The Influence of the Acyl Chain on the Transdermal Penetration-Enhancing Effect of Synthetic Phytoceramides

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Abstract

Background/Aims: The skin has become very attractive as a route for drug administration. Optimization of topical drug formulations by the addition of penetration enhancers may facilitate the passage of drugs through the stratum corneum. Methods: In this paper, the skin penetration effect of phytosphingosine and 9 derived phytoceramides (PCERs) on 3 transdermal model drugs (i.e. caffeine, testosterone, ibuprofen) was investigated via Franz diffusion cell experiments using split-thickness human skin. Azone was included as a positive control. Results: The main finding in our study was that the PCERs exerted a compound-dependent penetration-enhancing effect. Some of the investigated PCERs exhibited a penetration-enhancing ratio of more than 2 (mean ± SE): for caffeine PCER1 (2.48 ± 0.44), PCER2 (2.75 ± 0.74), PCER3 (2.62 ± 0.93) and PCER6 (2.70 ± 0.45) and for testosterone PCER1 (2.08 ± 0.56), PCER2 (2.56 ± 0.13), PCER3 (3.48), PCER4 (2.53), PCER5 (2.04 ± 0.14), PCER6 (2.05 ± 0.48) and PCER10 (4.84 ± 0.79), but none of them had an influence on ibuprofen. Conclusion: The investigated PCERs exhibited a penetration-enhancing effect on caffeine and testosterone but not on ibuprofen.

Key Words
Phytoceramide · Azone · Penetration enhancer · Skin penetration

Introduction

The skin has an important protective function against environmental damage, in which the outermost layer, the stratum corneum (SC), plays an important role. The 10- to 20-μm-thick layer acts as a penetration barrier, which protects the body against bacterial, enzymatic or chemical impacts and prevents excessive water loss. The SC is mainly composed of corneocytes surrounded by multilamellar organized lipids [1, 2]. Ceramides are the major lipid class of the extracellular matrix of the SC and are essential components of the SC barrier function. They are found in lamellar bodies of differentiating keratinocytes [3]. Ceramides not only contribute to the skin barrier function but also play physiological roles in signal transduction and cell regulation. When the cell membrane is...
altered during several biological processes, ceramide levels increase, and they induce cell differentiation and/or apoptosis and reduce cell proliferation [4, 5].

Chemically, a ceramide (2-N-acylsphingosine) is an acyclic 2-amino-1,3-diol, composed of a sphingoid base with a double bond between carbons 4 and 5, which is linked to a fatty acid through an amide bond. Depending on the nature of the sphingosine moiety and the fatty acid chain, 12 different classes of ceramides can be distinguished in human skin [6, 7]. Ceramides with a saturated sphingosine base containing an additional hydroxyl group at carbon 4 are known as phytoceramides (PCERs) [3].

Recently, ceramides have gained much attention as possible transdermal penetration enhancers, which are capable of increasing the amount of drugs permeating through the skin by reducing the barrier resistance of the SC. Skin penetration enhancers are present in several dermatological and cosmetic products to enhance the local and/or systemic absorption of the active compound. Only a few fragmental studies demonstrated the penetration-enhancing properties of selected ceramides [8–12]. In these studies, the effect of the ceramide acyl chain length on the skin permeability was investigated [13], indicating that short-chain ceramides increase the skin permeability of theophylline and indomethacin and exhibit a maximal effect with an acyl chain length between 4 and 6 carbons. However, studies using PCERs are lacking. This led us to assess the penetration-enhancing effect of phytosphingosine and a series of 9 PCERs with different acyl chains, via transdermal Franz diffusion cell (FDC) experiments on the 3 transdermal model compounds obtained by classification and regression trees, i.e. caffeine, testosterone and ibuprofen [14]. The well-known penetration enhancer azone served as a positive control. In our previous studies, these model compounds were already investigated in the presence or absence of other penetration enhancers, such as spilanthol [15, 16].

**Materials**

*Products Examined*

Caffeine and ibuprofen (Ph. Eur. grade) were obtained from ABC Chemicals (Vemedia, Wouters-Brakel, Belgium), while testosterone (≥99% grade) was purchased from Sigma-Aldrich (Bornem, Belgium). The investigated PCER types 2–9 (PCER2–PCER9) and azone were prepared in-house (see section Synthesis of Phytosphingosine and PCERs). Phytosphingosine (PCER1) and salicyloyl phytosphingosine (PCER10) were obtained from Evonic Industries (Essen, Germany). The structures of the PCERs are presented in table 1, and they will further be indicated as PCER1–PCER10.

**Table 1. Structures of the investigated phytosphingosine (PCER1), PCERs and azone (AZ)**

<table>
<thead>
<tr>
<th>Code</th>
<th>Structure</th>
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<tr>
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<td>AZ</td>
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</table>
Synthesis of Phytosphingosine and PCERs

D-ribo-Phytosphingosine (PCER1) was transformed into PCER2 by treatment with a 1:1 mixture of acetic anhydride and pyridine, followed by hydrolysis of the acetate esters. PCER3–PCER9 were prepared under Schotten-Baumann reaction conditions using the appropriate acyl chlorides. The latter were obtained by treating the corresponding fatty acids with thionyl chloride. A solution of phytosphingosine (1 g, 3.15 mmol PCER1) in 40 ml acetic anhydride/pyridine (1:1) was stirred for 24 h at room temperature. The reaction was quenched under ice-cold conditions with saturated sodium hydrogen carbonate solution and extracted with CH2Cl2. The organic layer was washed with saturated sodium hydrogen carbonate and dried over sodium sulphate, filtered, and evaporated to dryness to afford crude phytosphingosine tetra-acetate. This crude product was dissolved in MeOH (20 ml) and triethylamine (10 ml) and was stirred for 4 days at room temperature. The solvents were removed under reduced pressure, and the residue was purified by column chromatography (CH2Cl2:MeOH 9:1) to give PCER2 (0.63 g, 56% yield) as a white solid.

1H-NMR (300 MHz, pyridine): δ = 0.88 (t, J = 6.5 Hz, 3 H, CH3), 1.15–1.51 (m, 22 H, CH2), 1.57–1.80 (m, 1 H, H-6a), 1.81–2.07 (m, 2 H, H-5a, H-6b), 2.13 (s, 3 H, CH3), 2.17–3.25 (m, 1 H, H-5b), 2.13 (s, 3 H, CH3), 2.17–3.25 (m, 1 H, H-5b), 4.23–4.31 (m, 1 H, H-4), 4.33–4.39 (m, 1 H, H-3), 4.48 (d, J = 5.1 Hz, 2 H, CH2, 1), 5.10 (ddd, J = 9.3 Hz and 5.0 Hz, 1 H, H-2), 6.19 (br, s, 1 H, OH), 6.50 (br, s, 2 H, 2 × OH), 7.86 (d, J = 8.5 Hz, 1 H, NH).

Exact mass (ESI-MS) for C20H41NO4 [M + Na]+ found, 382.2931; calcd, 382.2928.

General Schotten-Baumann Procedure: PCER3–PCER9

A 1-molar solution of fatty acid (2 equivalents) in thionyl chloride was stirred overnight at room temperature. The mixture was concentrated and co-evaporated with toluene to afford the crude acid chloride. This was added dropwise to a 0.06-molar solution of phytosphingosine (1 equivalent) in tetrahydrofuran/10% aqueous sodium carbonate solution (1:1) at 0 °C. The aqueous layer was extracted with CH2Cl2, and the combined organic layers were washed with 1 M sodium hydroxide and brine, dried over magnesium sulphate, filtered, and evaporated to dryness. The residue was purified by column chromatography. PCER3–PCER9 were obtained using this general Schotten-Baumann procedure with phytosphingosine (1 g, 3.15 mmol PCER1) and butyric acid, hexanoic acid, octanoic acid, decanoic acid, dodecanoic acid, tetradecanoic acid, and benzoylchloride, respectively. Column chromatography (CH2Cl2:MeOH 95:5) gave PCER3 (671 mg, 55%), PCER4 (555 mg, 42%), PCER5 (106 g, 76%), PCER6 (181 mg, 12%), PCER7 (759 mg, 48%) and PCER9 (305 mg, 23%) as a white solid, while PCER8 (1.05 g, 63%) was a white electropowder.

**Chemicals and Reagents**

Ultrapure water (H2O) of 18.2 MΩ·cm quality was produced by an Arium 611 purification system (Sartorius, Göttingen, Germany), while triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromoalkane, while triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromoalkane, while triethylamine, methanol, sodium carbonate, 1-bromoalkane, while triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexane, triethylamine, methanol, sodium carbonate, 1-bromohexan...
N-[(2S,3S,4R)-1,3,4-Trihydroxyoctadecan-2-yl]butyramide (PCER3)

1H-NMR (300 MHz, CD3OD): δ = 0.92 (t, J = 6.7 Hz, 3 H, CH3), 0.98 (t, J = 7.4 Hz, 3 H, CH3), 1.29–1.41 (m, 24 H, CH2), 1.50–1.74 (m, 4 H, CH2), 2.23 (t, J = 7.4 Hz, 2 H, CH2), 2.35–3.61 (m, 2 H, H-4, H-1a), 3.68–3.82 (m, 2 H, H-3, H-1b), 4.12 (dd, J = 5.4 and 10.0 Hz, 1 H, H-2).

Exact mass (ESI-MS) for C32H64NO4 [M + H]+ found, 500.4677; calcd, 499.4673.

N-[(2S,3S,4R)-1,3,4-Trihydroxyoctadecan-2-yl]hexanamide (PCER4)

1H-NMR (300 MHz, pyridine): δ = 0.86 (t, J = 6.7 Hz, 3 H, CH3), 0.85–0.93 (m, 3 H, CH3), 1.17–1.55 (m, 26 H, CH2), 1.64–1.86 (m, 3 H, CH3), 1.87–2.03 (m, 2 H, CH2), 2.19–2.32 (m, 1 H, H-5a), 2.43 (t, J = 7.5 Hz, 2 H, CH2), 4.24–4.33 (m, 1 H, H-4), 4.36–4.43 (m, 1 H, H-1a), 4.45–4.54 (m, 2 H, H-3, H-1b), 5.10 (dddd, J = 5.0 and 9.6 Hz, 1 H, H-2), 6.17 (br. s, 1 H, OH), 6.53 (br. s, 2 H, 2 × OH), 8.45 (d, J = 8.4 Hz, 1 H, NH).

Exact mass (ESI-MS) for C26H53NO4 [M + Na]+ found, 438.3427; calcd, 438.3421.

N-[(2S,3S,4R)-1,3,4-Trihydroxyoctadecan-2-yl]octanamide (PCER5)

1H-NMR (300 MHz, pyridine): δ = 0.82 (t, J = 6.8 Hz, 3 H, CH3), 0.85–0.95 (m, 3 H, CH3), 1.09–1.53 (m, 30 H, CH2), 1.65–2.03 (m, 5 H, CH2, H-5a), 2.14–2.35 (m, 1 H, H-5b), 2.45 (t, J = 7.4 Hz, 2 H, CH2), 4.26–4.34 (m, 1 H, H-4), 4.37–4.43 (m, 1 H, H-3), 4.50 (d, J = 4.3 Hz, 2 H, CH2), 5.10 (dddd, J = 4.8 and 9.7 Hz, 1 H, H-2) 6.51 (br. s, 1 H, OH), 8.47 (d, J = 8.5 Hz, 1 H, NH).

Exact mass (ESI-MS) for C24H49NO4 [M + H]+ found, 388.3427; calcd, 388.3421.

Synthesis of Azone (1-Dodecylazacycloheptan-2-One)

Substitution of 1-bromododecane by ε-caprolactam gave access to azone (fig. 2). To a solution of ε-caprolactam (250 mg, 2.2 mmol) in toluene (6 ml) at 0 °C, sodium hydride (110 mg, 2.65 mmol) in toluene (6 ml) was added, and the mixture was stirred for 30 min at this temperature. 1-Bromododecane (0.64 ml, 2.65 mmol) was added, and the reaction mixture was heated to reflux for 20 h. MeOH (3 ml) was added to quench the reaction, and the solvents were removed under reduced pressure. The residue was taken into diethyl ether and washed with brine, the organic layer was dried over magnesium sulphate, filtered and evaporated to dryness. Purification by column chromatography (hexane:ethyl acetate 3:1) gave the title compound (629 mg, quantitative) as a colourless oil.

1H-NMR (300 MHz, pyridine): δ = 0.83–0.96 (m, 3 H, CH3), 1.14–1.51 (m, 22 H, CH2), 1.58–1.81 (m, 1 H, H-6a), 1.85–2.07 (m, 2 H, H-5a, H-6b), 2.22–2.37 (m, 1 H, H-5b), 4.26–4.43 (m, 1 H, H-4), 4.47–4.55 (t, J = 5.1 Hz, 1 H, H-3), 4.59 (app. d, J = 5.1 Hz, 2 H, CH2), 5.29 (dddd, J = 5.0 and 10.1 Hz, 1 H, H-2), 6.28 (br. s, 1 H, OH), 6.69 (br. s, 1 H, OH), 7.31–7.45 (m, 5 H, ar. H), 8.19 (d, J = 7.1 Hz, 1 H, NH).

Exact mass (ESI-MS) for C34H68N2O4 [M + H]+ found, 422.3271; calcd, 422.3264.

Preparations of Franz Diffusion Dose Solutions

Dose solutions were prepared at 80% of the maximal solubility in a 50/50 ethanol/H2O (% V/V) solution of caffeine, ibuprofen and testosterone to obtain the same thermodynamic activity for the 3 transdermal model compounds. The solubility at 32°C (i.e. the temperature of the skin) in the 50/50 ethanol/H2O solutions (mean ± SE, n = 3) amounts to 67.31 ± 7.01, 9.71 ± 0.12 and 53.80 ± 3.00 mg/ml for caffeine, testosterone and ibuprofen, respectively. In these dose solutions, the PCERS were added to obtain a final concentration of 1% (W/V). The dose solutions were kept at 32 ± 0.5°C until they were applied on the skin. Azone (1% W/V) was included in the experiments as a positive control, while negative controls to azone (fig. 2). To a solution of ε-caprolactam (250 mg, 2.2 mmol) in toluene (6 ml) at 0 °C, sodium hydride (110 mg, 2.65 mmol) in toluene (6 ml) was added, and the mixture was stirred for 30 min at this temperature. 1-Bromododecane (0.64 ml, 2.65 mmol) was added, and the solubility at 32 °C (i.e. the temperature of the skin) in the 50/50 ethanol/H2O solutions (mean ± SE, n = 3) amounts to 67.31 ± 7.01, 9.71 ± 0.12 and 53.80 ± 3.00 mg/ml for caffeine, testosterone and ibuprofen, respectively. In these dose solutions, the PCERS were added to obtain a final concentration of 1% (W/V). The dose solutions were kept at 32 ± 0.5°C until they were applied on the skin. Azone (1% W/V) was included in the experiments as a positive control, while negative controls.
controls were included as well (dose solutions without penetration enhancer). Azone and some PCERs were not tested as penetration enhancer on ibuprofen, as no effects were observed in the first experiments with selected PCERs in combination with ibuprofen.

**In vitro Permeation Study**

Static FDCs (Logan Instruments Corp., N.J., USA) with a receptor compartment of 5 ml and an available diffusion area of 0.64 cm² were used to determine the skin permeation of the 3 model compounds in the different dose formulations. Human skin was used, and the analyses were minimally done in duplicate using a randomized blocked design. The skin samples were obtained after a cosmetic reduction surgery from 7 healthy female patients (52 ± 6 years old, mean ± SD) which were supplied by the Department of Plastic and Reconstructive Surgery of the University Hospital (Ghent, Belgium). Confidentiality procedures with informed consent were applied. Immediately after the surgical procedure, the skin was cleaned with 0.01 M PBS, pH 7.4, the subcutaneous fat was removed and the skin was subsequently stored at −20°C for not longer than 6 months. Just before the start of the FDC experiments, the full-thickness skin was thawed, mounted on a template and sliced to obtain a skin thickness of approximately 400 μm, using an electrical powered dermome. An actual skin thickness of 340 ± 11 μm (mean ± SE, n = 70), 399 ± 9 μm (n = 40), 414 ± 11 μm (n = 20), 259 ± 5 (n = 40), 269 ± 8 (n = 20), 317 ± 13 μm (n = 40) and 403 ± 23 μm (n = 20) was experimentally determined with a micrometer (Mitutoyo, Tokyo, Japan) from the different patients. The receptor chambers were filled with 0.01 M PBS for caffeine and ibuprofen and with 5% W/V BSA in PBS for testosterone. The skin samples were visually inspected for skin damage and were mounted on the FDC between the donor and the receptor chambers, with the epidermis side upwards ensuring that no air was present under the skin. A Teflon-coated magnetic stirring bar (400 r.p.m.) allowed that the receptor fluid was continuously mixed. Skin integrity was controlled by measuring the skin impedance using an automatic microprocessor-controlled Tinsley LCR Impedance Bridge (Croydon, UK). Skin pieces displaying an impedance value below 10 kΩ were discarded and replaced by a new skin piece [17]. 500 μl of the dose solutions were applied on the skin surface with a micropipette. The donor chamber was covered with parafilm to prevent evaporation of the dose formulations. During the FDC experiment, the temperature of the receptor compartment was kept constant at 32°C by a water jacket. 200-μl samples of receptor fluid were taken at regular time intervals (0, 1, 2, 4, 8, 12, 18, 21 and 24 h) from the sample port and were immediately replaced by 200 μl fresh receptor fluid. This was taken into account for the calculation of the cumulative permeated concentrations. Immediately after the last sample had been drawn, the remaining dose formulation was removed from the skin surfaces using a cotton swab. The epidermis and dermis were separated with forceps, and the model compounds were extracted from the skin layers with ethanol to construct a mass balance and to obtain the ratios

\[
\frac{C_{24 h}^{\text{epidermis}}}{C_{24 h}^{\text{rechicum}}} \quad \text{and} \quad \frac{C_{24 h}^{\text{dermis}}}{C_{24 h}^{\text{epidermis}}}
\]

The concentration of the model compounds in the (epi)dermis was obtained by dividing the amount of extracted model compound (experimentally determined) by the volume of the (epi)dermis [thickness of (epi)dermis (cm) × skin surface (0.64 cm²)]. An epidermis thickness of 50 μm was always taken, and the thickness of the dermis was calculated from the total skin thickness minus the thickness of the epidermis. The concentration of the model compounds in the remaining dose solution after 24 h was calculated by dividing the amount of the dose solution left after 24 h (experimentally determined) by the applied volume of the dose solution.

A linear relationship of the individual cumulative amount of the model compounds versus time was observed. All individual runs confirmed the steady state as the model compounds were released at a constant rate into the receptor fluid. Sink conditions were also achieved: the receptor fluid did not contain more than 10% of the saturated concentration of testosterone (adsorbed to BSA), ibuprofen (fully ionized in PBS) and caffeine (water solubility of 29 mg/ml at 32°C) after 24 h.

**Sample Preparation**

Before HPLC-UV analysis, the BSA present in the 200 μl samples with testosterone was precipitated with 200 μl acetonitrile. The tubes were vortexed for 10 s, and after 10 min of incubation at room temperature, the tubes were centrifuged for 10 min (14,243 g). The clear supernatant was used for HPLC-UV analysis.

**High-Performance Liquid Chromatography**

The model compounds in the different samples taken from the FDC experiment were analysed using a validated high-throughput HPLC-UV method. A Waters Alliance 2695 separation module and a dual absorbance detector 2487 equipped with Empower 2 software (Waters) were part of the HPLC apparatus. 25 μl of each sample was injected on a Symmetry C₁₈ column (75 mm × 4.6 mm, 3.5 μm particle size; Waters) with an appropriate guard column. The sample compartment was kept constant at 20°C, while the column temperature was maintained at 30°C. A degassed isotropic mobile phase consisting of a preset composition of: 0.1% FA in H₂O (A) and 0.1% FA in MeOH (B) at a flow rate of 1.0 ml/min was used. A mobile phase composition of 75/25 A/B (% V/V) was used for the determination of caffeine and ibuprofen, while a 30/70 A/B (% V/V) composition was used for the testosterone determination. UV detection was performed at 272, 254 and 220 nm for caffeine, testosterone and ibuprofen, respectively, with peak areas used for quantification [15].

**Calculation of Skin Permeation Parameters**

The cumulative amounts of the model components (in micrograms) permeated through human skin were plotted as a function of time (in hours). For the calculations of the transdermal parameters, the individual graphs were used. Steady-state flux \(J_{ss} = \mu g/(cm²·h)\) was calculated from the slope of the linear portion of the cumulative amount versus time curve divided by 0.64 to correct for the exposed skin area (in square centimetres). The lag time (in hours) was obtained by setting \(y = 0\) in the individual linear regression equation. The \(Q_{lt}\) is the cumulative quantity, expressed as percentage of the effective dose applied, obtained after 1 day. The indicated parameters are the secondary parameters. The primary parameters are calculated in accordance with ECETOC, CEFIC [18]; the permeability coefficient \(K_p, cm/h\) was calculated as follows:

\[
K_p = \frac{J_{ss}}{C_{ss}},
\]

where \(C_{ss} (\mu g/ml)\) is the concentration of the model compound in the dose formulation. Furthermore, the apparent diffusion

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(D_m, cm²/h) and partition (K_m) coefficients were determined using the following equations:

\[ D_m = \frac{d^2}{6 \times t_{lag}} \]

\[ K_m = \frac{K_p \times d}{D_m} \]

where \( d \) and \( t_{lag} \) are the measured skin thickness (cm) and the lag time (h), respectively. The transdermal penetration-enhancing ratio (ER) was calculated as follows:

\[ ER = \frac{K_p, \text{with penetration enhancer}}{K_p, \text{without penetration enhancer}} \]

**Results**

**Penetration-Enhancing Effect of PCERs on Caffeine**

A linear relationship of all individual cumulative amounts of caffeine versus time was observed. The mean cumulative amounts versus time curves for caffeine with 1% of the different PCERs are visually presented in figure 3. Two groups of PCERs are visible in the graph: PCER4, 5 and 7–10 with cumulative amounts between 130 and 250 μg after 24 h and another group (PCER1–3 and 6) with cumulative amounts between 340 and 410 μg. The curves of this latter group exhibit higher slopes than the curves of the first group. The highest cumulative amount of caffeine after 24 h was obtained in the presence of the positive control azone (1,609 μg). For the PCERs, the highest cumulative amount was obtained with PCER1 (phytosphingosine), as observed in figure 3, and PCER2, PCER3 and PCER6 also provided an enhanced cumulative amount of caffeine compared to the control.

All \( J_{ss} \), \( t_{lag} \) and \( Q_{1d} \) data can be found in the online supplementary table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000365730). The skin flux of caffeine (mean ± SE, \( n = 10 \)) without the PCERs was 11.27 ± 1.84 μg/cm²·h. Neither PCER8 nor PCER9 had any effect on this value, and the flux values (mean ± SE, \( n = 2 \)) were determined as 11.53 ± 2.50 and 10.89 ± 1.26 μg/cm²·h, respectively. These values contrast the much higher flux values in the presence of PCER1, PCER2 and PCER3 of 35.44 ± 14.98 (\( n = 4 \)), 29.26 ± 8.94 (\( n = 3 \)) and 31.98 ± 13.97 μg/cm²·h (\( n = 4 \)), respectively.

The percentage of caffeine of the applied dose solution penetrated through the skin after 24 h (mean ± SE) for azone is 5.98 ± 1.97% (\( n = 4 \)) and for PCER1, 2 and 3 1.60 ± 0.76 (\( n = 4 \)), 1.45 ± 0.47 (\( n = 3 \)) and 1.44 ± 0.73% (\( n = 4 \)). The overall apparent permeability coefficients (K_p), as well as the derived diffusion (D_m) and partition (K_m) coefficients for caffeine are given in table 2. The experimentally determined K_p of the negative control, i.e. caffeine without any PCER (2.11 ± 10⁻⁴ cm/h), is comparable with our previous result (2.23 ± 10⁻⁴ cm/h) [15]. The highest K_p values of all the PCERs were seen with PCER2, PCER3 and PCER6. The enhanced permeability of caffeine in the presence of PCERs mainly originated from the partitioning of caffeine out of the dose solution into...
the skin, as evidenced by the overall increased $K_m$ values, as well as the ratio

$$\frac{C_{24\text{h}}^{\text{epidermis}}}{C_{24\text{h}}^{\text{vehiculum}}}.$$

The higher the ratio

$$\frac{C_{24\text{h}}^{\text{epidermis}}}{C_{24\text{h}}^{\text{vehiculum}}}.$$

the higher the concentration of caffeine in the epidermis, indicating the increased partition of caffeine from the dose solution into the epidermis.

The distribution of caffeine in the dose solution, the different skin layers and the receptor fluid is graphically represented in figure 4. The concentration of caffeine was overall higher in the remaining dose solution than in the epidermis. The concentrations in the dermis were approximately 10 times lower than in the epidermis. The receptor fluid concentration of caffeine after 24 h was more than 100 times lower than in the epidermis.

PCER1, PCER2, PCER3 and PCER6 exhibited penetration ERs for caffeine of 2.48, 2.75, 2.62 and 2.70, respectively, which are shown in table 3. The PCERs with an ER higher than 2 correspond to the upper group of
PCERs in the graph presented in figure 3. Azone had the highest ER for caffeine, namely 11.40.

Penetration-Enhancing Effect of PCERs on Testosterone

For testosterone, a linear relationship of all individual cumulative amounts versus time was observed. Figure 5 shows the mean amounts of testosterone accumulated in the receptor fluid as a function of time for all the investigated PCER penetration enhancers. Azone provided the highest cumulative amount for testosterone after 24 h (127 μg). As can be observed, PCER2 and PCER10 resulted in the highest accumulation of testosterone in the receptor compartment after 24 h. On the other hand, PCER7, PCER8 and PCER9 did not modify to a significant extent the amount of permeated testosterone with respect to that observed with the control. Three groups of PCERs are observed in figure 5: PCERs giving the highest cumulative amount of testosterone after 24 h (82–104 μg), PCERs with a moderate (57 μg) and PCERs with the lowest cumulative amount of testosterone (26–37 μg).

The secondary transdermal parameters for testosterone are given in supplementary table 1. The testosterone flux (mean ± SE, n = 9) without penetration enhancer was 2.95 ± 0.48 μg/cm² · h, while the $J_{ss}$ values (mean ± SE, n = 4) for testosterone with PCER2 and PCER10 were 9.06 ± 3.22 and 9.10 ± 1.23 μg/cm² · h, respectively.

The permeability, diffusion and partitioning coefficients for testosterone are given in table 4. The experimentally determined $K_p$ of the negative control (3.92 × 10⁻⁴ cm/h) is almost the same as our previous result (3.97 × 10⁻⁴ cm/h) [15]. There is no clear trend noticeable between high partition coefficient values of testosterone and the ratio

$$\frac{C_{epidermis}^{24\text{ h}}}{C_{vehiculum}^{24\text{ h}}}$$

Therefore, in contrast to caffeine, we cannot unambiguously conclude that high concentrations of testosterone in the epidermis can be explained by a high partitioning of testosterone from the dose solution into the epidermis.

Table 3. ERs for caffeine (CF, n = 2–4), testosterone (TES, n = 1–4) and ibuprofen (IBU, n = 2) (mean ± SE)

<table>
<thead>
<tr>
<th>Penetration enhancer</th>
<th>ER CF</th>
<th>ER TES</th>
<th>ER IBU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azone</td>
<td>11.40±3.30</td>
<td>4.13±1.32</td>
<td>–</td>
</tr>
<tr>
<td>PCER1</td>
<td>2.48±0.44</td>
<td>2.08±0.56</td>
<td>0.98±0.31</td>
</tr>
<tr>
<td>PCER2</td>
<td>2.75±0.74</td>
<td>2.56±0.13</td>
<td>0.92±0.03</td>
</tr>
<tr>
<td>PCER3</td>
<td>2.62±0.93</td>
<td>3.48</td>
<td>1.19±0.09</td>
</tr>
<tr>
<td>PCER4</td>
<td>1.39±0.05</td>
<td>2.53</td>
<td>–</td>
</tr>
<tr>
<td>PCER5</td>
<td>1.81±0.59</td>
<td>2.04±0.14</td>
<td>–</td>
</tr>
<tr>
<td>PCER6</td>
<td>2.70±0.45</td>
<td>2.05±0.48</td>
<td>1.25±0.21</td>
</tr>
<tr>
<td>PCER7</td>
<td>1.13±0.11</td>
<td>1.08</td>
<td>–</td>
</tr>
<tr>
<td>PCER8</td>
<td>0.96±0.11</td>
<td>1.12±0.11</td>
<td>1.42±0.05</td>
</tr>
<tr>
<td>PCER9</td>
<td>0.92±0.01</td>
<td>1.19±0.16</td>
<td>–</td>
</tr>
<tr>
<td>PCER10</td>
<td>1.55±0.16</td>
<td>4.84±0.79</td>
<td>–</td>
</tr>
</tbody>
</table>

Fig. 5. Cumulative amount versus time curve of testosterone (TES) with 1% (W/V) of the PCERs (mean ± SE, n = 2–9).
Figure 6 presents the distribution of testosterone into the dose solution, the different skin layers and the receptor fluid. The concentration of testosterone remained higher in the epidermis than in the remaining dose solution: the epidermis functioned as a skin reservoir for testosterone, the concentration gradient decreased towards the dermis and receptor fluid.

The enhancing ratios of the various PCERs for testosterone are given in table 3. A remarkable ER of 4.8 is observed for PCER10, followed by an ER of 4.1 for azone and 3.5 for PCER3, while PCER1, PCER2, PCER4, PCER5 and PCER6 have a moderate penetration-enhancing effect between 2 and 3 for testosterone through the skin. The other PCERs with an ER value lower than 2, belonging to the lowest group in figure 5, have no pharmaceutically relevant effect on the permeability of testosterone.

Penetration-Enhancing Effect of PCERs on Ibuprofen
All individual cumulative amounts of ibuprofen versus time showed a linear relationship. The curves of the mean cumulative amounts of ibuprofen in the receptor fluid as a function of time for all PCERs assayed are illustrated in figure 7.
The secondary transdermal parameters for ibuprofen can be found in the online supplementary table 1. The flux (mean ± SE, n = 4) of ibuprofen without the PCERs was 82.41 ± 6.95 μg/cm² · h, while in the presence of PCER8, PCER3 and PCER6 the flux (mean ± SE, n = 2) was 99.65 ± 14.94, 84.35 ± 9.29 and 87.67 ± 0.72 μg/cm² · h, respectively. Besides, the other investigated PCERs did not result in a significant increase in the ibuprofen flux through the skin.

The primary transdermal parameters for ibuprofen (Kₚ, Dₘ, Kₘ) are given in table 5. The experimentally determined Kₚ of the negative control (2.05 × 10⁻³ cm/h) is comparable with our previous result (2.19 × 10⁻³ cm/h) [15]. The distribution of ibuprofen into the different skin layers is given in figure 8. The highest concentration of ibuprofen after 24 h was located in the remaining dose solution. A decrease in ibuprofen concentration is observed towards the dermis and the receptor fluid.

The ERs of the PCERs are presented in table 3, but no penetration-enhancing effect was observed for ibuprofen. All ratios are below the value of 2.

**Discussion**

The shielding function of the human SC executes physicochemical limitations to the nature of molecules which can be passed through the barrier lipid matrix. Hence, it is important to use an appropriate penetration enhancer in the development of transdermal drug delivery systems to increase the skin permeation rate (flux) of permeants without significantly affecting toxicity and/or harming the skin [10, 19–21].

The most widely explored enhancement strategy involves the use of lipid-protein-partitioning chemicals that decrease the skin barrier properties and subsequently allow...
the entry of even poorly penetrating molecules into the deeper layers. It appears that interactions with the SC intercellular lipids between the corneocytes is the most effective one [9, 22]. The lipid interaction can occur near the polar head and between the hydrophobic tail of the lipid bilayer [23]. An example of such a penetration enhancer is the lipophilic molecule azone, which was the first chemical designed as a transdermal penetration enhancer. Azone partitions into the lipids of the SC and disrupts the lipid bilayer structure, producing a more fluid environment, by preventing chain crystallization. In addition, azone reduces the diffusional resistance of the skin. Drugs could pass the skin more efficiently through this less rigid environment. The efficacy of azone is dependent on the used concentration: it appears to be most effective at low concentrations (0.1–5%) and is often used in concentrations between 1 and 3% [20, 24, 25]. PCERs are amphiphilic molecules which penetrate with their hydrophobic part near the lipid fatty acid chains and with their hydrophilic part near the polar head of the lipids, indicating that they disrupt the lipid packing and induce a lateral fluidization of the lamellae [23].

The optimal chain length for chemical penetration enhancers derived from amino acids was found to be 10–12 carbons [9]. This chain length causes disorder between the much longer hydrophobic chains of ceramides present in the SC, leading to easier drug permeation. These general findings are in contrast with studies about ceramides as penetration enhancers [13]. In the latter study, ceramides with a sphingosine chain length of 18 carbons and fatty acid chains with various carbon atoms were investigated, and it seemed that the optimal acyl chain length for the penetration behaviour was between 4 and 6 carbons. In the current study, we investigated PCERs, featuring a common 18-carbon sphingoid base and different acyl chain lengths. The investigated PCERs exerted a compound-dependent penetration-enhancing effect. Some PCERs enhanced the penetration of caffeine and testosterone, but lack an enhancing effect on ibuprofen. PCERs with an ER of more than 2 having an effect on caffeine as well as on testosterone are PCER1, PCER2, PCER3 and PCER6. The PCERs containing a fatty acid chain with 4 (PCER3) and 10 (PCER6) carbons are in correspondence with the previous findings for ceramide-like compounds, reported in the literature [9, 13]. However, in contrast to what was found for earlier reported ceramides, we observed that PCERs with different acyl chain lengths also exhibited a penetration-enhancing effect, indicating that the extra OH group on C4 of the sphingoid base may play a role as well. Our study confirms the ceramide findings that there is no linear relationship between the acyl chain length and the skin permeability [13].

Caffeine is a hydrophilic compound with a logP value of –1.06 (calculated using HyperChem). The overall observation that the highest concentration of caffeine was found in the remaining dose solution seems logical, as the epidermis, with the SC as outer layer, is a more lipophilic skin layer. Caffeine will thus hardly diffuse to and penetrate into this skin layer, remaining in the hydrophilic dose solution.

Fig. 8. Concentration of ibuprofen (IBU; mean ± SE, n = 2–4) in different compartments 24 h after dermal application in FDC.
The highest permeability ($K_p$) was observed for PCERs with 2 (PCER2) and 10 (PCER6) carbons in the acyl chain. Co-administration of caffeine with PCER3 (4 carbons) and, strikingly, also PCER1 (phytosphingosine) gave a biomedically significant penetration-enhancing effect (ratio of more than 2).

In contrast to caffeine, the highest amount of testosterone after 24 h was found in the epidermis. Testosterone is a more lipophilic compound with a logP of 3.84 (calculated using HyperChem) and prefers to partition in the epidermis, out of the dose solution. On the other hand, the skin layer following the epidermis is the more hydrophilic dermis. Hence, testosterone will concentrate in the epidermis, and will penetrate with difficulty from the epidermis into the dermis.

Co-administration with the PCER containing a 2-OH-phenyl functional group (PCER10) resulted in the highest penetration-enhancing effect on testosterone, while PCER10 did not have a biomedically significant enhancing effect on caffeine. Phytosphingosine (PCER1) and the other PCERs having an acyl chain with 2–10 carbons had also a penetration-enhancing effect on testosterone with an ER of more than 2, while with PCER7, PCER8 and PCER9, containing 12 carbons, 14 carbons and a phenyl group, respectively, no significant enhancing effect occurred.

Ibuprofen, with a logP of 3.83 (calculated using HyperChem), contains also an acidic carboxyl function with a pKa between 4.4 and 5.7, depending on the ethanol concentration of the dose solution [26]. At the skin surface, ibuprofen will thus be partly ionized. Nevertheless, there is still an appreciable flux of ibuprofen through the skin. After 24 h, the highest concentration of ibuprofen was overall found in the remaining dose solution, different from testosterone with a similar logP, but no ionizable group. Nevertheless, none of the investigated PCERs had a penetration-enhancing effect on ibuprofen, similar to the situation with spilanthol as penetration enhancer.

**Conclusion**

The investigated PCERs exhibited a compound-dependent penetration-enhancing effect on caffeine and testosterone but not on ibuprofen. In combination with caffeine, PCER1 (phytosphingosine), PCER2 (2 carbons), PCER3 (4 carbons) and PCER6 (10 carbons) exhibited an ER of 2.48, 2.75, 2.62 and 2.70, respectively. PCER1–PCER6 and PCER10 (hydroxylated benzene function) gave an ER of more than 2 for testosterone, with PCER10 showing a remarkable ER of 4.84. None of the investigated PCERs modified the transdermal behaviour of ibuprofen. This study indicates that selected PCERs are potentially useful in transdermal formulations to facilitate transport of the active ingredient through the SC.

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**Disclosure Statement**

No competing financial interests exist.


