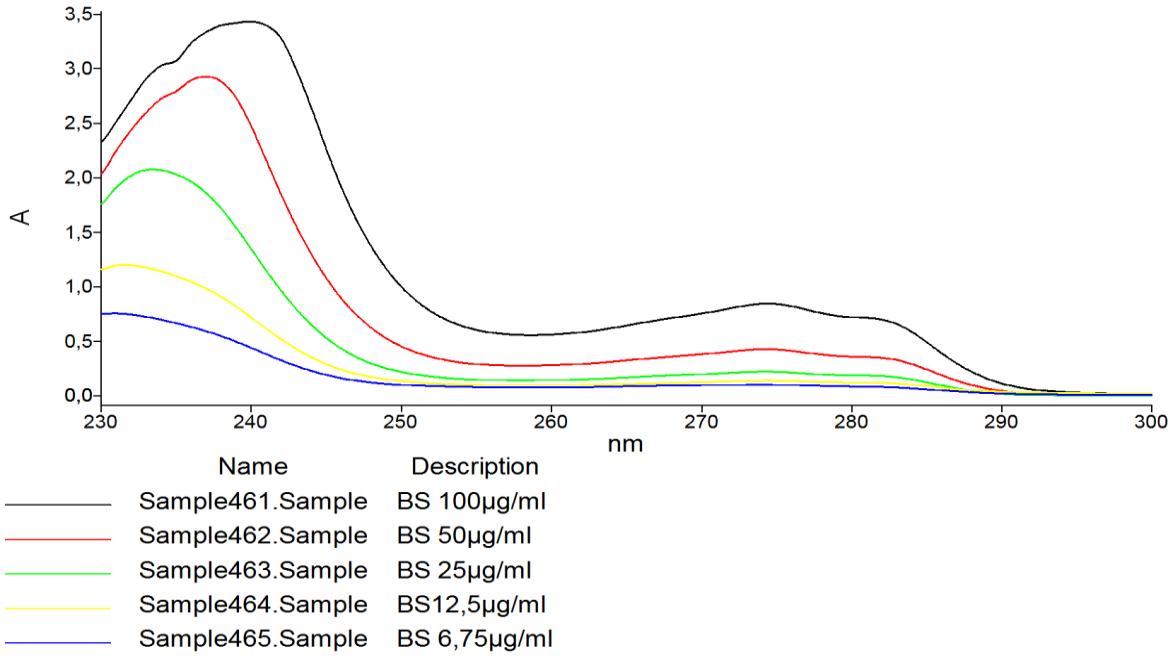


Figure 17: Scan of benzoic acid



From spectrum of all substances, we could find out the drug's concentration and required wavelength for measurement of calibration curve according to Lambert-Beer law.

Benzoic acid poses an exception. We could not take into account its main maximum of spectrum, because benzoic acid has to high absorption in low concentration there. Concerning our analytic possibilities, we measured calibration curve of benzoic acid by second maximum of scan.

Table 1: Results of scan experiments

	Limit of detection (µg/ml)	**Limit of determination (µg/ml)**	**Wavelength Λ_{max}(nm)**
Testosterone	1.25	2.50-15.00	238
Caffeine	1.88	3.75-20.00	277
Flufenamic acid	1.56	3.13-15.00	287
Benzoic acid	6.25	12.5-100.00	274

From calibration curves were determined slope of plots and saturation solubility of drug measuring absorptions of samples according chap. 3.4. (Tab.2)

Table 2: Slope of curves and saturation solubility for each drug

	$E^{1\%}_{1\ cm}$ (M±SD)	**Saturation solubility** (M±SD) µg/ml
Testosterone	427±47	498.15±55.29
Caffeine	290±48	134.06±3,31
Flufenamic acid	525±39	1176.65±52.20
Benzoic acid	116±32	9852.38±826.34

From the donor concentration and the saturation solubility the thermodynamic activity (A) of the drugs could be calculated.

Table 3: Thermodynamic activity of drugs

	Testosterone	Caffeine	Flufenamic acid	Benzoic acid
A	0.80	0.77	0.34	0.04

4.2. Partition coefficient

4.2.1. Kp-sc

Partition coefficient between liquid paraffin and SC was determined separately for each skin and each drug according chap. 3.5..

For testosterone had to be considered, that SC itself contains a small amount of testosterone. Therefore, it was necessary to prepare blank samples, which were incubated and measured at the same time with the other samples. Blank samples contained SC with liquid paraffin only.

After the experiment, measured paraffin showed released amount of testosterone from SC. Skin 2 contained 3416.27 μg of testosterone in 1g of SC. Skin 3 released 13481.79μg of testosterone from 1g of SC.

Considering these released amount of testosterone from SC, Kp-sc for testosterone could be calculated.

Table 3: Kp-sc for each skin and each drug (M±SD)

	Skin 2	Skin 3
Testosterone	168.27±47.90	455.51±311,96
Caffeine	103.74±39.09	330.03±198.53
Flufenamic acid	52.49±23.37	115,92±79.14
Benzoic acid	61,70±34,36	103,53±39,95

Active concentration of drug in SC could be calculated from eq. 1.

Table 4: Active concentration of drug in SC

C_{sc}(mg/cm^3)	Skin 2	Skin 3
Testosterone	67.31±19.16	182.20±124.78
Caffeine	10.37±3.91	33.00±19.85
Flufenamic acid	20.99±9.35	46,37±31.66
Benzoic acid	24,68±13,74	41.41±15,98

4.2.2. Ksoe-sc

Our measurements were performed only with benzoic acid according chap.3.6. For all other drugs, Ksoe-sc was found from different sources.

Ksoe-sc of testosterone ranges between 33 and 47 in literature.[38, 39] The reported results represent partitioning between water and SC. In case of testosterone, it is possible to consider this value, because partitioning of testosterone is not depending on pH of environment.

Determination of Ksoe-sc for caffeine and flufenamic acid was performed by Steffi Hansen in her Ph thesis work. Her method for determination of Ksoe-sc was similar to our method described above in chap. 3.6..

Table 5: Ksoe-sc for each drug (skin 2 / skin 3)

	Ksoe-sc (M±SD)
Testosterone	~40
Caffeine	4.51±2.73
Flufenamic acid	16.2±4.89
Benzoic acid	10.67±5.35/14.77±9.40

4.3. Permeation experiments

Excised human skin from donor 1 and 2 was used as experiment material, which is characterized nearer in chap. 3.1.. Skin from two donors was taken to see the inter-individual differences between various skin donors on drug permeation.
All experiments were performed in accordance to the above-described instructions in chap. 3.7. and all values were calculated by course of equations in chap. 3.9.

4.3.1. Testosterone

Testosterone as lipophilic substance should release slowly from paraffin due to its high affinity to lipophilic vehicle. It partitions rapidly to SC, where it diffuses trough lipid part of bilayer. We expected slow partition into viable epidermis and slow permeation there. Permeation of testosterone could decelerate high binding on proteins, also to keratin of the corneocytes.
Fig.18 illustrates the relation between amount of testosterone permeated per area and time for both skins.
All permeation values are summarized in table 5.

Figure 18: Penetration experiments with testosterone for skin 1 and 2

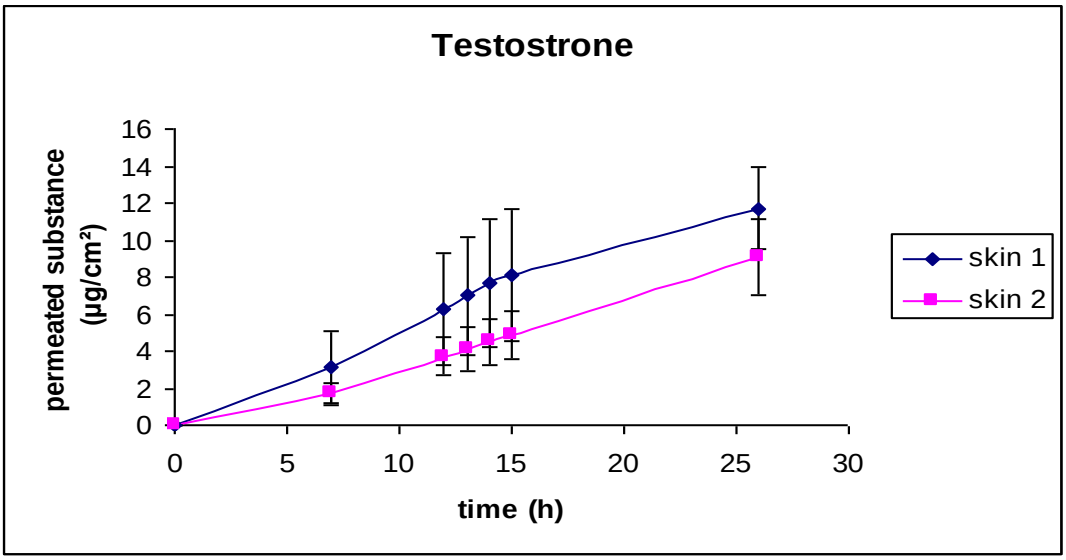


Table 6: Permeation values of testosterone

Testosterone	Skin 1	Skin 2	Skin 1+2
Kp(cm/s) ×10-7	4.39±1.52	2.50±0.56	3.45±1.47
J (µg/cm²/h)	0.63±0.22	0.36±0.08	0.50±0.21
J_{MAX} (µg/cm²/h)	0.79	0.45	0.62
Lag time (h)	2.27	1.68	1.97

4.3.2. Caffeine

Caffeine as most hydrophilic substance from our model drugs should release fast from lipophilic formulation. It partitions well to SC, permeates in hydrophilic part of SC bilayer, and relatively fast in hydrophilic epidermis. Possible transappendage route have to be take into consideration by caffeine as polar substance.

Figure 19: Penetration experiments with caffeine for skin 1 and 2

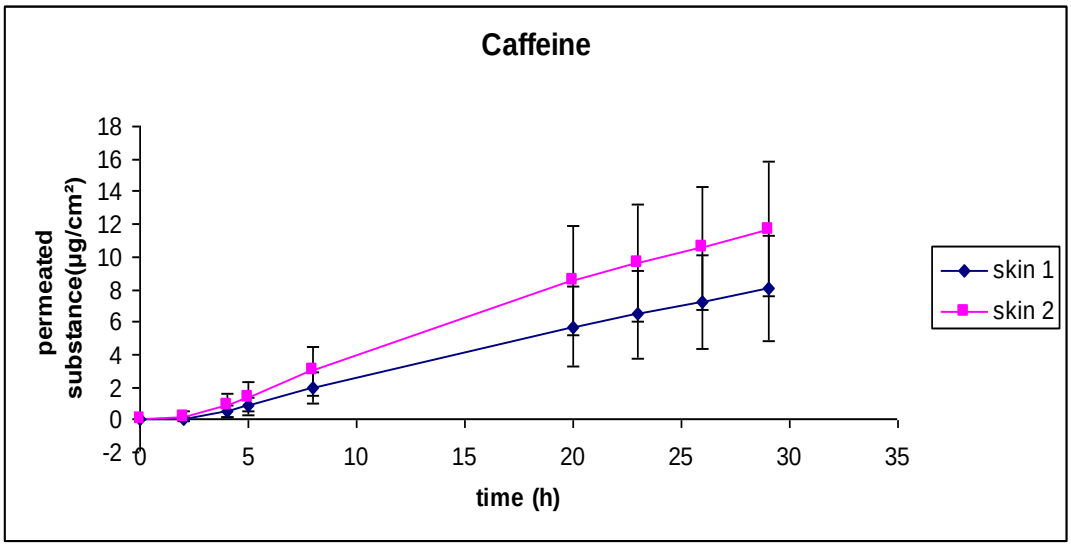


Table 7: Permeation values of caffeine

Caffeine	Skin 1	Skin 2	Skin 1+2
Kp(cm/s) ×10-7	8.43±3.69	11.43±5.22	9,91±4.57
J (µg/cm²/h)	0.30±0.13	0.41±0.19	0.36±0.16
J_{MAX} (µg/cm²/h)	0.40	0.53	0.46
Lag time (h)	2.04	1.44	1.91

4.3.3. Flufenamic acid

Flufenamic acid is a relatively lipophilic substance. We expected, that it will release slow from lipophilic vehicle. It partitions slowly into SC from all our substances and permeates trough lipophilic part of SC bilayer. As testosterone, the rate-limiting step should be the partition from SC into viable epidermis and it shows high protein binding activity, what it could induce the binding to keratin.

Figure 20: Penetration experiments with flufenamic acid for skin 1 and 2

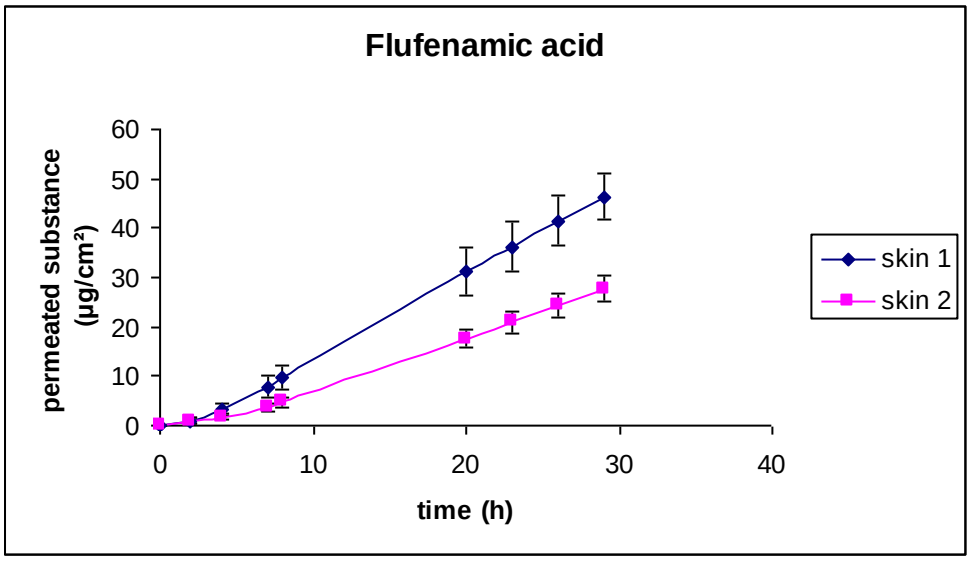


Table 8: Permeation values of flufenamic acid

Flufenamic acid	Skin 1	Skin 2	Skin 1+2
Kp(cm/s) ×10-7	11.86±1.29	7.11±0.77	9.70±2.68
J (µg/cm²/h)	1.71±0.19	1.02±0.11	1.40±0.39
J_{MAX} (µg/cm²/h)	5.04	3.02	4.12
Lag time (h)	1.93	2.48	2.20

4.3.4. Benzoic acid

Benzoic acid represents a moderate hydrophilic substance. Its release from paraffin should occur rapidly, but its partition into SC slowly. Through SC it permeates by hydrophilic tortuous route. We expected easy partition into viable epidermis and fast diffusion there. By benzoic acid as polar substance, there has been possibility of permeation through hair follicles.

Figure 21: Penetration experiments with benzoic acid for skin 1 and 2

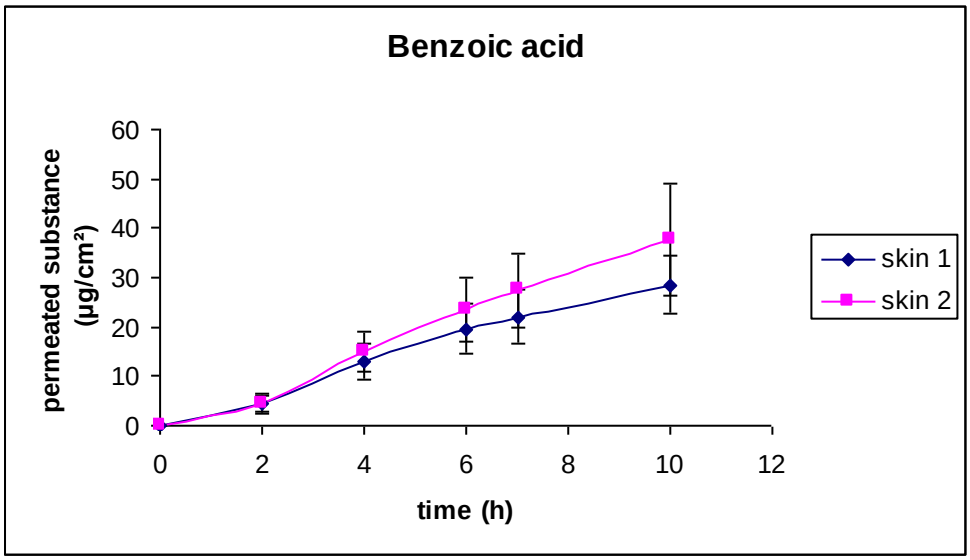


Table 9: Permeation values of benzoic acid

Benzoic acid	Skin 1	Skin 2	Skin 1+2
Kp(cm/s) ×10-7	23.22±6.49	29.68±9.87	25.53±8.70
J (µg/cm²/h)	3.34±0.94	4.28±1.42	3.81±1.22
J_{MAX} (µg/cm²/h)	82.34	105.25	90.53
Lag time (h)	0.3	0.51	0.40

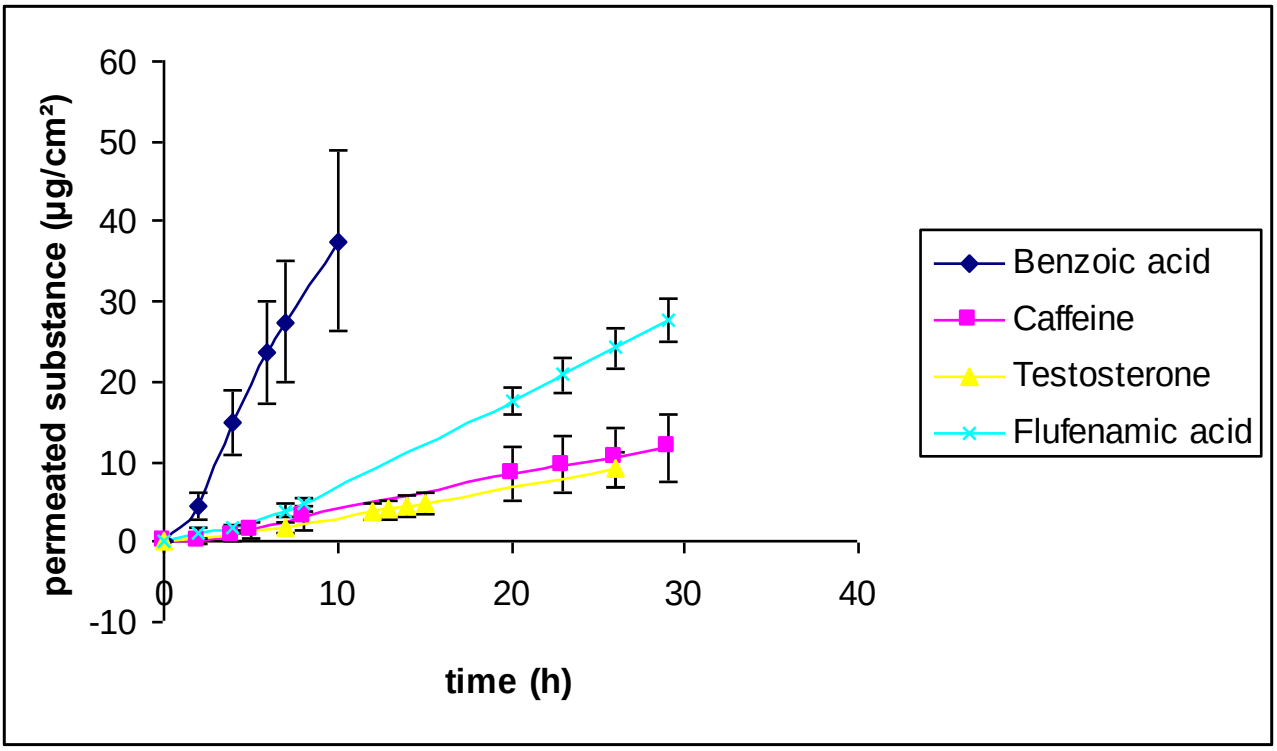
Different permeation profiles were obtained using two skins from various donors. To reduce the influence of inter-individual differences between various skin donors, the next calculation and discussion are performed only with skin 2, which was used for permeation experiments as well as for determination of partition coefficients. In the following table are summarized all values obtained from literature and in experiments with skin 2.

Table 10: Summarized data for all substances performed with skin 2

	Testosterone	Caffeine	Flufenamic acid	Benzoic acid
Molecular Weight	288.4	194.19	281.24	122.12
pK_a	-	1.39	3.9	4.19
Log P	3.47	-0.08	4.8	1.9
C_S (µg/ml)	498.15±55.29	134.06±3,31	1176.65±52.20	9852.38±826.34
C_D (µg/ml)	400	100	400	400
A	0.80	0.77	0.34	0.04
J (µg/cm²/h)	0.36±0.08	0.41±0.19	1.02±0.11	4.28±1.42
J_{MAX} (µg/cm²/h)	0.45	0.53	3.02	105.25
Kp(cm/s)×10-7	2.50±0.56	11.43±5.22	7.11±0.77	29.68±9.87
Kv-sc	168.27±47.90	103.74±39.09	52.49±23.37	61,70±34,36
C_{SC} (µg/ml)	67.31±19.16	10.37±3.91	20.99±9.35	24,68±13,74
Lag time (h)	1.68	1.44	2.48	0.51
D / h (cm/s)	0.02	0.11	0.14	0.48

D/h is diffusion coefficient of drug divided by thickness of SC. It can be calculated in accordance eq. 3 and 5. This value describes diffusion of drugs trough SC and viable epidermis.

Figure 22: Comparison of permeation profiles for all substances performed with skin 2



5. DISCUSSION

5.1. Saturation solubility

The values of saturation concentration of the drugs in highly liquid paraffin are decreasing in following order: benzoic acid> flufenamic acid > testosterone > caffeine.

The saturation solubility of the drugs in paraffin corresponds well to the lipophility of the drugs expressed as the octanol /water partition coefficient (log $K_{oct/\ water}$), which is as follows: flufenamic acid (4.80) > testosterone (3.47) > benzoic acid (1.9) > caffeine (-0.08).

The rank order of the drugs is nearly the same, except benzoic acid, which is a special substance. Bencoic acid shows a higher saturation concentration than we expected from the log $K_{oct/\ water}$ coefficient, what could be due to constituting of multi crystal structure.

5.2. Comparison of permeation of our model drugs out of paraffin

All of the permeation experiment values with skin 2 are summarized in table 10.

When we take a look at maximum flux, we can see that the highest value has benzoic acid, followed by flufenamic acid, than caffeine and the lowest value has testosterone. The maximum flux is primarily dependent on the solubility of the drug in the vehicle and not so much affected by the partition coefficients[40]. The relation between solubility of the drug and maximum flux seems to be applicable, with exception of testosterone. Considering its solubility and partition coefficient between stratum corneum and paraffin, testosterone should show a higher J_{MAX} value. We assume that the lower J_{MAX} can be due to its higher binding ability to proteins and high lipophilicity, which induce low partition to epidermis and a slow diffusion there. High binding ability to proteins could evoke the binding of testosterone to keratin in corneocytes and part of testosterone could remain in the SC. Calculated active concentration of drug in the SC confirms this assumption.

The active concentrations of the drugs correspond with the maximal flux of the substances, except testosterone.

If we compare the apparent permeability coefficient of the drugs, we see highest value for benzoic acid, than for caffeine, followed by flufenamic acid and testosterone. Altogether, the skin permeability of the drugs out of paraffin is determined by their hydrophilicity determining the ability of partitioning between the vehicle and the SC and the diffusion in the different phases. Considering hydrophilicity of drugs expressed as the octanol /water partition coefficient (log $K_{oct/\ water}$), it convinces us that the hydrophilic drugs permeate out of paraffin faster than lipophilic substances.

If we consider partitions coefficients of drugs, we have to notify, that two partitions coefficient influence permeation of drug through HSE. These are the partition coefficient between SC and paraffin and the partition coefficient between SC and viable epidermis. The last one is particularly important for lipophilic substances. The partition coefficient between SC and paraffin descends in the following order: testosterone > caffeine > benzoic acid> flufenamic acid. Testosterone has a high partition coefficient between SC and paraffin, but we do not know the partition coefficient between SC and viable epidermis. Considering testosterone lipophilicity, we can imply, that it will be low, what explains the lowest permeation from all of our tested drugs. Concerning caffeine and benzoic acid, we imply a high partition coefficient between SC and viable epidermis, what facilitates their permeation.

The reduced ability of partition may explain why the apparent permeability coefficient of testosterone is the lowest from all drugs. The diffusion coefficient of benzoic acid is the highest in comparison to all drugs used, which corresponds with its hydrophilicity, smallest molecular weight and therefore, with highest permeation coefficient. From apparent permeability coefficient, we would have expected that caffeine, as a hydrophilic substance will have a higher diffusion coefficient than flufenamic acid. However, this was not the case. Flufenamic acid shows a higher diffusion coefficient, although its permeability coefficient is lower than caffeine. An explanation could be that caffeine's partition coefficient is two times higher than of flufenamic acid, what improves naturally the permeation of caffeine. Furthermore, for caffeine as a hydrophilic substance not only the

intercellular permeation pathway must taken into consideration, but also the appendage and transcellular route may play an additional role.

We can only compare the flux values of different drugs if these substances are used in the experiments with the same activity in the vehicle. Unfortunately, we have not used our drugs at the same activity and therefore we cannot compare the flux values. However dividing the flux by activity, we get the permeability coefficients, which are already compared.

Lag time of all substances is very similar, only benzoic acid shows a little bit shorter lag time, which can be explained by its low molecular weight and the ability of easy releasing from paraffin to the SC. Large molecules tend to diffuse slowly.

5.3. Comparison of permeation of our model drugs out of paraffin versus out of Soerensen buffer

Data of partition coefficient between SC and Soerensen phosphate buffer pH 7.4 is shown in table 5.

F. Netzlaff and co-workers worked with the same substances as in our experiments. However, the substances were applied in Soerensen phosphate buffer and not in paraffin. In table 11 Kp values of the four test compounds in human HSE are depicted.

Table 11: Kp values of the four test compounds in human HSE in cm/s ×10-^{7}

		Caffeine	Benzoic acid	Testosterone	Flufenamic acid
Human	Individual 1	0.85	1.01	2.29	3.86
	Individual 2	0.15	10.70	2.34	5.37
	Individual 3	0.29	0.14	2.30	2.19
	Individual 4	0.06	-	-	-
	Individual 5	0.76	-	-	-
	Individual 6	-	-	-	-
	Mean	0.42	4.03	2.31	3.81
	B. b. precision	0.36 (86%)	5.79 (152%)	0.03 (1%)	1.59 (42%)
	W. b. precision	0.36 (85%)	2.38 (63%)	0.56 (24%)	1.43 (38%)

Table 11 shows, that the values of Kp for benzoic acid and caffeine significantly vary with skin from different individuals. For testosterone and flufenamic acid the Kp values are almost constant. The dependence of results on particular skin donor was present also in our work.

The explanation of this variability can be the fact, that the skin from various individuals have different amount of hairs. Hydrophilic substances like caffeine can permeate the transapendageal route too, meaning that the amount of drug in the acceptor phase will dependent on how hairy the skin is.

Comparing the mean values of the apparent permeability coefficients for all four substances, we can see that the highest value was found for benzoic acid, followed by flufenamic acid and testosterone, and the lowest value was shown for caffeine.

The sequence of the Kp values for the substances in hydrophilic vehicle is the same as with our results from lipophilic vehicle, except caffeine. Permeation of caffeine is considerable higher from lipophile vehicle than from hydrophile vehicle due to its low solubility in lipophilic media.

Generally, we can say, that the same drugs show higher permeabilities out of lipophilic vehicle compared to aqueous vehicle. Only the Kp values for testosterone are very similar. Kp for paraffin is 2.5×10^{-7} cm/s. Kp for Soerensen buffer is 2.3×10^{-7} cm/s. It might be speculated, that testosterone permeation does not depended on the donor, but partitioning between SC and epidermis and permeation there are critical for testosterone. Further, the binding ability of testosterone to keratin in corneocytes may influence testosterone permeation.

Comparing the partition coefficient of all drugs between vehicle and SC, it is noticeable, that the partition coefficients of testosterone are the highest as well as for Soerensen buffer as well as for parraffin. Testosterone as high lipophilic substance permeates easy to SC, where it is accumulated. In contrast caffeine partitiones from paraffin very easy to SC, however from Soerensen buffer partitioning occures very slowly. It validates the Blanks theory with homologous alkanols, that the lipophilic drugs show high permeabilities out of aqueous solutions and the permeabilities of lipophilic drugs out of lipophilic vehicles should be low. The skin permeabilities of homologous alkanols decrease with their chain lenght out of lipophilic vehicles (isopropyl palmitate, mineral oil, olive oil) but increase out of hydrophilic vehicle[41].

Benzoic acid and flufenamic acid have very similar value for partitioning from Soerensen phosphate buffer and from paraffin, whereas benzoic acid as hydrophilic substance is faster from paraffin and flufenamic acid as lipophilic acid is faster from Soerensen buffer.

According eq. 1 and 5, we calculated the diffusion coefficient by dividing the permeation coefficient by the thickness of SC for the drugs, which permeate out of Soerense buffer. The sequence of drugs is as follows: benzoic acid (0.37)> flufenamic acid (0.24) > caffeine (0.09) > testosterone (0.06). The rank order of the drugs is the same like for paraffin. This is a hint that the diffusion of drug may be independent of the vehicle, which drug permeates from.

The vehicle influences primarily the release of drugs and partitioning to the SC.

However we have to consider additional effect of auqueous vehicle on SC. Working with vehicle containing water swelling of SC may occur. Corneocytes contain more water than keratin. With lower water of hydratation, the corneocyte phase comprises a greater fraction of protein, which is more favorable to the drug than water[42]. This happens when using paraffin as a donor vehicle. Consequently SC could become a larger reservoir of lipophilic substances e.g. testosterone and flufenamic acid. This can be an additional reason, why the permeation of these two substances is low from praraffin.

For ionisable drug like benzoic acid in aqueous vehicles, the permeation depends on the degree of ionization and how the ionization influences the solubility in the applied phase and its partition into skin. This problem can we overcome by using paraffin as donor.

6. CONCLUSION

The purpose of this thesis was to investigate and determine the permeability of model drugs from a lipophilic vehicle (highly liquid paraffin) and consequently to compare permeation parameters of these drugs. The permeation of these drugs should be compared with results from hydrophilic vehicle obtained from literature.

Permeability is generally the outcome of several intertwined partitioning and diffusion process. Inter-individual differences between skins from various donors are well known. To minimize this problem, hence the last calculation and discussion is concerned only with skin 2, which was used for permeation experiments as well as for the determination of partition coefficients.

The potential of vehicle to influence the permeation of model drugs is present in our work. The above-described results have shown that the same drugs show high permeability out of both vehicles, lipophilic and aqueous. Normally the permeabilty was higher if using the lipophilic donor in comparison to the aqueous. However, for testosterone the permeation was very similar. This may lead to the conclusion, that skin absorption of testosterone is not dependeding on the vehicle used. It might be speculated that partitioning between SC and epidermis and the permeation in epidermis seems to be critical the step for the permeation of testosterone in HSE, because partitioning between both vehicle and SC is particularly high. A further explanation might be that the ability of testosterone to bind to keratin in the corneocytes may affect testosterone skin absorption. Caffeine shows a particulary higher permeation from lipophile than from hydrophile vehicle. The reason might be, that caffeine partitions from paraffin to the SC very easy however, from Soerensen buffer partitioning occurs very slowly. Generally, we can assert that hydrophilic drugs show high permeabilities out of lipophilic vehicle and the permeabilities of lipophilic drugs out of lipophilic vehicles should be low.

Benzoic acid as hydrophilic substance permeates very well from both vehicles. This might be explained, that for benzoic acid not only the intracellular pathway must be taken into consideration but also transappendageal and intracellular route may play and additional role. This might also the reason, why results from different individuals vary so significantly.

From the reported results, it seems to be evident that the diffusion of drug into human skin is independent of the applied vehicle.
Nevertheless, the universal validity of these arguments must be shown by further investigations.

7. LIST OF USED ABBREVIATIONS

NSAID	Non-steroidal anti-inflammatory drug
Kp	Apparent permeability coefficient
HSE	Heat separated epidermis
SC	Stratum corneum
FDC	Franz diffusion cell
V/V	volume to volume

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10. STATUTORY DECLARATION

Hereby I affirm in lieu of an oath, that I made the present thesis autonomously and without other than the indicated auxiliary means. The data used indirectly or from other sources, and concepts are characterized with lists of sources.

The thesis has been not been submitted for academic degree consideration either nationally or internationally in identical or similar from to date.

Saarbruecken, 30th March 2007_____

Koláčková Lenka