Unexpected Clobetasol Propionate Profile in Human Stratum Corneum After Topical Application *in Vitro*

Beat Mueller,[1] Yuri G Anissimov,[1] Michael S Roberts[1,2]

Received July 2, 2003; accepted August 1, 2003

Purpose. The validity of using drug amount-depth profiles in stratum corneum to predict uptake of clobetasol propionate into stratum corneum and its transport into deeper skin layers was investigated.
Methods. *In vitro* diffusion experiments through human epidermis were carried out using Franz-type glass diffusion cells. A saturated solution of clobetasol propionate in 20% (V/V) aqueous propylene glycol was topically applied for 48 h. Steady state flux was calculated from the cumulative amount of drug permeated vs. time profile. Epidermal partitioning was conducted by applying a saturated drug solution to both sides of the epidermis and allowing time to equilibrate. The tape stripping technique was used to define drug concentration-depth profiles in stratum corneum for both the diffusion and equilibrium experiments.
Results. The concentration-depth profile of clobetasol propionate in stratum corneum for the diffusion experiment is biphasic. A logarithmic decline of the drug concentration over the first four to five tape strips flattens to a relatively constant low concentration level in deeper layers. The drug concentration-depth profile for the equilibrium studies displays a similar shape.
Conclusions. The shape of the concentration-depth profile of clobetasol propionate is mainly because of the variable partitioning coefficient in different stratum corneum layers.

KEY WORDS: stratum corneum; drug profile; clobetasol propionate; maximal flux.

INTRODUCTION

For effective topical treatment of skin diseases and transdermal drug therapy, it is imperative that the topically applied drug enters the epidermis or the dermis of the skin and the peripheral blood supply, respectively. Skin penetration and permeation of the drug after topical administration depend on the physicochemical properties of the drug molecule and on the function of the skin as a transport barrier and can be influenced by the applied formulation (1,2). These factors, along with skin first pass metabolism and the hemodynamic parameters of the cutaneous tissue, determine the bioavailability of topically applied drugs. The experimental determination of bioavailability and, hence, bioequivalence of dermal formulations presents a formidable challenge because of the difficulty entailed in the measurement of drug concentration in a compartment of cutaneous tissue, relevant to the pharmacologic action of the drug (3).

In vivo dermatopharmacokinetic studies for topically applied corticosteroids, based on the tape stripping methodology as once proposed by the Food and Drug Administration (FDA) (4) are today only accepted if appropriate validation is provided. Validating this approach, using a correlation between pharmacodynamic measurements and drug amounts in stratum corneum (SC), is a challenge caused by the non-linear relationship between drug amount at the site of action and its pharmacologic effect and the small window between minimal effective concentration and the concentration causing maximal pharmacologic effect.

Our goal was to investigate the relationship between the corticosteroid concentration-depth profile in SC and transport of the drug through human epidermis after topical application *in vitro*, as established by Pirot (5) for 4-hydroxybenzonitril *in vivo*. Clobetasol propionate (CP) was chosen as a model corticosteroid based on the short lag time of its *in vivo* pharmacologic effect after topical application and its high pharmacologic potency (6). *In vitro* maximal fluxes (J_{ss}) at steady state through heat-separated human epidermis were established from diffusion experiments carried out in Franz-type diffusion cells under occlusion. Normalized drug amount-depth profiles in SC were calculated using the tape stripping technique at the end of each experiment. Equilibrium partition studies were carried out to investigate partitioning and/or diffusion inhomogeneities in SC.

MATERIALS AND METHODS

In vitro diffusion studies were carried out using Franz-type glass diffusion cells, with a diffusion area of 1.3 cm^2 and a receptor volume of 3.5 ml, at a temperature of 35°C over 48 h. Donor solutions were applied after 60 min of equilibration between the human epidermal membrane and the receptor, which consisted of 4% bovine serum albumin (BSA) in phosphate buffered saline. 0.5 ml of a saturated solution of CP (SIGMA Chemical Co., St. Louis, MO, USA) in 20% (V/V) aqueous propylene glycol was applied under occlusion. Samples of 0.2 ml were taken from the receptor at predetermined time points and replaced by receptor medium. J_{ss}, and the lag time to reach steady state flux (t_{lag}) were calculated from the linear part of the cumulative drug amount-time profile in the receptor.

Equilibrium experiments were carried out in the same way, except that saturated CP in 20% (V/V) aqueous propylene glycol was used as receptor and samples from the receptor were not taken during the experiment.

Human epidermal membranes were obtained by separation from full-thickness skin using heated water at 60°C for 60 s. Female abdominal skin samples, from donors ranging in age from 29 to 45 years, were obtained from plastic surgery. Epidermal membranes were hydrated in water for 60 min before mounting in Franz-type glass diffusion cells.

At the end of the diffusion and equilibrium experiments the Franz-type glass cells were dismantled and both surfaces of the epidermal membrane were dried by blotting with paper tissues. The membranes were placed on aluminum foil, SC side facing up, and dried for 30 min at room conditions. A stencil made of a sheet of polyethylene with a hole of the size

[1] Department of Medicine, University of Queensland, Princess Alexandra Hospital, Woolloongabba, Australia.
[2] To whom correspondence should be addressed. Princess Alexandra Hospital, TAFE 3 building, Wooloongabba, QLD 4102, Australia. (e-mail: m.roberts@mailbox.uq.edu.au)

ABBREVIATIONS: BSA, bovine serum albumin; CP, clobetasol propionate; FDA, food and drug administration; HPLC, high pressure liquid chromatography; M, molar [mol/l]; SC, stratum corneum; J_{ss}, steady state flux; t_{lag}, lag time; λ, wavelength.

0724-8741/03/1100-1835/0

of the diffusion area was fixed over the membrane and pinned down on a corkboard. Panfix® tape (Union Thai-Nichiban Co. Ltd., Bangkok, Thailand) was applied by a D-Squame® Tape Applicator (Cuderm Co., Dallas, TX, USA) to the skin and 10 strips were taken. Strips were extracted by 0.5 ml acetonitrile including 2 mg/l p-hydroxybenzoic acid propyl ester as an internal standard for 12 h. To normalize the drug amount-depth profile in SC, the weight of SC on the tape was quantified by the amount of protein on the tape using a modified Lowry protein assay (7,8). Assuming homogenous SC density and protein content, the ratio between the weight of SC on a particular tape strip and the total weight of the corresponding area of the SC represents the relative thickness of the SC layer on that particular tape strip. The concentration of CP was plotted against the relative thickness of SC, whereby the actual drug concentration was plotted in the middle of the particular SC layer.

To quantify the weight of SC in the corresponding tape strip, strips were placed in 8 ml glass vials facing the sticky side upwards and extracted by 0.6 mL of a 1 M NaOH solution for 24 h. During extraction samples were treated three times for 30 min in an ultrasonic bath (Unisonics FX P 120 H, Unisonics PTY, Sydney, Australia). A modified Lowry protein assay was carried out using 0.5 ml of the extract (7,8). The coloration of the samples was measured by spectrometry at the wavelength (λ) of 750 nm (Hitachi 557 spectrophotometer, Hitachi Sciences System Ltd., Ibaraki Pref, Japan). Tape strips without epidermal contact were used as the control.

Isolated SC membranes were used for calibration. A sheet of heat-separated epidermis was floated in a 0.02% trypsin (SIGMA Chemicals Co., St. Louis, MO, USA) solution in phosphate buffered saline (PBS, SIGMA Chemicals Co., St Louis, MO, USA) overnight. Viable cells transformed into a slime, which was gently removed under running distilled water by wiping off the viable cells with wet cotton sticks. Pieces of SC weighing from 1.5 mg to 3.3 mg were placed on aluminum foil, dried to a constant weight over silica gel in a desiccator, dissolved in 1 M NaOH, and diluted to standard solutions, which were analyzed in the same way as the tape strip extracts. Pure 1 M NaOH solution was used as a control. The weight per unit area for each donor SC was established by weighing dried SC samples of known area using microbalance (Mettler ME22, Mettler Instrumente, Switzerland).

BSA in receptor samples was precipitated using an equal volume of acetonitrile with 2 mg/l p-hydroxybenzoic acid propyl ester as internal standard. After 3 h, samples were centrifuged (Clements Orbital 100, Clements Medical Equipment Pty Ltd, Rydalmere, NSW, Australia) for 20min at high speed and their supernatant analyzed by HPLC (Shimadzu, Kyoto, Japan). Samples from the tape stripping extract were mixed with an equal volume of distilled water, centrifuged for 30 min at high speed (Clements Orbital 100) and their supernatant analyzed by HPLC.

The mobile phase consisting of 10% (V/V) methanol, 40% (V/V) of 0.1 M KH_2PO_4 in distilled water pH = 3, and 50% (V/V) acetonitrile was pumped with a flow rate of 1 mL/min through a reverse phase C18 column (Phenomenex® Luna C18 (2), 5 μm, 150 mm × 4.6 mm, Torrance, CA, USA) including a guard column (Phenomenex® C18 (ODS), 4 mm × 3 mm). The solution of p-hydroxybenzoic acid propyl ester (internal standard) and CP was analyzed UV-photometrically at λ = 239 nm. The injection volume was 50 μl and samples were kept at 20°C.

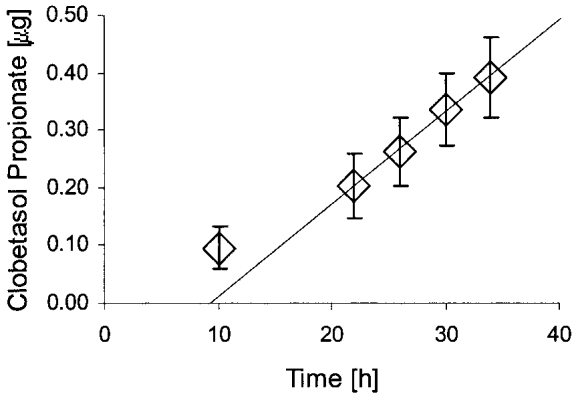

Fig. 1. Clobetasol propionate–time profile in the receiver. Error bar represents standard error of the mean (n = 9).

RESULTS AND DISCUSSION

The cumulative drug amount permeated vs. time profiles of CP in the receptor (see Fig. 1) are characterized by a steady state flux after a t_{lag} of 10.5 +/– 2.5 h (n = 9). The measured J_{ss} is 12 +/– 0.9 ng/ (h cm^2). This t_{lag} emphasizes that diffusion steady state conditions are achieved within 48 h.

The shape of CP concentration-depth profile in SC after permeation studies is biphasic (see Fig. 2). In the first phase it declines logarithmically. The CP concentration in the first strip is low in relation to strips two to five and probably reflects the cleaning of the SC surface at the end of the diffusion experiment. The second phase is a relatively constant value at a low amount of CP. Whether this second phase has a slope is difficult to ascertain as the levels approach HPLC analysis detection limit.

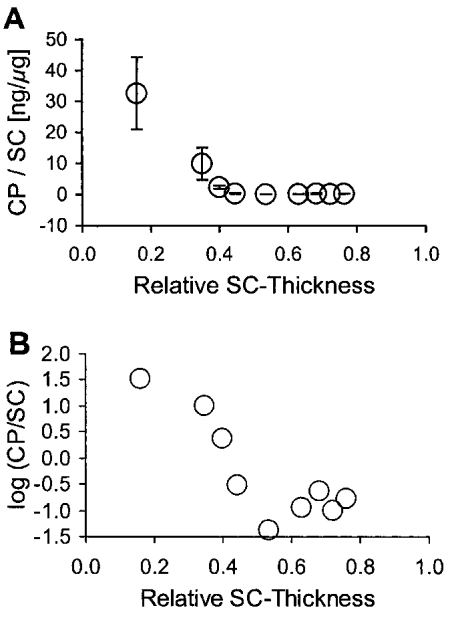

Fig. 2. (A) CP concentration-depth profile in SC after a 48 h diffusion experiment. (B) shows the same profile in a logarithmic scale. Points are shown with standard deviation (n = 3).

A biphasic concentration-depth profile is inconsistent with the notion of the SC being a homogenous membrane with a constant diffusivity and partition profile between layers. The biphasic CP-depth profile in SC was somewhat unexpected at the well-maintained steady state conditions. We had expected a linear CP concentration-depth profile in the permeation study throughout the SC layers.

The shape of CP concentration-depth profiles in SC for the equilibrium experiments over 48 h is similar to the shape of the profile for the diffusion experiments (see Fig. 3). Similar shapes for the two studies (one permeation, the other equilibrium) demonstrate that the shape of the CP-concentration depth profile in SC is predominately caused by a variable partitioning coefficient phenomenon. There is no direct relationship between propylene glycol partitioning into SC and the CP concentration-depth profile in SC. After 48 h diffusion experiment propylene glycol was distributed uniformly over the different SC layers with an average concentration of 15 ng/μg.

Possible explanations are a different partitioning behavior of CP into corneocytes between the *stratum disjunctum* and the *stratum compactum* caused by an increased permeability of the corneocyte cell envelope in the superficial layers, as suggested by Bodde for $HgCl_2$ (9) and a change of the solubility of CP in the intercellular lipid matrix between these layers. The compositions of the main groups of intracellular lipids along a SC depth axis changes mainly in the ratio of free fatty acids, from 35% to a level of 20% in the first 5 of 18 to 20 tape strips in total, and in the ratio of cholesterol, with increases from about 10% to 18% over the first 10 tape strips (10). Evidence for an increased permeability of the corneocyte cell wall envelope (and thus more partitioning into the corneocyte) are provided by corneodesmosome and water concentration profiles (11,12).

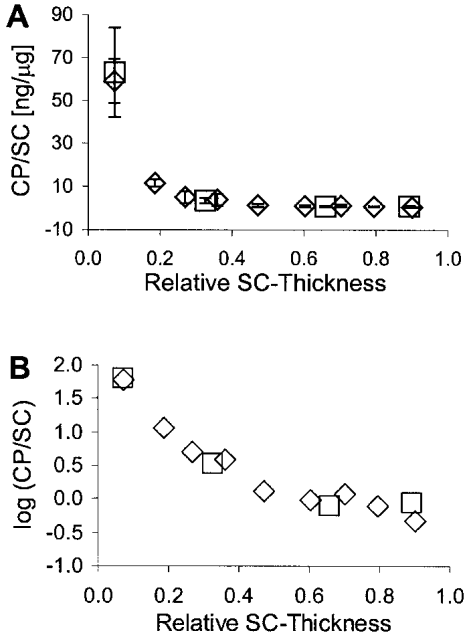

Fig. 3. (A) CP concentration-depth profile in SC after a 48 h equilibrium experiment. (B) shows the profile in a logarithmic scale. □ and ◊ represent different skin donors. Points are shown with standard deviation (n = 3).

Using a homogenous membrane model to predict drug flux through epidermis based on drug concentration-depth profiles in SC (5) could be problematic, because of strong partitioning inhomogenities in SC. Our studies were performed with CP at a likely steady state (48 h) of diffusion and partitioning processes. It remains to be seen whether this unexpected partitioning behavior is specific to CP and a long period of exposure.

ACKNOWLEDGMENT

We are grateful for the financial support of the National Health & Medical Research Council of Australia and the Queensland and New South Wales Lions Medical Research Foundation. Support for this project in the form of scholarships to B.M. from the "Novartis Stiftung vormals Ciba-Geigy-Jubilaeums-Stiftung", Basel Switzerland and the "Freiwillige Akademische Gesellschaft", Basel Switzerland is thankfully acknowledged.

REFERENCES

1. C. S. Leopold and H. I. Maibach. Effect of lipophilic vehicles on in vivo skin penetration of methyl nicotinate in different races. *Int. J. Pharm.* **139**:161–167 (1996).
2. M. Bach and B. C. Lippold. Percutaneous penetration enhancement and its quantification. *Eu. J. Pharm. Biopharm.* **46**:1–13 (1998).
3. Code of Federal Regulations. US Government Printing Office, Washington DC, 1999.
4. Guidance. *Topical Dermatologic Corticosteroids: in Vivo Bioequivalence*, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Rockville, MD, USA, 1995.
5. F. Pirot, Y. N. Kalia, A. L. Stinchcomb, G. Keating, A. Bunge, and R. H. Guy. Characterization of the permeability barrier of human skin in vivo. *Proc. Natl. Acad. Sci. USA.* **94**:1562–1567 (1997).
6. G. J. Singh, W. P. Adams, L. J. Lesko, V. P. Shah, J. A. Molzon, R. L. Williams, and L. K. Pershing. Development of in vivo bioequivalence methodology for dermatologic corticosteroids based on pharmacodynamic modeling. *Clin. Pharmacol. Ther.* **66**:346–357 (1999).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin Phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
8. F. Dreher, A. Arens, J. J. Hostynek, S. Mudumba, J. Ademola, and H. I. Maibach. Colorimetric method for quantifying human Stratum corneum removed by adhesive-tape stripping. *Acta Derm. Venereol.* **78**:186–189 (1998).
9. H. E. Bodde, M. A. M. Kruithof, J. Brussee, and H. K. Koerten. Visualization of normal and enhanced mercuric chloride transport through human skin in vitro. *Int. J. Pharm.* **53**:13–24 (1989).
10. A. Weerheim and M. Ponec. Determination of stratum corneum lipid profile by tape stripping in combination with high-performance thin-layer chromatography. *Arch. Dermatol. Res.* **293**:191–199 (2001).
11. M. Simon, D. Bernard, A. M. Minondo, C. Camus, F. Fiat, P. Corcuff, R. Schmidt, and G. Serre. Persistence of both peripheral and non-peripheral corneodesmosomes in the upper stratum corneum of winter xerosis skin versus only peripheral in normal skin. *J. Invest. Dermatol.* **116**:23–30 (2001).
12. B. Querleux, S. Richard, J. Bittoun, O. Jolivet, P. I. Idy, R. Bazin, and J. L. Leveque. In vivo hydration profile in skin layers by high-resolution magnetic resonance imaging. *Skin Pharmacol.* **7**: 210–216 (1994).