4. DISCUSSION

For drug development but also for hazard assessment in toxicology, predictive test models are under development, which aim at the estimation of the drug levels in the patient. With respect to transdermal uptake this may be done by in vitro tests using excised skin of human and animal origin [221-222]. However, specimens of human skin of adequate size and quality for the cutaneous uptake experiments are not readily accessible to all investigators and only available in limited amounts. Therefore, commercially available reconstructed human skin equivalents could, by principle, offer an interesting alternative, if the permeation barrier of these models reflects that of the healthy human skin. The morphological appearance, differentiation markers as well as lipid patterns and the developed barrier function for these reconstructed skin equivalents are well characterised [137-138, 167]. The less developed barrier function of these models as compared to human skin corresponds to the difference in the lipid pattern of the Stratum corneum and should explain the higher permeability of these models as compared to human skin [163-165]. Nevertheless if the permeation / penetration of reconstructed epidermis by substances of different physicochemical characteristics exceed the uptake by human skin by a defined ratio, a higher permeability can be considered in the interpretation of the results.

Also, the use of such models for in vitro experiments should reduce the number of animal and human testing [135-139]. Alternative in vitro tests are demanded by European authorities introducing a new area of chemical policy to improve customer security and industrial health and safety. Skin models, in particular reconstructed human epidermis, have already been used to test skin uptake of substances in cosmetics and dermatology [136]. They are currently used to assess raw materials, drug formulations, and finished products under conditions approaching those of potential contact or intended use, respectively. However, for application of reconstructed epidermis in regulatory toxicology studies one has to keep in mind that neither the tissues nor the test procedures have been subjected to formal validation studies, although in 2004 the OECD has released a test guidline [223].

In order to contribute to the evaluation of the reconstructed epidermis for permeation / penetration experiments, we have compared these methods, additionally including an alveolar model as well as a perfusion model. To be suitable for studying intensely metabolised drugs, models should not only predict permeation and penetration but also drug metabolism.

To establish reconstructed human epidermis for routine skin permeation and penetration experiments, substances of different hydro-lipophilicity have to be tested. We, in this study focused on lipophilic substances. We selected two substances, which are slightly different in their lipophilic appearance; the more lipophilic E2 (log P= 4.13, M.Wt. 272) and the less lipophilic testosterone (log P= 3.47, M.Wt. 288) and the more hydrophilic hydrocortisone (log P 1.43, M.Wt. 362). These substances are extensively studied in other permeation and penetration studies [33, 43, 51, 135]. Moreover testosterone is even recommended as reference substance by the current OECD guidline [135] for in vitro skin absorption studies. The permeation study using testosterone was integrated in a nationwide BMBF (Bundes Ministerium für Bildung und Forschung)-funded study to validate skin models for routine skin absorption tests in drug development, cosmetics, and toxicology.

4.1. Testosterone

The testosterone experiments were a part of the nationwide study, to validate test protocols using the skin models for permeation studies. Here, the SkinEthic® reconstructed epidermis model was selected since previous prednisolone penetration experiments in our laboratory indicated a superior barrier function of the SkinEthic® model as compared to the EpiDerm and EpiSkin models [166].

4.1.1. Static versus dynamic set up

Evaluation of the static and dynamic set up resulted in almost identical curve progression of permeated testosterone independent of the used Franz-cell type. This result was in agreement with existing literature [224-225]. Based on the identical curve progression, we decided for the more economical static set up to decrease the expenses of the experimental work.

4.1.2. Donor vehicle and solubility restrictions of highly lipophilic drugs

It is now generally accepted that Stratum corneum lipids influence the barrier function of the skin. Anomalies in Stratum corneum lipid composition due to various disease states e.g. psoriasis or essential fatty acid deficiency or due to the use of organic solvents are accompanied by impaired barrier function and increased skin permeability [36, 50]. Thus, the organic solvents can affect the transport across the horny layer which is controlled by many physicochemical aspects, the most important of which are partition coefficient (K), diffusion coefficient (D), and solubility of the substance (Cs). To enhance the drug uptake, strategies are adopted to improve K, D, or Cs. Because of the difficulty to dissolve testosterone in water

based donor media, organic solvents had to be introduced. However, toxic effects of organic solvents to the skin increase with their concentrations [36, 75]. Testosterone is well dissolved in absolute ethanol and $\geq$ 60% ethanol-water solutions (v/v), but this ethanol concentration was very toxic to skin cells (Tab 4, 5) and decreased the skin permeability because of Stratum corneum dehydration. This was also described by Kanikkannan et al., Altenburger et al., and Pershing et al.

[226-228]. Testosterone is also soluble in oils e.g olive oil and miglyol (naturally occurring oil which derived from the coconut, a triglyceride of medium chain length, C_8-C_{10}, of saturated fatty acids). Oil based donors, however, prevented almost completely any testosterone permeation. The addition of BSA to the acceptor medium did not change this result. This could be explained by the higher solubility of testosterone in oils than in Stratum corneum lipids. An ethanol-miglyol mixture favoured testosterone penetration into the skin. Therefore, the amount of ethanol was thoroughly balanced versus the skin toxic effects and a compromise had to be made. Testosterone permeation from 10/90 ethanol-miglyol exceeded that from 100% and 60% ethanol solutions (Fig. 9) ensured the superiority of the ethanol-miglyol donor vehicle. We, therefore decided for 10/90 (v/v) ethanol/miglyol as donor vehicle. Ethanol in this concentration will act as permeation enhancer [226] and induces less toxicity. To avoid the toxic effect of the ethanol we were also introduced the miglyol-water based O/W and W/O emulsions into the experiments.

4.1.3. Effect of the presence of BSA in acceptor fluid

It is well known that cutaneous uptake includes: partitioning of the substance into the Stratum corneum from the donor vehicle and partitioning from the Stratum corneum into the viable epidermis. In the in vitro experiment, viable epidermis makes contact with a water based solvent as acceptor fluid. In general, a physiological medium maintains skin viability [145]. Concerning the appropriate acceptor medium enhancing permeation of lipophilic substances, we have found that addition of 5% BSA to the acceptor medium (PBS) doubled the flux of testosterone (Fig. 9). This is due to protein binding favouring mass balance to the acceptor side and was reported earlier, too [229]. The addition of 5% BSA is also recommended by the OECD [135]. An alternative could be to decrease the concentration of the applied testosterone by several orders of magnitude to overcome the solubility restrictions of testosterone in protein-free water-based solvents.

4.1.4. Viability of skin models

MTT test

Evaluating the viability of the reconstructed skin models after the treatment with the different donor vehicles, 10/90 ethanol-miglyol showed the highest safety when compared to 60% ethanol (moderately safe) and 100% ethanol, which induced toxic effects (Tab. 6). This result was expected due to the toxicity of the ethanol for keratinocytes and fibroblasts monolayers. Moreover, the viability of the model was slightly impaired by acceptor fluids containing BSA (Tab. 4).

Lactate and LDH monitoring

Lactate formation and LDH activity by the the SkinEthic® model were followed using the 10/90 ethanol/miglyol and 60% ethanol as donor vehicles. The increasing lactate concentrations in the acceptor medium during the experiment demonstrated the maintenance of anaerobic metabolic processes in the tissue. Therefore, the skin model was still metabolically active. The released LDH activity, however, indicated a damage of cell membranes, which is in accordance with our former findings using the MTT test.

Histological Evaluation

The morphology of reconstructed epidermis treated with different acceptor fluids, however, did not indicate a major damage of the tissue (Tab. 8). Comparing the harmful effect of both PBS+ 5%BSA and PBS media on the control skin tissues indicated the superiority of the former, however, this result is in contrast to the results of MTT test (Tab 4). This superiority of BSA addition to the acceptor medium histologically was in full agreement with the increased testosterone permeation (up to twofold) using this acceptor medium.

4.1.5. Effect of the cryopreservation of skin

Cryopreservation of the skin did not influence the flux of testosterone as compared to fresh skin. Since we focused on cutaneous uptake only and did not care for skin metabolism, fresh skin could be replaced by cryopreserved skin of human and animal origin, which simplified the work process. This result is in full agreement with the literature, which also stated that there is no difference in the permeability of cryopreserved skin as compared to fresh skin [142]. Although the use of cryopreserved skin is appropriate for the permeation experiments, it is not for the metabolism studies and in fact the OECD proposes to use fresh skin [135].

4.1.6. Permeation of heat separated epidermis

Comparing the flux of testosterone using the heated separated human epidermis (about 100 μm thickness) to that obtained by using human split skin (1mm thickness) showed that the testosterone permeation of both two models was rather similar, although both are significantly different with regard to thickness. This will ensure the fact that the Stratum corneum (20 μm thickness) in both models is the main barrier for the lipophilic testosterone, which was also described by Regnier et al. [163]. This result also indicates that the intercellular lipid pathway dominates for permeation of the lipophilic substances [163]. This result can be explained by a fact that a lipophilic molecule does not favourably partition out of the Stratum corneum into the more aqueous viable epidermis [40]. Although protein denaturation might occur during the process of heat separation, these proteins are probably less involved in the penetration of testosterone, since the permeation characteristics of both heat separated epidermis and split skin was similar.

4.1.7. Infinite dose approach for studying formulation effects

For the testosterone experiments, we have decided for the infinite dose approach to assess the pharmacokinetic parameters of cutaneous permeation which are the lag time, the steady state flux and the permeability coefficient (apparent permeability [P_{app}], [217]). We determined these parameters for all models. To quantify recovery is not relevant for the infinite dose approach [135].

Testosterone P_{app} values of the different skin models showed the following rank order EpiAirway^{TM} >> reconstructed epidermis (SkinEthic$^®$) >> split porcine skin $\geq$ heated separated human epidermis $\approx$ split human skin. This ranking reflects the differences in the barrier function of the tissues. Comparing the P_{app} of testosterone using the SkinEthic$^®$ model with respect to different donor vehicles demonstrated the higher permeability using 10/90 ethanol-miglyol as donor vehicle than 60% or 100% ethanol (Tab 7). This reflects that the testosterone permeation was highly dependent on the drug vehicle.

The transport of substance across stratum corneum is controlled by many physicochemical aspects, the most important of which is the partition coefficient (K) which is directly proportional to the lipophilicity of the permeated substance. The permeation of testosterone through the skin increases from a hydrophilic carrier or water-based solution. Because of the difficulty to dissolve testosterone in water based solvents we tested emulsions of the O/W and

W/O type in addition to the 10/90 ethanol-miglyol vehicle. Hydrocortisone served as a more hydrophilic reference steroid.

Investigating the effect of the donor vehicle on the permeability of the models to testosterone showed the following ranking order: O/W > W/O > 10/90 ethanol-miglyol solution which was in contrast to the more hydrophilic hydrocortisone, where the following rank order was observed 10/90 ethanol-miglyol solution > W/O > O/W. However, this result was expected, since hydrocortisone as a more hydrophilic substance does favourably partition out of the oil-based vehicles to the Stratum corneum lipids, whereas testosterone does not favourably partition out of the oil-based vehicles. The external phase in the W/O emulsion is the oil which makes it more similar to the ethanol-miglyol solution than to O/W in which the external phase is water. This was clear from the permeation data of both testosterone and hydrocortisone using three different skin models as depicted in Tab 15.

Tab 15 Comparison of the pharmacokinetic parameters of testosterone and hydrocortisone as a more hydrophilic control using three different models and three test formulations following application of 500 µl of 0.1% test substance for 26 h, n=3.

Skin model	Formulation	Hydrocortisone			
		Flux (µg/cm^2.h)	Lag time (h)	P_{app} x 10^{-8} (cm/s)	Permeation (%)
Pig skin	E/M	1.24±0.17	2.20±0.54	34.40±4.70	6.19±0.17
	O/W	0.63±0.08	2.40±0.26	17.60±2.30	3.11±0.20
	W/O	0,97±0.15	2.38±0.43	27.10±4.30	4.80±0.67
RHE	E/M	1.44±0.51	2.64±0.33	40.00±14. 00	6.61±2.20
	O/W	0.71±0.13	2.90±0.18	19.70±3.72	3.27±0.61
	W/O	1.16±0.37	2.63±0.09	32.30±10.30	5.41±1.49
EpiAirway	E/M	3.23±0.20	0.0	89.60±5.50	19.23±1.16
	O/W	2.70±0.35	0.0	75.00±9.60	18.45±2.00
	W/O	2.88±0,57	0.0	79.90±15.90	19.17±3.70
Testosterone					
Pig skin	E/M	0.27±0.07	0.0±0.21	7.52±2.02	1.53±0.40
	O/W	0.30±0.06	0.31±0.90	8.40±1.64	1.69±0.34
	W/O	0.30±0.10	0.11±1.02	8.33±2.67	1.72±0.39
RHE	E/M	0.88±0.03	0.0	24.40±0.83	4.43±0.16
	O/W	1.03±0.09	0.0	28.60±2.45	5.15±0.47
	W/O	1.06±0.09	0.0	29.60±2.39	5.50±0.54
EpiAirway	E/M	1.72±0.29	0.0	47.70±8.02	9.32±1.86
	O/W	1.90±0.49	0.0	52.80±13.60	10.61±3.10
	W/O	1.57±0.68	0.0	43.60±19.00	9.40±4.70

RHE: reconstructed human epidermis

4.1.8. Use of occlusion method

The hydration status of the skin is a major factor affecting the rate and extent of cutaneous uptake [52]. Skin hydration can influence the partitioning of a substance and therefore change the concentration gradient of the penetrating substance in the Stratum corneum. The hydration condition of the skin also influences tissue thickness and may finally alter the size of stratum corneum reservoir. This changed the permeation profile of different penetrants [230].

Occlusion prevents surface evaporation of endogenous water which results in stratum corneum hydration. It also prevents the evaporation of water or solvents from the formulations. Use of occlusion as a primary method to enhance skin penetration was described by Feldman and Maibach [230-231] investigating hydrocortisone uptake in man, and by Wurster and Kramer [232] investigating salicylates. Due to the practical difficulties which did not allow using the occlusion (the use of relatively large volume with regard to the surface area and use of Franz cell), our testosterone permeation experiments were run under protected conditions (Franz cell was covered by paraffin film) to prevent, to a level, surface evaporation of endogenous water but also water or solvent evaporation from the preparations. The protection also decreases the hazards of contact with radioactive substance.

4.1.9. Lag time of testosterone

In man substance initially penetrating into the skin may ultimately enter the circulation. Accordingly, in Franz cell experiments, drug concentration may be detected in the acceptor fluid [233-234] but increase after the lag time. We have decided to investigate the testosterone and hydrocortisone permeation in porcine split skin and reconstructed epidermis for as long as 26 h, in order to be able to quantify the lag time of testosterone and hydrocortisone. Using split porcine skin, the estimated lag time of testosterone was 0.3-0.8 h and of hydrocortisone was 2.2-2.4 h. The lag time of testosterone was identical to the one of a 6 hour experiment. Testosterone uptake after 6 h was 0.2 % and after 26 h was 3.1% of the applied dose. Using reconstructed epidermis, hydrocortisone showed a lag time of 2.6-2.9 h, whereas testosterone did not show any lag time. Absence of the dermal tissue in the reconstructed epidermis decreases the lag time of the more lipophilic substances, however, increases that of the hydrophilic substances, because the lipophilic testosterone does not favourably partition out of the Stratum corneum into the more aqueous dermis, while the hydrophilic hydrocortisone favours this transfer from the Stratum corneum into the dermis. The permeation decreased at 24 and 26 h.

It has been reported in a recent publication that the partial negative charge distribution on the polar head group of the permeation enhancing agent is important for its enhancing activity. If a smaller polar head group analogue with a partial negative charge on the opposite sides is used there is a potential to condense the skin lipids and retardation of cutaneous absorption may be observed [16]. Miglyol (triglyceride ester), which is used in the donor vehicle, can permeate into the skin and gets partially hydrolysed by the esterases in skin [135] resulting in

this charge distribution. According to the above mentioned hypothesis miglyeol might condense the skin lipids and inhibit the permeation of more testosterone during the advanced permeation time (24-26 h).

4.1.10. Pig skin: Regional effects

Regional variation is well known to influence the cutaneous uptake of the topically applied substances [2-4]. Rougier et al. had ranked the benzoic acid uptake in men according to the anatomical sites as follows: forehead > abdomen > thigh > chest > arm > back [3]. Uptake parallel thickness of the epidermis which is different at different anatomical sites [4], but also differences in the amount and composition of human skin surface lipids may be of relevance [2]. Testosterone permeation of abdomen skin exceeded the permeation of back skin which is in accordance with the previous data generated in man.

4.1.11. Inter-individual variations

Comparing the variability of testosterone permeation (standard deviation of flux) through all used skin models indicated less inter-batch-variation with reconstructed epidermis as compared to inter-individual variations in normal skin of human and animal origin. With all models, the intra-individual or intra-batch reproducibility exceeded inter-individual and inter-batch reproducibilities. This conclusion was in accordance with existing literature [234]. Moreover, inter-individual variation of fresh human skin was much higher than that of cryopreserved human skin (Tab. 9). This might be due to the inter-individual variation of cutaneous testosterone metabolism of fresh skin influencing the concentration gradient. Cryopreservation declines this enzymatic activity [142] resulting in a more constant uptake.

4.1.12. Comparison of epidermis and alveolar model

As expected, owing to absence of the horny layer, the EpiAirway model was more permeable to both testosterone and hydrocortisone than the cornified tissues which also contain epidermal lipids. As compared to reconstructed epidermis, the permeation through alveolar tissue was increased by about twofold and 3-5fold for testosterone and hydrocortisone, respectively. This indicates the importance of the horny layer lipids as barrier against the uptake of agents from the environment but also the specific limitations of transdermal drug application. The increase in the permeation of testosterone by only two fold shows that the viable epidermis (hydrophilic) still represents a barrier against testosterone permeation. The

more pronounced uptake of hydrocortisone in the alveolar model may be due to better solubility of the more hydrophilic drug in the viable tissue but also the acceptor medium.

4.2. Estrogens

A lot of effort has been undertaken over the last two decades in order to develop a TTS. These maintain the premenopausal E2 plasma level as well as the E2/E1 ratio close to 1.0 in postmenopausal women [131-133]. E2-TTS application may overcome disadvantages of the oral E2 replacement therapy which are mainly the increased risk of thromboembolic complications and breast cancer [119-121]. These side effects appear to result from the progestogen medroxy progesterone but also from the intense hepatic estrogen metabolism during the first liver passage [122-128]. Yet prospective clinical studies of larger size are to be awaited.

4.2.1. Cutaneous E2 metabolism and E2/E1 ratio

The present data demonstrate that E2 is highly metabolised in the skin mainly to E1 by 17β-hydroxysteroid dehydrogenase type II with any used test model. Most importantly, E2/E1 ratio in the acceptor medium was always close to 1.0. Therefore, the in vitro models reflect the in vivo situation. The perfusion model even reflected the slightly delayed increase in E1 plasma levels of postmenopausal women treated with an E2-TTS. Yet, the sophisticated perfusion model does not allow to generate data clearly surmounting the less elaborate Franz cell studies, given the tissue viability is maintained by an appropriate acceptor fluid and the system fits to the needed test surface. Other than with the restricted area of reconstructed epidermis, excluding the evaluation of estrogen uptake from the E2-TTS patch, this was not a problem with the perfusion model. Human studies comparing E2 gels and patches indicate peak plasma levels and E2/E1 ratio to be rather close [235].

The models should be also predictive for e.g. testosterone uptake studies since 17β-hydroxysteroid dehydrogenase is also of relevance for testosterone metabolism in keratinocytes and fibroblasts [74]. Transferases in reconstructed epidermis and pig skin formed E2 and to a small extent also E1 conjugates. E2 conjugates should mainly constitute of glucuronides while E1 is mainly formed as sulphates.

4.2.2. Effect of ethanol on uptake and metabolism of E2

The efficacy of topically applied drugs is often limited by their poor penetration into the skin. Therefore, chemical penetration enhancers have been used in the formulations to improve the cutaneous uptake of drugs [236]. Ethanol is a widely used solubiliser and permeation enhancer in the commercially available topical formulations of E2. Increasing drug solubility in the skin [237] and the diffusion through the intercellular lipid pathway [238] as well as extracting stratum corneum lipids [226], ethanol strongly enhances the permeation of E2 across the skin up to a concentration of 63%. Higher ethanol concentrations, however, reduce the permeation [226], which was also the case in our experiments using reconstructed epidermis. Following the application of an ethanolic E2 solution, the E2/E1 ratio increased in our experiments. This, however, should be an artefact, induced by the toxicity of the solvent inhibiting E2 metabolism which has also been described earlier [75]. Comparing a transdermal nitroglycerine formulation with and without ethanol, a reduced metabolism following the ethanol-based formulation compared to the ethanol-free one was described, too [239]. Comparing gel and ethanolic solution in reconstructed human epidermis confirmed that ethanol damaged the epidermal keratinocytes and the proteins resulting in an increased E2 tissue level and reduced permeability. With split pig-skin ethanol diminished uptake and metabolism was less pronounced. This should result from the thicker Stratum corneum protecting viable skin.

4.2.3. Effect of the presence of BSA in acceptor fluid on E2 permeation.

It has been reported that protein drug binding due to addition of BSA to the acceptor medium, especially for the highly lipophilic steroids, favours the drug transport from the skin tissue to the acceptor fluid [240]. This should result in increased drug permeation due to increased drug solubility. Moreover, cutaneous drug metabolism may also be affected by BSA [240]. Comparing the total permeation of E2 (ethanolic solution and a gel) without or with 5% BSA in the acceptor medium we observed an increase in the total permeation of E2 by 26.15 % and 14.29 %, for the gel and ethanolic solution, respectively. This increase, however, was statistically not significant in contrast to testosterone which exhibited a significant increased permeation (about twofold) upon addition of 5% BSA, although testosterone is less lipophilic than E2. This difference in the effect of BSA addition to the acceptor medium on both lipophilic testosterone and estradiol permeation may be due to the use of testosterone concentration (1% solution) higher than that of estradiol (0.1%) in investigating the effect of BSA addition. Also, using different acceptor medium (MEME) in investigating estradiol

permeation may be another cause for this differences in the effects where MEME contains several vitamins, amino acids, inorganic salts as well as glucose, phenol red and pyruvate. These different components may consume many of the binding sites of BSA decreasing its activity in binding the lipophilic substances.

4.2.4. Use of the mathematical models for prediction of in vivo plasma concentration

Mathematical models are under development, which predict in vivo absorption of compounds in man from the data of in vitro permeation / penetration experiments. These models even considering E2 [219] are founded on physiologically based pharmacokinetic programs [241]. While these models appear almost fully developed for hydrophilic compounds, the prediction of in vivo data for lipophilic substances proves to be more difficult [228-242]. Taking the species difference into account, estrogen levels obtained from our permeation data (both Franz cell experiments with porcine skin and perfused pig forelimb) were well in accordance with published plasma concentrations in femals after application of an E2 TTS [235]. Results obtained with reconstructed epidermis, however, overestimated the uptake. Nevertheless, if this should prove to be fixed factors, reconstructed epidermis which is commercially available in defined quality will be as predictive as the other models.

As expected, reconstructed epidermis once more turned out more permeable as compared to split pig skin which is well explained by the thickness of the horny layer and viable skin. Taking this in mind, the reconstructed epidermis is most convenient with respect to handling.