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Research paper

In vitro skin absorption and drug release – A comparison of six commercial prednicarbate preparations for topical use

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Abstract

Reconstructed human epidermis is a useful tool for in vitro skin absorption studies of chemical compounds. If this may hold true also for topical dermatics, we investigated the glucocorticoid prednicarbate applied by two sets (innovator and generic) of cream, ointment and fatty ointment using the commercially available EpiDerm™ model. Moreover, stability and local tolerability of the preparations as well as drug release were studied, to estimate an influence on prednicarbate absorption and metabolism. While release ranked in the order cream < fatty ointment < ointment for both sets of preparations, prednicarbate penetration and permeation of the EpiDerm model did not. Less PC uptake observed with the generic ointment and fatty ointment appeared to be linked to impaired enzymatic ester cleavage within the tissue. Thus with drugs subject to skin metabolism, cutaneous uptake is not to be derived from drug release studies, yet has to be studied experimentally with viable skin or reconstructed human epidermis.

Keywords: Prednicarbate; Topical dermatics; Drug release; Skin absorption; Skin metabolism; Reconstructed human epidermis

1. Introduction

For hazard analysis of chemicals making contact with the skin surface the Organisation for Economic Cooperation and Development (OECD) approved test guideline 428 to study percutaneous absorption *in vitro* using human and animal skin [1]. The guideline is accompanied by the technical guidance document 28 [2]. According to the OECD in vitro protocol, skin permeation of the standard substances testosterone, caffeine, and benzoic acid was compared in 10 laboratories [3]. In fact, permeation of these agents varied widely which is explained by rather openly defined procedures. Since reconstructed human epidermis (RHE) can be used, too, given absorption of standard compounds is comparable to absorption by the approved skins [2], a formal validation process was started and is now completed focusing on aqueous solutions. The experimental set-up and prevalidation results have been published [4–6]. Thus, in the future RHE may eliminate the shortage of human skin of sufficient size and quality for percutaneous absorption experiments in hazard assessment.

Above that, this technique may be used for drug development, too. In fact RHE was studied in parallel with human and animal skin to investigate percutaneous absorption and skin metabolism of glucocorticoids [7,8] and estradiol [9,10] as well as absorption of flufenamic acid [11,12], chlorpheniramine [13] and various other agents [14,15]. To evaluate the potential of RHE in drug development, however, additional investigations are needed focusing on the influence of dosage forms and comparing drug release and skin absorption. While drug release studied by the permeation of cellulose nitrate membranes revealed the superiority of analgesic loaded xyloglucan over Pluronic gels, it was only the skin which demonstrated improved

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absorption of ibuprofen as compared to ketoprofen [16]. Differences between various ibuprofen and ketopofen formulations were also detectable when using human epidermis [17]. The effects of penetration enhancers on flufenamic acid absorption from polyacrylate gels, too, were only to be detected if a biological membrane was used [11]. In fact, permeation of artificial membranes and human or animal skin and RHE is compared only rarely [11–13,15,18,19].

We now aimed to compare drug release, RHE absorption and metabolism in greater detail selecting a prednisolone diester as model drug. Available in various preparations glucocorticoids applied topically are first choice in acute dermatitis [20–22]. Bioavailability of generic and innovator products can differ considerably. Although glucocorticoids are extensively metabolised within the skin [23] formulation effects on skin metabolism have not been studied yet. While such an influence on absorption and ester cleavage has been seen with methyl 4-hydroxybenzoate [24] and ethyl 4-biphenylyl acetate [25] with hairless mouse skin and with estradiol permeation and metabolism in RHE and split porcine skin [9,10]. Additionally, glucocorticoid ester cleavage was shown to be sensitive to cytotoxicity induced by receptor fluid constituents such as albumin [26,27].

We decided to study prednicarbate (prednisolone 17-ethylcarbonate, 21-propionate; PC; $\mathrm{Log}\,P = 3.82 \pm 0.55$) which is commercially available both as innovator and generic cream, ointment and fatty ointment and thus allowed us to compare six preparations in total. Innovator formulations have been compared before [7,8,28]. In the skin PC is hydrolysed by esterases [7,26–29], the resulting monoesters prednisolone 17-ethylcarbonate (P17EC) and prednisolone 21-ethylcarbonate (P21EC) as well as prednisolone (PD; $\mathrm{Log}\,P = 1.69 \pm 0.48$) differ in receptor binding [22,30] and in their influence on keratinocytes and skin fibroblasts [31].

2. Materials and methods

2.1. Materials

Six preparations containing 0.25% prednicarbate (innovator: Dermatop® cream, ointment, and fatty ointment, Sanofi-Aventis, Berlin, Germany; generic: Prednitop® cream, ointment and fatty ointment, Dermapharm, Munich, Germany) were used as well as respective drug-free vehicles (base preparations) of the innovator (Dermatop®) series. The qualitative composition of preparations according to the manufacturers is given in Table 1; identical formulation types are made of identical ingredients. Concentration of PC and possible hydrolysis products was quantified by HPLC. The preparations were stored at ambient temperature.

RHEs were EpiDerm™ skin models (MatTek Corp., Ashland, MA, USA) with highly developed horny layer, which are Epi-606-X (surface area 4 cm^2), used for the absorption study and Epi-200-X-HCF (grown hydrocortisone free; surface area 0.63 cm^2) used in the toxicity tests.

Prednicarbate (PC) and prednisolone (PD) were donated by Sanofi-Aventis; betamethasone (internal standard), phosphate-buffered saline, pH 7.4 (PBS), Eagle's minimum essential medium, gentamicine, L-glutamine, D-glucose, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and isopropanol were supplied by Sigma–Aldrich (Munich, Germany). Amphotericin B was from Biochrom KG (Berlin, Germany), Dulbecco's phosphate-buffered saline (D-PBS) and 1% Triton X-100 solution from MatTek. Adenylate kinase (AK) kits (Toxi-Light® Bio Assay kits, Ref: LT07-217) were obtained from Cambrex Bio Science Rockland Inc. (Rockland, ME, USA), Elisa kits for IL-1α (Ref: SLA50) were from R&D systems (Minneapolis, MN, USA). All other reagents were obtained from Merck (Darmstadt, Germany) and were of the highest quality available.

2.2. Physical stability of preparations

Phase separation was quantified by measuring the amount of the liquid phase after centrifugation. About 2 g of each preparation, exactly weighted, was placed in Eppendorf tubes and centrifuged at $1.42 \times 10^4 g$ for 10 and 40 min, respectively. Then the separated liquid phase was removed and tubes were weighted to calculate by subtraction the amount of the liquid phase which is reported in % of total mass. PC concentrations in ointment and fatty ointment liquid phases were quantified by HPLC.

Bleeding number for ointment and fatty ointment was quantified as described [11]. The preparations (ointment 1.9 g, fatty ointment 1.7 g) were placed in a plastic container (1.5 cm in diameter) and stored for 1 h at 32 °C. After smoothing the surface of the preparation and setting the container up-side–down on a filter paper (qualitative standard cellulose filter grade 595 Whatman, Dassel, Germany) the preparations were kept for another 8 h at 32 °C. Then the area of the wet surface, which was approximated to have the shape of an ellipse, was recorded. Experiments were performed in triplicate.

2.3. Drug release, absorption and metabolism

PC release was derived from the permeation of cellulose nitrate membranes (pore size 0.1 μm, diameter 25 mm, Schleicher & Schuell, MicroScience, Dassel, Germany). The experimental set-up was close to previous protocols [32,33]. Skin absorption (penetration plus permeation) and metabolism were followed in RHE (Epi-606-X, three batches). Static Franz diffusion cells (diameter 15 mm, volume 12 ml, PermeGear Inc., Bethlehem, PA, USA) and the infinite dose approach [6] were used. Thus, a mass balance was not established. Experiments were performed in triplicate.

PBS was the receptor fluid for drug release studies while supplemented Eagle's minimal essential medium (20 μg/ml

Table 1
Qualitative composition of innovator and generic preparations

Cream (oil-in-water)	Benzyl alcohol, EDTA, fatty alcohols (cetyl alcohol, myristyl alcohol, stearyl alcohol), 2-octyldodecanol, polysorbate 60, purified water, semi-liquid paraffin, sorbitan stearate
Ointment (water-in-oil)	EDTA (generic: sodium EDTA), glyceryl monooleate, magnesium sulphate (generic: magnesium heptahydrate), 2-octyldodecanol, purified water, white vaseline
Fatty ointment (water free)	Glyceryl monooleate, 2-octyldodecanol, white vaseline

Identical formulation types are made of identical ingredients.

gentamicin, 50 ng/ml amphotericin B, 2 mM glutamine, 0.1% glucose; MEME) was used for experiments with reconstructed epidermis [26,27]. PC stability (5.00 μg/ml) was tested in both receptor media when kept at 37 °C for up to 24 h (PBS three, MEME two independent experiments).

Cellulose nitrate membranes were soaked in bi-distilled water for 24 h and then in PBS for 1 h. EPI-606-X was kept at +4 °C overnight and used within 24 h, as suggested by the manufacturer. The day of the experiment, RHE was transferred from the delivery plate into a 6-well plate, each well of it containing 0.9 ml of pre-warmed maintenance medium (MatTek), taking care to remove all adherent agarose sticking to the cell culture inserts. Tissues were then pre-incubated for 1 h at 37 °C, 5% CO_2 before being mounted into Franz cells without removing the supporting membrane.

Cellulose nitrate membranes or RHEs were positioned between the receptor and donor compartment of Franz cells, the latter receiving the test preparation and the (support) membrane making contact with the receptor medium maintained at 37 ± 1 °C and magnetically stirred at 500 rpm. The system was allowed to equilibrate for 30 min before a sample of receptor fluid was collected. Using a syringe 500 μl of the test preparations was applied on the membrane/RHE surface and spread with a bulb-headed probe. Then, the donor compartments were sealed with parafilm. Receptor fluid was collected in fractions (PBS 0.5 ml, MEME 1.0 ml) and replaced by fresh fluid at 0.33–7 h for drug release studies and at 1–8 h when measuring skin absorption and metabolism. At the end of the experiment, the RHE surface was wiped clean with cotton balls soaked in water. Subsequently, punch biopsies (11 mm in diameter) were removed from the treated area and transferred into 600 μl of a PBS:NaF saturated solution in water 5:1. All samples were immediately kept at −80 °C until subjected to drug analysis.

2.4. HPLC analysis

Native PC and its metabolites were quantified in PBS, MEME and in RHE by HPLC using betamethasone as internal standard [7,29]. While receptor fluid samples were extracted immediately after defrosting, tissues were first subjected to five freeze-thaw cycles to disrupt membranes before extraction. Following the addition of the internal standard, the samples were extracted three times by 500 μl of ethyl acetate. The combined extracts were exsiccated by vacuum rotation. Residues were dissolved in 1 ml of methanol, exsiccation was repeated. Following dissolution in 100 μl of methanol, 50 μl was injected into the HPLC system (LaChrom™ HPLC system; Merck-Hitachi, Darmstadt, Germany).

A modified sample preparation was used to quantify PC and its metabolites in preparations and liquid phases. One hundred milligrams of the preparation was dispersed in 10 ml of chloroform. Following the addition of the internal standard, 1 ml was withdrawn and exsiccated by vacuum rotation. Residues were extracted three times by 200 μl of methanol. The combined extracts were once more exsiccated by vacuum rotation and then dissolved in 600 μl of methanol. Fifty microliters was injected into the HPLC system.

The glucocorticoids were quantified by online UV-detection at 254 nm. Prednisolone 17-ethylcarbonate (P17EC) and prednisolone 21-ethylcarbonate (P21EC) were identified according to their retention times determined previously [29] and quantified by relating UV absorbance (peak area) to PC. Retention times for PD, BM, P17EC, P21EC and PC were 6.6, 7.9, 9.5, 10.7, 13.2 min, respectively. A linear correlation was observed between peak areas and concentrations from 100 ng/ml (limit of quantification) up to 50 μg/ml (correlation coefficient > 0.994). Recovery of steroids was 96 ± 13% from preparations and liquid phases, 82 ± 9% from PBS and 61 ± 13% from MEME. A correction factor was used to calculate steroid amounts in preparations, PBS and MEME.

2.5. Local tolerability

Local tolerability was tested adapting a protocol as previously suggested for skin irritation by chemicals [34] based on measuring in vitro viability of RHE by the mitochondrial dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction, adenylate kinase (AK) activity and IL-1α release in the culture medium. The MTT reduction assay [35] is a standard colorimetric method for the determination of cell viability based on the reduction of the yellow MTT salt to blue insoluble formazan crystals via the mitochondrial dehydrogenase activity of viable cells. Adenylate kinase is a cytosolic enzyme which is released following cell membrane damage [34]. The damage can be quantified by measuring the AK activity of the culture medium by a bioluminescent reaction. In response to inflammatory stimuli (chemicals, infections, microbial endotoxins) keratinocytes release the inflammatory

mediator IL-1α. A commercial sandwich enzyme immunoassay technique was used for the determination of IL-1α in the culture medium [31,36].

Toxicity tests (each in duplicate) were performed on RHE (Epi-200-X-HCF cultures, three batches, stored at +4 °C overnight and prepared as described above) treated with PC preparations, base preparations and liquid phases (freshly obtained by centrifugation the day of each experiment) in order to detect a potential lack of local tolerability. After removal of maintenance medium and replacement by fresh one, 140 μl of the preparations was applied on the RHE surface by using a positive displacement pipette, spread with a bulb-headed probe and left in place for 8 h. Untreated RHE served as negative control, RHE receiving Triton X-100 solution 1% as positive control. When needed excessive material was removed from the epidermal surface, then the tissues were repeatedly washed with PBS buffer and immediately subjected to MTT assay [5,6]. Culture medium was collected repeatedly for AK and IL-1α analysis and replaced by fresh medium. Medium was immediately frozen at −25 °C until analysed. AK activity and IL-1α release were determined as suggested by the manufactures.

2.6. Data evaluation and statistics

For drug release experiments glucocorticoid permeated was calculated by summing the amount of PC and PC plus its hydrolysis products (total PC) in the receptor medium. Mean cumulative PC or total PC amounts were plotted versus square-root of time (Eq. (1); [37,38]):

$$Q_t = K * t^{(1/2)} \quad (1)$$

where, Q_t is the cumulative drug amount recovered in the receiving compartment (μg), K is the kinetic constant indicative of the release rate (μg/h$^{(1/2)}$) and $t^{(1/2)}$ is the square-root of time (h$^{(1/2)}$).

The kinetic constant K (slope of the plot) and the release Lag-time (abscissa intercept, Lag-time$_{rel}$,) were calculated by linear regression.

With reconstructed epidermis, glucocorticoid permeated was calculated by summing the amounts of the metabolites (P17EC and P21EC) recovered in the receptor medium. The apparent permeability coefficient P_{app} (Eq. (2)) and Lag-time were calculated for the individual experiments as described [5] assuming constant epidermal thickness and diffusion area resulting in an equation only consisting of the slope of the linear portion of the permeation curve divided by the applied donor concentration

$$P_{app} = (V/A * C_i) * (dC_a/dt) \quad (2)$$

where, P_{app} is the apparent permeability coefficient (cm/s), V is the volume of the receiver chamber (12 cm^3), A is the area of the skin surface exposed to the receptor medium (1.767 cm^2), C_i is the initial concentration of the applied substance in μg/cm^3 and dC_a/dt is the increasing concentration of the substance in the receptor medium with increasing time.

To quantify steroid penetration into reconstructed epidermis the amount of total PC at 8 h was calculated which is defined as the sum of PC, P17EC, P21EC and PD amounts in the tissue.

Results of toxicity testing were reported as percentage of control (MTT test) and as × fold increase of AK activity (luminescence) and IL-1α release (cumulative amount in pg).

All data (given as means ± SE) were evaluated by explorative data analysis. Thus $p \leq 0.05$ was regarded to indicate a difference. First data of release (K; PC, total PC), permeation (P_{app}; P17EC+P21EC), penetration (PC, P17EC, PC+P17EC) and results of toxicity tests obtained with cream, ointment and fatty ointment were subjected to Kruskal–Wallis analysis. If a significant difference ($p \leq 0.05$) between the respective outcome parameters was obtained or the H value was close to significance, comparison was performed by pair-wise Wilcoxon test. Moreover, in the series of permeation experiments using reconstructed epidermis Dixon's test or Grubb's test was used to eliminate outliers from the data set (1 out of 6 experiments).

3. Results and discussion

3.1. Physical stability of preparations

Lipophilic ointments may show phase separation with storage. This phenomenon known as bleeding was detected with generic PC ointment and fatty ointment when opening the tube while bleeding was not observed with the corresponding innovator preparations and both creams.

PC creams also appeared to be quite stable when subjected to limited centrifugation (Table 2). No liquid phase was present after 10 min while prolonged centrifugation

Table 2
Results of the stability tests. Phase separation occurring after 10 and 40 min centrifugation and bleeding number (mean values ± SE; $n = 3$, $n = 2$ for vehicle preparations)

	Phase separation (%)		Bleeding number (cm^2)
	10 min	40 min	
Cream			
Vehicle[a]	0	4.3 ± 2.1	nd
Innovator	0	6.6 ± 2.3	nd
Generic	0	15.3 ± 2.7	nd
Ointment			
Vehicle[a]	0	5.3 ± 5.9	15.9 ± 1.1
Innovator	0	11.5 ± 2.8	15.2 ± 1.0
Generic	12.8 ± 4.6	25.3 ± 4.2	48.2 ± 7.8
Fatty ointment			
Vehicle[a]	0	2.9 ± 4.6	29.1 ± 3.4
Innovator	0	6.9 ± 8.0	23.2 ± 7.9
Generic	2.9 ± 4.9	21.4 ± 17.9	62.5 ± 30.7

[a] Innovator only.

unravelled a better stability of the innovator cream (+/− PC loading). Centrifugation of ointments and fatty ointments produced a highly viscous semi-transparent liquid floating on the semisolid phase below. Considering the composition of preparations, this liquid phase should be composed mainly of 2-octyldodecanol which has a lower density (0.837 ± 0.06 g/cm^3) than glyceryl monooleate (0.969 ± 0.06 g/cm^3) and white vaseline ($\sim$0.9 g/cm^3). The generic ointment showed extensive phase separation ($12.8 \pm 4.6\%$ after 10 min, $25.3 \pm 4.2\%$ after 40 min), innovator ointments presented limited phase separation (5.3 ± 5.9 and $11.5 \pm 2.8\%$, respectively) after 40 min. The generic fatty ointment showed minor phase separation after 10 min centrifugation, while the percentage of liquid phase after centrifugation for 40 min ($21.4 \pm 17.9\%$) was close to that observed with the generic ointment. Once more the respective innovator preparations presented limited phase separation only.

Subjecting the liquid phases (innovator and generic preparations) to HPLC analysis revealed an increase in PC concentration with the ointments ($0.46 \pm 0.02\%$) which was not true with any native preparation ($0.26 \pm 0.03\%$) and the liquid phase obtained from the fatty ointments ($0.26 \pm 0.06\%$). The increase in PC concentration in the liquid phase is well explained by steroid distribution between aqueous and lipid media, the former is lacking with the fatty ointment.

Considering bleeding (Table 2), base preparations (available only for the innovator) did not differ from innovator ointment and fatty ointment, while stability of generic ointment and fatty ointment was inferior. Then bleeding was about 3-fold increased. In fact, the generic fatty ointment displayed the highest bleeding number. Thus, except for PC cream, generic ointment and fatty ointment appeared less stable than innovator preparations.

3.2. Prednicarbate release

PC is by far the dominating steroid recovered in the receptor medium. Products of spontaneous hydrolysis varied from $7.1 \pm 2.5\%$ of the total steroid following the innovator fatty ointment to $14.4 \pm 2.6\%$ following the innovator ointment at the end of the experiment. Initially, low amount of P17EC and P21EC dominated the spectrum of degradation products, while P21EC and PD were the most important hydrolysis products at the end of the release experiments ($47 \pm 11\%$ and $38 \pm 10\%$ of the total hydrolysis product, respectively). PC degradation observed in the drug release study is well in accordance with the spontaneous hydrolysis in PBS and MEME. When kept at 37 °C for 8 h recovery of intact PC was $79.0 \pm 12.5\%$ and $77.3 \pm 14.3\%$, respectively.

PC as well as total PC release (Fig. 1, total PC) from innovator and generic preparations range in the order of ointment > fatty ointment > cream ($p \leq 0.05$, Kruskal–Wallis analysis). The 6-fold variation of the kinetic constant K (Table 3, total PC) demonstrated that the release from the semisolid matrices was rate limiting and not the permeation of the cellulose membrane. Significant Lag-times ranging from 0.63 ± 0.07 to 1.32 ± 0.06 h (total PC) can be attributed to the time needed by the membrane to saturate in PC. High R^2 values, ranging from 0.9790 to 0.9959, demonstrate a good reproducibility of our data. The release order can be explained by the composition and inner structure of preparations together with the high lipophilicity of PC. PC which is solubilised in the fatty ointment and in the external phase of ointments (W/O emulsion) can freely diffuse through the membrane at the interface with the preparation. Moreover, PC thermodynamic activity should be increased in the ointment containing water in significant amounts. With the cream (O/W emulsion), however, the external water phase is in contact with the membrane and a low PC concentration at the interface together with a longer diffusion pathway may explain the lower release rate.

While PC loaded creams have congruent release profiles ($p > 0.05$), membrane permeation from generic ointment and fatty ointment exceeded total PC permeation from the respective innovator preparations by 26% and 48% ($p \leq 0.05$). This can be explained by phase separation which was seen with generic ointment and fatty ointment: liquid phase might favour release at the membrane interface due to its low viscosity and, following the generic ointment, due to its increased PC content.

3.3. Permeation of reconstructed epidermis

Next we studied the predictivity of PC release for skin absorption. Steroid permeation and penetration was studied using the Epiderm™ model which as well as EPISKIN® (L'OREAL, Paris, France) and SkinEthic® (Laboratoire SkinEthic, Nice, France) reflect the percutaneous absorption by human epidermis [4–6]. Moreover, esterase activity of EpiDerm™ appeared to be consistent with human skin as suggested by bioconversion studies of vitamin E acetate [39] and naltrexone-3-*O*-alkyl-esters [40]. Since PC spontaneous hydrolysis in MEME was comparable to the one observed in PBS, differences in the steroid profile following drug release and PC permeation of RHE can be ascribed to the enzymatic activity of the tissue. In fact, the comparison between the order of the recovered steroids following RHE permeation (P17EC, P21EC $\gg$ PD, PC) and cellulose nitrate membrane permeation (PC $\gg$ P21EC, PD > P17EC) proved the preponderant contribution of metabolism over the spontaneous hydrolysis in the skin absorption test.

Well in accordance with previous studies [7,8], the 17-monoester appeared first in the receiving chamber, followed by the 21-monoester with a delay of approximately 2 h (Fig. 2). As to be expected from previous experiments, viable keratinocytes forming the basal RHE layers cleaved PC with high activity. P17EC, in the tissue or more probable in the aqueous receptor fluid, rearranges spontaneously into the more stable P21EC. The very low concentration of

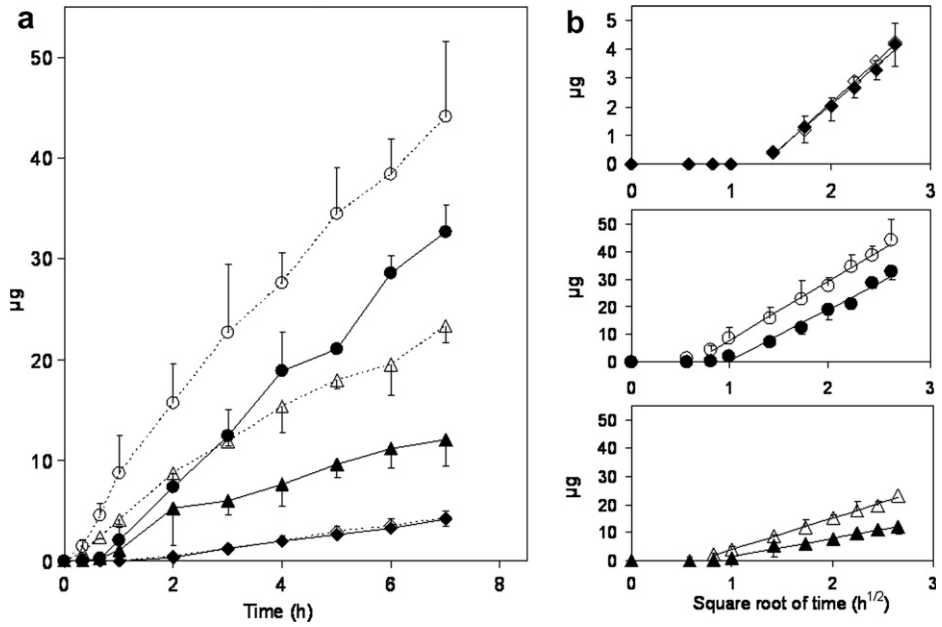

Fig. 1. Cumulative total PC release (mean values ±SE; (a) non-transformed data, (b) Higuchi plot). ◆,◇ Cream; ●,○ ointment; ▲,△ fatty ointment; closed symbols innovator product, open symbols generic product ($n = 3$).

Table 3
Total PC release data as derived by Higuchi plot from drug permeation of cellulose membranes following topical application of 1.25 mg PC (Lag-time$_{rel}$ and slope (K) are given as mean values ± SE; $n = 3$. R^2 are calculated from the average profiles)

	Cream		Ointment		Fatty ointment	
	Innovator	Generic	Innovator	Generic	Innovator	Generic
Lag-time$_{rel}$ ($h^{(1/2)}$)	1.31 ± 0.05	1.32 ± 0.06	1.00 ± 0.10	0.64 ± 0.10	0.78 ± .0.22	0.63 ± 0.07
K (µg/$h^{(1/2)}$)	2.98 ± 0.35	3.14 ± 0.50	18.71 ± 1.09	21.17 ± 1.83	6.48 ± 0.48	11.17 ± 1.17
R^2	0.9923	0.9953	0.9790	0.9953	0.9814	0.9959

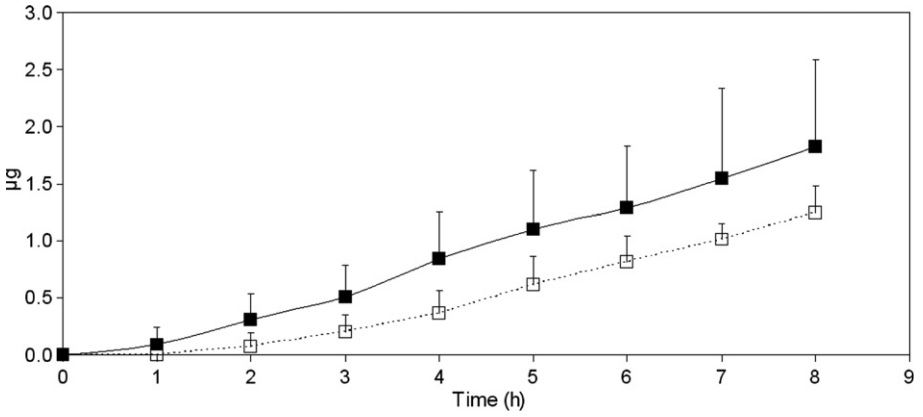

Fig. 2. Cumulative release of P17EC and P21EC (mean values ± SE) permeated through reconstructed epidermis following topical application of 1.25 mg PC (500 µl of innovator ointment); ■, P17EC; □, P21EC ($n = 3$).

the final metabolite PD, which is once more formed by enzymatic ester cleavage [7], suggests a rapid diffusion of the two monoesters into the receptor fluid which preserves P21EC from further metabolism. Although some enzyme may leak into MEME from damaged keratinocytes during the 8-h test period, the minor amount of PD to be detected excludes major tissue leakage. PC permeation was calculated from the sum of P17EC and P21EC amounts in the receiver chamber neglecting the only minor amounts of PC and PD.

Permeability of RHE (Fig. 3 and Table 4) was one order of magnitude lower as compared to the permeability of

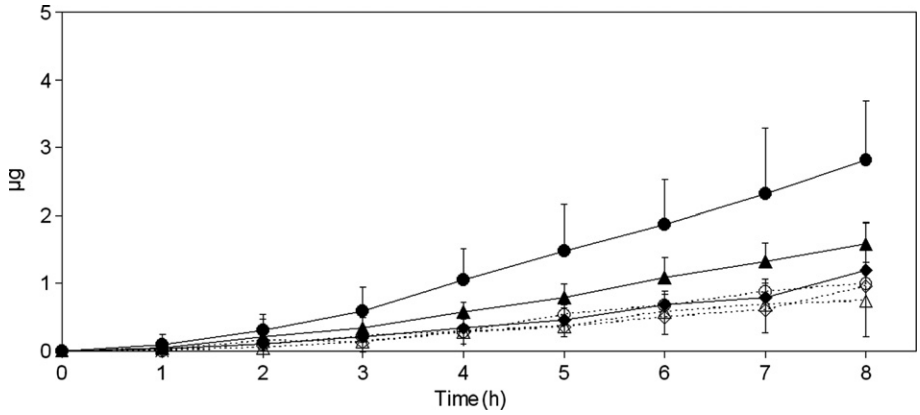

Fig. 3. Cumulative amount of PC17EC plus PC21EC (mean values ± SE) permeated through reconstructed epidermis following topical application of 1.25 mg PC. ◆,◇ Cream; ●,○ ointment; ▲,△ fatty ointment; closed symbols innovator product, open symbols generic product ($n = 3$ for all preparation except for generic ointment $n = 2$).

Table 4
P_{app} and lag-time and of the sum of PC metabolites (PC17EC plus PC21EC) permeated through reconstructed epidermis following topical application of 1.25 mg PC (mean values ± SE; $n = 3$ for all preparations except for generic ointment $n = 2$)

	Cream		Ointment		Fatty ointment	
	Innovator	Generic	Innovator	Generic	Innovator	Generic
Lag-time (h)	2.1 ± 0.6	1.0 ± 1.6	1.7 ± 0.5	1.8 ± 0.2	1.4 ± 1.1	2.1 ± 0.8
P_{app} ($X \times 10^{-8}$, cm/s)	1.2 ± 0.6	1.0 ± 0.8	2.8 ± 0.6	1.0 ± 0.2	1.5 ± 0.5	0.9 ± 0.3

cellulose membranes, which is in accordance with the results seen with flufenamic acid [12] and once more proves RHE to be an efficient barrier, although less so than human epidermis sheets [12] and human, rat and pig skin [5–7,15]. Mean Lag-times obtained with RHE exceeded mean Lag-times of the release experiments. This, too, should be due to the horny layer barrier, a slower saturation of the tissue but also to a possible delay due to PC metabolism.

As with the membrane, PC permeation of RHE followed the range order ointment > fatty ointment > cream with innovator preparations (Fig. 3), Kruskal–Wallis analysis indicated significance of the differences in permeation ($p \leq 0.05$). This is well in accordance with results of a previous study using an in-house model [28,41] which has currently not been subjected to formal validation for absorption testing. In contrast PC permeation of commercial RHE SkinEthic® was almost the same with cream and ointment [7,8], which may be explainable by the less advanced cell culture conditions [42]. Yet, even today SkinEthic RHE is more permeable than EpiDerm-606-X (e.g. caffeine and testosterone [5].

3.4. Penetration and metabolism of PC in reconstructed epidermis

Using RHE as permeation barrier allowed us to determine also the penetration into the organ of interest. As depicted in Fig. 4, PC penetrated into reconstructed epidermis to significant amounts when applied for 8 h. Besides, native PC and the first metabolite P17EC were detectable in high amounts while PD and P21C were present only in minor quantities or absent and thus neglected for

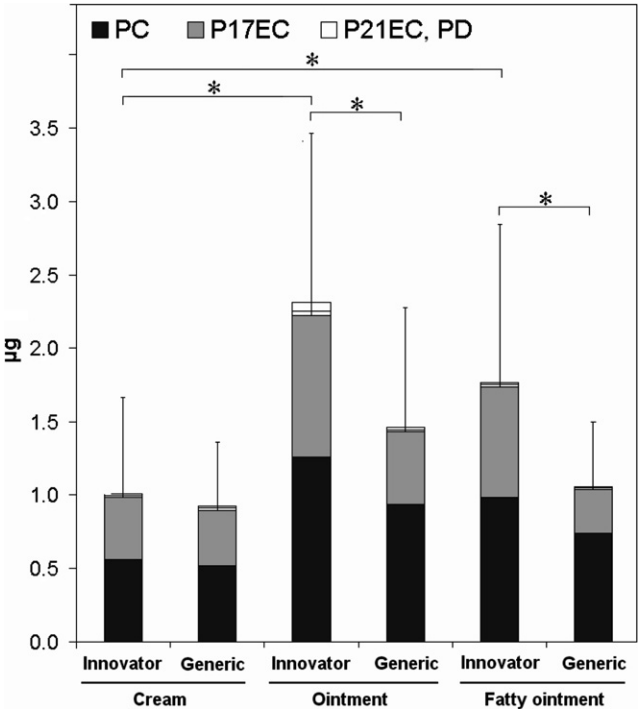

Fig. 4. Mean PC and metabolite amounts (PC plus P17EC, mean values ± SE) in reconstructed epidermis after 8 h when applying 1.25 mg PC ($n = 3$; $^*p \leq 0.05$).

statistical comparisons. This result is in accordance with previous studies. When applying PC cream or fatty ointment to RHE and to viable human skin for 6 h, only PC and P17EC were detected [7]. Kruskal–Wallis analysis indicated differences in P17EC concentrations ($p \leq 0.05$) and was close to significance with the sum of PC plus P17EC yet not with the native PC.

Recovery of total PC (native drug plus metabolites) was close with the creams (innovator cream 0.98 ± 0.59 μg, generic product 0.89 ± 0.42 μg, Fig. 4). The innovator ointment resulted in the highest penetration (2.22 ± 1.14 μg total PC) followed by the respective fatty ointment (1.74 ± 0.97 μg) both significantly exceeding the penetration following the cream ($p \leq 0.05$). An intermediate recovery was found from the generic ointment (1.43 ± 0.77 μg total PC, $p \leq 0.05$ as compared to the cream), while the respective fatty ointment (1.03 ± 0.41 μg total PC) did not differ from the cream. Importantly, PC penetration following generic ointment and fatty ointment as derived from the sum of native drug plus P17EC within the RHE was significantly less than with the corresponding innovator products ($p \leq 0.05$). Therefore, steroid penetration was well in accordance with the amounts of PC metabolites to be found in the receiver chamber. The reduced recovery of P17EC, not associated to an equal reduction in native PC (Fig. 4), may be due to a decreased metabolic activity of the reconstructed tissue resulting from cellular damage induced by components of generic ointment and fatty ointment, possibly because of phase separation. Liquid components having contact with stratum corneum in high amount can disrupt its barrier properties [43], may penetrate the skin in higher amounts and reduce its viability. In fact, 2-octyldodecanol has limited tolerability by the skin [44,45]. Moreover, the less developed horny layer of RHE as compared to normal human skin [46,47] may favour drug but also vehicle compounds to reach viable epidermis. This may render RHE supersensitive as compared to healthy human skin.

Considering skin atrophogenicity which is an important side effect of topical glucocorticoids though less so with prednicarbate [22], it has to be considered that fibroblast growth is more influenced by P17EC as compared to native PC [31]. While P17EC to PC ratios were close to 1 with four of our six test preparations, the ratio was even more favourable with the generic ointment and fatty ointment (Fig. 4). In viable human skin average P17EC to PC ratio was reported to be less than 1 [7,8].

3.5. Skin tolerability

Toxicity tests performed in almost identical RHE except for sizes as used for the uptake experiments, however, failed to reveal irritant effects of preparations. Moreover, skin irritation of liquid phases did not exceed the one observed with the corresponding preparations. Therefore, results did not substantiate a relation between phase separation and a possible reduction skin in metabolism due to an irritant effect of preparations. Following Triton X-100 treatment viability was $78.3 \pm 13.2\%$ ($p \leq 0.05$) of the untreated control, while for PC loaded and preparations and drug free vehicles the viability ranged between $95.9 \pm 10.7\%$ and $111.2 \pm 8.3\%$ (Table 5). Thus the MTT reduction test failed to detect any significant cytotoxicity of PC preparations. This holds also true with test procedures for membrane damage and irritation. Following 1% Triton X-100, AK activity increased 14.7 ± 4.6-fold and IL-1α release 7.26 ± 2.97-fold × control over the untreated control ($p \leq 0.05$) indicating a significant membrane and skin irritation damage which clearly exceeded the proposed cut-offs of 2.5- and 5-fold × control, respectively. With test preparations AK activity ranged between 0.69 ± 0.30- and 2.14 ± 1.12-fold and IL-1α release ranged between 0.86 ± 0.47- and 1.69 ± 0.50-fold × control ($p > 0.05$).

Yet it has to be kept in mind that the toxicity testing procedures used here have been established to detect major changes induced by chemicals [48] and not minor damage due to the vehicles intended for the use in patients. Currently, no in vitro test system specifically designed for the assessment of toxicity of dermatics is available. Moreover, the more sensitive Epi-200 model with a less developed skin barrier is generally used for skin corrosivity testing (http://www.mattek.com/pages/products/epiderm/skin_irritation).

3.6. Percutaneous absorption: formulation effects

Prednicarbate release ranks in the order cream < fatty ointment < ointment. While PC release from formulations supplied by two manufacturers was rather close, penetration and permeation of reconstructed human epidermis

Table 5
Viability (MTT test), membrane effects (adenylate kinase assay) and irritation (IL-1α release) of RHE treated for 8 h (mean values ± SE; $n = 3$)

	MTT test (% negative control)	AK release (fold × negative control)	IL-1α release (fold × negative control)
Positive control			
1% Triton X-100 (aq.)	78.3 ± 13.2	14.73 ± 4.58	7.26 ± 2.97
Cream			
Vehicle[a]	111.2 ± 8.3	1.14 ± 0.70	1.47 ± 0.33
Innovator	106.7 ± 20.5	0.69 ± 0.30	1.59 ± 0.53
Ointment			
Vehicle[a]	96.5 ± 15.4	1.33 ± 0.31	1.05 ± 0.25
Innovator	103.0 ± 20.9	1.61 ± 0.95	0.91 ± 0.57
Generic	102.5 ± 4.6	1.26 ± 0.27	1.25 ± 0.33
Liquid phase – innovator	104.6 ± 13.2	1.23 ± 0.06	0.99 ± 0.29
Liquid phase – generic	102.7 ± 0.5	1.07 ± 0.13	1.03 ± 0.01
Fatty ointment			
Vehicle[a]	95.9 ± 10.7	1.04 ± 0.20	0.90 ± 0.17
Innovator	96.5 ± 11.9	0.95 ± 0.19	0.86 ± 0.47
Generic	99.1 ± 5.6	2.14 ± 1.12	1.69 ± 0.50
Liquid phase – innovator	98.9 ± 6.7	1.02 ± 0.14	1.26 ± 0.69
Liquid phase – generic	97.2 ± 1.7	1.04 ± 0.02	1.17 ± 0.24

[a] Innovator only.

was not. Brinkmann and Müller-Goymann [28] suggested that the increased steroid permeation following the ointment as compared to fatty ointment and cream should be due to a permeation enhancing effect of ointment components. According to our results with innovator preparations, the permeation coefficient (P_{app}) and the amount of drug in the RHE appeared also to be linked to the increased thermodynamic activity of the drug in the ointments, as derived from total PC release (K). Surprisingly, however, PC uptake following the generic preparations was almost the same, independent of the increase in PC release from the ointment (Fig. 3). This result may be explained by phase separation: while alcohols with organic chains up to six carbon atoms can enhance skin absorption [49,50], alcohols of higher molecular weight as 2-octyldodecanol impaired indomethacin penetration [51].

Importantly, reduced PC penetration and permeation appeared to be linked to a lower enzymatic ester cleavage within RHE too, which may result from minor skin damage. Until now the influence of skin metabolism on percutaneous absorption has been studied only rarely, although there are first data indicating that skin metabolism can mediate absorption [24,25,52,53]. Delayed PC cleavage may have reduced total PC penetration by limiting the concentration gradient of active drug. Thus we decided to study local tolerability of the test preparations, too. Yet we failed to detect such a difference although the package insert of the generic products claims skin irritation to occur frequently (1–10% of the patients) while this side effect is observed only rarely with the innovator ointment and fatty ointment (<1%).

4. Conclusions

With drugs subject to skin metabolism release studies are not predictive for drug uptake, yet experiments have to be run with skin or reconstructed epidermis. Test procedures set up for regulatory toxicology appear applicable to topical dermatics. In contrast in vitro methods as suggested for skin irritation of chemicals are not suitable to detect very mild damages due to dermatics. An in vitro protocol specifically set up for a preclinical comparison of topical dermatics is highly desirable.

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