Skin Penetration and Metabolism of Topical Glucocorticoids in Reconstructed Epidermis and in Excised Human Skin

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Purpose. To investigate pharmacokinetic differences between the non-halogenated double ester prednicarbate (PC) and the fluorinated monoester betamethasone 17-valerate (BM17V) their metabolism in human keratinocytes and fibroblasts as well as their permeation and biotransformation in reconstructed epidermis and excised human skin was compared. Special attention was given to the 17-monoesters because of their high receptor affinity and antiproliferative effects.

Methods. Glucocorticoid penetration was determined using Franz diffusion cells, quantifying metabolite concentrations by HPLC. Chemical stability and reactivity of the monoesters was determined by molecular modeling analysis.

Results. PC accumulated in the stratum corneum. A considerable amount of penetrating PC was hydrolyzed by viable keratinocytes to prednisolone 17-ethylcarbonate (P17EC). P17EC permeated the skin very rapidly when compared to BM17V. Overall P17EC concentrations in viable tissue were low. Inside of the acceptor fluid, but not within the tissue, P17EC was converted to the more stable prednisolone 21-ethylcarbonate (P21EC).

Conclusions. The inactivation of highly potent, but also cell toxic, 17-monoesters to almost inactive 21-congeners seen with isolated cell monolayers appears less important in the skin. In vitro determination of the dermal 17-monoesters concentrations may allow the prediction of the atrophogenic risk in man. BM17V levels exceeding P17EC concentrations about 6-fold may contribute to its lower tolerance when compared to PC.

KEY WORDS: benefit/risk ratio; drug metabolism; prednicarbate; skin penetration; topical glucocorticoids.

INTRODUCTION

Topical glucocorticoid therapy of steroid-sensitive dermatoses demands a strong antiphlogistic action combined with minor local and preferably no systemic adverse effects. However, local adverse reactions, especially skin thinning phenomena, are still a severe problem.

Much effort was taken to separate the beneficial antiphlogistic and the unwanted atrophogenic actions. Numerous regimens adapting treatment to the type and severity of skin diseases have been developed. Moreover, corticosteroids based on the "prodrug principle" have been introduced into therapy, showing minor atrophogenic side effects than equipotent fluorinated congeners (1). They are pregnane esters in order to increase lipophilicity. They are activated (21-ester) or deactivated (17-ester) by ester-cleavage during skin permeation (2). As previously described, prednicarbate (prednisolone 17-ethylcarbonate, 21-propionate; PC) is hydrolyzed by human epidermal keratinocytes very rapidly to the monoester prednisolone 17-ethylcarbonate (P17EC). This converts nonenzymatically to prednisolone 21-ethylcarbonate (P21EC), again serving as a substrate for the esterases to be hydrolyzed to prednisolone (PD). However, in dermal fibroblasts, there is almost no ester cleavage (3).

PC and P17EC have a significantly stronger antiinflammatory potency than PD, when determining the suppression of TNFα-induced Il-1α and Il-1α-mRNA production in keratinocytes (4). Conversely, in fibroblasts proliferation assays, suppression of TNFα-induced Il-6 synthesis and ^{3}H thymidine incorporation showed antiproliferative effects only with P17EC, whereas PC and PD appeared significantly less potent.

In the present study, we extended our investigations to the molecular mechanism of the acylmigration from position 17 to 21 and to skin penetration itself. Since controlled clinical studies on efficacy and risks of topical glucocorticoid therapy for the most part use BM17V-formulations for reference, BM17V biotransformation was compared to PC metabolism.

MATERIALS AND METHODS

Materials

PC, P17EC, P21EC, and PD as well as PC cream and fatty ointment (Dermatop™), and the respective corticosteroid-free preparations were kindly donated by Hoechst Marion Roussel (Frankfurt, Germany). BM17V, betamethasone 21-valerate (BM21V), betamethasone (BM), BM17V cream, and ointment (Betnesol™) as well as the drug-free preparations were a gift of Glaxo Wellcome (Hamburg, Germany). Keratinocyte growth medium was obtained from Clonetics (San Diego, CA). Fibroblast growth medium, MTT, phosphate buffered saline (PBS), and minimum essential medium (Eagle) (MEME) was from Sigma (St. Louis, MO). All other reagents were obtained from Merck (Darmstadt, Germany) and were of the highest quality available.

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ABBREVIATIONS: AM1, Austin model 1; AM1-SM1, Austin model 1-solvation Model 1; Biquinoline, 4,4′-Dicarboxy-2,2′-biquinoline; BM, betamethasone; BM17V, betamethasone 17-valerate; BM21V, betamethasone 21-valerate; H/E, hematoxylin/eosin-staining; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; Il, interleukin; k_{el}, elimination rate constant; k_m, metabolite formation rate constant; LDH, lactate dehydrogenase; MEME, minimum essential medium (Eagle); MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; P17_cryst, crystal structure of P17EC; P17_dyn, dynamic structure of P17EC; P17_hbond, P17EC containing a hydrogen bond; P17EC, prednisolone 17-ethylcarbonate; P21_cryst, crystal structure of P17EC; P21_dyn, dynamic structure of P21EC; P21_hbond, P17EC containing a hydrogen bond; P21EC, prednisolone 17-ethylcarbonate; PBS, phosphate buffered saline, pH 7.0; PC, prednicarbate (prednisolone 17-ethylcarbonate, 21-propionate); PD, prednisolone; TNFα, tumor necrosis factor α; TEWL, transepidermal water loss.

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Cell Culture and Skin Preparation

Primary cultures of keratinocytes and fibroblasts were obtained from human juvenile foreskin and were cultivated as previously described (3).

The reconstructed epidermis (Skinethic™, Laboratoire Skinethic, Nice, France) was prepared within 24 h after shipping. The tissue samples were punched to circles of 15 mm diameter and transferred onto medium-soaked swabs. After an equilibration time of 30 min in a climate chamber (21 ± 1°C/ 60% humidity) (WTB™, Binder, Tuttlingen, Germany), transepidermal water loss (TEWL) was determined by an evaporimeter (Evaporimeter™ EP 1, Servomed, Kinna, Sweden) on the surface of the epidermis sample. TEWL-values above 15 $g \cdot m^{-2} \cdot h^{-1}$ were regarded as exclusion-criteria for a use in the penetration-assay because of possible defects of the stratum corneum.

Human abdominal skin was obtained from females aged 26 to 54 years subjected to cosmetic surgery. Fresh samples were placed in ice-cold acceptor medium consisting of Hepes-buffered MEME supplemented with gentamicin sulfate (20 µg/ml), amphotericin B (50 ng/ml), glutamine (2 mM), and glucose 0.1%. Within 1 h skin samples were sliced (Vibrating Microtome™ 500-202, TSE Systems, Bad Homburg, Germany) to 500 µm thickness and prepared as described above. Only samples with TEWL-values below 9 $g \cdot m^{-2} \cdot h^{-1}$ were admitted to the penetration studies.

Viability Assays

In parallel to the experiments, keratinocyte and fibroblast viability, and proliferation rate were surveyed by MTT-tests (3).

Lactate formation was monitored to demonstrate the maintenance of anaerobic metabolic processes. Samples of 100 µl were taken from the acceptor fluid at time points 0 and 6 h. Lactate concentrations were quantified using a commercially available kit (Sigma, Deisenhofen, Germany). Lactate concentrations had to exceed 1.30 mM and should not decline more than 10% during the experiment (5). LDH-activity serving as a signal for enzyme leaching through destroyed cell-membranes was determined in parallel by a commercially available kit (Sigma, Deisenhofen, Germany). Penetration studies with skin showing LDH-activities above the detection-limit of the kit (8 U/l) were excluded from the evaluation.

Penetration Experiments

Immediately following the TEWL-measurements the reconstructed epidermis and the excised human skin samples were mounted in Franz flow-through diffusion cells (Crown Scientific, Somerville, NJ, USA). The acceptor fluid (flow rate 6 ml/h) was magnetically stirred and the temperature maintained at 37 ± 1°C by a circulating water bath. 50 mg of PC-cream and fatty ointment (0.25 or 2.5%), and BM17V-cream and ointment (0.1 or 1.0%), respectively, were applied on the skin surface. Acceptor fluid collected every hour was immediately mixed with 1 ml of NaF-suspension (120 mg/ml PBS).

At the end of the experiment, the skin surface was wiped clean with ethanol and stripped twice for removal of surplus glucocorticoid formulation. Subsequently, the samples were punched into circles with a 11 mm diameter. In addition, excised skin was cut in a freeze-microtome (Frigocut™ 2800 N, Leica, Bensheim, Germany) to slices of 100 µm thickness. The tissue samples, as well as the tape strips, were mixed with 20 mg of crystalline NaF and homogenized under liquid nitrogen using a ball mixer mill (MM 2000, Retsch, Haan, Germany).

To screen for the accuracy of the microtome cutting procedure, protein measurement was performed using the method of Lowry *et al.* (6) as modified by Smith *et al.* (7). The coefficient of variation did not exceed 12% (n = 120).

HPLC–Method

All samples were extracted twice by equivalent amounts of ethylacetate (vortex, 1 min) and centrifugated (180·g, 5 min, 4°C). The remainder of combined organic phases exsiccated by vacuum rotation was reconstituted in 100 µl methanol. Samples of 20 µl were analyzed by RP HPLC (LaChrom™, Merck-Hitachi, Darmstadt, Germany) and UV-detection at 254 nm as described (3). The limits of detection (signal/noise ratio 3:1) ranged between 10.0 and 25.6 ng/ml, and the coefficients of variation for intra- and inter-assay precision of the quality control samples did not exceed 4.97 and 14.24%, respectively. Recovery of the steroids from the cell medium, reconstructed epidermis and excised human skin exceeded 95%, 85% and 75%.

The identification of the compounds was achieved by ^1H NMR spectroscopy following preparative thin layer chromatography as described (3).

Molecular Modeling

Cartesian coordinates for P17EC and P21EC were generated based on the crystal structure of the prednisolone skeleton (Cambridge Crystallographic Database, Cambridge, UK), using the SYBYL molecular modeling software (Tripos Associates, St. Louis, MO, USA).

The conformational analysis was performed by using a molecular dynamics procedure following a simulated annealing protocol (8). From the set of low energy conformers seven particular species were selected for further geometry refinement with quantum chemical methods. First of all, seven structures were subjected to a semiempirical geometry optimization employing the AM1 method (9). Since the calculation is performed in vacuo a second optimization was done using the AM1-SM1 method, which takes into account the solvation effect (10).

Data Analysis

The drug concentration-time curves were analyzed according to a one-compartment model. The terminal elimination and metabolite formation rate constant (k_{el}, k_m) were calculated by least square regression using TOPFIT™ 2.0 software (Thomae, Biberach, Germany).

Statistics

All data are presented as arithmetic mean values ± standard deviations ($\bar{x}$ ± SD). Significance of differences was analyzed using Shapiro-Wilk-, F-, and Student's t-test, $p \leq 0.05$ was considered significant.

RESULTS

Metabolism of BM17V in Keratinocytes and Fibroblasts

As there is a cell specific biotransformation of PC in human skin it was of interest, whether the BM17V metabolism depends on the cell-type, too. In contrast to PC, metabolism of BM17V appears similar in keratinocytes and fibroblasts (Fig. 1). BM17V converts nonenzymatically to the respective 21-compound since concentrations in the cell system and in the cell free medium decreased to the same extent (data not shown). BM21V is immediately hydrolyzed to the free alcohol BM. Cell-free controls served to correct for solvent-dependent hydrolysis of BM21V. Calculation of the respective kinetic parameters confirm, there is no significant difference between the cell-types, neither in the BM21V degradation (k_{cl} = 0.078 ± 0.008 and 0.074 ± 0.008, respectively) nor in the formation of the final metabolite BM (k_m = 0.143 ± 0.013 and 0.140 ± 0.013, respectively).

Toxic effects of BM17V and its metabolites are excluded as there were no significant differences in mitochondrial activity between glucocorticoid-treated cells and controls (MTT test).

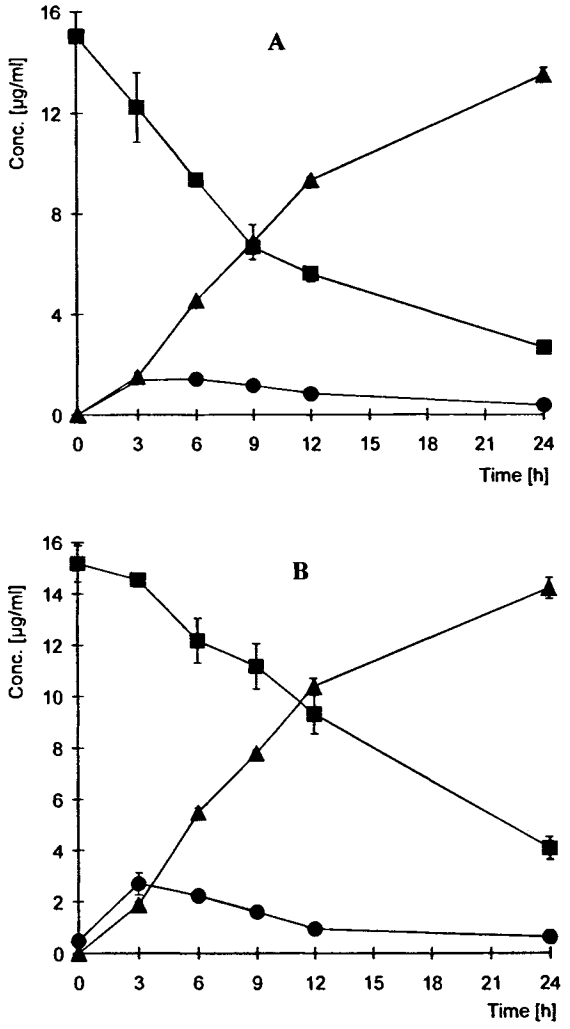

Fig. 1. BM17V metabolism, (A) keratinocytes, (B) fibroblasts. BM17V (■), BM21V (●) and BM (▲); $\bar{x}$ ± SD, n = 6.

Thus, enzymatic BM17V metabolism was considered nonsuppressed, too.

Molecular Mechanism of Nonenzymatic Conversion from P17- to P21EC

BM17V was originally described as converting nonenzymatically to the respective BM21V congener (3). In agreement with these results, we have recently found, the ethylcarbonate-group of the monoester P17EC also migrates to position 21. Moreover, the experimental data implicate a superior stability of the 21-compound to the 17-structure. This is strongly supported by the results obtained from quantum chemical calculations. This occurred not only when the calculation was performed in vacuo, which is the normal scenario, but also when it was done in solution. P21EC was found to be the far more stable isomer. The energy differences between the optimal P21EC conformer (P21 cryst) and all P17EC conformers range from 2.39 to 6.37 Kcal/mol.

Penetration and Metabolism of PC and BM17V in Reconstructed Epidermis

For topical treatment, glucocorticoid metabolism is not the only process of interest. The penetration plays a central role in the glucocorticoid efficacy and side effects. Therefore, we examined permeation and biotransformation in reconstructed epidermis following topical application of PC and BM17V formulations. Hematoxylin/eosin-staining (H/E) demonstrates an intact stratum corneum barrier and a multilayer epidermis of reconstructed skin (Fig. 2A).

The results, presented in Fig. 3A, clearly point at the high epidermal metabolic activity. The acceptor fluid contained remarkable concentrations of P17EC and shows P21EC increasing steadily with time. Most interesting, PC was not detected in the acceptor fluid. Also, BM17V permeates the epidermis. In contrast to PC, BM17V and BM21V levels in the acceptor fluid were almost constant after the first hour (Fig. 3B). With both drugs the permeation rate was the same as with the cream and ointment formulation.

To further substantiate the location of glucocorticoid metabolism, adhesive tape strips were taken for separation of stratum corneum and viable epidermis. In the strips representing the horny layer, only PC and BM17V were detected. In the viable epidermis, only P17EC was observed.

Penetration and Metabolism of PC and BM17V in Excised Human Skin

Figure 2B demonstrates the vertical slice of human abdominal skin. H/E-staining shows the red coloured viable epidermis with an intact horny layer and papillary inlets of dermis. Its total thickness exceeds that of reconstructed epidermis by about 2-fold.

Analogous to the results regarding the reconstructed epidermis, PC was metabolized mostly to P17EC and P21EC during its migration through the skin (Fig. 4A). Moreover, high amounts of BM17V as well as BM21V were detected in the acceptor fluid (Fig. 4B). As with BM17V, but in contrast to PC kinetics in reconstructed epidermis, there was not a steady increase of the overall steroid concentration when using the low dose ointment (continuous lines) or cream preparations

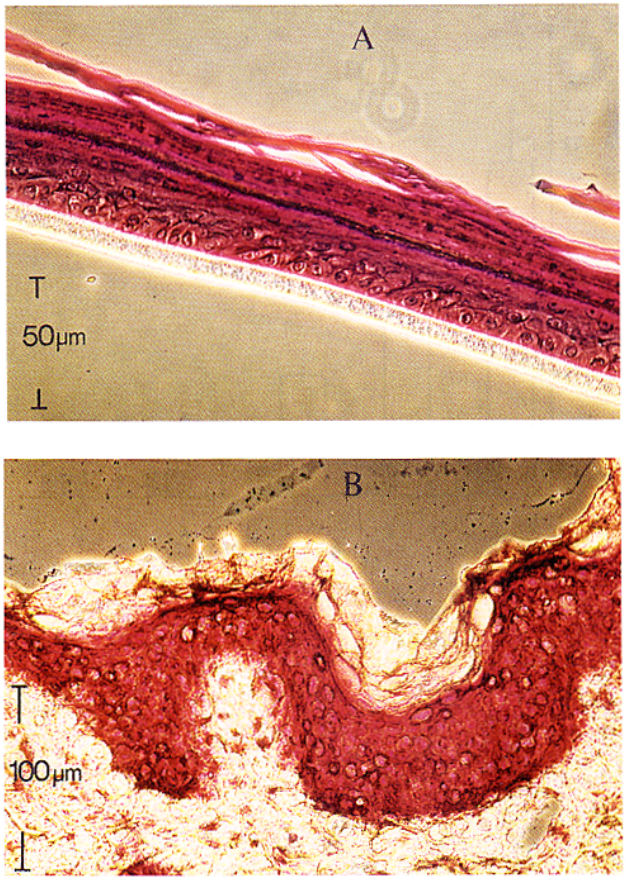

Fig. 2. Vertical slice of (A) reconstructed epidermis and (B) human abdominal skin (H/E-staining).

(data not shown). One hour after the application, the maximum level of glucocorticoid could be detected in the acceptor fluid, subsequently there was no further increase.

To verify if the absolute steroid permeation rate is limited by the physiological barrier (leading to the typical saturation curve) or rather dependent on the drug-concentration of the applied formulation, penetration studies utilizing tenfold concentrated preparations were performed. As depicted in Fig. 4 (broken lines), an increase in concentration leads to significantly higher values of the 17- and 21-monoesters in the acceptor fluid when applying the ointments. Applying the respective cream formulations did not result in enhanced permeation rates (data not shown). This finding seems in good accordance with the occlusive effects of ointments facilitating drug absorption (11).

Moreover, Fig. 4 indicates P17EC and BM17V to reach similar concentrations in the acceptor fluid, despite the fact that the PC ointment contained a 2.5-fold higher drug concentration. Obviously BM17V permeates the skin to a higher degree, resulting in increased amounts of the monoester in the dermis. Therefore we investigated the steroid-levels within the different layers of the skin. Figure 5 shows glucocorticoid concentrations in 100 µm slices of excised human skin after 6 h. The concentration of P17EC (Fig. 5A) compared to BM17V (Fig. 5B) was remarkably low in all slices whereas the acceptor fluid concentrations did not differ (Fig. 4A and B). The amount of PC was highest in the first slice representing the epidermis (Fig. 5A).

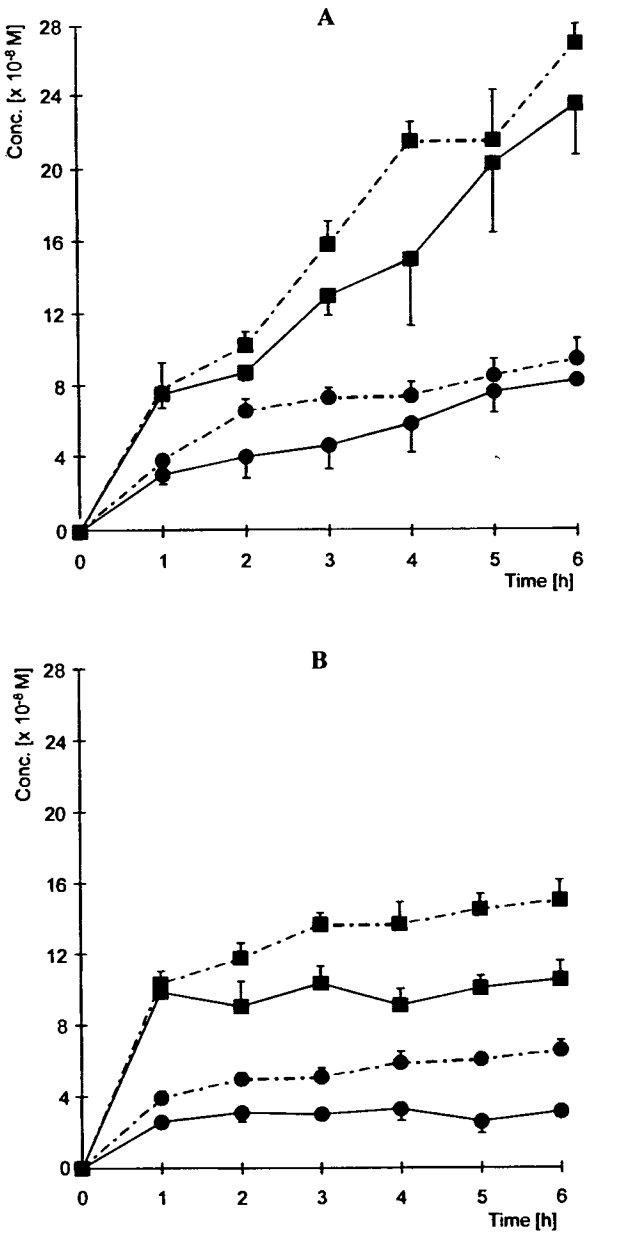

Fig. 3. Penetration study in reconstructed epidermis applying (A) PC [P17EC (■), P21EC (●)] and (B) BM17V [BM17V (■), BM21V (●)] as cream (continuous lines) or ointment formula (broken lines); $\bar{x} \pm SD$, n = 6.

Therefore, P17EC permeates human skin very quickly and is subsequently absorbed by the vascular system of the dermis, whereas PC accumulates in the stratum corneum. BM17V concentration exceeded P17EC levels in all layers of the skin.

DISCUSSION

In atopic eczema, one of the most common dermatoses, topical glucocorticoid treatment is well established. Much effort has been taken to develop topical corticosteroids with an improved benefit/risk ratio. A steroid highly effective against inflammation with a low risk of inducing skin atrophy would be especially desirable.

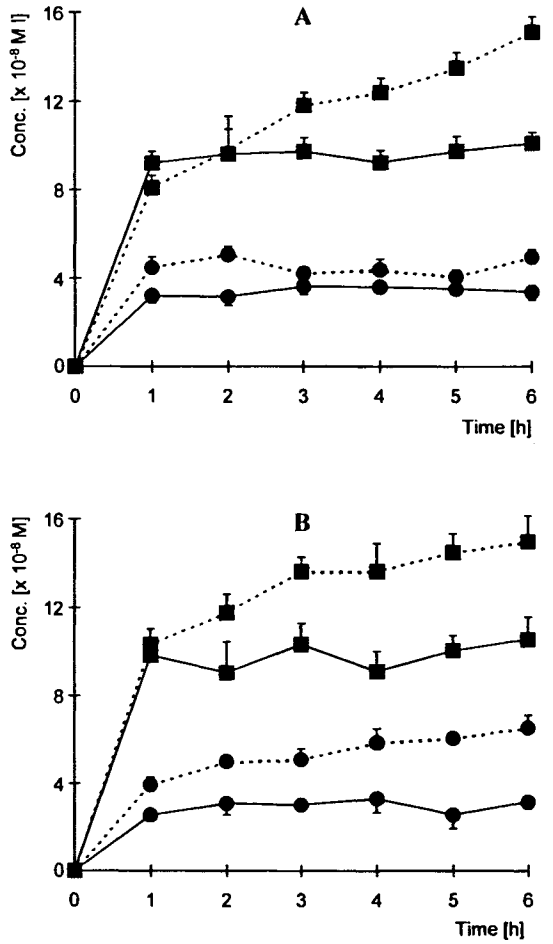

Fig. 4. Penetration study in excised human skin applying (A) PC ointment 0.25% (continuous lines) or 2.5% (broken lines) [P17EC (■), P21EC (●)] and (B) BM17V ointment 0.1% (continuous lines) or 1.0% (broken lines) [BM17V (■), BM21V (●)]; $\bar{x} \pm$ SD, n = 6.

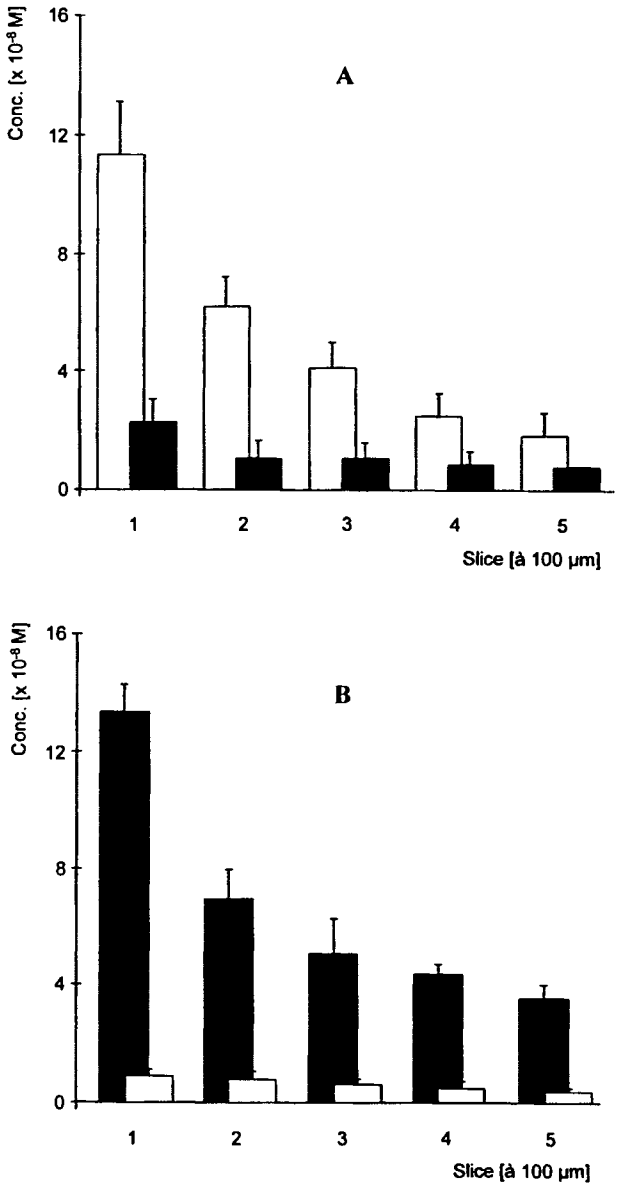

Fig. 5. (A) PC (open bars) and P17EC (full bars) concentrations and (B) BM17V (full bars) and BM21V (open bars) concentrations assessed in horizontal slices à 100 µm after 6 h incubation with PC (0.25%) and BM17V (0.1%) ointment. Slice No increase top to down, slice No 1 includes the epidermis; $\bar{x} \pm$ SD, n = 6.

Clinical investigations describe equal potency of the diester PC and conventional halogenated congeners but distinctively lower systemic and atrophogenic effects of the former (12). A possible explanation for this observation is the differential effect of PC in keratinocytes and fibroblasts. Recently Lange *et al.* (4) showed a high activity of PC in suppressing inflammation processes in keratinocytes, whereas its antiproliferative potency in fibroblasts was less marked. It is of great interest, how these different pharmacodynamic properties are caused despite the identical mode of action via intracellular receptor binding.

The present data demonstrate the specific pharmacokinetic behaviour of PC may be of central importance for its rather selective effects in the skin. With topical treatment, the skin itself influences drug effects by its metabolizing enzymes and its function in drug penetration. The stratum corneum of human skin is not only a barrier, but also a reservoir for topically applied glucocorticoids (13), resulting in a steady steroid release (14). PC is cleaved in position 21 to the monoester P17EC (3), whereas BM17V cannot be hydrolyzed at position 17 by epidermal esterases (15). When taking receptor binding studies and pharmacodynamic investigations into consideration, the 17-monoesters appear to be the active compounds with respect to their antiphlogistic, but also antiproliferative effects (4,16). In particular, the study by Lange *et al.* (4) revealed a stronger inhibition of fibroblast proliferation by P17EC when compared to the parent compound PC. However, this antiproliferative active metabolite is formed at very low rates in fibroblasts (3).

In the present penetration study, P17EC concentrations in epidermal and dermal tissue are considerably lower than BM17V levels, although values in the acceptor fluid are nearly the same. The prednisolone monoester seems to permeate the tissue much faster due to its more hydrophilic properties: Log k′ P17EC = 1.68, log k′ BM17V = 4.46 (17). The lower levels of the antiproliferative congener P17EC especially in the dermis may result in a lower atrophogenic risk for the tissue.

Our data revealed no difference in glucocorticoid penetration and metabolism following ointment and cream preparations when applied in conventional concentrations. Only when applying 10-fold higher concentrated ointments a significantly higher amount of the 17-monoesters can be observed in the acceptor fluid, maybe due to an occlusive effect of the vehicle and/or ingredients of the ointment rendering the skin more permeable. When taking into consideration that most cases of topical therapy occurs non-occluded, no serious vehicle effects on wanted and unwanted glucocorticoid effects have to be excepted. This may change by repeated drug application modifying the hydration status, and thus, drug penetration. Indeed, PC ointment may induce some atrophy/pre-atrophy especially under occlusive conditions (18).

In a variety of investigation modes reconstructed epidermis has proved to be a suitable model in testing phototoxicity or irritancy induction by topically applied drugs (19,20). Using a commercially available skin equivalent with excellent barrier function (21), esterase activity in the reconstructed epidermis and excised human skin correlates very well. Drug amounts in the acceptor fluid were only 1.7-fold higher with the reconstructed tissue. Stratum corneum turns out to be slightly less compact and tight as in healthy human skin, thus simulating the decreased barrier function of chronically diseased skin. Nevertheless the metabolism of PC and BM17V is well reflected by this skin equivalent which makes it useful in studies of topical dermatologic agents and ingredients of cosmetics.

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