

2. MATERIALS AND METHODS

2.1. Materials and reagents

17 β -Estradiol (E2), estrone (E1), testosterone, hydrocortisone, anti-E2 and anti-E1 antibodies, E2-glucuronide (sodium salt), E1-sulphate (sodium salt), dextran coated charcoal (100-400 Mesh), dextran (approximate average molecular weight 70000), β -glucuronidase and sulphatase mixture (105000 + 4300 units respectively), bovine serum albumin fraction V (BSA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), amphotericin B, gentamycin sulphate, glutamine, trypsin, ethylene - diamine tetra-acetic acid (EDTA), sodium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate, potassium chloride, ethanol, ether, glycerol, propylene glycol, miglyol, Dulbecco's modified Eagle medium (DMEM), and minimum essential medium Eagle (MEME) were obtained from Sigma (St. Louis, MO). 2,4,6,7-³H-E2 (88 Ci/mmol), 2,4,6,7-³H-E1 (94 Ci/mmol), and 2,4,6,7-³H-testosterone (100 Ci/mmol) were purchased by Ammersham, Freiburg). Dialysis membrane (MW-cutoff 10000, very high permeability) was obtained from Diachema (Heidelberg). Biopsy punches (diameter: 4 mm and 8 mm) were obtained from Stiefel (Offenbach). The purity of E2, E1, and testosterone was checked by thin layer chromatography before use. Stock solutions of estrogens (1 μ g/ml) and testosterone (100 mg/ml) in ethanol kept at 4°C were stable for at least 4 weeks. MTT solution, 5mg/1 ml sterile PBS, was kept in the dark and stored at -20°C. Scintillation cocktail (Optiphase Supermix) was purchased by Wallac (Turku, Finland). Dimethylsulfoxide and ethylacetate were obtained from Merk (Darmstadt). McIlvaine buffer, pH 5.0 was prepared in our laboratory. Estraderm[®] TTS (E2 patches, release rate 100 μ g/24 h) was obtained from Novartis Pharma (Basel, Switzerland). Sisare[®] gel (1 mg E2/g gel) was obtained from Noury Pharma GmbH (Oberschleißheim).

Reconstructed epidermis (Skinethic[™]) was purchased by Laboratoire Skinethic (Nice, France). EpiAirway[™] tissue model was purchased by MatTek (MatTek Corp., Ashland, MA, USA).

The nutrition media and solutions were used under sterile and aseptic conditions. Keratinocytes growth medium (KGM) was composed of keratinocyte basal medium supplemented with hydrocortisone, 0.5 μ g/ml; bovine pituitary extract, 30.0 μ g/ml; human epidermal growth factor, 0.1 μ g/ml; insulin, 5.0 μ g/ml; amphotericin B, 25.0 μ g/ml; and

gentamycin sulphate, 25.0 µg/ml. Fibroblast growth medium (FGM) was DMEM supplemented with fetal calf serum, 10 % (v/v); glutamine, 2 mM; amphotericin B, 50.0 ng/ml; and gentamycin sulphate, 20.0 µg/ml.

2.2. Instruments

Franz-flow-through diffusion cells with 9 mm diameter and 5 ml volume (Crown Scientific, Somerville, NJ, USA). Flow-through diffusion and static Franz cells with 15 mm diameter and 12 ml volume (PermGear, Bethlehem, PA, USA). β-Scintillation Counter 1450 Microbeta Plus (Wallac, Turku, Finland). Vortex Genie (Bender-Hobein, Zürich, Schweiz). Ultraturrax with Rotor S-25 N-18G (Carl Roth, Karlsruhe). Freeze-Microtome (Frigocut™ 2800 N, Leica, Bensheim, Germany). Dermatome™ Aesculab (Tuttlingen, Germany). Incubator BB 6220 (Heraeus, Hanau, Germany). ELISA-reader (Berlin, Bornheim, Germany). Phase contrast microscope Axiover 13 (Zeiss, Jena, Germany). pH-meter (Knick, Nürnberg, Germany). Peristaltic pump Sarah® (Crown Scientific, Somerville, NJ, USA). Reference Eppendorf® pipette (Eppendorf, Hamburg, Germany). Vacuum centrifuge Speed Vac® SC 110 A (Life Sciences International, Frankfurt, Germany). Ultrasound Sonorex® RK 100 (Bandelin, Berlin, Germany). Steril work bank LaminAir® (Heraeus, Hanau, Germany). Agitator IKA® MTS2 (Janke & Kunkel, Staufen, Germany).

2.3. Methods

2.3.1. Test formulations

In addition to testosterone, hydrocortisone was used as reference drug for uptake studies investigating formulation effects. Besides aqueous and ethanol solutions, three semi-solid vehicles were tested.

60% Ethanol solution

50 mg of testosterone is dissolved in 3.0 ml ethanol (100 %), and spiked with 10 µCi ³H-testosterone. The solution is diluted to 5.0 ml with water and mixed well to produce 1% solution which allows applying 1 µCi radioactivity pro Franz cell.

100% Ethanol solution

50 mg of testosterone is dissolved in 4.990 ml ethanol and spiked with 10 µCi ³H-testosterone to produce 1% solution. The solution is stable for at least 4 weeks.

For Estradiol, 50 mg is dissolved in 50 ml ethanol (96%) to produce 0.1% solution without using radioactive material. The solution is stable for at least 4 weeks.

10/90 Ethanol/migleol solution (E/M)

50 mg of testosterone or hydrocortisone is dissolved firstly in 5.0 ml ethanol. 500 µL of this ethanol solution is spiked with 10 µCi ³H-testosterone or hydrocortisone which allowed applying 1 µCi/ Franz cell. Then the solution is diluted to the required volume of 5.0 ml with miglyol to receive a final solution of 1 mg/ml (0.1% solution). The solution is thoroughly mixed by vortex for 10-15 seconds before use. The steroid concentration of the E/M solution can be increased to 1% by increasing the amount of non-labelled testosterone and hydrocortisone to 10 fold without increasing the radioactive amount.

Oil/Water emulsion (O/W)

4.0 mg of testosterone or hydrocortisone is dissolved in 2 ml (≈1.95 g) miglyol by vortexing for 15 minutes. Then 0.1 g of tween 80 emulsifying agent (about 2.5 %) is added. Mixture is spiked with 8 µCi ³H-testosterone or hydrocortisone which allowed applying 1 µCi/ Franz cell. Finally, 2.0 ml of distilled water is added and the solution is mixed thoroughly using ultraturrax to form O/W emulsion which is stable for about 48 h.

Water /Oil emulsion (W/O)

W/O emulsion is prepared as the O/W emulsion, but 0.08 g of span 20 plus 0.02 g of tween 80 are used as emulsifying agent instead of 0.1 g tween 80. The W/O emulsion was stable for about 5 days.

Stability of formulations

The stability of all formulations was observed visually for several days and the results are summarized in the following table (Tab.1). Permeation experiments were performed with stable preparations.

Type of formulation	Ethanol content	Water content [g]	Miglyol content [g]	Polysorbate (Tween 80) content [g]	Span 20 content [g]	Stability time
E/M solution	10 %	-	90 %	-	-	At least 4 weeks
O/W emulsion		9.75	9.75	0.5 (100%)	0.00	48 ± 5 h
W/O emulsion		9.75	9.75	0.00	0.5 (100%)	17 ± 4 h
		9.75	9.75	0.1 (20%)	0.4 (80%)	5 ± 1 days
		9.75	9.75	0.2 (40%)	0.3 (60%)	5 ± 1 days
		9.75	9.75	0.3 (60%)	0.2 (40%)	6 ± 1 days

2.3.2. Preparation of skin models and skin

For the estimation of the steroid permeation and penetration, abdominal skin was used which was excised from the abdomen or back of pigs of the "Deutsche Landrasse Hybride". The skin was supplied by a local abattoir being able to avoid the procedure of scalding. Excised human skin (abdomen or breast) was obtained from females aged 20 to 62 years subjected to cosmetic surgery. The skin was immediately placed in ice-cold cloth-sheets and transferred to the laboratory. Fat and connective tissues were removed to avoid the contamination of the surface by subcutaneous lipids. For cryo-preservation both pig and human skin were frozen at $-25\text{ }^{\circ}\text{C}$ for at least 24 h after trimming for fat and connective tissues.

After arrival of the reconstructed tissue, reconstructed epidermis and the EpiAirway model are incubated overnight in growth medium at $37\text{ }^{\circ}\text{C}$, 5% CO_2 , and saturated humidity. The tissues are now ready for the Franz cell experiment as described before.

Excised human and porcine skin

Freshly excised pig skin was placed in transport medium consisting of HEPES-buffered MEME supplemented with gentamicin sulphate (20 $\mu\text{g}/\text{mL}$), amphotericin B (50 ng/mL), glutamine (2 mM), and glucose (0.1%) to maintain its viability. Fresh split pig or human skin

(1000 μm) was prepared using a Dermatome™ within 2-4 h after the excision. Cryopreserved skin of both pig and man was also used, but not for the metabolic studies. Circles of 22 mm (in case of 15 mm Franz cell) or 15 mm diameter (in case of 9 mm Franz cell) of the fresh or cryopreserved tissue were carefully punched out and mounted into Franz flow-through cells. The horny layer faced the air and the dermis was in contact with the acceptor medium (Fig. 6).

Heat separated human epidermis

Circles of 25-30 mm of the cryopreserved human skin tissue were carefully punched out. Epidermis was separated from punch biopsies by heating the skin at 60 °C in a water bath for 60 - 90 seconds [78]. Then the epidermis sheets were carefully removed by forceps, inspected for integrity and transferred to a supporting polycarbonate membrane, which has been previously soaked in water for 24 h, then in acceptor medium for 1 h.

2.3.3. General procedure for Franz cell experiment

The Franz cells, either static or dynamic design, are connected to the circulating water bath, which is maintained at 37 °C in order to maintain the temperature of the skin surface at 32 °C. The Franz cells are filled with an appropriate acceptor medium which must have an adequate capacity to dissolve the test substance and is magnetically stirred by using a magnetic bar (Fig.6). The skin model is then mounted to the Franz cell after examination of its integrity. The underside of the skin model or the dermal side of the skin is maintained in contact with the acceptor medium, air bubbles have to be removed before applying the test substance. After an equilibration time of 30 minutes, the test preparation is applied to the upper surface of the skin (model) and remains on the skin for a specified period of time according to the protocol of the work. The acceptor fluid is sampled at time points throughout the experiment to determine the amount and rate of the test substance passing through the skin. At the end of the experiment, the remaining of the dose on the skin surface, the amount penetrated into the skin and the amount which permeated the skin in the acceptor fluid is determined and is to be found. Therefore, the remaining dose on the skin surface was carefully removed, and the skin surface was wiped twice by a cotton wool and washed with the ethanol. For the estimation of the penetration into skin model, 8 mm punches of treated skin areas were taken immediately after the experiment using biopsy puncher.

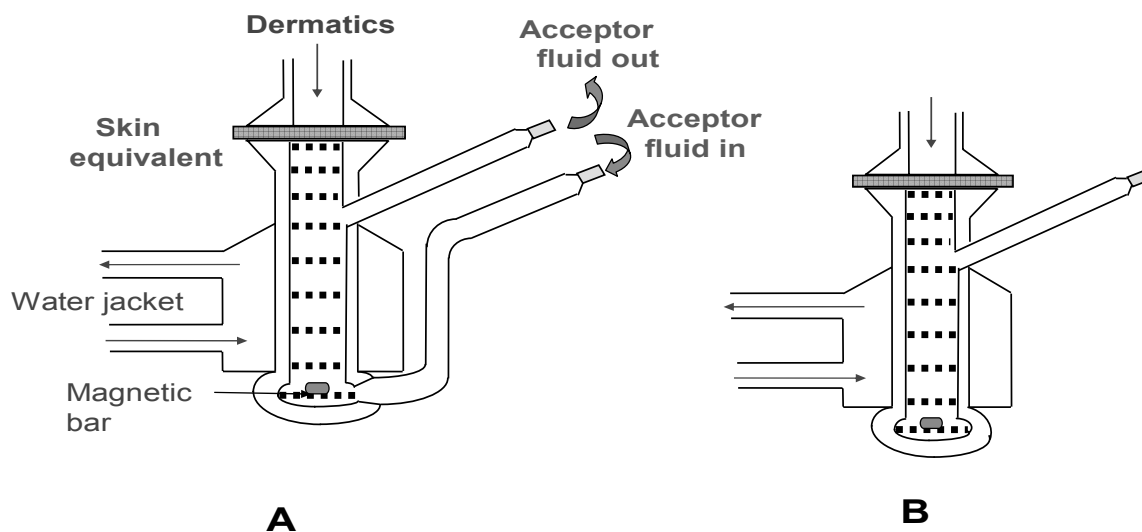


Fig. 6. Schematic presentation of Franz flow-through diffusion cell (A) and static Franz cell (B)

2.3.4. Permeation of testosterone

Testosterone permeation of various skin types was compared using the Franz cell approach. After the end of the incubation, tissue integrity was determined. As acceptor fluids served phosphate buffered saline pH 7.4 (PBS), or PBS containing 5% BSA, continuously flowing through dynamic Franz cells with a flow rate of 8 ml/h. Alternatively, 4 ml medium were replaced by fresh medium each h for static Franz cells. After an equilibration for 30 min, 4 ml medium were taken from the acceptor medium as blank value for substance analysis and for analysis of lactate and lactate dehydrogenase (LDH). Then 500 μ l of 1% testosterone solution was applied to the skin surface for 6 h. Testosterone vehicles were 60% ethanol, 100% ethanol or ethanol/miglyol, 10/90 (v/v), respectively. For drug analysis, acceptor fluid was collected hourly for 6 h. For the viability assay and histological examination, 4 mm or 8 mm punches of skin samples, respectively, were taken immediately after the experiment using biopsy punches. All samples were stored at 1-8 °C.

2.3.5. Cutaneous uptake and metabolism of estrogens

2.3.5.1. Franz cell experiments

To investigate not only the estrogen permeation and penetration, but also estrogen metabolism, an acceptor fluid favoring skin viability for several hours had to be used. This is possible using minimum essential medium Eagle, MEME. Moreover, freshly excised split skin was used instead of cryopreserved skin. All estrogen investigations were performed with the dynamic set up (9 mm diameter, the flow rate was adjusted to 6 ml/h). 100 mg Sisare[®] gel and 100 μ L of 0.1% ethanolic E2 solution were used as test substance. The experiments were carried out, as described before, for 6 h. The acceptor medium was continuously collected in fractions of 1 h for analysis. At the end of the experiment, the remaining E2 formulation was removed by wiping the treated area twice with ethanol-soaked cotton wool, punch biopsies of the treated skin were taken and stripped for four times with self-adhesive tape strips for the removal of the most superficial horny layer. The remaining pig skin was then cut in a freeze-microtome into horizontal slices of 100 μ m thickness. All samples collected for analysis were stored at -80 °C until further use.

2.3.5.2. Isolated perfused porcine forelimb

In co-operation with Mediport Biotechnik, Berlin, estrogen uptake and metabolism was also studied using perfusion model. Freshly removed limbs (female pig, 5-8 months, and 70-100 kg) were transported at 10-15 °C and connected to a perfusion circuit via the arteria brachialis for 6-8 h (Fig. 8). Standardized computer-assisted perfusion with a blood-based perfusion medium (PBS, 5% BSA, 4% erythrocytes, O₂ + CO₂, 37°C) supplied the limb with nutrients and oxygen. Flow rate, arterial pressure, temperature, pH, O₂ saturation, electrolytes (K⁺, Ca²⁺, Na⁺), blood glucose, haemoglobin, LDH were continuously monitored and adjusted if necessary [152]. Venous perfusion medium was sampled before the application of the estrogen TTS (Estraderm[®]) for the determination of the base line estrogen concentrations. Following the E2-TTS application to the limbs, venous perfusion medium was removed hourly for analysis. At the end, skin and muscle biopsies were taken from treated as well as untreated areas. The latter served for control. All samples were stored frozen and transferred from Mediport Biotechnik to the Institute of Pharmacy for analysis.

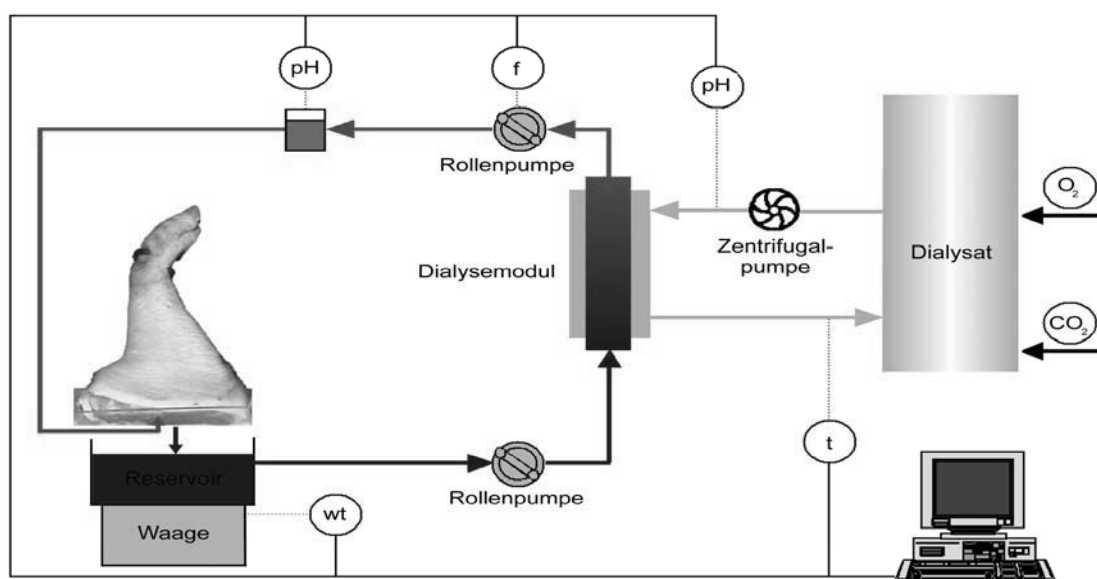


Fig. 8. The perfusion model for porcine forelimb produced by Mediport Biotechnik GmbH

2.3.6. Cutaneous versus alveolar uptake: formulation effects

Testosterone permeation of various skin types and alveolar tissue model was compared using static PermGear Franz cell model. As acceptor fluids served PBS containing 5% BSA. 400 μl medium were replaced by fresh medium. 500 μl of 0.1% testosterone spiked with 1 μCi radioactive testosterone solution was applied to the skin surface for 24 h. For drug analysis, acceptor fluid was collected hourly for 6 h then 8, 10, 12, 22, 24, and 26 h. Testosterone formulations were ethanol/miglyol, 10/90 (v/v), O/W or W/O emulsion, respectively. For determination of the drug penetration into cutaneous or alveolar models, 8 mm punches of the treated cutaneous or alveolar model were taken immediately after the experiment using biopsy puncher.

Testosterone permeation was performed separately in parallel to hydrocortisone as reference drug.

2.3.7. Tissue integrity and viability tests

Histology: Samples of human skin, pig skin and reconstructed epidermis were pre-fixed in Karnovsky solution at 4-8 $^{\circ}\text{C}$ for transport, fixed in synthetic resin [214] and stained with 1% pyronin G / 1% toluidine. 1 μm slices were inspected by light microscopy and classified by a histological score. Histological score is expressed in 4 degrees of harmful effects on the skin tissues; normal (N), light (L), mild (M), and hard (H).

Viability test: In parallel to the drug permeation experiments, epidermal viability was measured using the MTT-test as described earlier [215]. Briefly, skin biopsies of 4 mm diameter were cut out from the treated area and control reconstructed epidermis, placed each in a well of a 24-well plate and incubated with 200 μ l MTT solution for 4 h at 37 °C and 5% CO₂. Mitochondrial enzymes of the viable cells reduce the MTT forming the blue-violet coloured Formazan dye which was quantified spectrophotometrically at 540 nm after extraction from the skin biopsies by shaking the well plate gently with a 400 μ l of lysis solution, isopropanol (Fig.7).

Calculation:

Viability was expressed as percentage of control [100%].

$$\text{Viability in \%} = \frac{A_{\text{experiment}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}}$$

$A_{\text{experiment}}$ = light absorbance of the treated skin

A_{control} = light absorbance of the untreated skin

A_{blank} = light absorbance of the blank (isopropanol)

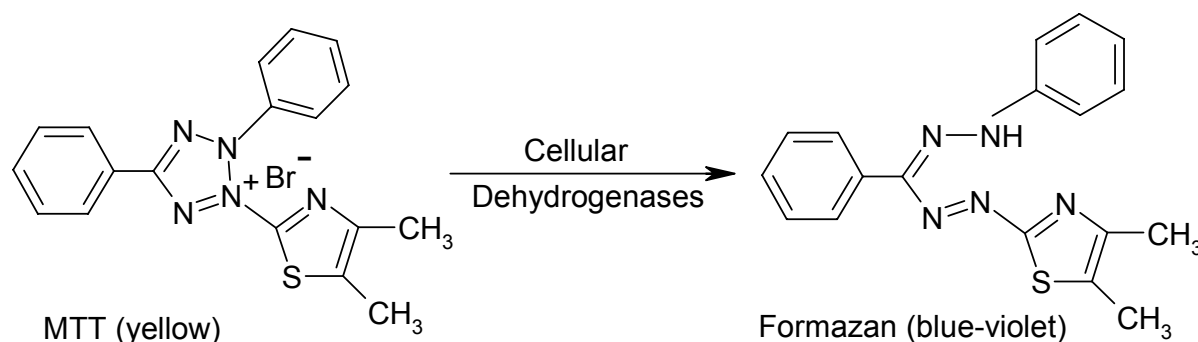


Fig.7. Schematic presentation of the MTT-test: cellular dehydrogenases reduce the MTT substrate forming a formazan dye.

In addition, the toxicity of ethanol and of estrogens on keratinocytes and fibroblasts in culture was determined using the MTT-test. Briefly, 10⁵ cells/well of keratinocytes or fibroblasts were grown in 6-well plates for 24 h at 37 °C and 5% CO₂. After the addition of different concentrations of ethanol and/or estrogens the cells were incubated for further 6 h. Then MTT test was performed as described above.

Lactate and LDH determination: To investigate the tolerability of donor vehicles by reconstructed human epidermis lactate concentration and LDH activity were determined. After 30 min equilibration of the skin model, 500 µl of the donor vehicles 60% ethanol/water (v/v) and 10/90 ethanol/miglyol (v/v) were applied to the skin surface. At 0 h, 6 h and 24 h, 4 ml acceptor medium were removed for the analysis and replaced by fresh medium.

Lactate was quantified by an enzymatic colorimetric method based on the lactatoxidase dependent conversion of lactate to pyruvate and H₂O₂. H₂O₂ induces an enzymatic oxidation of 4-aminoantipyrin resulting in a coloured dye with an absorption maximum at 540 nm. The colour intensity is directly proportional to the lactate concentration of the sample.

A method recommended by the International Federation of Clinical Chemistry, which is based on the LDH catalysed conversion of lactate to pyruvate was used for the determination of the LDH. Pyruvate absorption intensity at 340 nm is directly proportional to LDH activity in the sample.

2.4. Steroid analysis

2.4.1. Determination of the testosterone and hydrocortisone concentrations

For the estimation of the testosterone and hydrocortisone concentrations, the acceptor media and the ethanol extracts of the cotton wool were quantified directly by liquid scintillation counting following the addition of a liquid scintillation cocktail to the samples. To overcome the quenching effect of BSA, calibration curves were obtained dissolving radiolabeled drug in identical solvents. Moreover, the same volume of the sample and scintillation cocktail was used for all measurements. The cotton wool was extracted twice with 1.0 ml ethanol, the extraction rate was about $72.5 \pm 2.7 \%$ and $71.6 \pm 2.2\%$ for testosterone and hydrocortisone, respectively. The limit of detection (LOD) depends on the concentration of the non-labeled testosterone. i.e. the LOD is different for 1% testosterone solution and for 0.004 % solution labelled with the same amount of radioactivity (1 µCi). However, generally, the LOD for any pure radioactive substance is less than one pg, the LOD in our experimental conditions is 0.03 and 0.1 µg/ml for testosterone and hydrocortisone, respectively and the linear range was between 0.2 µg and 5000 µg testosterone and between 0.5 and 500 µg for hydrocortisone. Because of safety consideration, we can not use more than 1 µCi radioactivity/Franz cell

(about 3 ng ^3H -testosterone or ^3H -hydrocortisone). Therefore, we had to use non-labelled testosterone solution (1 %, 0.1 %, or 0.004 %) spiked only with 1 μCi of radioactivity/ Franz cell.

2.4.2. Determination of the estrogen concentrations

Estrogen extraction and cleavage of conjugate: In order to identify the solvent with the best extraction rate for free estrogens, ether, ethyl acetate, chloroform, and dichloromethane were tested. Free E2 and E1 were extracted from 1 ml acceptor medium (MEME) by adding 5 ml diethyl ether. Samples were vortexed for 1 min and centrifuged at 2000 rpm, 4°C for 5 min for phase separation. After removing the organic layer the extraction was repeated twice. The combined ether phases were evaporated under reduced pressure and the dried extract was dissolved in 1 ml 0.9 % (w/v) sodium chloride solution.

To determine the time and amount of the enzyme which resulted in complete cleavage of the estrogen conjugates, we have incubated 50 ng of conjugated E2 with a mixture of sulphatase/glucuronidase increasing amounts (0.5 to 400 units) for 0.5-24 h. As described in the results section, incubation for 6-12 h with at least 20 units of enzyme ensured complete conjugate cleavage. Therefore, for the following quantification of estrogen conjugates, 20 units of sulphatase/glucuronidase was added to the samples which were then incubated 6-12 h.

In order to quantify the fraction of conjugated E2 and E1, 1 ml of the acceptor medium was first incubated overnight with a mixture of sulphatase/glucuronidase (20 units / 400 units) in 2 ml McIlvaine buffer pH 5.0 at 37 °C. Since the enzymes interfered with the following antibody reaction for the estrogen determination resulting in high blank values, the enzymes had to be purified by charcoal treatment before the hydrolytic procedure [216]. This procedure resulted in complete conjugate cleavage and low blank values allowing to quantify total E2 and E1. Conjugated estrogens were derived by subtracting the concentrations of free drug from the total drug value.

Horizontal slices of 100 μm thickness of treated and untreated pig skin and muscle were chopped into small slices. Following the addition of 4 ml 0.9% sodium chloride solution, the tissue was homogenised by an ultraturrax for 1 min at 25000 rpm and centrifuged at 4°C,

1000 rpm for 5 minutes. The supernatant was subjected to estrogen extraction and conjugate cleavage as described above.

The cotton wool and self-adhesive tape strips were extracted twice with 5 ml ether. The solvent of the combined extracts was evaporated under reduced pressure. The dry residue was dissolved in 0.5 ml of ethanol and then diluted 20-40fold by 0.9% sodium chloride solution.

Radioimmunoassay: Commercially available radioimmunoassay (RIA) kits (Sigma-Aldrich, St. Louis, MO) were adapted for the determination of E1 and E2 extracted from acceptor medium, tissue, and cotton wool. Different buffers (Tris-HCl buffer, KBM) and 0.9% NaCl were tested to find the buffer which favours binding of estrogens with their specific antibodies. 0.9% NaCl was found to be most suitable.

The RIA was carried out according to the following protocol.

RIA protocol:

1. In polypropylene test tubes, 0.1 ml sample or standard solution and 0.5 ml diluted antiserum were vortexed and then incubated for 30 minutes at room temperature.
2. 0.1 ml tritiated radioactive tracer diluted in 0.9% sodium chloride solution was added, and the tubes were vortexed and incubated for 1 h at 37 °C.
3. The tubes were cooled for 15 minutes at 4 °C and 0.2 ml cold dextran coated charcoal suspension was rapidly added to each tube. The mixture vortexed was incubated for 10 minutes at 0°C.
4. After centrifugating the tubes at 2000 x g for 15 minutes at 4 °C, the supernatant was transferred to a fresh tube, scintillation cocktail was added and the amount of radioactivity present was determined.

2.5. Statistics and data analysis

Since testosterone and hydrocortisone concentration following the permeation of human and porcine skin but also reconstructed epidermis was studied using Permgear cells (diameter 15 mm and 12 ml volume), while estrogen studies was performed using Crown glass cells (diameter 9 mm and 5 ml volume), permeation is given as $\mu\text{g}/\text{cm}^2$ surface. This allows comparing estrogen results.

Testosterone and hydrocortisone permeation was performed using human and pig skin of 3 donors, and reconstructed epidermis and the EpiAirway model of 3 batches. Each experiment was performed in duplicate. (Reconstructed epidermis of 2 batches was used to optimise the operating protocol. Each experiment was performed in triplicate.)

For estrogens; with pig skin 2 independent experiments were run in triplicate. Reconstructed human epidermis of 2 batches served for 2 independent experiments run in duplicate. With regard to isolated porcine forelimb model, 3 independent experiments were performed.

For a model independent comparison of E2 permeability the slopes of the cumulated amounts of free E2 and total estrogens (E2 + E1 + conjugates; $\mu\text{g}/\text{h}$) in acceptor media were calculated.

The permeability constant ($P_{\text{app}} = [V/A \cdot C_i] \cdot dC_A/dt$) was calculated as described earlier [217-218] and is expressed as [cm/sec] where V is the volume of the receiver chamber (Crown cells 5.0 cm^3 , Permgear cells 12.0 cm^3), A the area of the skin surface (Crown cells 0.636 cm^2 , Permgear cells 1.768 cm^2), C_i the initial drug concentration in donor compartment [$\mu\text{g}/\text{cm}^3$] and dC_A/dt represents the increasing drug concentration in the acceptor medium with time.

Absorption is derived from the sum of penetration plus permeation. The quantity of estrogens estimated in self-adhesive tape strips was considered as absorbed.

For all used models plasma E2 concentrations in man [ng/ml] were calculated as described by Rohr et al. [219].

$$C_p(\text{ss}) = \frac{\text{Flux } (\mu\text{g} / \text{day} \times \text{cm}^2 \text{ patch}) \times A \times [1-P]}{Cl_p (\text{l} / \text{day})}$$

Where C_p (ss): the steady state plasma concentration, A: surface area of the patch, P: metabolic factor for estradiol which equal to 0.2 and Cl_p : average plasma clearance (1120 l / day).

The sensitivity of the analytical methods was determined by their slopes (S). The limit of detection (LOD) was determined as $3S_b/S$ where S_b is the SD of the blank experiment [220]. The accuracy of the methods was determined by the percentage recoveries and precision. Intra- and inter-batch (day) SD were determined.

All data are presented as the arithmetic mean values \pm standard deviation (mean \pm SD). Significance of differences was analysed using the Stateasy program. The F-test served for the comparison of variances, and Student's t-test for the comparison of mean values. The Shapiro-Wilk test was used in case of the normal distribution of the data, while the U-test was used if this was not true. $p \leq 0.05$ was considered to be significant.