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Use of artificial skin constructs in permeation studies of clindamycin phosphate

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Penetration and permeation of topically applied substances may be studied *in vitro* either by using excised human and animal skin or with a 3-dimensional artificial skin construct (ASC). In the present study, ASC consisting of dermal and epidermal layers were cultivated with human dermal fibroblasts and spontaneously transformed human epidermal cells from the HaCaT-cell line. For comparative purposes the permeation barrier of ASC was also evaluated using a commercial skin model (AST-2000). Higher drug permeabilities were achieved with ASC in comparison with excised human stratum corneum (EHSC). The factors between permeation coefficients of ASC and EHSC depend on the respective drugs and also on different types of the formulation. Due to higher permeation coefficients obtained, the running time of the experiments could be reduced in the case of ASC. A further advantage of ASC is the independency of skin donations. Additionally, cultivation of ASC for the use in permeation studies is possible in advance, because storage conditions under nitrogen freezing do not affect ASC quality negatively.

1. Introduction

Since Rheinwald and Green (1975) introduced cultivation of human epidermal keratinocytes great progress in cultivation has been made. In the beginning cultivation of human keratinocytes was only possible on 3T3 feeder layer, later keratinocytes could be cultivated as a multilayered and differentiated epidermis on dermis equivalents (Parentau et al. 1991). The resulting skin models resembled the *in vivo* conditions.

Different research groups described organotypic cultures of epidermal cells with or without a dermal layer beneath for the application in drug permeation studies and cytotoxicity studies in order to replace human stratum corneum (Asbill et al. 2000; Zghoul et al. 2001; Monteiro-Riviere et al. 1997; Faller and Bracher 2002). Since drug development requires studying the influence of topical formulations and their excipients on drug transport, excised human stratum corneum or animal skin are normally used (Poulsen et al. 1968). Disadvantages of excised human stratum corneum are the differences in permeability depending on sex and age of the donor and the body site the sample was taken from. Skin equivalents do not show these differences in permeability if culture conditions are standardized and if they are composed of the same cell species as human skin *in vivo*.

In a previous study an organotypic skin construct consisting of transformed epidermal cells was compared with a skin construct with cells of native cultures (Specht et al. 1998). Concerning the barrier properties similar permeation coefficients were obtained for the model drug ibuprofen. Therefore an artificial skin construct composed of human dermal fibroblasts in a collagen-type-I-based matrix and spontaneously transformed human epidermal cells from HaCaT-cell line was used in further studies with other drugs and drug formulations (Specht et al. 1998; Wassermann and Müller-Goymann 2000a; Winkler and Müller-Goymann 2002). The use of an established cell line will not only allow unlimited access of cells during passaging and cryopreservation, but may also improve the reproducibility and consistency of skin models (Boelsma et al. 1999). Furthermore studying lateral diffusion in human skin (Schicksnus and Müller-Goymann 2004) is possible.

In the present study ibuprofen from a commercial cream formulation (Ibutop® Creme) was used for permeation studies to evaluate the influence of prior nitrogen freezing on ASC and to compare ASC with commercial constructs. Furthermore permeation of clindamycin phosphate as an antiinfective model drug was studied from two different commercial formulations across ASC and EHSC to rank the influence of the excipients on drug transport. Clindamycin is generally considered the most effective topical antibiotic in acne therapy (Rumsfield and West 1992). The topical administration of antibiotics is well tolerated compared with the systemic use which can cause considerable side effects.

2. Investigations, results and discussion

The artificial skin construct consists of dermal and epidermal layers. Microscopic examination of hematoxylin and eosin stained sections of ASC was performed after 4 weeks of cultivation. In contrast to normal skin (Fig. 1) ASC (Fig. 2) shows only marginal stratification. This finding corresponds with literature reports (Boelsma et al. 1999), showing a similarity of air-exposed HaCaT cultures to im-

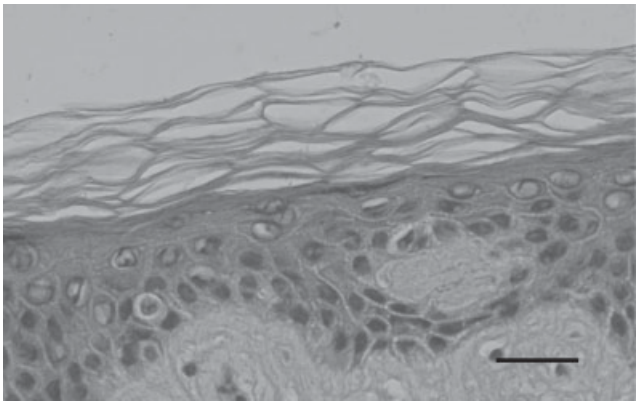

Fig. 1: Cross section of exised human skin, H&E staining, magnification 400, bar = 20 μm

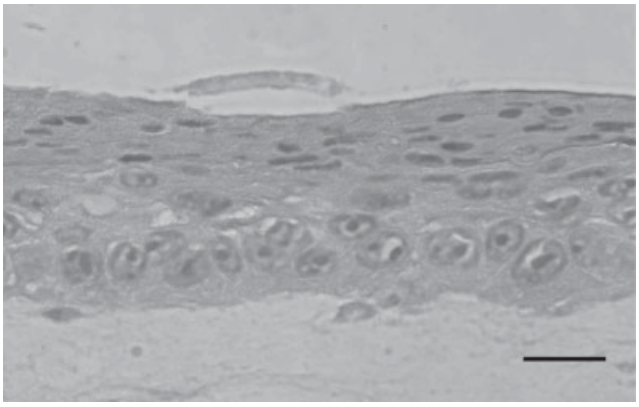

Fig. 2: Cross section of ASC, H&E staining, magnification 400, bar = 20 μm

mature and "wet" epithelia. Furthermore the authors describe an inability or at least difficulty of HaCaT cells to generate a mature cornified epithelium. In contrast an advantage of using HaCaT cells for the cultivation of ASC is that immortalized cell lines do not cause problems in terms of a short culture lifetime and quality variations between passages (Schürer et al. 1993).

To rank the barrier properties of ASC presented in this study it was compared with a commercial skin model i.e. AST-2000. AST-2000 consists of human keratinocytes in a differentiated epidermis and of human dermal fibroblasts in the dermis equivalent. According to the provider's general information AST-2000 provides an excellent *in vitro*/*in vivo* correlation together with a high reproducibility of results.

For the present permeation experiments ibuprofen from a commercial formulation (Ibutop® Creme) was used as model drug. Fig. 3 shows ibuprofen permeation profiles versus time for ASC and the commercial skin model AST-2000. The permeation coefficients K_P (cm/s) were calculated as flux/drug concentration from the linear ascents of the curves as to $8.2 \times 10^{-7} \pm 0.8 \times 10^{-7}$ cm/s and $7.3 \times 10^{-7} \pm 0.8 \times 10^{-7}$ cm/s, respectively. Considering statistics of 6 determinations with either skin model there is no significant difference between permeation coefficients. This means that there is also no difference between the different epithelia (AST-2000 and ASC), which are assumed to be responsible for the barrier properties of skin in context with drug permeation. Thus the ASC with an epidermis equivalent consisting of immortalized HaCaT cells is well comparable in permeation studies with a commercial skin model consisting of native cells only.

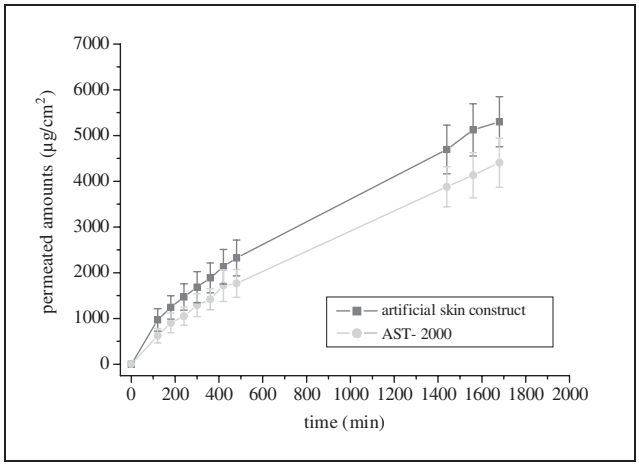

Fig. 3: Permeation profile of ibuprofen through ASC and AST-2000, n = 6, mean ± standard deviation

ASC cultivation of larger quantities is an interesting concept instead of starting cultivation every week to obtain sufficient ASC for permeation studies. This might become possible if freezing does not destroy ASC. Therefore the influence of freezing on the permeability of ASC was studied. In Table 1 the permeation coefficients are presented summarizing data of ASC and of those frozen for 24 h and 6 months, respectively. There is no statistically significant difference in permeation coefficients between ASC from normal treatment and cryo-treatment. Moreover, the duration of freezing does not show any influence on permeation barrier of ASC. Hence cultivation of ASC for permeation studies in advance is possible.

A further series of experiments was aimed at studying the influence of different formulations on the permeation be-

Table 1: Influence of nitrogen freezing on permeation coefficients of ibuprofen across ASC barrier, mean ± standard deviation

	Unfrozen ASC n = 5*	Frozen ASC (24 h) n = 8*	Frozen ASC (6 months) n = 6
Kp (cm/s)	7.70×10^{-7} $\pm 0.28 \times 10^{-7}$	7.87×10^{-7} $\pm 0.44 \times 10^{-7}$	7.17×10^{-7} $\pm 0.70 \times 10^{-7}$

* Wassermann and Müller-Goymann (2000b)

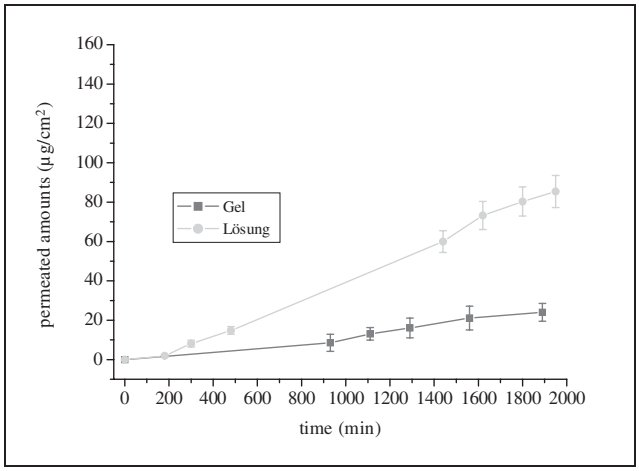

Fig. 4: Permeation profile of clindamycin phosphate through EHSC (donor: female, 63 years old, abdomen), n = 4, mean ± standard deviation

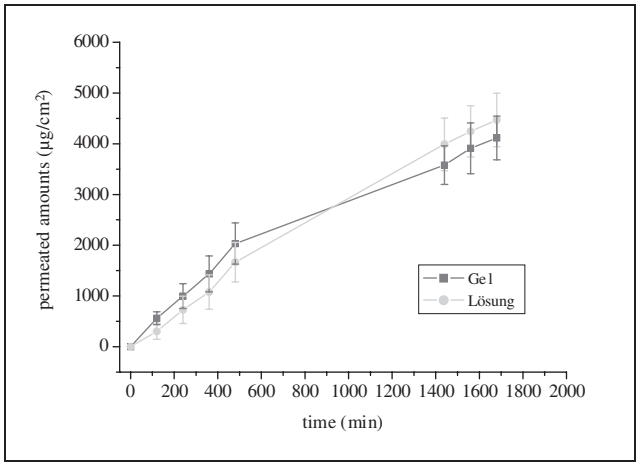

Fig. 5: Permeation profile of clindamycin phosphate through ASC, n = 6, mean ± standard deviation

haviour of clindamycin phosphate. Fig. 4 and 5 show the permeation profiles versus time from two different formulations containing clindamycin phosphate across EHSC and ASC, respectively. The permeation profiles of both formulations through ASC are very similar (factor 1.4), whereas there is a major difference between the permeation profiles through EHSC (factor 3.6). The permeation coefficients K_P (cm/s) are summarized in Table 2.

The permeation coefficient of clindamycin phosphate from Basocin® Akne Gel is 125-fold higher comparing ASC with EHSC whereas the permeation coefficient from Basocin® Akne Lösung results only in a 48-fold higher permeation rate. Permeation rate is always higher from the solution than that from the gel, but the increase in permeation rate is bigger with EHSC. A possible explanation is the enhancing effect of the adjuvants of Basocin® Akne Lösung, particularly of isopropyl alcohol.

Isopropyl alcohol interacts with the lipids of the stratum corneum, whereby it exerts a direct influence on the aqueous regions between the polar lipid head groups of the bilayer. Enhancer molecules penetrate into these regions to such an extent that they alter the solubilizing ability of this site; thereby promoting drug partitioning into the skin which subsequently results in an increased flux of penetrant (Bennet and Barry 1985). Because of fewer layers of stratum corneum of ASC in comparison with those of EHSC there would also be a reduced amount of stratum lipids in the ASC for the interaction with isopropyl alcohol, which results in a lower influence of the permeation enhancer on ASC than on EHSC.

In comparison to permeation data from a variety of different drugs (Specht et al. 1998; Winkler and Müller-Goymann 2002) permeation behaviour can be well described by means of both artificial skin constructs and excised human stratum corneum, although ASC always shows a higher permeability than EHSC.

Table 2: Comparison of the permeation coefficients K_P of CP, mean ± standard deviation

	EHSC (n = 4)	ASC (n = 6)	Factor
Kp (cm/s)	2.27×10^{-8}	2.83×10^{-6}	
Basocin® Akne Gel	$\pm 0.39 \times 10^{-8}$	$\pm 0.24 \times 10^{-6}$	125
Kp (cm/s)	8.17×10^{-8}	3.93×10^{-6}	
Basocin® Akne Lösung	$\pm 0.70 \times 10^{-8}$	$\pm 0.61 \times 10^{-6}$	48
Factor	3.6	1.4	–

The factor between the permeation coefficients (through ASC or EHSC) depends on the physicochemical properties of the respective drug molecules and, as shown in this study with clindamycin phosphate, also on different types of formulations. Due to a limited number of stratum corneum layers of ASC and thus a small amount of lipid matrix within ASC's stratum corneum, penetration enhancement of additives such as isopropyl alcohol is less pronounced with ASC in comparison to EHSC, although permeation rate across ASC is always higher than that across EHSC. This latter phenomenon, although assumed to be a disadvantage at first sight, actually allows minimizing the running time of the permeation experiments.

Therefore ASC is an appropriate tool to rank the permeation behaviour of drugs and different drug formulations especially in the early stages of drug development. It is useful to reduce animal testing and to minimize the running time of the experiment. Furthermore it could be shown with the model drug ibuprofen that the permeation barrier of the presented model (ASC) is comparable with a commercial skin model (AST-2000) and finally that cryo freezing of ASC allows a cultivation in advance.

3. Experimental

3.1. Materials

The formulations used for permeation experiments are as follows: Ibutop® Creme (Deutsche Cefaro Pharma GmbH, Waltrop, Germany) containing 5% ibuprofen, Basocin® Akne Gel and Basocin® Akne Lösung (Galderma, Freiburg i. Br., Germany) both containing 1% clindamycin phosphate. The clindamycin formulations are different in terms of composition. Basocin® Akne Gel is based on a polyacrylate gel whereas the main component of Basocin® Akne Lösung is isopropyl alcohol.

3.2. Cell lines

Human epidermal keratinocytes and human fibroblasts were used in the present study. HaCaT cell line (human adult keratinocytes, low calcium condition, elevated temperature) was provided by Fusenig (DKFZ, Heidelberg, Germany). HaCaT cell line has been derived from normal abdominal skin and described to exhibit a differentiation profile comparable with normal human keratinocytes when cultured under submerged conditions. Expression of a number of differentiation-specific protein markers, such as keratins K1 and K10, involucrin and filaggrin has been demonstrated in these cultures, although stratification was incomplete (Ryle et al. 1989). In contrast to virus transfected cells this cell line has been transformed spontaneously.

Fibroblasts of native human origin were isolated from skin samples taken from circumcisions according to standard protocols (Johnson 1992).

Both cell lines were cultivated as monolayers using Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% new born calf serum and 2mM L-Glutamine (PAA, Paschings, Austria). Media were renewed three times a week.

The cell suspensions obtained after trypsinisation were used for the cultivation of the organotypic cultures.

3.3. Organotypic cultures

ASC was constructed step-by-step in Transwell® (Costar, Fernwald, Germany) following the method of Specht and Müller-Goymann (1998) with modification of Wassermann and Müller-Goymann (2000a) (Fig. 6). Human dermal fibroblasts and spontaneously transformed human epidermal cells from the HaCaT-cell line were used to reconstruct the skin consisting of dermal and epidermal layers. The fibroblasts were cultivated in a collagen gel extracted from rat tails. The gel contraction was caused by the fibroblasts and occurred after 4–7 days. After one week, when the gel contraction was complete, the keratinocytes were seeded on the top. After cultivation submerged for 7 days to confluence, the cultures were lifted at the air liquid interface using metal disks. For permeation studies, cultures grew for 14 days at the air-liquid interface. During the latter period a multilayered epidermis (Fig. 2) with significant barrier properties was formed (Specht et al. 1998).

In addition to ASC from our own group, AST-2000 (CellSystems®, St. Katharinen, Germany) was used as a commercial skin model for comparative purposes. It is also a three-dimensional skin model and consists of dermal cells in a collagen gel and an epidermal part (Graeve and Fuchs 1999; Merkle et al. 1999; Nolte et al. 1999).

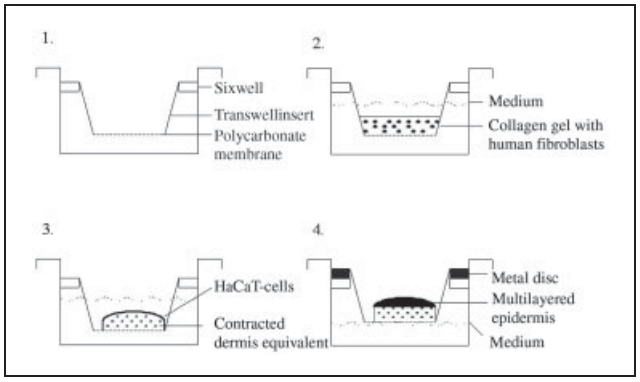

Fig. 6: Reconstruction of ASC

3.4. Nitrogen freezing

For cryo preservation experiments, ASC in Transwell®-Inserts were covered with sterile aluminium foil, frozen in the vapour phase over liquid nitrogen for 1 h and subsequently lowered into liquid nitrogen for a storage period of 24 h. Then the foil-covered frozen ASC were thawed in the incubator atmosphere for 10 min and set atop medium of 37 °C in Sixwell-Plates for another 10 min (Wassermann and Müller-Goymann 2000b). In a further experiment ASC were frozen for a period of 6 months to investigate a longer period of nitrogen freezing.

3.5. Isolated stratum corneum

Isolated stratum corneum from skin donations of plastic surgery was obtained using an aqueous trypsin solution in concentration of 2% (w/w) (Merck KgaA, Darmstadt, Germany). For this purpose the fatty tissue was first removed from the skin samples. Then the skin was transferred, dermal side down, into a Petri dish, containing the trypsin solution. After an incubation time of 24 h at 32 °C the stratum corneum could be removed carefully with forceps. The isolated stratum corneum pieces were washed once in an aqueous trypsin-inhibitor solution containing 0.02% (w/w) trypsin-inhibitor (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) followed by three times washing with distilled water. They were stored in a desiccator until use.

3.6. Permeation studies

The most widely accepted permeation testing system is the Franz-diffusion cell (FD-C) (Franz 1975). Permeation experiments with this model lead to data which provide information about the drug amount permeated through the skin depending on time.
The FD-C consisted of a donor compartment and a receiver compartment. The donor compartment was filled with the drug formulation (Ibutop® Creme, Basocin® Akne Gel, Basocin® Akne Lösung), whereas the receiver compartment contained an isotonic phosphate buffer pH 7.4. The barrier used (EHSC or ASC) was sandwiched on top of a polycarbonate membrane between the two compartments. Sampling was done at given time intervals from the receiver compartment. For the permeation experiments with ASC, aliquots of 250 µl were taken between 2 h and 28 h and with EHSC between 3 h and 32.5 h. They were immediately replaced by fresh medium. The whole apparatus was kept at 37 °C in a water bath while the receiver fluid was stirred with a magnetic stirring bar.

3.7. HPLC Analysis

Permeated amounts of the model drug ibuprofen were analyzed by HPLC using a column of Hypersil® ODS 5 µm 125 × 4 mm (Grom, Rottenburg –Hailfingen, Germany). Double distilled water : acetonitril : acetic acid 54 : 46 : 2 (v/v) served as mobile phase with a flow rate of 1.7 ml/min. UV-detection was performed at 246 nm.
Permeated amounts of the model drug clindamycin phosphate were analyzed by HPLC using a column of Nucleosil® 100-10 C_8 250 × 4.6 mm (Macherey-Nagel, Düren, Germany), isotonic phosphate buffer pH 2.5 : acetonitril 77.5 : 22.5 (v : v) as mobile phase with a flow rate of 1.0 ml/min and a UV-detection at 210 nm.

3.8. Data analysis

The permeation parameters of the model drugs were calculated by plotting the cumulative amounts (µg/cm^2) of drug permeated through ASC or EHSC versus time (min). The slope of the linear portion of the graph provided maximum flux values at steady state (µg/cm^2 h). The permeation coefficients K_P (cm/s) were calculated as flux/drug concentration.

3.9. Statistical methods

In order to re-examine the significance of differences in results, a two-sided t test was conducted.

3.10. Morphological analysis

For histological evaluation the cultures were fixed in 8% formalin, dehydrated in a graded isopropyl alcohol series and embedded in paraffin. Cross sections (6 µm) were stained with haematoxylin and eosin (H & E) for light microscopical examination.

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References

Asbill C, Kim N, El-Kattan A, Creek K, Wertz P, Michniak B (2000) Evaluating of a human bio-engineered skin equivalent for drug permeation studies. Pharm Res 17: 1092–1097.
Bennett SL, Barry BW, Woodford R (1985) Optimization of bioavailability of topical steroids: non-occluded penetration enhancers under thermodynamic control. J Pharm Pharmacol 37: 298–304.
Boelsma E, Verhoeven MCH, Ponec M (1999) Reconstruction of a human skin equivalent using a spontaneously transformed keratinocyte cell line (HaCaT). J Invest Dermatol 112: 489–498.
Faller C, Bracher M (2002) Reconstructed skin kits: Reproducibility of cutaneous irritancy testing. Skin Pharmacol Appl Skin Physiol 15 (suppl 1): 74–91.
Franz TJ (1975) Percutaneous absorption- on the relevance of *in vitro* data. J Invest Dermatol 64: 190–195.
Graeve T, Fuchs H (1999) Dreidimensionale zelluläre Testsysteme. Biofo-rum 22: 178–180.
Hoffmann C, Müller-Goymann CC (2002) Arch. Pharm. Pharm. Med. Chem. 335 (Suppl. 1): 113.
Johnson EW, Meunier SF, Roy CJ, Parenteau NL (1992) Serial cultivation of normal human keratinocytes: a defined system for studying the regulation of growth and differentiation. In vitro Cell Dev Biol 28A: 429–435.
Merkle ML, Kandsberger M, Noll M, Matthes TH, Fuchs H, Graeve T (1999) Dreidimensionales Hautäquivalent als Testsystem (AST-2000), ALTEX 16: 52.
Monteiro-Riviere NA, Inman AO, Snider TH, Blank JA, Hobson DW (1997) Comparison of an in vitro skin model to normal human skin for dermatological research. Microsc Res Tech 37: 172–179.
Noll M, Merkle ML, Kandsberger M, Matthes TH, Fuchs H, Graeve T (1999) Reconstructed human skin (AST-2000) as a tool for pharmacotoxicology. ATLA 27: 302.
Parentau NL, Nolte CM, Bilbo P, Rosenberg M, Wilkins LM, Johnson EW, Watson S, Mason VS, Bell E (1991) Epidermis generated in vivo: practical considerations and applications. J Cell Biochem 45: 245–251.
Poulsen BJ, Young E, Coquilla V, Katz M (1968) Effect of topical vehicle composition on in vitro release of fluocinolone acetonide and its acetate ester. J Control Release 6: 85–97.
Rheinwald JG, Green H (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell 331–344.
Rumsfield JA, West DP (1992) Common skin disorders: Acne and psoriasis; In DiPiro JT et al., Pharmacotherapy, a Pathophysiologic Approach, 2nd ed., Elsevier, Amsterdam pp. 1387–1403.
Ryle CM, Breitkreutz D, Stark HJ, Leigh IM, Steinert PM, Roop D, Fusenig NE (1989) Density-dependent modulation of synthesis of keratins 1 and 10 in the human keratinocyte line HaCaT and in transfected tumorigenic clones. Differentiation 40: 42–54.
Schicksnus G, Müller-Goymann CC (2004) Lateral diffusion of ibuprofen in human skin during permeation studies, Skin Pharmacol Physiol 17: 84–90.
Schuerer N, Köhne A, Schliep V, Barlag K, Goerz G (1993) Lipid composition and synthesis of HaCaT cells, an immortalized human keratinocyte cell line, in comparison with normal human adult keratinocytes. Exp Dermatology 2: 179–185.
Specht C, Stoye I, Müller-Goymann CC (1998) Comparative investigations to evaluate the use of organotypic cultures of transformed and native dermal and epidermal cells for permeation studies. Eur J Pharm Biopharm 46: 273–278.
Wassermann K, Müller-Goymann CC (2000a) Arch Pharm Med Chem. 333 (Suppl. 1): 34.
Wassermann K, Müller-Goymann CC (2000b) Arch Pharm Med Chem. 333 (Suppl. 2): 17.
Winkler A, Müller-Goymann CC (2002) Comparative permeation studies for δ-aminolevulinic acid and its n-butyl ester through stratum corneum and artificial skin constructs. Eur J Pharm Biopharm 53: 281–287.
Zghoul N, Fuchs R, Lehr CM, Schaefer U (2001) Reconstructed skin equivalents for assessing percutaneous drug absorption from Pharmaceutical formulations. ALTEX 18: 103–106.