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Cutaneous Estradiol Permeation, Penetration and Metabolism in Pig and Man

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Key Words
Estrogen metabolism · Skin penetration · Reconstructed epidermis · Perfused porcine forelimb · Alternative methods

Abstract
Aim and Methods: Drug development in dermatotherapy and also development of transdermal therapeutic systems (TTS) demand high-predictive in vitro models to estimate drug levels in skin and systemic uptake. Here we compare three ready-to-use models, reconstructed human epidermis, split porcine skin and the perfused porcine forelimb. 17β-Estradiol (E_2), which is highly metabolized by skin cells, serves as model drug since E_2 application is of high relevance in hormone replacement therapy while topical E_2 may promote wound healing. E_2 TTS, gel and an ethanolic solution were investigated for cutaneous penetration, permeation and metabolism. ***Results:*** E_2 TTS enabled an E_2 uptake of 42.9% of the applied dose accompanied by a high percentage of E_2 metabolism (30% of the penetrated dose) in the perfused porcine forelimb. In Franz cell experiments with reconstructed human epidermis and split porcine skin, the gel allowed an E_2 uptake of 41.7 and 22.9% of the applied dose accompanied by a high E_2 metabolism (42.6 and 28.6% of the penetrated dose). Due to toxic effects of the vehicle, this was not true with an ethanolic solution, then E_2 permeation and metabolism were clearly diminished. Most importantly, the in vitro models proved to be predictive with respect to the E_2/estrone ratio in female plasma under transdermal hormone replacement therapy. ***Conclusion:*** In vitro tests should reduce the need for both animal and human studies for cutaneous uptake and metabolism in the future.

Introduction

Systemic estrogens are widely used in hormonal replacement therapy (HRT) for the treatment of menopausal symptoms and osteoporosis prophylaxis. Due to the early stop because of side effects (increased risk of breast cancer and strokes) of the Women's Health Initiative study subjecting more than 8,000 postmenopausal females to conjugated estrogens plus medroxyprogesterone acetate in a placebo-controlled trial, however, oral HRT has been seriously questioned by now [1, 2]. Although investigated in a limited number of patients only, trans-

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dermal estrogen application may be of lower risk compared to oral application since coagulability and C-reactive protein concentrations did not increase [3–5]. Recently, estrogens have also attracted interest because of their ability to promote wound healing [6, 7]. With respect to wound healing, a local effect is preferred. If systemic uptake is largely avoided, wound healing may become another indication for estrogens even in men and in women with increased breast cancer risk.

The main barrier in cutaneous estrogen uptake is the stratum corneum which also acts as reservoir [8, 9]. Therefore transdermal application results in a sustained constant drug release, overcomes the short half-life of estradiol (E_2) and avoids the hepatic and gastrointestinal first-pass metabolism [2]. Yet also the skin can affect the estrogen actions by E_2 metabolism. The metabolizing enzymes and their activities in the skin need to be considered. While oral estrogen replacement increases estrone (E_1 [10]) and estrone sulfate plasma levels [11, 12], this holds not true with transdermal systems [13–15]. Inhibition of cutaneous enzymes involved in the metabolism of steroids, however, may influence the systemically available steroids. This has been observed with ethanol, a widely used permeation/penetration enhancer and excipient in transdermal delivery systems of E_2 [16, 17].

To evaluate the predictability of in vitro tests for drug levels following transdermal but also local treatment in man, we compared test protocols including reconstructed human epidermis and excised porcine skin [18, 19] but also the recently described isolated perfused porcine forelimb [20]. E_2 was selected as model drug because of its extensive use and intense metabolism. Predictive in vitro models are economical and result in a reduction or elimination of animal experiments.

Materials and Methods

Materials

17β-Estradiol (E_2), estrone (E_1), anti-E_2 and anti-E_1 antibodies, E_2 glucuronide (sodium salt), E_1 sulfate (sodium salt), dextran-coated charcoal (100–400 mesh), dextran (approximate average molecular weight 70,000), β-glucuronidase and sulfatase mixture (105,000 and 4,300 units, respectively) and Eagle's minimum essential medium (MEME) were obtained from Sigma (Taufkirchen, Germany). 2,4,6,7-^{3}H-E_2 (88 Ci/mmol) and 2,4,6,7-^{3}H-E_1 (94 Ci/mmol) were obtained from Amersham (Freiburg, Germany). The purity was checked by thin-layer chromatography before use. Stock solutions of estrogens (1 µg/ml) in ethanol kept at 4°C were stable for at least 4 weeks. Scintillation cocktail (Optiphase Supermix) was purchased from Wallac (Turku, Finland). All other reagents were obtained from Merck (Darmstadt, Germany) and were of the highest quality available. McIlvaine buffer, pH 5.0, was prepared in our laboratory. Estraderm® TTS (transdermal therapeutic system, E_2 patches, release rate 100 µg/24 h) was obtained from Novartis Pharma (Basel, Switzerland). Sisare® gel (1 mg E_2/g gel) was obtained from Noury Pharma GmbH (Oberschleissheim, Germany).

Reconstructed epidermis (Skinetic™) was purchased from Laboratoire Skinetic (Nice, France). Porcine skin was excised from the inner side of forelimbs of female 'Deutsche Landrasse Hybride' pigs at a local abattoir being able to avoid the procedure of scalding.

Cutaneous Uptake

Franz Cell Experiments

Freshly excised pig skin was placed in transport medium consisting of HEPES-buffered MEME supplemented with gentamicin sulfate (20 µg/ml), amphotericin B (50 ng/ml), glutamine (2 m*M*) and glucose (0.1%). Contact of the skin surface with medium was avoided. Within 2–4 h after excision, split skin (1,000 µm) was prepared using a Dermatome™ (Aesculab, Tuttlingen, Germany). The reconstructed human epidermis was used for the experiments within 24 h after delivery according to the instructions of the manufacturer. Tissue samples of 15 mm in diameter were carefully punched out and mounted onto 9-mm Franz flow-through cells (Crown Scientific, Somerville, N.J., USA). The horny layer faced the air and the dermis or the polycarbonate membranes supporting the skin models were in contact with the acceptor medium. The acceptor medium MEME (flow rate 6 ml/h) was magnetically stirred and the temperature of the skin surface was maintained at 32 ± 1°C by a recirculating water bath. After an equilibration for 30 min, 100 mg Sisare gel or 100 µl of 0.1% ethanolic solution was applied to the skin surface for 6 h. The acceptor medium was continuously collected in fractions of 1 h for analysis. At the end of the experiment, the skin surface was wiped twice with ethanol using cotton wool. Punch biopsies of treated skin were stripped twice using self-adhesive tape strips for the removal of the most superficial horny layer and remaining E_2 formulation. The excised pig skin was then cut in a freeze microtome (Frigocut™ 2800 N, Leica, Bensheim, Germany) into horizontal slices of 100 µm thickness.

Isolated Perfused Porcine Forelimb

Freshly removed limbs (female pigs, 5–8 months old and weighing 70–100 kg) were prepared for perfusion as described [20] and transported at 10–15°C. Limbs were connected to a perfusion system via the arteria brachialis for 6–8 h. Standardized computer-assisted perfusion with a blood-based perfusion medium (bicarbonate-buffered saline, 4% bovine serum albumin, 4–10% erythrocytes, O_2 + CO_2, 37°C) supplied the limb with nutrients and oxygen. Flow rate, arterial pressure, temperature, pH, O_2 saturation, electrolytes (K^+, Ca^{2+}, Na^+), blood glucose, hemoglobin and lactate dehydrogenase were continuously monitored and adjusted if necessary [20]. Venous perfusion medium was collected before application of the TTS for the determination of basal estrogen concentrations. Following the E_2 TTS application to the limbs, venous perfusion medium was collected hourly, centrifuged and the supernatant kept for analysis. At the end, skin and muscle biopsies were taken from treated as well as untreated areas which served as control.

Analysis

Material collected for estrogen analysis was stored at –80°C for further use.

Estrogen Extraction

Free E_2 and E_1 were extracted from the acceptor medium (1 ml) using 5 ml diethyl ether. The samples were vortexed for 1 min and centrifuged at 2,000 rpm, 4 °C for 5 min for phase separation. After removing the organic layer, extraction was repeated twice. The combined ether phases were evaporated using a centrifugal vacuum concentrator (SpeedVac SC110A, Savant, Thermo Electron, Dreieich, Germany), and the dry residue was dissolved in 0.9% (w/v) sodium chloride solution. To quantify conjugated E_2 and E_1, the acceptor medium was first incubated with a sulfatase/glucuronidase mixture (20 units/400 units) in 2 ml McIlvaine buffer, pH 5.0, for 12–24 h at 37 °C. Because of interferences of the enzyme with the antibody reaction resulting in high blank values, the enzyme had to be purified by charcoal treatment before the hydrolytic procedure [21]. This procedure resulted in complete conjugate cleavage and low blank values allowing to quantify total E_2 and E_1, respectively. Conjugated estrogens were estimated by subtracting the concentrations of free drug from total drug level.

Horizontal slices of 100 μm thickness of treated and untreated porcine skin and muscle were chopped into small slices and placed into 4 ml 0.9% sodium chloride solution. The tissue was homogenized using an ultraturrax (S-25 N-18G, Carl Roth, Karlsruhe, Germany) for 1 min at 25,000 rpm and centrifuged at 4 °C, 1,000 rpm for 5 min. The supernatant was subjected to estrogen determination as described above.

The cotton wool and self-adhesive tape strips were extracted twice with diethyl ether. The solvent of the combined extracts was evaporated using a centrifugal vacuum concentrator and the dry residue was dissolved in 0.9% sodium chloride solution.

Radioimmunoassay

A commercially available radioimmunoassay kit from Sigma-Aldrich (St. Louis, Mo., USA) was adapted for the determination of E_1 and E_2 in the ether extracts of the acceptor medium, tissue and cotton wool. The radioimmunoassay was carried out according to the instructions of the manufacturer except for the replacement of the buffer solution by 0.9% (w/v) sodium chloride solution to improve the binding of the antibodies. A β-Scintillation Counter 1450 Microbeta Plus (Wallac) served for the measurements of radioactivity. The limit of detection was 3.75 pg/tube (total tube volume is 0.9 ml), and the linear range was between 5.0 and 500 pg/tube. Reproducibility of the technique was tested with spiked E_2 and E_1 levels in the acceptor medium within 0.15–10 ng/ml. The extraction yield exceeded 90%. The maximum intraday and interday variation coefficients were 9.99 and 19.67% for E_2 and 4.82 and 11.06 % for E_1 (n = 6).

MTT Test

The toxicity of ethanol on keratinocytes and fibroblasts in culture was determined using the MTT test [22].

Statistics and Data Analysis

Using porcine skin, 2 independent experiments were performed in triplicate. Reconstructed human epidermis of 2 batches served for 2 independent experiments in duplicate. For a model-independent comparison of E_2 permeability, the slopes (fluxes) of the cumulated amounts of free E_2 and total estrogens (E_2 + E_1 + conjugates; μg/h per Franz cell) were calculated for the different models used.

For all used models, plasma E_2 concentrations in man (ng/ml) were calculated as described by Rohr et al. [23].

All data are presented as the arithmetic mean values ± standard deviation (means ± SD). Significance of differences was analyzed 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.

Results

For topical treatment, both drug penetration and cutaneous metabolism are important for efficacy of treatment and adverse effects. With respect to transdermal drug application, skin permeation and liver metabolism are of relevance, too, but the latter less than with oral application. Therefore, we focussed not only on the E_2 uptake but also the cutaneous estrogen metabolism. To obtain reliable data in this complex study, all methods had to be validated carefully. This holds true with respect to estrogen extraction (>90%) from the different matrices, release of estrogen from their conjugates and the radioimmunoassay. Complete cleavage of conjugates was verified by varying the incubation time and the amount of sulfatase/glucuronidase enzyme mixture. Most importantly, except for collecting the washing fluids, a complete mass balance of Franz cell experiments was obtained. Total estrogen recovery was 88.3–100.5% which demonstrates the quality of our experiments.

Reconstructed Human Epidermis

Using reconstructed human epidermis, estrogen concentrations in the acceptor medium before drug application were close to the detection limit. Levels increased within 1 h after treatment. The application form influenced E_2 uptake and metabolism. A pronounced difference in the estrogen concentrations in the acceptor fluid became obvious for both native E_2 and its metabolites. Following the application of 100 μg E_2 using a commercially available gel (fig. 1a), the permeation of native and conjugated E_2 amounted to 12.8 and 2.4%, respectively. Moreover E_1 and conjugated E_1 were in the same order of magnitude as E_2. Taking estrogens extracted from the skin and tape strips into account, 41.7% of the applied dose penetrated reconstructed human epidermis after application as gel, and 42.6% of the penetrated amount was found to be metabolized. E_1 was the dominating metabolite (36.3%). Most importantly, the E_2/E_1 ratio was 0.96 (table 1) which is in the range of the ratios seen in postmenopausal women receiving transdermal HRT [13–15].

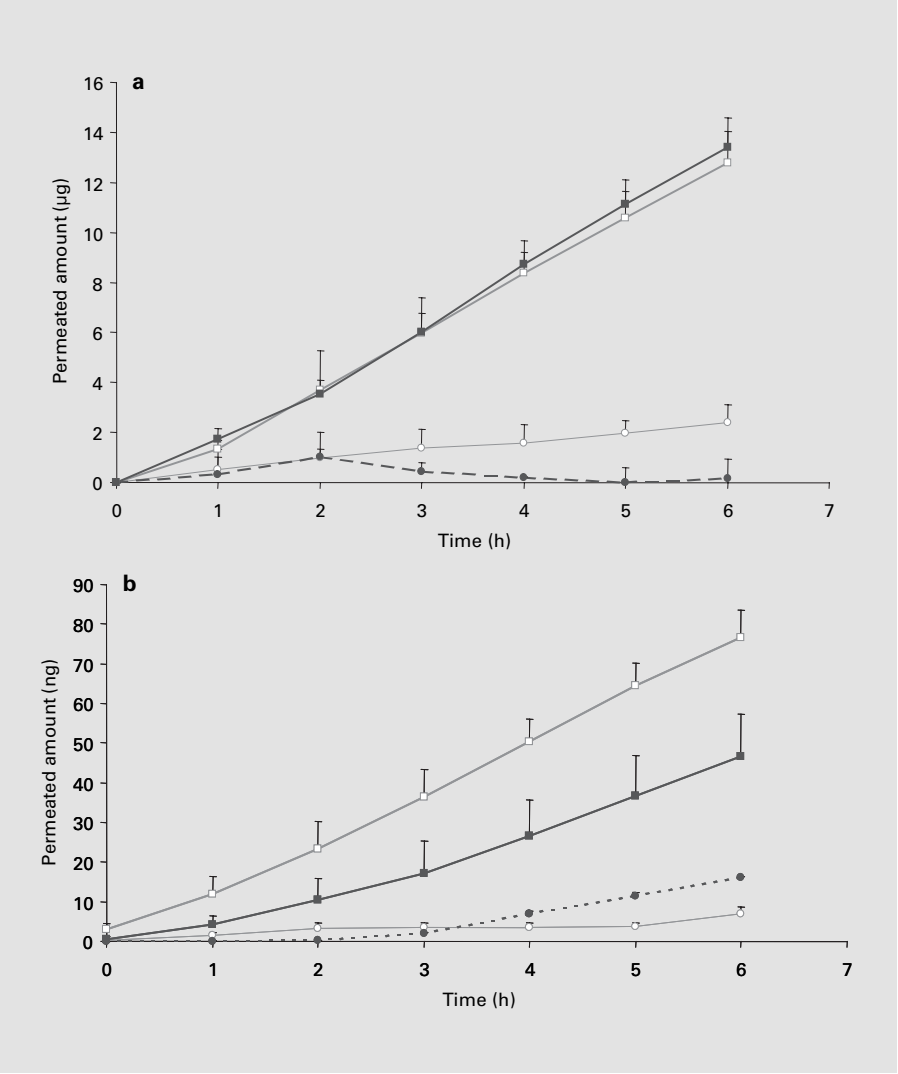


Fig. 1. Estrogen permeation using reconstructed epidermis following the topical application of 100 µg E_2 as an ethanolic solution and gel. □ = E_2; ○ = E_2 conjugate; ■ = E_1; ● = E_1 conjugate; mean permeation per Franz cell ± SD, n = 2. **a** Gel. **b** Ethanolic solution.

When applying the identical dose as a 0.1% ethanolic solution for 6 h (fig. 1b), however, less than 0.20% of the dose permeated into the acceptor medium, and 15.1% were recovered from the epidermis. The very low E_2 permeation and metabolism following the application of an ethanolic solution (table 1) suggest that the solvent may damage the tissue. Indeed, 2% ethanol concentration in the medium of monolayer cultures is toxic for keratinocytes reducing their viability by 52% and that of fibroblasts even by 97%. The pronounced differences in estrogen permeation following the applications of gel and ethanolic solution are also reflected by the slopes summarized in table 1.

Split Porcine Skin

Using porcine skin, the estrogen concentrations in the acceptor medium before drug application were barely detectable and increased rapidly thereafter. As compared to reconstructed epidermis (fig. 1), estrogen permeation through split porcine skin was clearly lower after application of Sisare gel (0.23 vs. 28.8%, fig. 2a) and slightly reduced after application of the ethanolic solution (0.09 vs. 0.15%, fig. 2c). Moreover, using split porcine skin, the influence of ethanol on E_2 metabolism was less pronounced (table 1). 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 effects.

Table 1. Estradiol pharmacokinetics in reconstructed epidermis and split pig skin (Franz flow-through cell experiments; means ± SD, n = 2)

		Reconstructed epidermis		Pig skin	
		Sisare gel	ethanolic solution	Sisare gel	ethanolic solution
E_2	Surface	53.33 ± 0.43	73.22 ± 22.99	77.71 ± 9.21	82.98 ± 18.01
	Strips 1 + 2	3.55 ± 0.06	8.85 ± 5.35	4.80 ± 3.40	7.12 ± 0.73
	Strips 3 + 4	5.00 ± 0.56	5.13 ± 1.17	1.80 ± 0.70	5.00 ± 1.10
Skin	Free E_2	0.49 ± 0.01	1.02 ± 0.47	9.70 ± 2.90	2.17 ± 0.21
	E_2 conj.	not determined		3.80 ± 1.40	1.07 ± 0.44
Acceptor medium	Free E_2	12.79 ± 1.23	0.077 ± 0.007	0.060 ± 0.019	0.033 ± 0.005
	E_2 conj.	2.42 ± 0.69	0.007 ± 0.002	0.070 ± 0.021	0.022 ± 0.004
E_1	Surface	n.d.		n.d.	
	Strips 1 + 2	n.d.		n.d.	
	Strips 3 + 4	n.d.		n.d.	
Skin	Free E_1	0.26 ± 0.01	0.06 ± 0.01	1.90 ± 1.10	0.27 ± 0.01
	E_1 conj.	not determined		0.70 ± 0.36	0.04 ± 0.01
Acceptor medium	Free E_1	13.41 ± 1.19	0.047 ± 0.011	0.071 ± 0.023	0.021 ± 0.003
	E_1 conj.	0.16 ± 0.79	0.016 ± 0.000	0.025 ± 0.001	0.015 ± 0.004
E_2/E_1 ratio		0.96	1.66	0.79	1.57
E_2 + conj./E_1 + conj. ratio		1.12	1.34	1.31	1.53
Total recovery		91.40	88.29	100.54	98.71
Slope, µg/h	Free E_2	2.196	0.013	0.009	0.005
	Total estrogen	4.840	0.024	0.036	0.015
Calculated plasma level of E_2 in man, ng/ml		37.65	0.22	0.16	0.09

Conj. = Conjugate. 100 µg E_2 was applied as an 0.1% ethanolic solution and as a gel. Percentages of dose of native E_2 + metabolites in the tissue, acceptor medium, skin strips and removable from the tissue surface are given; n.d. = the concentration was below the detection limit.

Perfusion Model

With untreated porcine skin, intrinsic estrogen concentrations were low in the perfusate (21 pg E_2/ml, 43 pg E_1/ml) as well as in the skin (180 pg E_2/g or 2,870 pg E_1/g tissue) and muscle tissue (table 2). Following the application of an E_2 TTS for 6 h, the concentrations of E_2 as well as the metabolites increased considerably in the perfusion medium (fig. 3). While E_2 increased within the first hour, E_2 conjugates were first detectable after 2 h. E_1 and E_1 conjugate concentrations increased after a lag time of about 3 h (fig. 3). After 6 h, the E_2/E_1 ratio amounted to 0.49.

In order to compare the different methods we decided to introduce the slopes of the cumulated amounts of E_2 and total estrogens. These data revealed the superiority of the E_2 TTS method with respect to permeation (tables 1, 2). This result was confirmed by in vivo plasma E_2 concentrations calculated from our in vitro penetration data.

As to be expected, not only estrogen levels in the perfusion medium increased but also those in the tissue. In skin and muscle of the treated forelimbs, the concentration of E_2 and its metabolites exceeded those of the control limbs about 1,000-fold (table 2). A considerable increase in tissue levels was also seen if E_2 and E_2 conjugate concentrations of the treated area were compared to those in distant skin areas (3.9 and 0.12 ng/g, respectively) of the same forelimb. Taking the size of the treatment area into account, E_2 uptake was 42.9% of the incorporated dose. Moreover, 30% of the penetrated amount of E_2 in the skin is subjected to metabolization, 11% of the estrogens were identified as conjugated E_2, 19% as E_1 and 0.5% as conjugated E_1.

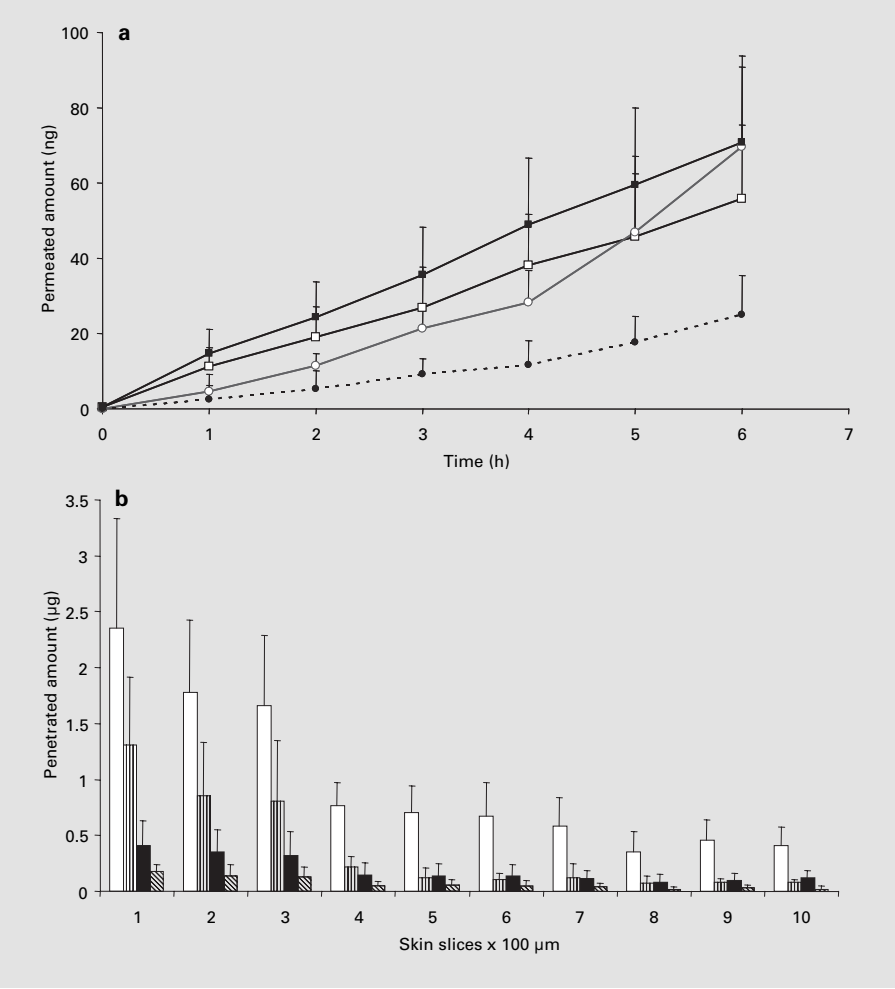
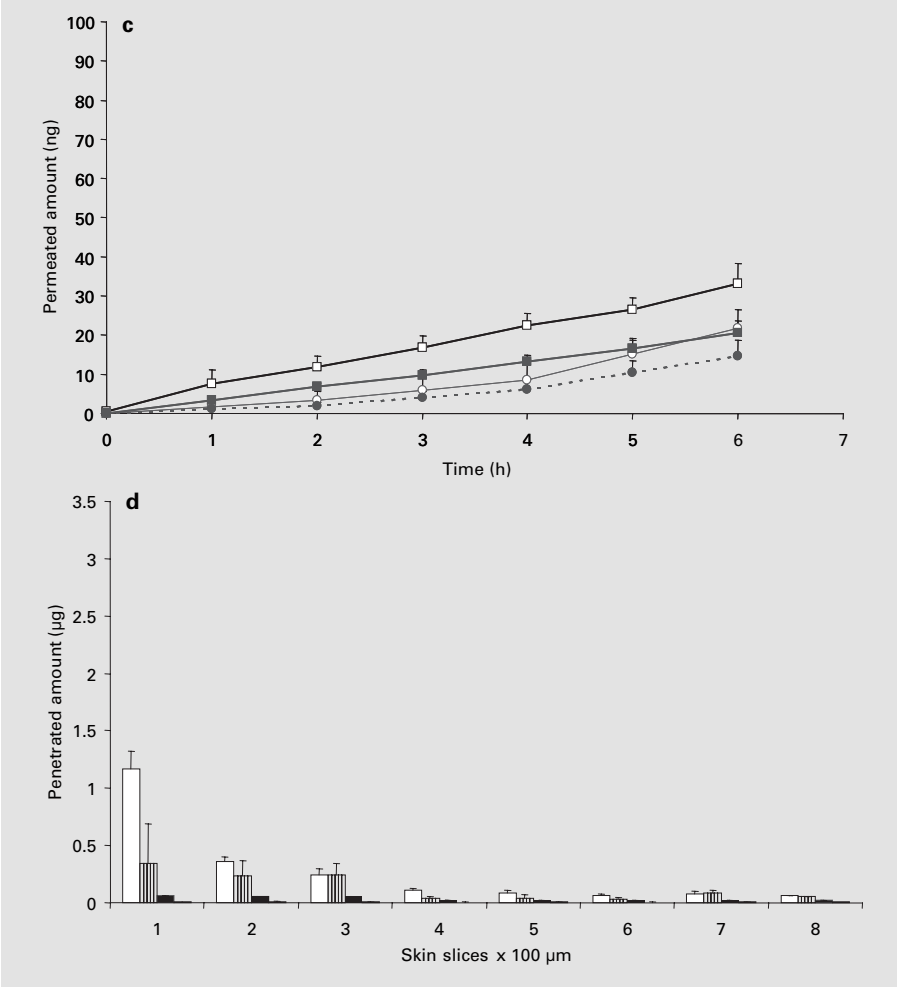


Fig. 2. Franz cell experiments using split porcine skin. Estrogen levels following 100 µg E_2 were compared for a commercially available gel (**a**, **b**) and an ethanolic solution (**c**, **d**). **a** and **c** represent estrogen concentrations in acceptor medium; □ = E_2; ○ = E_2 conjugate; ■ = E_1; ● = E_1 conjugate. **b** and **d** show estrogen concentrations in horizontal skin slices; □ = E_2; ▥ = E_2 conjugate; ■ = E_1; ▨ = E_1 conjugate; mean permeation per Franz cell ± SD, n = 2.

Table 2. E_2 and its metabolites in perfusate and tissues

		Perfusate pg/ml	Skin, ng/g (application site)	Muscle, ng/g (application site)
Estraderm TTS	Free E_2	310 ± 79	7,510 ± 480	3.08
	Conjugated E_2	171 ± 60	1,160 ± 560	1.21
	Free E_1	623	1,990 ± 480	
	Conjugated E_1	84	50 ± 10	
Control	Free E_2	21 ± 11	0.18 ± 0.22	0.14 ± 0.01
	Conjugated E_2	n.d.	0.17 ± 0.28	0.11 ± 0.21
	Free E_1	43 ± 26	2.87 ± 0.91	2.56 ± 0.68
	Conjugated E_1	n.d.	0.58 ± 0.72	0.28 ± 0.56
Slope, µg/h	Free E_2		0.0303	
	Total estrogen		0.1363	
Calculated plasma level of E_2 in man, ng/ml			0.52	

Porcine forelimbs were perfused with blood-based medium for 6 h with and without the application of E_2 TTS (means ± SD, n = 3); n.d. = the concentration was below the detection limit.



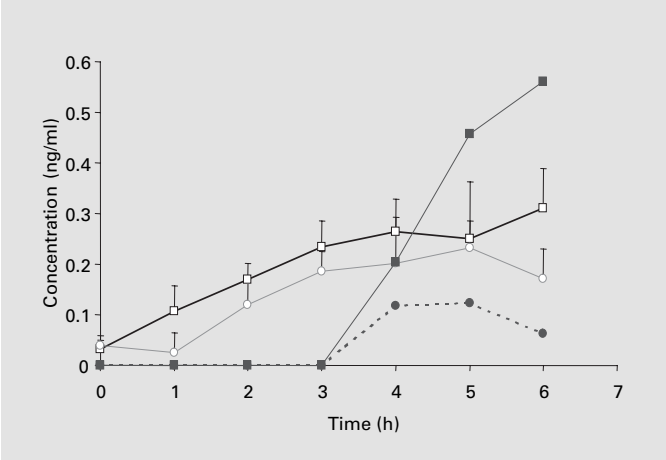


Fig. 3. E_2 and its metabolites in the perfusate of the porcine forelimb after application of an E_2 TTS. □ = E_2; ○ = E_2 conjugate; ■ = E_1; ● = E_1 conjugate; mean ± SD, n = 3.

Discussion

Over the last two decades a lot of effort has been undertaken in order to develop TTS which maintain premenopausal E_2 levels as well as the E_2/E_1 ratio in postmenopausal women. E_2 TTS application may overcome disadvantages of the oral E_2 replacement therapy – in particular the increased risk of thromboembolic complications and breast cancer [3–5] – and does not change the hepatic protein pattern [2, 14]. In fact, increased plasma levels of E_1 and estrogen overloading the liver changing protein synthesis due to oral E_2 replacement have been suspected to be a reason for adverse effects [10, 14]. Besides influencing the E_2/E_1 ratio in plasma, cutaneous E_2 metabolism may also influence permeation/penetration of the native entity, since metabolites with different physicochemical properties distribute independently from native drug [24].

For drug development, predictive test models are looked for allowing to estimate drug levels in the patient. This may be done as for chemicals by studying in vitro uptake by human or porcine skin; test procedures have been approved by the OECD [25]. Recently, also reconstructed human epidermis has become available [25, 26], which will also be acceptable for uptake studies as soon as equivalence of the results to those generated with human or porcine skin is established [25]. To be suitable for studying intensely metabolized drugs, models should not only predict permeation and penetration but also drug metabolism. We have compared these methods, additionally including a perfusion model. In vitro data served to roughly estimate plasma levels in patients; calculations where performed as described by Rohr et al. [23] for estradiol studies.

The present data demonstrate that E_2 is metabolized in the skin mainly to E_1 by 17β-hydroxysteroid dehydrogenase with any test model. Most importantly, E_2/E_1 ratios in the acceptor media are close to 1.0. Human studies comparing E_2 gels and patches indicate peak plasma levels and E_2/E_1 ratio to be rather close to our data generated [15, 27] with porcine skin and perfused pig forelimb (tables 1, 2), while calculated plasma levels from studies with reconstructed epidermis overestimate the uptake (table 1). Nevertheless, if these should prove to be fixed factors, reconstructed epidermis is as predictive as the other models. Although the perfusion model even reflected the slightly delayed increase in E_1 plasma levels of postmenopausal women following the application of E_2 patches [14], the sophisticated perfusion model did not allow to generate data clearly surmounting the results of the less elaborate Franz cell studies, given the tissue viability is maintained by an appropriate acceptor fluid and the system fits the needed test surface. Other than with the restricted area of reconstructed epidermis excluding the evaluation of estrogen uptake from the patch, this was not a problem with the forelimb model.

Following the application of E_2 ethanolic solution, the E_2/E_1 ratio (table 1) appeared to increase. This, however, may be an artifact induced by the toxicity of the solvent inhibiting E_2 metabolism which has also been described earlier [16, 17]. Nevertheless, ethanol as a widely used vehicle in commercially available topical formulations of E_2 had to be considered in our study. Extracting stratum corneum lipids, ethanol up to a concentration of 63% strongly enhances the permeation of E_2 across the human skin, while higher ethanol concentrations reduce its permeation [16]. This was also the case in our experiments. Comparing transdermal nitroglycerine formulations, a reduced metabolism following an ethanol-based formulation as compared to an ethanol-free one was described, too [28]. Therefore uptake data generated with ethanol-containing vehicle should be interpreted carefully.

Conclusion

Here we compare three in vitro techniques based on human and porcine skin to estimate the transdermal availability of estradiol and its metabolites in man. In vitro studies may reduce the need for both animal and human volunteer studies in drug development in the future given there is a validation of the existing penetration/permeation models with respect to in vivo predictions in man. This will be possible by in vitro/in vivo comparisons of a broad spectrum of compounds widely differing in physicochemical properties.

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