3. RESULTS

3.1. Testosterone

While regulatory toxicology aims at the characterisation of the risk potential of chemicals, animal experiments should be avoided as far as possible. To establish a most universal test protocol for the estimation of cutaneous uptake we compared testosterone permeation (as a strong lipophilic drug) of human skin equivalent (SkinEthic®), human epidermis and split human and porcine skin. Experimental conditions such as the effect of BSA in the acceptor medium and vehicle effects were varied systematically. The effects of the donor vehicles and acceptor media on tissue integrity were also studied. The presented results are part of a nationwide BMBF-funded validation study comparing the in vitro uptake of chemical compounds by reconstructed human epidermis to already accepted in vitro techniques and by this to improve predictability to man but also reducing animal testing for risk assessment.

3.1.1. Validation of the liquid scintillation counting

Tab. 2a Accuracy and precision of LSC method for testosterone (n=6).

Added conc (µg)	Ethanol		PBS only		PBS + 5% BSA	
	Found amount	percentage recovery	Found amount	percentage recovery	Found amount	Percentage recovery
0.7			0.37 ± 0.02	52.86± 5.40		
2.0			1.80 ± 0.22	90.00± 12.22	2.05 ± 0.10	102.5± 4.88
4.0			3.95 ± 0.19	98.75± 4.81	4.31 ± 1.20	107.7± 27.8
6.0	4.24 ± 0.18	70.60± 4.25	6.35 ± 0.09	105.83± 1.42	5.63 ± 0.99	93.83± 17.6
8.0			8.37 ± 0.23	104.63± 2.75	7.61 ± 0.23	95.13± 3.02
10.0	10.07 ± 0.27	100.70± 2.68	9.69 ± 0.03	96.90± 0.31	10.4 ± 0.98	104.0± 9.42
25.0	23.95 ± 0.29	95.80± 1.21				
50.0	49.11 ± 0.05	98.22± 1.02				
125.0	124.2 ± 1.65	99.36± 1.33				
250	160.37 ±1.26	64.15± 0.79				
500	464.58 ±2.23	92.92± 0.48				
1000	1081.4 ±2.98	108.14± 0.28				
2000	2108.7 ±3.29	105.44± 0.16				
3000	2983.4 ±1.30	99.45± 0.04				
4000	3944.0 ±7.85	98.60± 0.20				

Tab. 2b Accuracy and precision of LSC method for Hydrocortisone (n=6).

Added conc (µg)	Ethanol		PBS + 5% BSA	
	Found amount	% recovery	Found amount	% recovery
0.5	0.40 ± 0.0	79.59 ± 0.4	0.40 ± 0.02	79.9 ± 4.8
1	0.99 ± 0.0	98.70 ± 4.1	0.96 ± 0.05	95.8 ± 5.2
2	1.98 ± 0.0	99.04 ± 0.1	1.98 ± 0.09	99.0 ± 4.3
4	4.01 ± 0.0	100.19 ± 0.0	4.01 ± 0.04	100.2 ± 1.1
8	8.19 ± 0.1	102.33 ± 0.7	8.22 ± 0.44	102.8 ± 5.3
16	15.76 ± 0.4	98.49 ± 2.5	15.48 ± 0.68	96.7 ± 4.4
32	31.88 ± 0.0	99.62 ± 0.1	32.41 ± 1.20	101.3 ± 3.7
64	61.52 ± 2.3	96.13 ± 3.6	60.93 ± 1.41	95.2 ± 2.3
125	125.96 ± 3.9	100.77 ± 3.2	131.01 ± 2.61	104.8 ± 2.0
250	245.78 ± 4.4	98.31 ± 1.8	248.91 ± 7.23	99.6 ± 2.8
500	480.38 ± 3.5	96.08 ± 0.7	477.91 ± 13.66	95.6 ± 2.9

Limit of detection of LSC method for testosterone was 3.03 ng/ml and for hydrocortisone was 0.1µg/ml. The reproducibility of the technique was tested with testosterone concentrations in the acceptor medium between 0.7 µg and 5 mg/ml and for hydrocortisone between 0.5 and 500µg/ml. The intraday variation coefficient did not exceed 3.25% for testosterone and 5.2% for hydrocortisone, whereas interday variation coefficient of testosterone read 0.04-4.25, 0.31-12.22, and 3.02-27.8 % for ethanol, PBS and PBS+ 5% BSA solutions, respectively, and for hydrocortisone did not exceed 6.6% for both media (Tab. 2). LSC is not specific for testosterone, because it can not differentiate between testosterone and its metabolites.

3.1.2. Influence of BSA on testosterone permeation

With respect to the effect of BSA on the permeation of highly lipophilic drugs, the results revealed an increased permeation of testosterone by addition of 5% BSA to the acceptor medium compared to PBS alone (Fig. 9). Permeation after 6 h amounted to 3.69 % and 1.77 % of the applied dose of testosterone following the application of 500 µl of 1% testosterone

Tab.3. Percentage of testosterone permeation through reconstructed epidermis after application of 500 µl of 1% testosterone in different donor vehicles for 6 h

Donor vehicle	PBS + 5% BSA		PBS	
	Static set up	Dynamic set up	Static set up	Dynamic set up
60 % EtOH	2.79	2.08	2.39	1.26
100 % EtOH	0.93	1.24	0.58	0.79
10/90 E/M	3.69 ± 1.14	2.86	1.77 ± 0.08	1.46

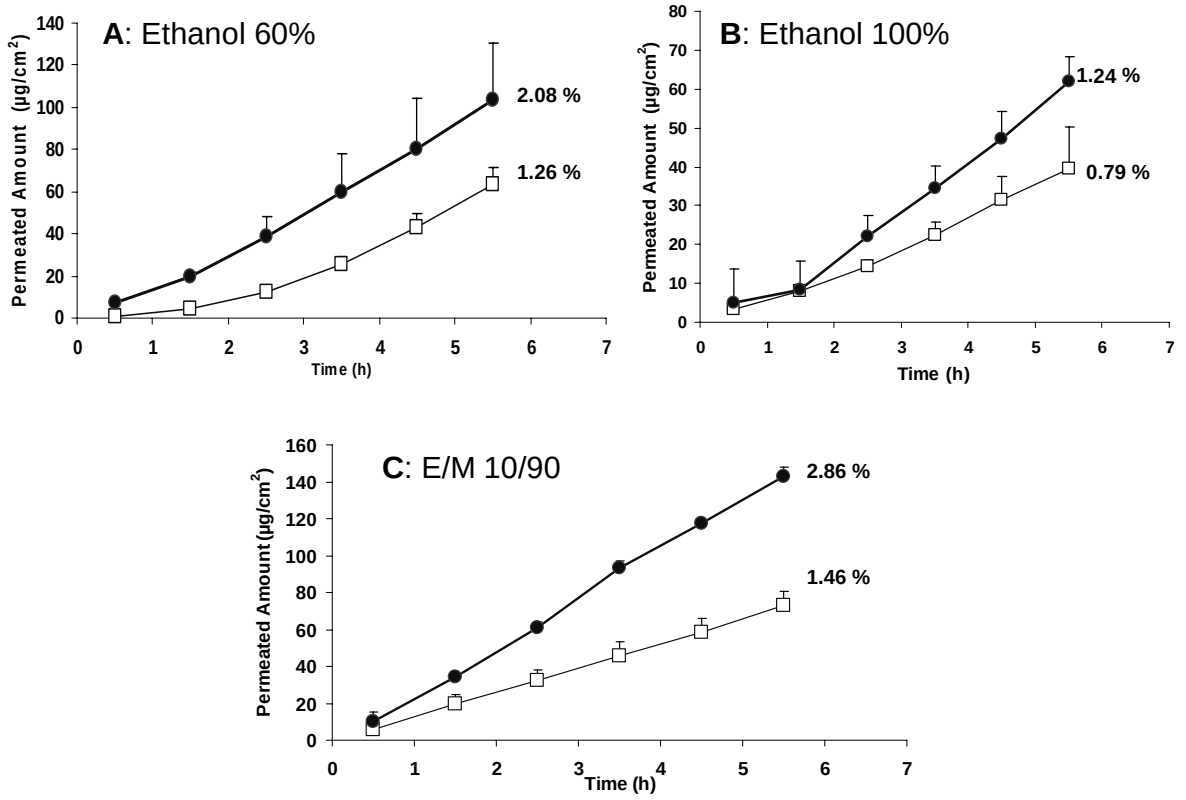

Fig. 9 Influence of BSA on the permeation of testosterone through reconstructed epidermis. 500 µl of 1% testosterone solution and dynamic Franz cell set up was used (Acceptor medium PBS -□- and acceptor medium PBS + 5% BSA -●-). Experiments were run using reconstructed epidermis from one batch, in triplicate.

3.1.3. Experimental set up: Static versus dynamic approach

Comparing the static with the dynamic Franz-cells showed a slightly higher testosterone permeation using the static experimental set up as shown in Fig. 10. The permeation of reconstructed epidermis amounted to 3.69% and 2.86% of the applied dose in static and dynamic Franz-cells, respectively. These minor differences between the static and the dynamic experimental set up were confirmed using split porcine skin as presented in Fig. 10C.

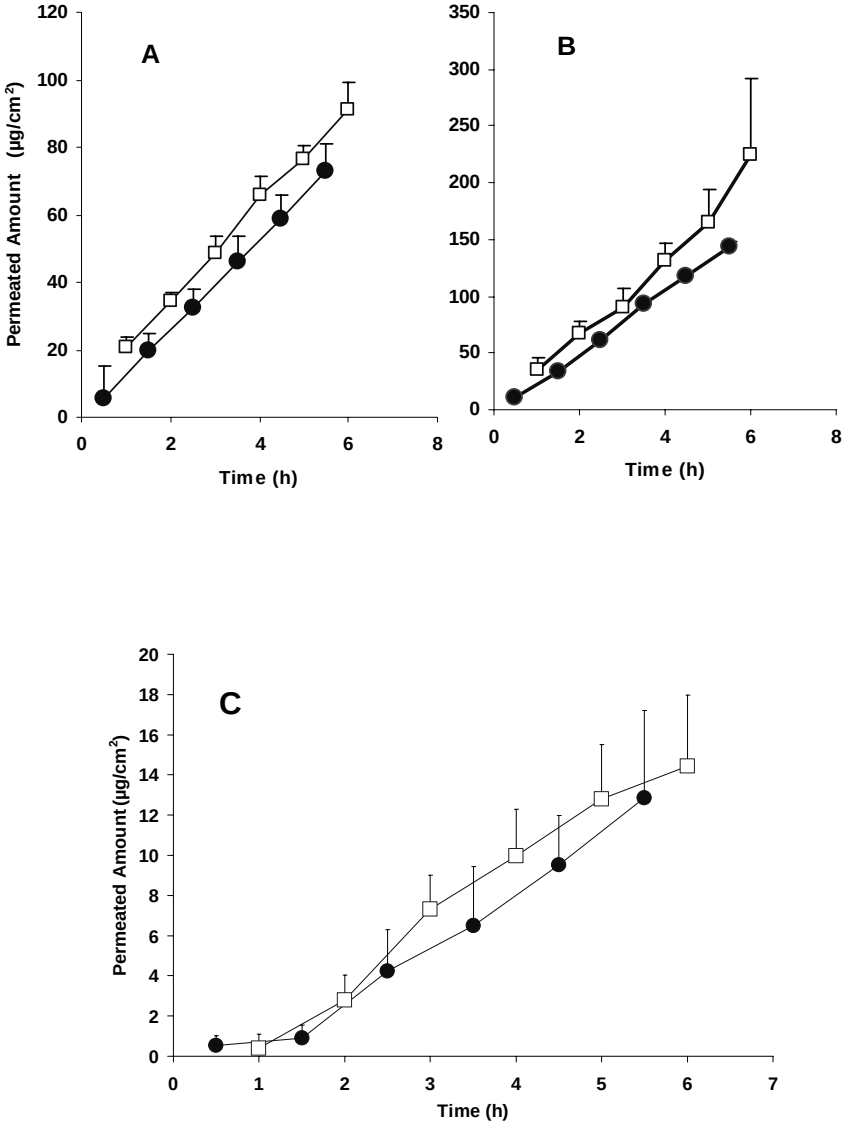

Fig. 10 Influence of the static (-□-) and dynamic (-●-) set up of Franz cell experiments. 1% testosterone solution (10/90 E/M) was applied for 6 h to A, B: reconstructed epidermis (2 batches) C: pig skin (3 donators). Each experiment was run in triplicate as described under materials and methods. A: The acceptor medium contains PBS B, C: The acceptor medium contains PBS + 5% BSA.

All differences between the static and the dynamic experimental set up failed to reach statistical significance.

In the following, it had to be studied whether testosterone adherence to the rubber tubes caused the slightly reduced recovery in the dynamic set up. As depicted in Fig 11, testosterone concentrations in the acceptor medium were identical, if rubber tubes and teflon tubes were used as transferring tubes. According to this result and the easier handling of the static Franz cell system, we have decided to use the static Franz cell set up in the following experiments.

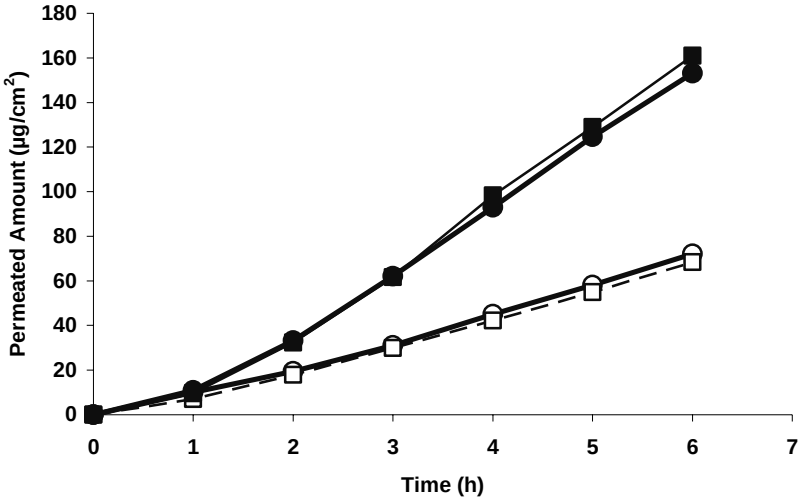

Fig. 11 Influence of the tube material (rubber, -□- and teflon, -○-) on the permeation of testosterone through reconstructed epidermis using 500 µL of 1% testosterone solution (10/90 v/v E/M) in the dynamic Franz cell set up. The acceptor medium was PBS (open symbols) and PBS + 5% BSA (closed symbols).

3.1.4. Pig skin: the influence of cryopreservation and regional effects

Comparing testosterone permeation through split porcine skin fresh and cryopreserved, a significant difference was not to be detected (Fig. 12). Investigating regional effects, a tendency (not significant) for higher permeation of abdominal skin as compared to the back skin was observed. This result was expected because of a higher thickness of back epidermis, especially the horny layer.

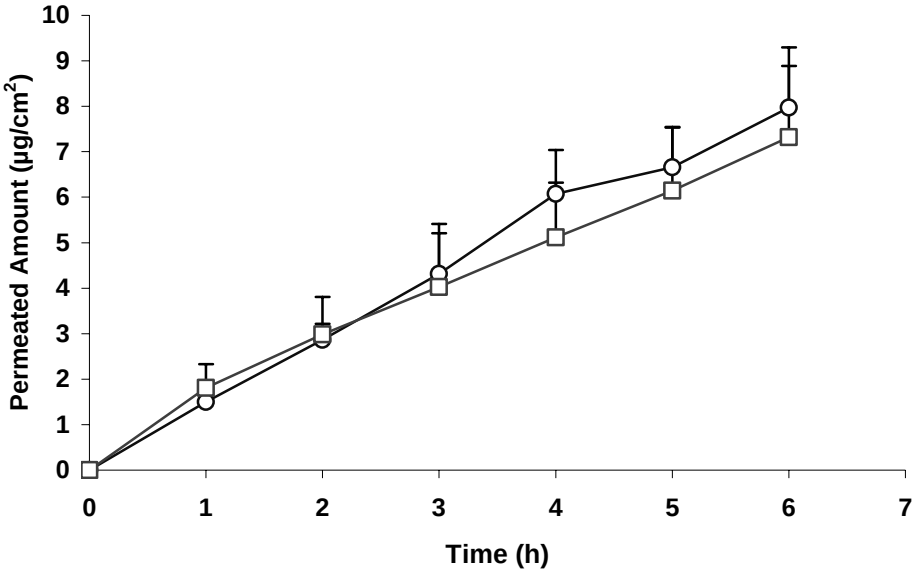

Fig. 12 Influence of cryopreservation on testosterone permeation (fresh, -○- and cryoconservered -□- pig skin). 500 µl of 1% testosterone solution (100 % ethanol) was applied to back skin of 2 pigs. All the experiments were run in triplicate.

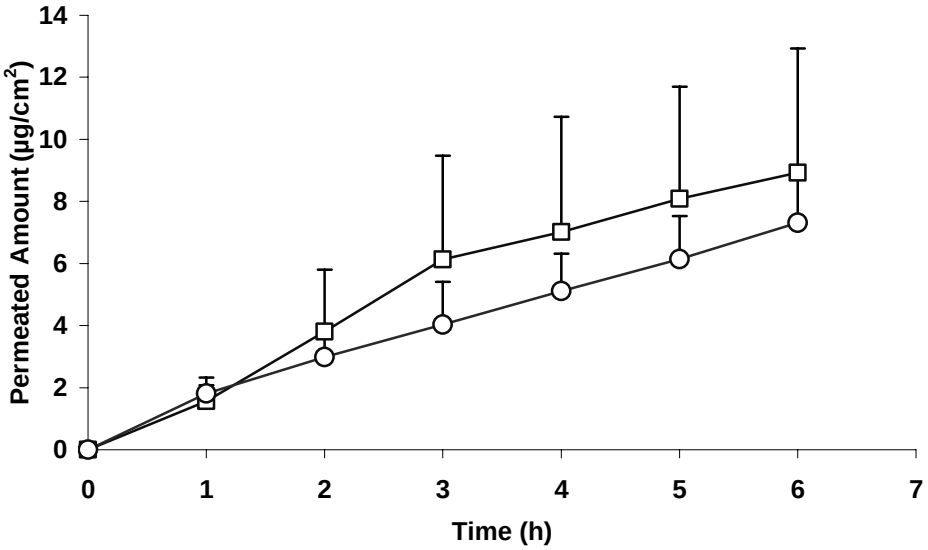

Fig. 13 Influence of the skin region [abdomin, (-□-) and back, (-○-)] on the permeation of testosterone through cryopreserved skin. 500 µl of 1% testosterone solution (100% ethanol) was applied to the skin of two pigs. All the experiments were run in triplicate.

3.1.5. Human skin: split skin versus epidermis sheet

In the following, fundamental parameters of the permeation of human skin were studied. Comparing the permeation of isolated human epidermis and cryopreserved human split skin, significant differences were not detected (Fig. 14). This result indicates the importance of the

stratum corneum for testosterone permeation. The stratum corneum is the rate limiting step for the permeation of lipophilic drugs.

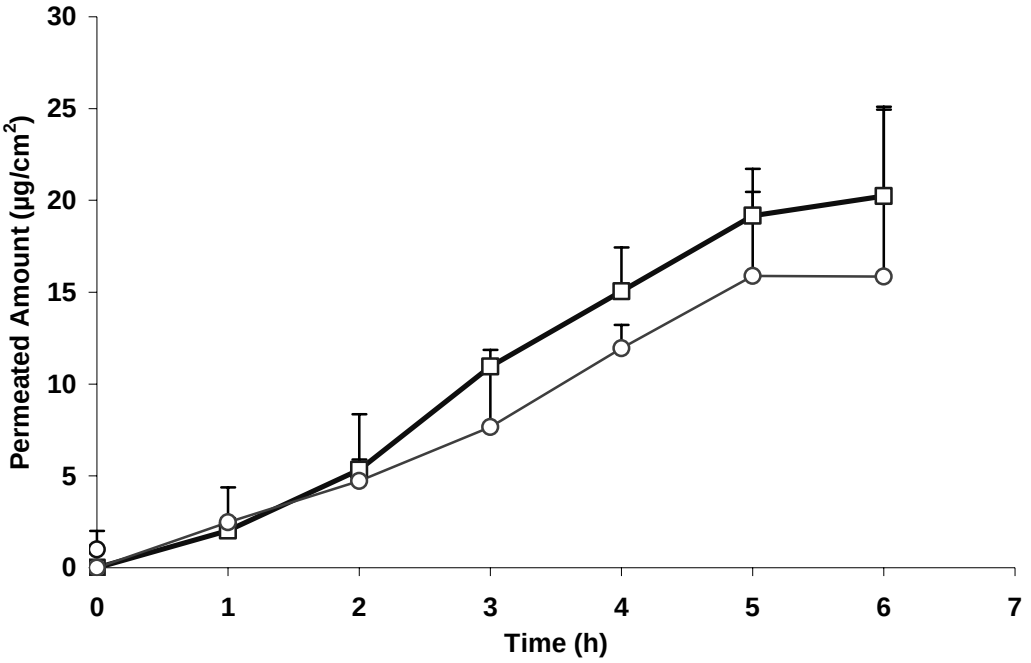

Fig. 14 Permeation of testosterone through cryopreserved human split skin (-□-) and isolated human epidermis (-○-) using 500 µl of 1% testosterone solution (10/90 v/v E/M). All the experiments were run in parallel using the skin of three donators, each experiment was run in triplicate.

3.1.6. Reconstructed epidermis: the influence of donor and acceptor vehicles

With respect to reconstructed human epidermis, the donor vehicle influenced not only on the viability of the skin but also testosterone permeation. Using 10/90 (v/v) E/M as donor vehicle testosterone permeation amounted to 3.69 % of the applied dose. Using 60 % and 100% ethanol as donor vehicles, however, permeation was only 2.79 % and 0.93 %, respectively (Fig. 9).

The MTT-test showed that 10/90 E/M is less toxic for the skin than 60% and 100% ethanol as depicted in Tab. 3. Moreover, the results showed also that the acceptor medium PBS plus 5 % BSA is more toxic than PBS alone. Since we aimed at a ready to use test protocol for permeation only, skin viability was not a matter of concern. This is different, however, if skin metabolism is to be investigated.

Tab.4. MTT-test results of reconstructed epidermis after application of 500 µl of 1% testosterone for 6 h and testosterone-free solution for 6 and 24 h in different donor vehicles. All the experiments were run using reconstructed epidermis of 2 batches each experiment was run in duplicate.

Type of vehicle	Viability (%) of RHE after application of testosterone-free donor vehicle		Viability (%) of RHE after application of 1% testosterone-in donor vehicle	
	PBS (6 h)	PBS (24 h)	PBS (6 h)	PBS + 5 % BSA (6 h)
60 % EtOH	89.00 ± 37.30	29.70 ± 6.22	74.15 ± 10.60	63.35 ± 3.32
100 % EtOH			41.39 ± 30.99	22.96 ± 14.81
10/90 E/M	120.70 ± 24.47	118.9 ± 11.17	117.33 ± 3.79	75.76 ± 6.17

Besides formazan formation also lactate concentration and lactate dehydrogenase (LDH) activity were determined in the acceptor media in separate experiments, in order to obtain information about the skin integrity during the experiment ts. The release of lactate and LDH by reconstructed human epidermis was measured for drug free donor vehicles applied for 6 and 24 h. Lactate and LDH levels increased with time following 60% ethanol as donor vehicle, while 10/90 E/M increased lactate levels only (Tab. 5). Therefore 60 % ethanol is more toxic for reconstructed human epidermis than 10/90 E/M which is well in accordance with the results of the MTT test.

Tab.5. Lactate accumulation and LDH activity in the acceptor medium 0, 6, and 24 h after application of the drug free vehicle. All the experiments were run using reconstructed epidermis of 2 batches each experiment was run in duplicate.

Vehicle	**Lactate (mmol/L)**			**LDH (U/L)**		
	0 h	6 h	24 h	0 h	6 h	24 h
60 % EtOH	0.00	2.59±0.80	6.65±0.11	2.75 ± 5.91	60.0 ± 22.6	103 ± 7.07
10/90 EtOH-Miglyol	0.00	1.99±0.15	6.41±0.40	5.50 ± 3.11	5.50 ± 2.12	2.0 ± 4.24

Histological examinations of the reconstructed epidermis after the experiments, however, revealed that the donor vehicle has no major effect on the histology of reconstructed tissue, if

PBS or PBS plus 5%BSA served as acceptor media. Moreover, according to histological examinations of a limited number of treated samples PBS appears more toxic for the tissue than PBS plus BSA. This outcome is not in accordance with the results of MTT test increasing viability. This may suggest that miglyol and ethanol affect the lipid content of the SC as permeation enhancer, while miglyol, but not ethanol, has no effect on mitochondrial enzymes of epidermal cells. (Fig. 15 C, D).

Tab. 6 Results of the histological examination of reconstructed epidermis of the control and of reconstructed epidermis after permeation experiments. The results are expressed in 4 degrees of harmful effects on the skin tissues; normal (N), light (L), mild (M), and hard (H).

Vehicle	Media	control	Static Franz-cell	Dynamic Franz-cell
60 % Ethanol-water	PBS	M	L	
	PBS +5% BSA	L	M	
100% Ethanol	PBS	M	H	H
	PBS +5% BSA	N-L	M	M
Ethanol-Miglyol-10/90	PBS	M	H	M
	PBS +5% BSA	M	M	L

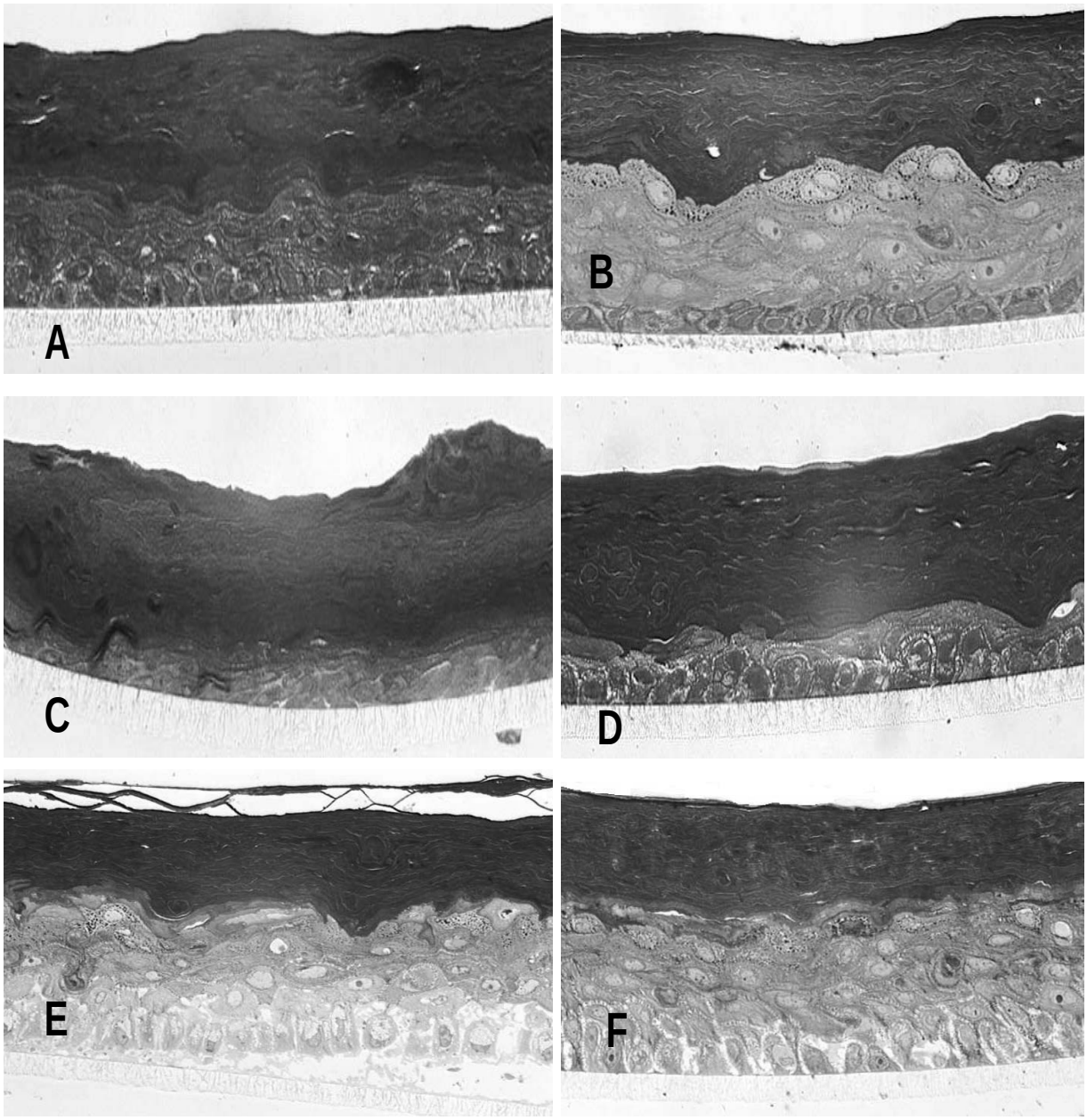

Fig. 15 Reconstructed epidermis untreated with test solution (control) and treated with 1% testosterone in different donor vehicle (test). All are in contact with acceptor media for 6 h in a Franz cell: **A**. control using PBS revealed signs of mild damage. **B**. control using PBS + 5% BSA revealed normal appearance **C**. test using 100% ethanol and PBS revealed signs of hard damage. **D**. test using 100% ethanol and PBS + 5% BSA revealed signs of mild damage. **E**. test using 10/90 E/M and PBS revealed signs of hard damage. **F**. test using 10/90 E/M and PBS + 5% BSA revealed signs of mild damage.

3.1.7. Inter-individual and batch-to-batch variation

In a following series of experiments the inter-individual and batch-to-batch variations of testosterone permeation were tested. The results confirmed the individual variations between the animals and human individuals as depicted in Fig. 16A-17C. Permeation through different batches of reconstructed human epidermis however, varied clearly less (Fig. 16D). Therefore, with respect to the reproducibility of the results, reconstructed epidermis appears superior over the excised skin of human and animal origin.

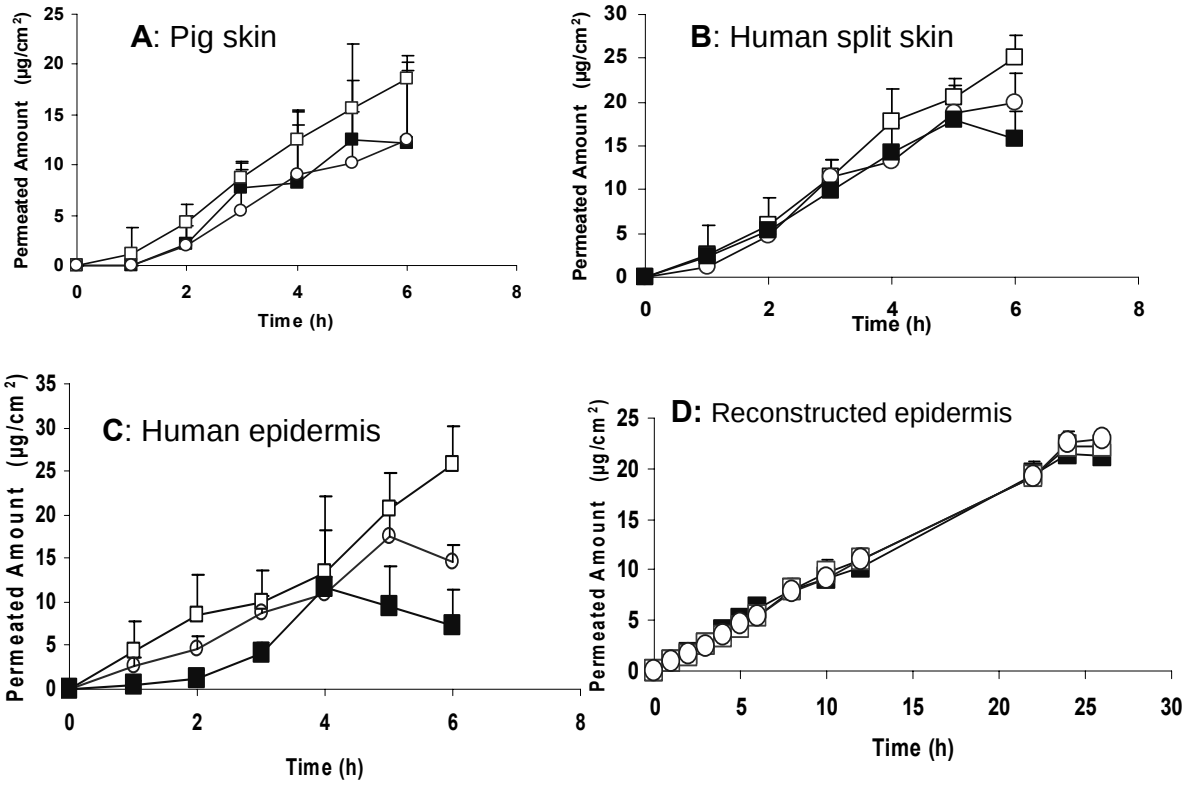

Fig. 16. Inter-individual variation of the testosterone permeation. Using the static Franz cell model, 500 µL of 1% testosterone in 10/90 E/M was applied on the skin surface. **A**. cryopreserved pig skin (Animal A, -■- B, -□- and C, -○-); **B.** cryopreserved human skin and **C.** isolated human epidermis, both abdominal skin of females (50, -■-, 58, -□-, and 62 years,-○-); or **D.** reconstructed human epidermis (Batch A, -■-, B, -□-, and C, -○-). Split skin and isolated human epidermis were studied in parallel for skin of the same donors. All experiments were run in triplicates.

3.1.8. Pharmacokinetic parameters of the evaluated skin models

In the following, pharmacokinetic parameters of cutaneous uptake of testosterone (lag time, steady state flux and permeability coefficient) are summarized (Tab 7). The results revealed that reconstructed human epidermis has the highest flux and permeation with the shortest lag time. Flux of split skin of animal and human origin was rather close. With split porcine skin, the lag time was almost one hour.

Tab. 7 Pharmacokinetic parameters of testosterone permeation after 6 h.

Skin tissue	**Donor vehicle**	**Flux (µg/cm2.h)**	**Papp x E-08 (cm/s)**	**Lag time (h)**	**Permeation (%)**
RHE	60%EtOH	21.5	59.8	0.3	2.8 ± 0.2
RHE	100%EtOH	8.8	24.3	0.6	0.9 ± 0.2
RHE	10/90 EM	27. 0 ± 3.5	75.0 ± 9.7	0.1	3.7 ± 1.1
PS cryo	10/90 EM	1.7 ± 0.3	4.6 ± 0.9	0.8 ± 0.2	0.2 ± 0.04
PS cryo*	10/90 EM	8.2	11.4	0.8	3.1
HS fresh	10/90 EM	2.5 ± 1.7	6.8 ± 4.8	0.1 ± 0.2	0.39 ± 0.2
HS cryo.	10/90 EM	2.1 ± 0.4	5.9 ± 1.0	0.2 ± 0.2	0.2 ± 0.1
HSE	10/90 EM	1.6 ± 0.8	4.4 ± 2.2	0.2 ± 0.2	0.2 ± 0.1

*The permeation experiment was carried out for 26 h. RHE, reconstructed human epidermis, PS, porcine skin, HS, human skin and HSE, heat separated epidermis

3.1.9. Evaluation of the test protocol: Testosterone versus hydrocortisone

For a first evaluation of the test protocol, we compared three semi-solid formulations of the lipophilic testosterone to identical hydrocortisone (lipophilicity, logP 1.43) preparations in the reconstructed epidermis and pig skin. Comparing the lipophilic testosterone to the more hydrophilic hydrocortisone should lead to the identification of permeation patterns which result in a proper characterisation of the epidermis models. Moreover, the experiments were also performed using an alveolar model (EpiAirway) for comparison.

First using the same reconstructed epidermis as in the previous experiments, testosterone uptake was compared to hydrocortisone absorption (Fig.17). Both O/W and W/O emulsion were compared to the ethanol/miglyol solution. With respect to testosterone, both O/W and W/O emulsion increased permeation to 5.2 and 5.5 % of the applied dose, respectively, whereas 10/90 E/M yielded in a permeation reading 4.4 % (Fig.17A). The difference in

permeation between O/W and W/O was not significant, but that between 10/90 E/M and either O/W or W/O was significant. (0.1% Testosterone preparations were applied for 26 h).
In contrast to testosterone, hydrocortisone permeation using 10/90 E/M (6.6 % of the applied dose) was higher than that of both O/W and W/O emulsion (3.8 and 5.4 %, respectively). The difference in permeation between 10/90 E/M and W/O was not significant, but that between O/W and either 10/90 E/M or W/O was significant (Fig.17B). These results were expected, because hydrocortisone is better to permeate from lipophilic vehicle than from hydrophilic one according to its hydro-lipophilicity parameter. As compared to testosterone, permeation of hydrocortisone was slightly higher.

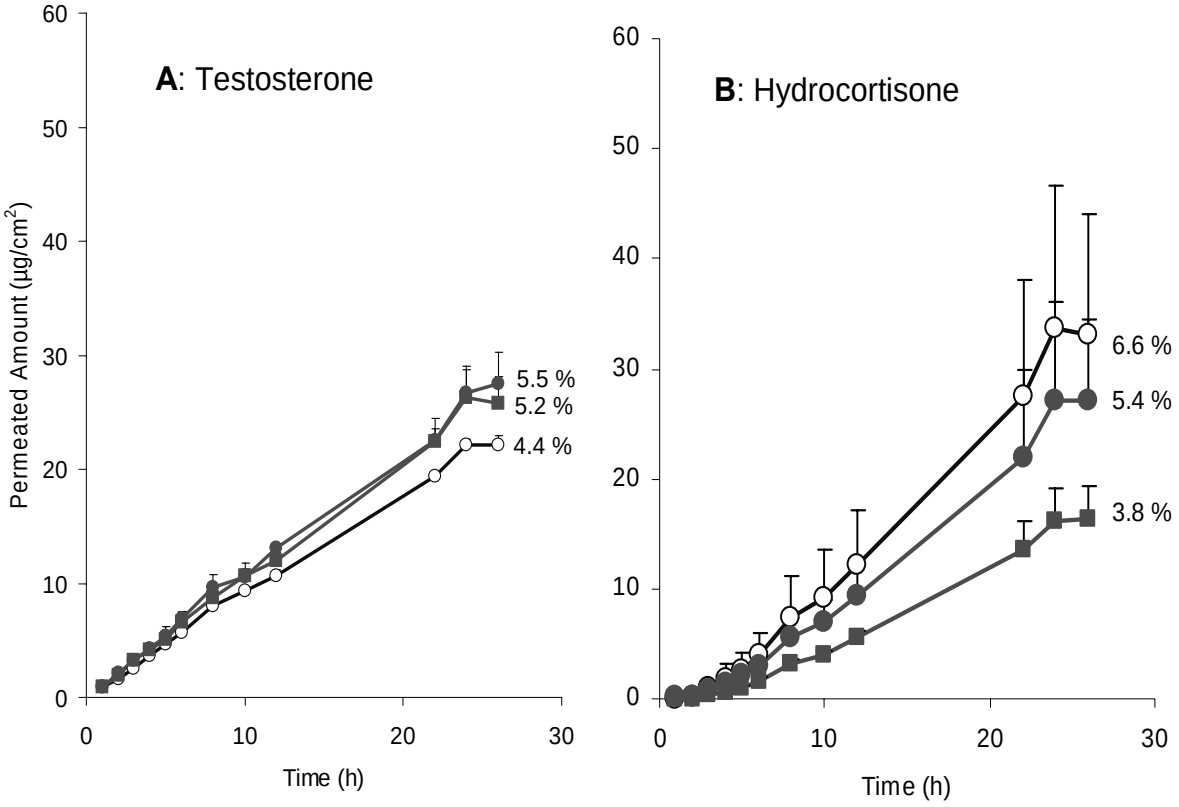

Fig. 17 Permeation of testosterone (A) and hydrocortisone (B) was investigated using three batches of reconstructed human epidermis (Skinethic). 500 µl of 0.1% test solution was applied as 10/90 E/M solution (○), O/W emulsion (■), or W/O emulsion (●) using PBS + 5% BSA as acceptor medium. All experiments were run in duplicate.

Performing the identical experiment with pig skin, once more the O/W and W/O emulsion turned out nearly similar and resulted in the highest testosterone permeation reading 1.72 and 1.69 % of the applied dose, respectively. With 10/90 E/M, the permeation read only 1.53 % (Fig. 18A). The difference in permeation between O/W and W/O was not significant, but that between 10/90 E/M and either O/W or W/O was significant.

Investigating hydrocortisone permeation across porcine skin, again 10/90 E/M resulted in the highest permeation (6.2 % of the applied dose) among the test formulations. Permeation from 10/90 E/M exceeded uptake from O/W and W/O emulsion (3.1 and 4.8 %, respectively). The difference in permeation among all used formulations was significant. As compared to testosterone, permeation of hydrocortisone was higher by about four-fold (Fig.18B).
While hydrocortisone uptake by pig skin was close to the uptake from the reconstructed epidermis, testosterone permeation of the latter exceeded pig skin permeation about fourfold.

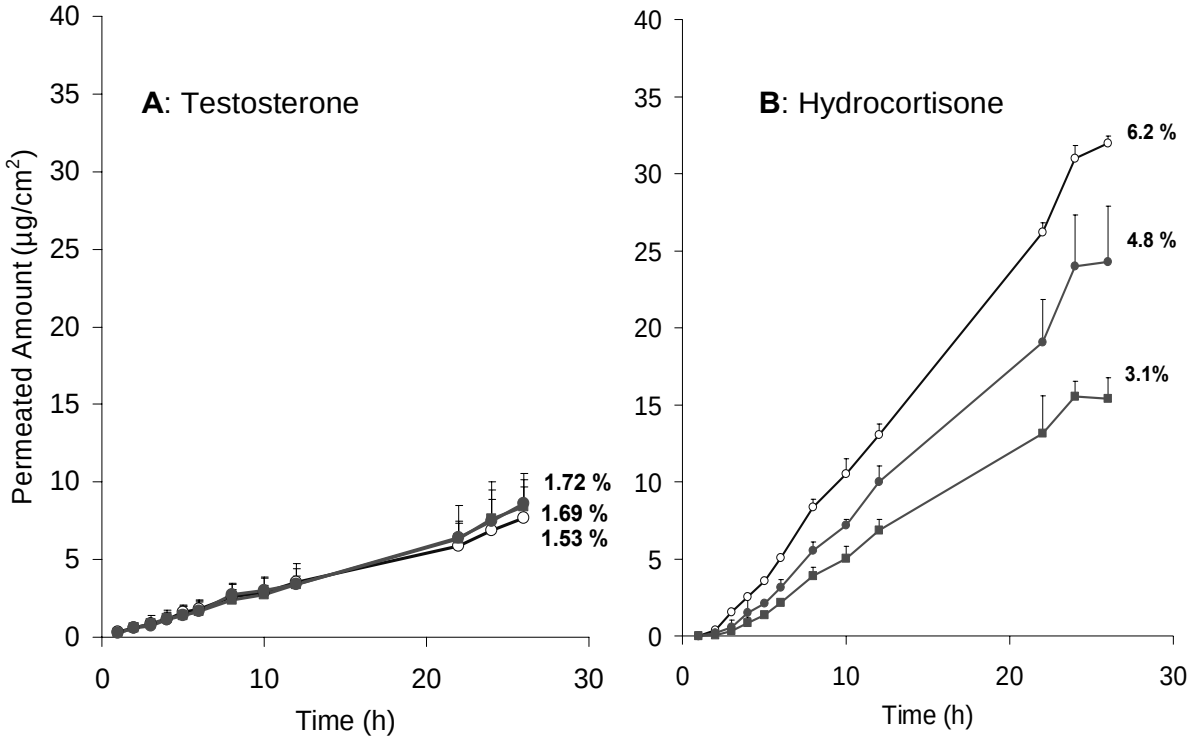

Fig. 18 Permeation of testosterone (A) and hydrocortisone (B) was investigated using split skin of three pigs. 500 µl of 0.1% test solution was applied as 10/90 E/M solution (○), O/W emulsion (■), or W/O emulsion (●) using PBS + 5% BSA as acceptor medium. All experiments were run in duplicate.

With both steroids, permeation of the alveolar model exceeded uptake by split porcine skin about fivefold, and uptake by reconstructed epidermis 2-(testosterone) to 3fold (hydrocortisone). With respect to the EpiAirway model, hydrocortisone exceeded testosterone permeation about twofold. The O/W emulsion resulted in the highest testosterone permeation (10.61% of the applied dose), whereas uptake was less with W/O emulsion (9.40%) and 10/90 E/M (9.32 %, Fig.19A). With the latter both are lipophilic in their external phase. Since a lipophilic substance is more soluble in lipophilic donor vehicle than in viable epidermis but is more soluble in alveolar than hydrophilic donor vehicle, the better testosterone uptake from the O/W emulsion was anticipated.

With regard to hydrocortisone absorption from W/O emulsion and 10/90 E/M were identical (19.2 %) and exceeded the uptake from O/W emulsion (18.45%, Fig.19B).

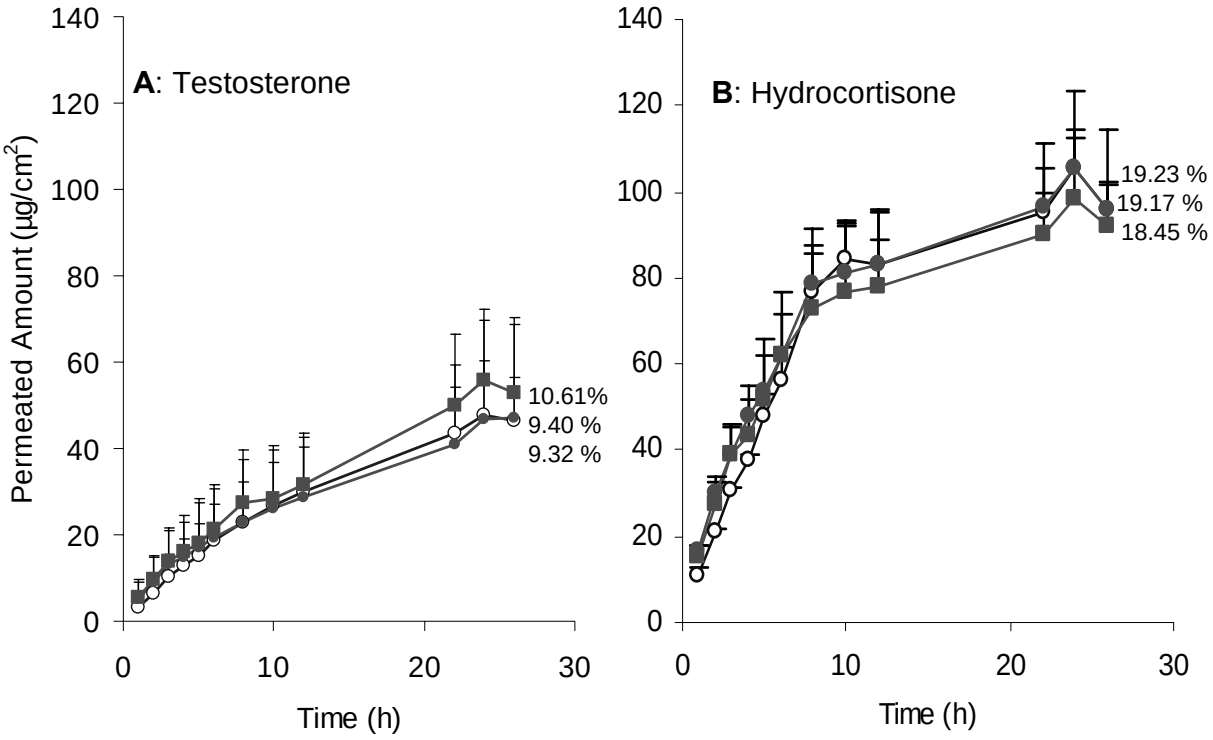

Fig. 19 Permeation of testosterone (A) and hydrocortisone (B) was investigated using three batches of EpiAirway model. 500 µl of 0.1% test solution was applied as 10/90 E/M solution (○), O/W emulsion (■), or W/O emulsion (●) using PBS + 5% BSA as acceptor medium. All experiments were run in duplicate.

3.2. Estrogens

For topical estrogen application as with other drugs used as TTS, both drug penetration and cutaneous metabolism are important for efficacy of treatment and unwanted side effects. Therefore, we focussed not only on the E2 uptake but also on the cutaneous estrogen metabolism. Consequently estrogen analysis had to allow quantifying E2 metabolites which are E1 but also E2 and E1 conjugates.

3.2.1. Optimisation of the RIA procedure for determination of estrogens in different matrices

Investigating the effect of Tris-HCl buffer, keratinocyte basal medium (KBM), 5% BSA solution, and 0.9% NaCl solution on the binding of estrogen antibodies revealed that 0.9% NaCl solution favoured the binding of the estrogen antibodies as compared to KBM, 5% BSA, and Tris-HCl buffer (Tab. 8). This resulted in higher sensitivity of the RIA method. Although this improvement was not significant as compared to Tris-HCl buffer, 0.9% NaCl solution was selected since it resulted in also a clear supernatant after centrifugation.

Tab.8. Estrogen binding to estrogen antibodies (% B/B°) in different buffer solutions.

Estrogen	Conc. (pg/tube)	Tris-HCl Buffer	0.9 % NaCl Solution	KBM Solution	5% BSA Solution
E2	0	100 (45.7 % of total activity)	100 (59.8 % of total activity)	100 (42.8 % of total activity)	100 (39.5% of total activity)
	25	65.9	69.6	77.8	72.9
	50	50.1	50.2	61.5	51.3
	100	33.6	35.9	53.6	43.4
	200	21.0	22.2	47.7	41.6
	500	16. 0	17.7	38.6	36.0
E1	0	100 (49.3 % of total activity)	100 (55.7 % of total activity)	100 (45.6 % of total activity)	100 (43.4 % of total activity)
	15	81.7	72.5	88.6	78.7
	31	62.2	51.6	72.9	72. 0
	63	49.7	43.4	44.6	63.5
	125	46.9	38. 0	43.9	61.6
	250	35.2	23.2	38.0	57.8
	500	20.2	13.8	23,2	48.7

First experiments to quantify free estrogens in presence of their conjugates revealed that both the conjugates and skin proteins interfere with the determination of the free estrogens (Fig. 20).

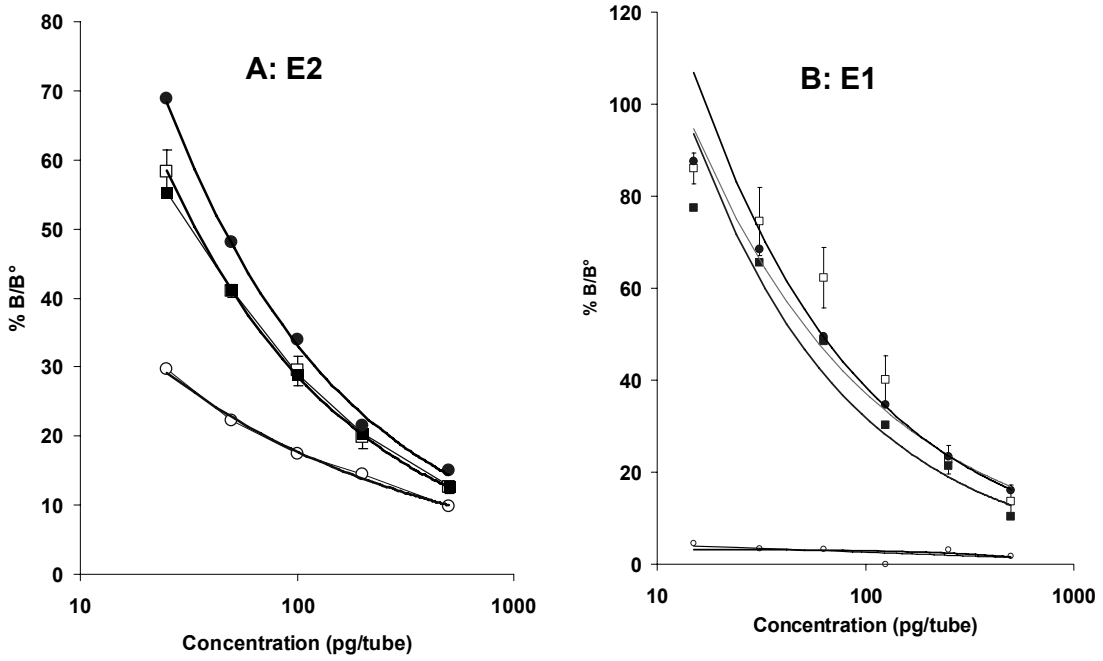

Fig. 20 Standard curve for A: E2 in presence of 100 pg of E1, E2-conjugate, and E3 (-□- E2, -○- E2 + E2 conjugate, -■- E2 + E1 and -●- E2 + E3) and B: E1 in presence of 100 pg of E2, E1-conjugate, and E3 (-□- E1, -○- E1 + E1 conjugate, -■- E1 + E2 and -●- E1 + E3)

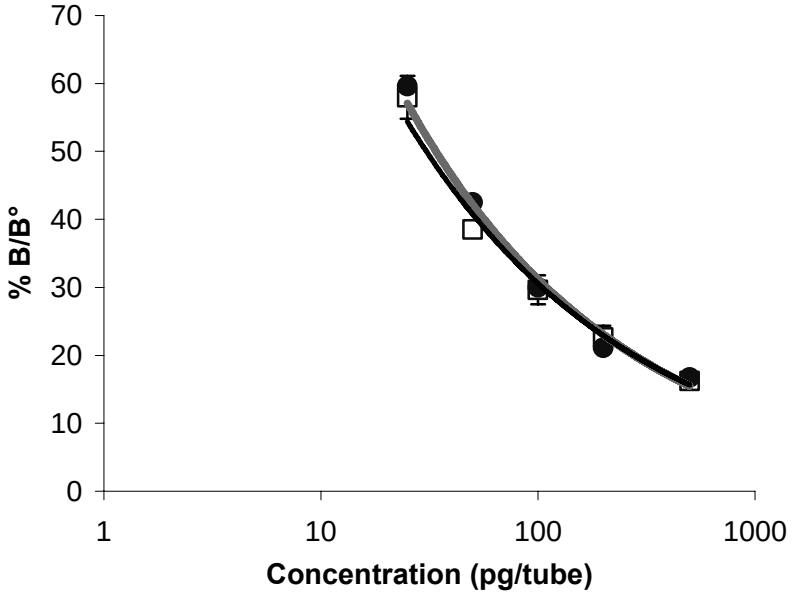

Fig. 21 Standard curve for E2 in presence of skin tissue homogenate after ether extraction (- - -●- - -) compared to the corresponding E2 curve in 0.9% NaCl solution (-□-).

Solvent extraction of the steroids prior to submission to the RIA procedure proved to overcome this problem. Different organic solvents were investigated to find the one enabling selective estrogen extraction with high recovery (Tab. 9). Extraction with ethyl acetate yielded the highest recovery, because of its ability to extract also the more polar estrogen conjugates, ether was selected for extraction.

Tab. 9. Estrogen recovery after the extraction with different solvents. 1 μg E2 or E1 was added to 1ml acceptor medium and extracted three times with 5 ml of organic solvent.

Estrogen		Ether	Ethyl acetate	Chloroform	Dichloromethane
E2	**Found**	0.901 ± 0.083	1.046 ± 0.119	0.853 ± 0.071	0.828 ± 0.104
	% recovery	90.14 ± 9.21	104.61 ± 11.4	85.30 ± 8.31	82.77 ± 12.6
E1	**Found**	0.831 ± 0.063	0.846 ± 0.11	0.755 ± 0.081	0.726 ± 0.094
	% recovery	83.11 ±7.58	84.60 ± 13.00	75.50 ± 10.73	72.67 ± 12.95

The sensitivity was 3.75 and 10.0 pg/tube for E2 and E1, respectively. The linear range was 5.0 - 500 and 15.0 – 500 pg E2/tube for E2 and E1, respectively (Tab. 10). Specificity of the RIA procedure for E2 and E1 was determined by estimating the percentage of cross reactivity to their metabolites (Tab.10) (ratio of E2 or E1 concentration to cross reactant concentration at 50% inhibition of maximum binding)

Tab. 10 Sensitivity and specificity of the RIA method

Estrogen	Sensitivity	Linear range (pg)	Cross reactivity (%) to				
			E1-conj	E2-conj	E2	E1	E3
E2	3.75	5.0 - 500	-	> 100	100	4.8	1.3
E1	10.0	15 - 500	> 100	-	< 0.1	100	< 0.1

Sensitivity is defined as the 90% intercept of a B/B° standard curve.

Reproducibility of the technique was tested with spiked E2 and E1 levels in the acceptor medium in range of 0.15-10 ng/mL. The intraday variation coefficients were 0.61 - 9.99 and 1.88 - 4.82 % whereas the interday variation coefficients were and 7.89 - 19.65 and 0.86 - 11.06 % for E2 and E1, respectively (Tab. 11).

Tab.11. Validation of radioimmunoassay procedures

Concentration (ng)	Estradiol		Estrone	
	amount	percentage	amount	percentage
Intraday variation coefficient				
0.15	0.14 ± 0.01	92.7 ± 6.3		
0.30	0.33 ± 0.01	111.3 ± 4.2	0.29 ± 0.01	95.7 ± 3.2
0.60	0.66 ± 0.04	109.7 ± 5.5	0.60 ± 0.03	99.3 ± 4.3
1.25	1.19 ± 0.02	95.4 ± 2. 0	1.24 ± 0.06	98.9 ± 4.8
2.50	2.42 ± 0.02	97. 0 ± 0.6	2.42 ± 0.05	96.8 ± 1.9
5.00	4.67 ± 0.35	93.5 ± 7.6	4.71 ± 0.11	94.1 ± 2.3
10.00	9.64 ± 0.96	96.4 ± 10.	9.27 ± 0.36	92.7 ± 3.8
Interday variation coefficient				
0.15	0.12 ± 0.02	79.3 ± 19.7		
0.30	0.26 ± 0.05	85.7 ± 18.5	0.29 ± 0.03	97.3 ± 9.6
0.60	0.54 ± 0.10	89.3 ± 17.9	0.57 ± 0.04	95.7 ± 7.5
1.25	1.17 ± 0.22	93.6 ± 18.5	1.29 ± 0.09	103.5 ± 6.7
2.50	2.55 ± 0.41	101.8 ± 16.1	2.45 ± 0.02	98.0 ± 0.9
5.00	4.61 ± 0.85	92.2 ± 18.5	4.68 ± 0.39	93.5 ± 8.4
10.00	9.21 ± 0.73	92.1 ± 7. 9	9.44 ± 1.04	94.4 ± 11.1

3.2.2. Determination of E2 conjugates.

The complete cleavage of the conjugates was verified by varying the incubation time and the amount of enzyme. The results showed that the enzyme glucuronidase / sulphatase mixture amounts required for the complete hydrolysis of the conjugates is at least 20 units and incubation 6-12h at 37 °C (Fig. 22, 23).

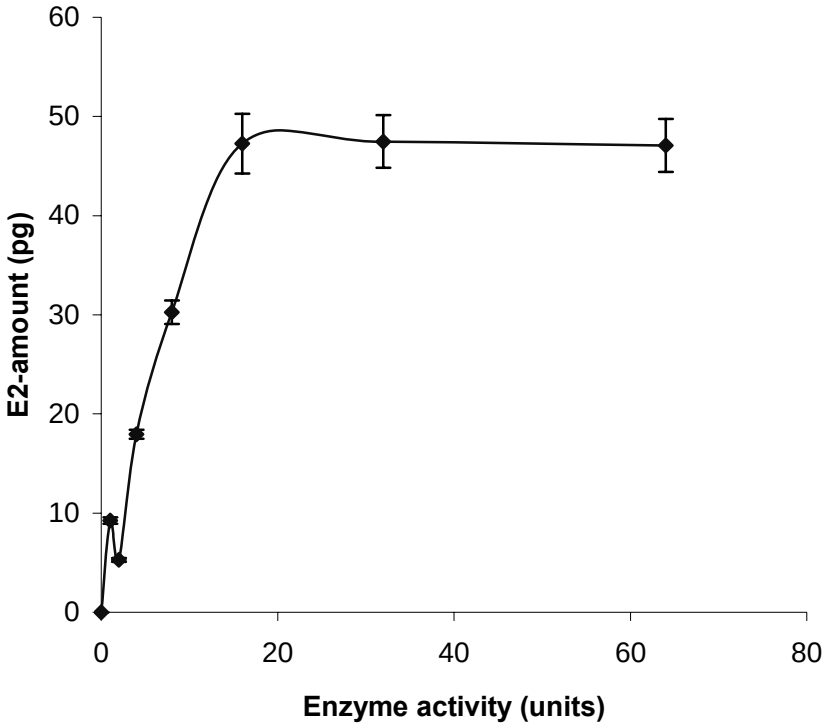

Fig. 22 Hydrolysis of 50 pg E2-conjugates by varying amounts of glucuronidase/sulphatase. Samples were incubated for 6-12 h as described in the materials and methods section.

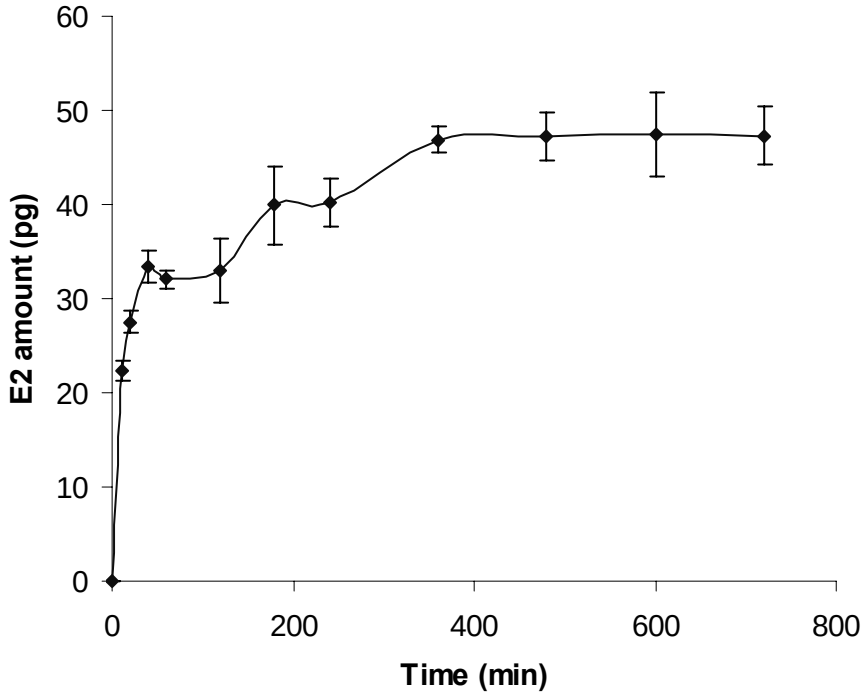

Fig.23 Effect of the incubation time on the hydrolysis of 50 pg E2-conjugate. 400/20 units of glucuronidase/sulphatase were added to the samples and incubated from 10 min to 12 h as described in the materials and methods section.

The validated methods served for estrogen analysis in the samples drawn from Franz cell and perfusion model experiments.

3.2.3. Influence of BSA on E2 permeation

The addition of 5% BSA to the acceptor fluid is recommended by the OECD to improve the permeation of highly lipophilic substances. We therefore tested the effect of BSA on the permeation using reconstructed human epidermis and two E2 formulations, an ethanolic solution and a gel. Addition of BSA to the acceptor medium (MEME) slightly increased the permeation of E2 by 14.29 % and 26.15 % for the ethanolic solution and the gel, respectively (Fig. 24).

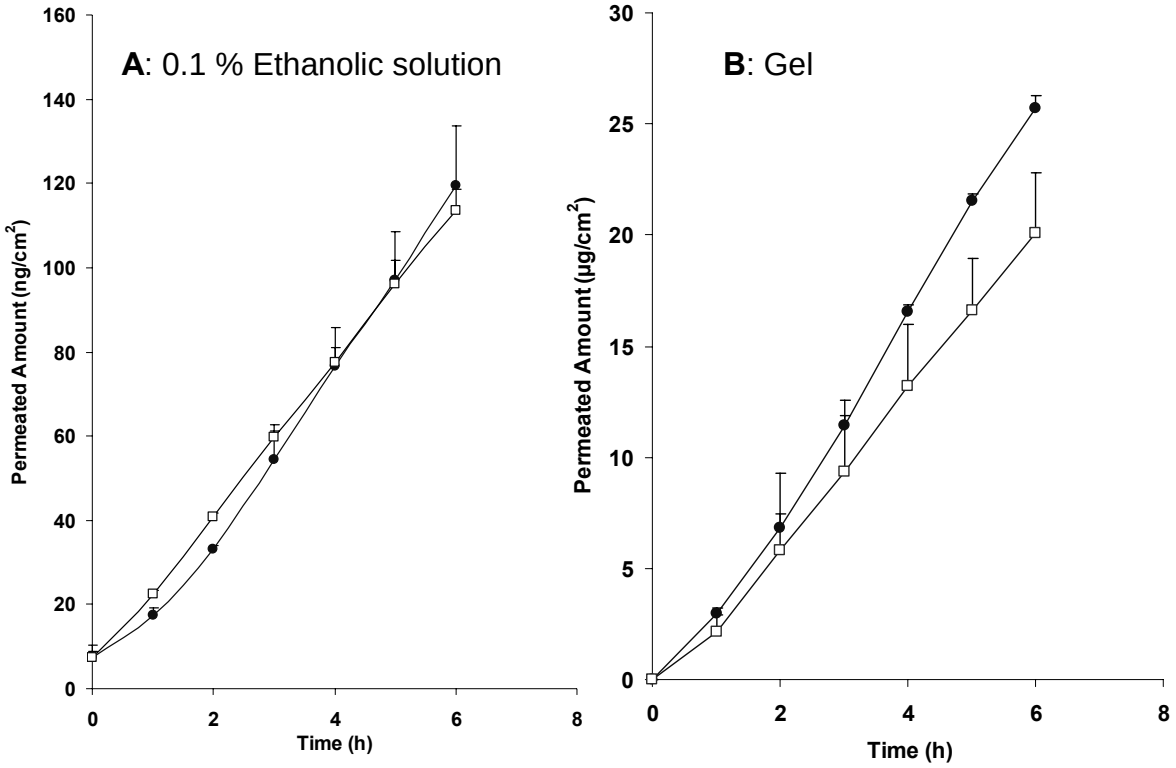

Fig. 24 Influence of BSA in the acceptor medium on the permeation of 17 ß-estradiol through reconstructed human epidermis. A. Applying 100 µg E2 as 0.1% ethanolic solution B. Applying 100 µg E2 as 100 mg gel (Acceptor medium MEME -□- and acceptor medium MEME + 5% BSA -●-)

3.2.4. Estrogen permeation / penetration and metabolism

3.2.4.1. Reconstructed human epidermis

With reconstructed human epidermis, estrogen concentrations in the acceptor medium before drug application were close to the detection limit. This changed within 1 h after E2 application. The application vehicle influenced the E2 uptake and metabolism. Differences in the estrogen concentrations in the acceptor fluid depending on the vehicle form became obvious for both, native E2 and its metabolites. Following 100 µg E2 applied as Sisare®Gel the permeation of native and conjugated E2 amounted to 12.8 % and 2.41 %, respectively (Fig. 25 A). Moreover E1 and conjugated E1 were in the same order of magnitude as E2 and its conjugates.

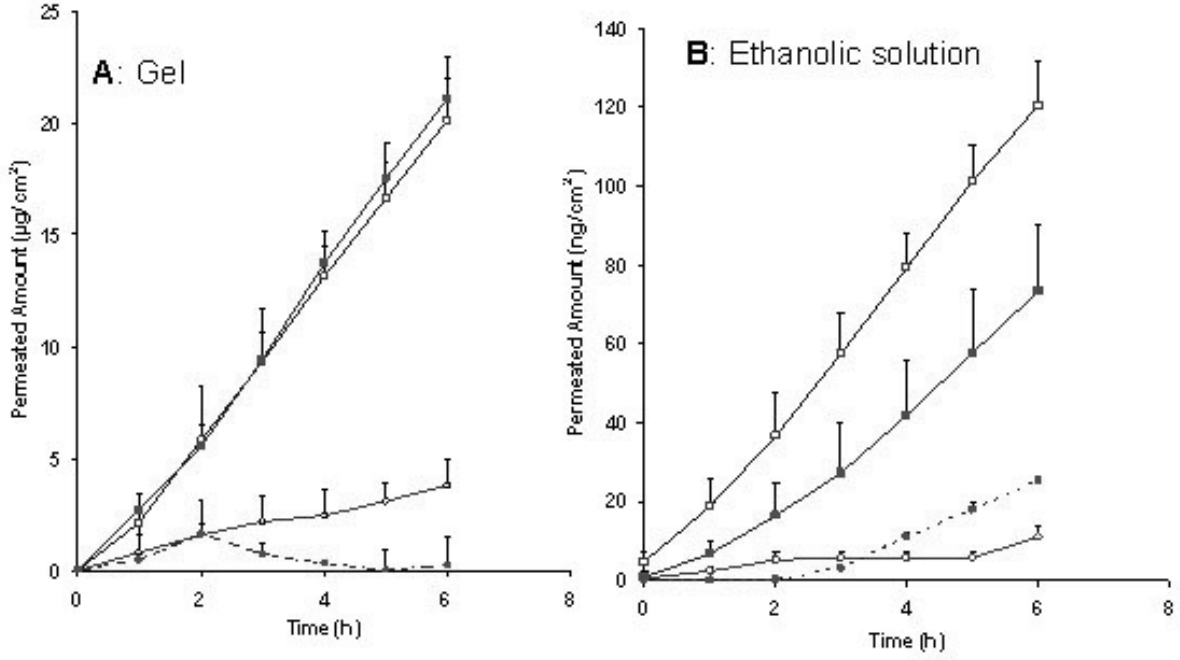

Fig. 25 Estrogen permeation through reconstructed epidermis following the topical application of 100 µg E2 as an ethanolic solution (B) and gel (A). (-□- E2, -○- E2 conjugate, -■- E1 and -●- E1 conjugate, mean ± SD, n = 2)

Taking the estrogens extracted from the skin into account, 41.7 % of the applied dose was absorbed by reconstructed human epidermis using the gel. 42.6 % of the totally absorbed amount was found to be metabolized. E1 was the dominating metabolite (36.3%). Comparing conjugate formation E2 appeared to be more actively transformed as compared to E1. Most importantly, the E2/E1 ratio was 0.96 (Tab. 12), which is in the range of the ratios seen in postmenopausal females receiving transdermal E2 replacement therapy [124, 126-127].

Applying the identical E2 dose as a 0.1% ethanolic solution (Fig. 25B), however, less than 0.2% of the dose permeated into the acceptor medium (0.08% permeated as E2, 0.01% as E2 conjugate, 0.05% as E1, and 0.02% as E1 conjugate) and 15.2 % were recovered from the epidermis. The concentrations of Estrogens recovered from the epidermis following application of 0.1% ethanolic solution were more than following application of the gel (9.3%). The very low E2 permeation and metabolism following the ethanolic solution (Tab. 12) suggests that the solvent damages the tissue. Indeed, a concentration of 2% ethanol in the medium of monolayer cultures is toxic. Keratinocytes viability is reduced by 52 % and for fibroblasts even by 97 %. The pronounced differences in estrogen permeation following the gel and ethanolic solution are also reflected by their slopes summarised in Tab. 12.

3.2.4.2. Excised pig-skin

Also with pig skin, the estrogen concentrations in the acceptor media before drug application were barely detectable and rose rapidly thereafter. As compared to reconstructed epidermis, estrogen permeation of split pig skin was clearly less following the application of sisare gel (0.23 % versus 28.78 %, Fig. 26A and Fig. 25A, respectively) and slightly reduced following the ethanolic solution (0.091 % versus 0.15 %, Fig. 26C and Fig. 25B, respectively). With split pig skin also the influence of ethanol on E2 metabolism was less pronounced (Tab. 5). This should be due to a superior barrier function of the horny layer of split skin as compared to reconstructed epidermis, protecting viable cells from ethanol toxicity. E2 tissue levels following the application of ethanolic solution were clearly lower than E2 levels following the application of the gel (Tab. 13 and Fig. 26B & 26D), which is in contrast with reconstructed epidermis (Tab.12).

Tab. 12 Estradiol pharmacokinetics using reconstructed epidermis in the Franz-flow-through cell system. 100 µg E2 was applied as an 0.1% ethanolic solution and as a gel. Data are presented as [%] of the totally recovered drug. (mean ± SD, n = 2). * N.D. means that the concentration was below the detection limit.

		Sisare Gel	Ethanolic Solution
E2	Surface	53.33 ± 0.43	73.22 ± 22.99
	Strip 1+2	3.55 ± 0.06	8.9 ± 5.3
	Strip 3+4	5.00 ± 0.56	5.13 ± 1.17
Skin	Free E2	0.49 ± 0.01	1.02 ± 0.47
	Conj. E2	Not determined	
Acceptor medium	Free E2	12.79 ± 1.23	0.077 ±0.007
	Conj. E2	2.42 ± 0.69	0.007 ± 0.002
E1	Surface	N.D*	
	Strip 1+2	N.D	
	Strip 3+4	N.D	
Skin	Free E1	0.26 ± 0.01	0.06 ± 0.01
	Conj. E1	Not determined	
Acceptor medium	Free E1	13.41 ± 1.19	0.047 ± 0.011
	Conj. E1	0.16 ± 0.79	0.016 ± 0.000
E2/E1 ratio		0.96	1.66
E2+Conj/E1+Conj ratio		1.12	1.34
Total Recovery		91.40	88.29
Slope [µg/h]	**Free E2**	2.196	0.013
	Total estrogen	4.840	0.024
Calculated plasma level of E2 in man		37.65 ng/ml	0.22 ng/ml

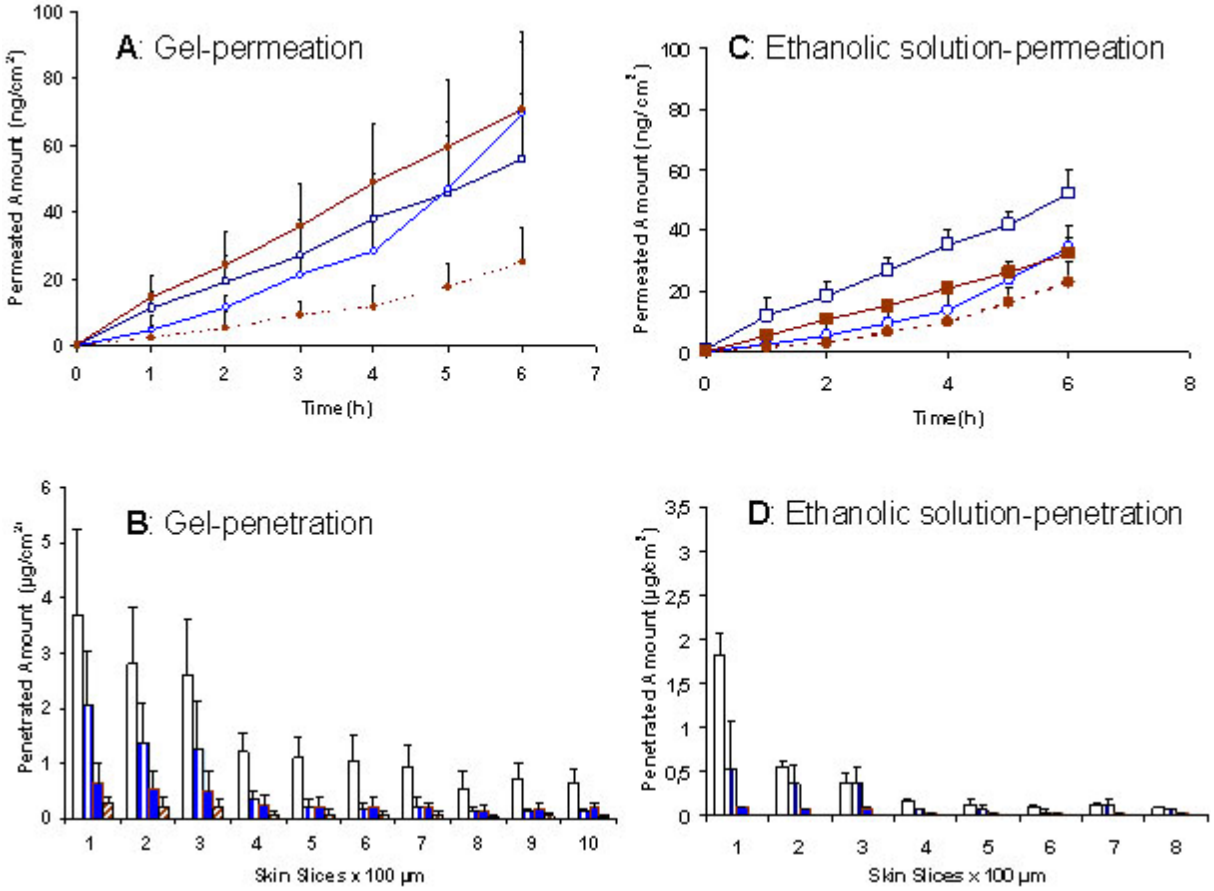

Fig. 26 E2 permeation/penetration and metabolism were investigated using split pig skin and the Franz-flow-through cell system. 100 µg E2 was applied as gel (A, B) and an ethanolic solution (C, D). A and C show the estrogen concentrations in the acceptor media (MEME) (-□- E2, -○- E2 conjugate, -■- E1 and -●- E1 conjugate), B and D in horizontal skin slices (□ E2, ▥ E2 conjugate, ■ E1 and ▧ E1 conjugate; mean ± SD, n = 2)

Tab. 13 Estradiol pharmacokinetics using split pig skin in the Franz-flow-through cell system. 100 µg E2 was applied as an 0.1% ethanolic solution and as a gel. Data are presented as [%] of the totally recovered drug. (mean ± SD, n = 2). * N.D. means that the concentration was below the detection limit.

		Sisare Gel	Ethanolic Solution
E2	**Surface**	77.71 ± 9.21	82.98 ± 18.01
	Strip 1+2	4.80 ± 3.40	7.12 ± 0.73
	Strip 3+4	1.80 ± 0.70	5.00 ± 1.10
Skin	**Free E2**	9.70 ± 2.90	2.17 ± 0.21
	Conj. E2	3.80 ± 1.40	1.07 ± 0.44
Acceptor medium	**Free E2**	0.06 ± 0.019	0.033 ± 0.005
	Conj. E2	0.07 ± 0.021	0.022 ± 0.004
E1	**Surface**	N.D	
	Strip 1+2	N.D	
	Strip 3+4	N.D	
Skin	**Free E1**	1.90 ± 1.10	0.27 ± 0.01
	Conj. E1	0.70 ± 0.36	0.04 ± 0.01
Acceptor medium	**Free E1**	0.071 ± 0.023	0.021 ± 0.003
	Conj. E1	0.025 ± 0.001	0.015 ± 0.004
E2/E1 ratio		0.79	1.57
E2+Conj/E1+Conj ratio		1.31	1.53
Total Recovery		**100.54**	**98.71**
Slope [µg/h]	**Free E2**	0.009	0.005
	Total estrogen	0.036	0.015
Calculated plasma level of E2 in man		0.16 ng/ml	0.09 ng/ml

One important quality parameter of uptake experiments is the mass balance. Total estrogen recovery was 88.3 to 100.5 % (without collecting the washing fluids), which demonstrates the quality of our experiments.

3.2.4.3. Perfusion model

With untreated skin, estrogen concentrations were low in the perfusat (21 pg E2, 43 pg E1/ml) as well as in the skin (180 pg E2, or 2870 pg E1/g tissue) and muscle (Tab. 14). Following the application of an E2-TTS for 6 h, the E2 concentration as well as those of E2 conjugates increased considerably in the perfusion medium. While E2 already increased within the first hour, E2-conjugates were first elevated after 2 h. There was even a lag time of about 3 h until E1 and E1 conjugate concentrations raised (Fig. 27). At 6 h the E2/E1 ratio amounted to 0.49.

Tab. 14 E2 pharmacokinetics. Porcine forelimbs were perfused with blood-based medium for 6 h with and without the application of E2-TTS (rate of release of E2 ≈ 100 µg/24h). Data are presented as estrogen concentrations. (mean ± SD, n = 3). * N.D. means that the concentration was below the detection limit.

	Metabolites	**Perfusate (pg/ml)**	**Skin as ng/g (application site)**	**Muscle as ng/g (application site)**
Estraderm-TTS	Free E2	310 ± 79	7510 ± 480	3.08
	Conjugated E2	171 ± 60	1160 ± 560	1.21
	Free E1	623	1990 ± 480	
	Conjugated E1	84	50 ± 10	
Control	Free E2	21 ± 11	0.18 ± 0.22	0.14 ± 0.01
	Conjugated E2	N.D*.	0.165 ±0.275	0.107 ± 0.205
	Free E1	43 ± 26	2.87 ± 0.91	2.56 ± 0.68
	Conjugated E1	N.D.	0.578 ± 0.718	0.283 ± 0.565
Slope [µg/h]	Free E2	0.0303		
	Total estrogen	0.1363		

Yet, not only estrogens in the perfusion medium increased but also those in the tissue. In skin and muscle of forelimbs beneath the application side, the concentration of E2 and its metabolites exceeded those of the control leg about 1000fold (Tab. 14). A considerable increase of tissue levels was also seen if E2 and E2-conjugate concentrations of the treated area was compared to E2 and E2-conjugate concentrations in distant skin (3.9 and 0.12 ng/g, respectively) of the same forelimb. Taking the size of the treatment area into account, E2 uptake was 42.85% of the incorporated dose. Moreover, 30 % of the penetrated amount of E2

in the skin is subjected to metabolization, 11 % of the estrogens were identified as conjugated E2, 19 % as E1, and 0.5 % as conjugated E1.

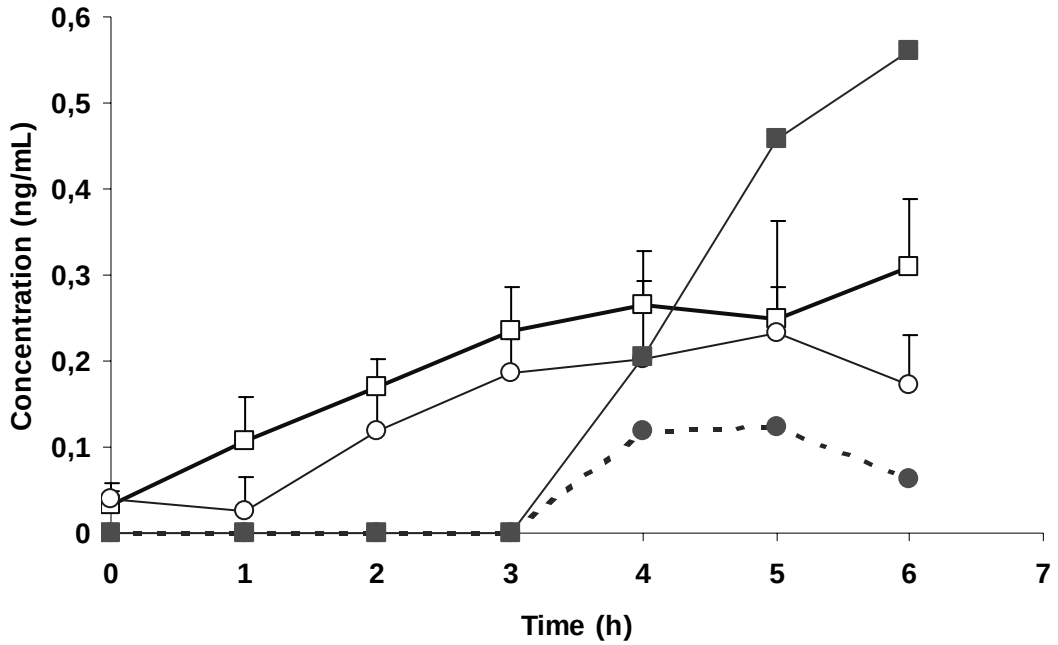

Fig. 27 Estrogen concentrations in the perfusate of the pig forelimb after application of an E2-TTS (rate of release of E2 ≈ 100 µg/24h, -□- E2, -○- E2 conjugate, -■- E1 and -●- E1 conjugate, mean ± SD, n = 3)

In order to compare the different methods we decided to introduce the slopes (fluxes) of the cumulated amounts of E2 and total estrogens. With respect to pig skin these data revealed the superiority of the E2-TTS-method with respect to estrogen permeation (Tab. 13, 14).