

# Development and Validation of Analytical Methods for the Detection and Quantification of a Novel Dimeric Ceramide in Stratum Corneum and Other Layers of the Skin

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**Abstract** Ceramides (CERs) are integral parts of the intercellular lipid lamellae of the stratum corneum (SC), which is responsible for the barrier function of the skin. Many skin diseases such as atopic dermatitis and psoriasis are associated with depletion or disturbance of the level of CERs in the SC. Administration of an exogenous novel dimeric ceramide (dCER) deep into the SC may help to stabilize the SC barrier substantially and to treat some skin disease conditions and with the help of the existing technology it might be possible to formulate various pharmaceutical dosage forms that can facilitate penetration of dCER into the SC. However, assessment of the rate and extent of permeability of the exogenous dCER involves appropriate analytical techniques which can discriminately quantify the exogenous dCER in the SC and in the other skin layers. Therefore, an attempt was made to develop an AMD-HPTLC and an HPLC/APCI-MS method for the detection and quantification of exogenous dCER in the SC as well as other skin

layers. The method involved synthesis of the dCER and development of appropriate HPTLC and LC/ESI-MS methods for the separation and quantification of the dCER in the SC and deeper layers of the skin. The methods developed were optimized for quantification of a novel dCER. In comparison to the AMD-HPTLC method, the HPLC/MS method offers a higher sensitivity. Both methods could be used for the quantification of dCER in presence of a complex matrix (e.g., skin extract). The developed methods are complementary and could be used for the quantification of dCER in any further stage of substance research and industrial application. The methods developed are robust, linear and sensitive with a low limit of detection (LOD) and low limit of quantification (LOQ).

**Keywords** Novel dimer ceramide · HPTLC · LC-APCI-MS · Validation · Skin samples

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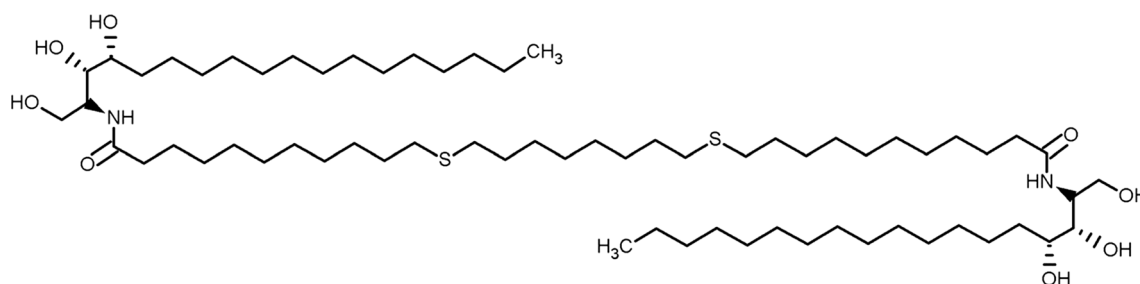
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## Introduction

Ceramides (CERs) are integral parts of the intercellular lipid lamellae of the stratum corneum (SC), which fulfills the role of barrier function in the mammalian skin [1–3]. Many skin disease conditions, such as psoriasis [4], atopic dermatitis [5–7] and irritant/allergic contact dermatitis [5] are associated with depletion or disturbance of the level of CERs in the SC. Therefore, incorporation of a novel dimeric ceramide (dCER; see Fig. 1) into the lipid lamellae of the SC might help to stabilize the SC and to treat such skin conditions. Molecules are not described in the literature for stabilizing the SC lipids [8].

Some analytical methods were developed for the identification and quantification of CERs in the SC, which include different HPTLC, HPLC and LC/MS hyphenation



**Fig. 1** Structure of the dimeric ceramide

techniques [9–14]. However, none of these methods can be used to discriminate identify and quantify the amount of exogenous CERs permeated into the SC, which is necessary for ex vivo or in vivo dermal bioavailability.

LC/MS technique is highly sensitive and selective technique that can be used for identification and quantification of minute quantities of analytes in biological fluids [15]. Besides, quantification of compounds using HPTLC and LC/MS demands adequate degree of substance separation. However, in APCI/MS a sufficient separation of the potential analytes in a biological fluid is necessary to avoid significant of ion suppression, which is referred as “matrix effect”, and the reproducibility and accuracy of the method could be highly compromised [15, 16]. Thus, analyses of biological fluids like SC extract needs development of appropriate HPTLC or HPLC methods for maximum separation of the substance to be quantified from the rest of the matrix.

Therefore, the aim of this work was to develop a sensitive analytical assay for a newly synthesized extremely lipophilic and difficult to handle molecule such as dCER (Fig. 1) which has been distinguished from the endogenous CERs using an HPTLC and a sensitive and selective LC/APCI-MS method which helps to quantify trace amounts of the exogenous dCER in SC and other skin layers. This is a real analytical challenge.

## Materials and Methods

### Materials

Carbamazepine (CBZ) was obtained from Sigma-Aldrich (St. Louis, USA). Ethanol, chloroform, *n*-hexane, ethylacetate and copper(II) sulfate pentahydrate were obtained from Carl ROTH GmbH & Co. KG (Karlsruhe, Germany). Ceramides for lipid mix were purchased from: Evonik Industries AG (Essen, Germany) (CER [AP], [AS], [NP], [EOP], [EOS]) and Croda International Plc (East Yorkshire, UK) (CER [NS]). A minimal two-letter code was used to describe the CER subspecies. The first letter stands for the N-acetylated fatty acid, i.e., N stand for a non-hydroxy fatty acid and A indicates an  $\alpha$ -hydroxy fatty acid. Furthermore,

for several subspecies, the installation of a third letter was necessary to describe an additional esterified fatty acid (E). For the description of the sphingoid bases the following letters were recommended: P (phytosphingosine), and S (sphingosine). Methanol, *n*-heptane, *n*-hexane and isopropanol were obtained from VWR International S.A.S. (Fontenay-sous-Bois, France). 4 M acetic acid solution and acetone were purchased from Merck KGaA (Darmstadt, Germany). All solvents used for extraction and HPLC purpose were of HPLC grade. For synthesis all materials and reagents were purchased from Sigma-Aldrich (St. Louis, USA). The solvents were dried before use. For HPTLC aluminum sheets coated with silica gel 60 F254 from Merck KGaA (Darmstadt, Germany) were used and the detection was obtained with bromothymol blue solution. Silica gel 60 (0.036–0.200 mm) from Merck KGaA (Darmstadt, Germany) was used for column chromatography. The mass spectrometry data were recorded on a Finnigan MAT 710C from Thermoseparation Products (San Jose, USA) for ESI-MS and on an LTQ–Orbitrap XL hybrid mass spectrometer from Thermo Fisher Scientific (Bremen, Germany) for high-resolution mass spectrometry (HR-MS). The  $^1\text{H-NMR}$  and the  $^{13}\text{C-NMR}$  spectra were recorded on a Varian Gemini 2000 and a Varian Inova 500.  $\text{CDCl}_3$ ,  $\text{CD}_3\text{OD}$   $\text{DMSO-D}_6$  and their mixtures were used for the NMR spectroscopy.

### Methods

#### Synthesis of the dCER

The synthesis of 12,21-dotriacontanedioic acid (**1**) in detail is described in the literature [17].

Preparation of the *N,N'*-bis[(2S, 3S, 4R)-1,3,4-trihydroxyoctadec-2-yl]-12,21-dithiadotriacontanediamide (**2**): to a suspension of 7 mmol (3.83 g) of compound **1** in 300 mL  $\text{CH}_2\text{Cl}_2$  was added under stirring 14.7 mmol (7.65 g) of benzotriazol-L-yl-oxy-tris-pyrrolidino-phosphonium-hexafluorophosphat (PyBOP<sup>®</sup>) and 28 mmol (4.76 mL) of diisopropylethylamine. The mixture was stirred for 20 min at room temperature. After that 14.7 mmol (4.67 g) phytosphingosine was added to the solution. The stirring was

continued for 16 h at the same temperature. The precipitate was filtered off and the filter was washed two times with 15 mL  $\text{CH}_2\text{Cl}_2$ . The crude ceramide was purified by column chromatography using chloroform/methanol/ammonia as eluent. The obtained data for MS,  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$  and HR-MS are shown in the supplementary material (see Fig. S1–S4).

Yield: 6.09 g (76%); white substance;  $R_f = 0.22$  ( $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{NH}_3$ , 90:10:1, v/v/v); mp 132–135 °C;  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ , 40 °C):  $\delta = 0.89$  (t,  $^3J(\text{H,H}) = 6.8$  Hz, 6, 1.27–1.39 (m, 80 H,  $(\text{CH}_2)_6(\text{CH}_2)\text{S}(\text{CH}_2)_2(\text{CH}_2)_4(\text{CH}_2)_2\text{S}(\text{CH}_2)_2(\text{CH}_2)_6$ ), 2, 1.51–1.71 (m, 16 H, 222 overlaid by  $\text{H}_2\text{O}$ ), (t,  $^3J(\text{H,H}) = 7.7$  Hz, 4 (t,  $^3J(\text{H,H}) = 7.4$  Hz, 8  $^{13}\text{C-NMR}$  (125 MHz,  $\text{CDCl}_3/\text{CD}_3\text{OD}$ , 35 °C):  $\delta = 13.66$  (2  $\text{CH}_3$ ), 22.42 (2, 25.60, 25.63, 28.61, 28.71, 28.89, 29.03, 29.08, 29.11, 29.14, 29.23, 29.29, 29.42, 29.46, 29.51, 29.52 (2, 31.70 ( $2\text{CH}_2\text{CH}_2\text{CH}_3$ ), 31.95 and 31.97 (2, 32.76 (2, 36.30 ( $2\text{CH}_2\text{CONH}$ ), 51.83 (2, 61.01 (2, 72.32 (2, 75.44 ( $2\text{CH}_2\text{CHOHCHOH}$ ), 174.49 (2 ppm; MS:  $m/z$ : 1144.2  $[\text{M}-\text{H}]^-$ ; HRMS calcd for  $\text{C}_{66}\text{H}_{133}\text{N}_2\text{O}_8\text{S}_2$   $[\text{M}+\text{H}]^+$  1145.9498; found 1145.9488.

#### Development of Stock and Working Solutions

For HPTLC and HPLC the dCER and internal standard (IS) carbamazepine (CBZ) were weighted on an analytical balance Mettler-Toledo XA105 Dual Range (Mettler-Toledo GmbH, Gießen, Germany) in glass tubes and further diluted with chloroform/methanol 9:1 (v/v) solvent to a concentration of 250  $\mu\text{g mL}^{-1}$ .

The lipid mix was prepared from standard stock solutions of CER [AP], [AS], [NP], [EOP], [NS], [EOS] [1 mg  $\text{mL}^{-1}$  in chloroform/methanol 1:1 (v/v)] by adding 1000  $\mu\text{L}$  of each standard stock solution in a 10-mL volumetric flask and then adjusting the volume till final 10 mL with chloroform/methanol 1:1 (v/v). This results in a final concentration of 100  $\mu\text{g mL}^{-1}$  for each CER. The lipid mix was transferred into separate screw cap glass bottles and frozen until use.

#### HPLC/APCI-MS

The development and validation of a quantification method for dCER was performed by means of HPLC-system Agilent Technologies 1220 (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler. A normal phase HPLC column Zorbax RX-SIL "Agilent" 2.1  $\times$  150 mm, 5 micron, P.N. 883700-901, S.N. USK1002248 (Agilent Technologies, USA) was used as stationary phase. The mobile phase consists of Solvent A [*n*-heptane/isopropanol 95:5 (v/v)] and Solvent B [*n*-heptane/isopropanol/ethanol 50:25:25 (v/v/v)]. The following gradient elution mode was applied: 0 min 100% A; 15 min 25% A, 75% B; 16 min

100% B; 20 min 100% B; 21 min 100% A. Stop time of the gradient was set at 28 min with 1 min of post time. A flow rate of 0.4  $\text{mL min}^{-1}$  was applied till the 16th min of the gradient. Afterwards a 0.6  $\text{mL min}^{-1}$  flow rate was used for a proper washing of the column and faster back equilibration to the starting parameters. The maximum pressure was set to 400 bar. The injection volume and the temperature of the column were 10  $\mu\text{L}$  and 30 °C, respectively. The Agilent OPENLAB software for HPLC was applied.

MS-detector Infinity LC MS-System/LCQ (Thermo Finnigan AG, Waldbronn, Germany) was used for quantification of dCER after chromatographic separation. The detector was set to positive APCI ((+) APCI) mode (source heat temperature 500 °C, sheath gas flow rate 70 arb, aux gas flow rate 10 arb, discharge current 7  $\mu\text{A}$ , capillary temperature 200 °C, capillary voltage 3 V and tube lens offset at 15 V).  $\text{N}_2$  was used as nebulizer and as drying gas.

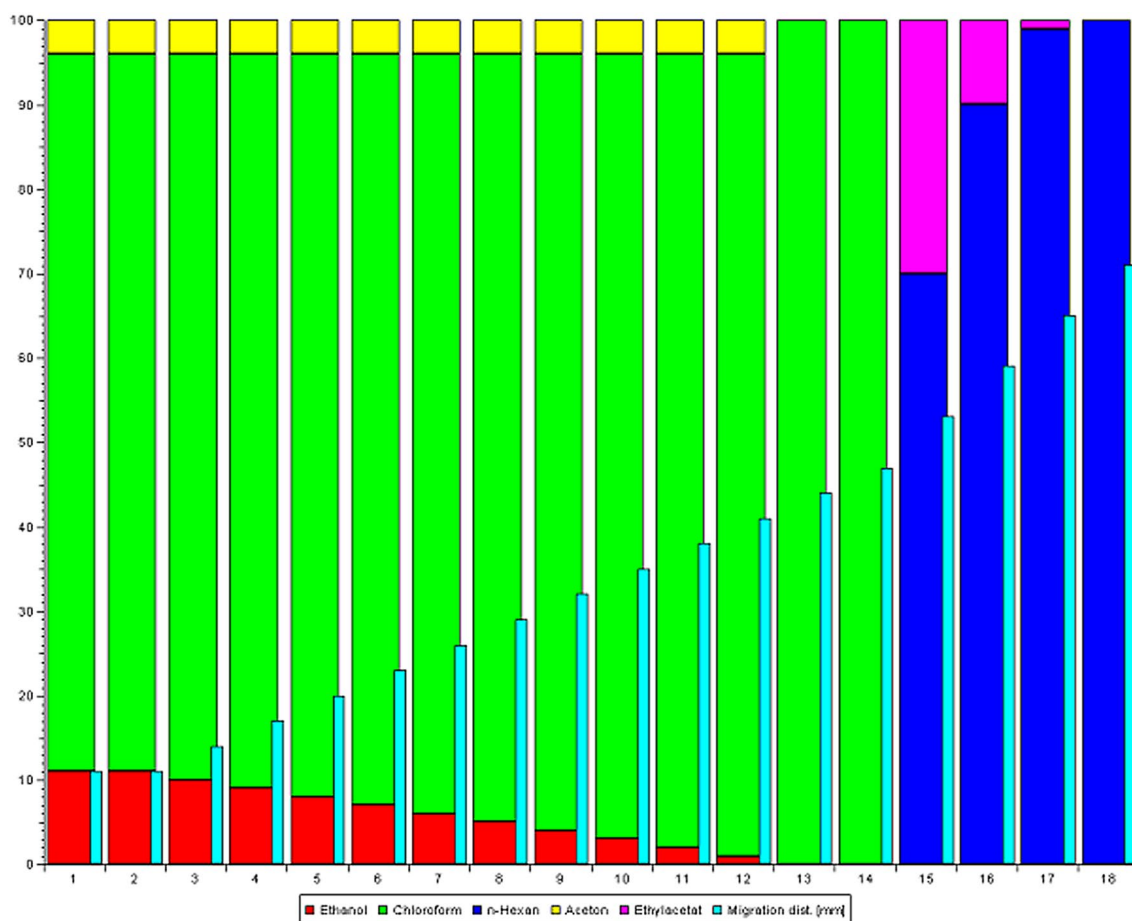
The acquisition with MS-detector was performed for 28 min with 0 min start delay. Two scan events were used: Scan event 1 (full scan) at 100.00–2000.00  $m/z$  range; Scan event 2 (single ion monitoring (SIM)) with center mass 237.20  $m/z$ , width 2.00 (for CBZ) and 1146.00  $m/z$ , width 4.00 (for dCER). Normal mass range, positive polarity and centroid data type were applied.

The Xcalibur 2.0 software was used for MS-spectra recording and evaluation. A special layout for the evaluation of the obtained data was developed:

1. Full scan with Base Peak F: (+) APCI corona full scan MS [100.00–2000.00];
2. CBZ SIM  $m/z = 236.77$ – $237.77$  F: (+) APCI corona SIM MS [236.2–238.2, 1144.00–1148.00];
3. DC SIM  $m/z = 1145.39$ – $1146.39$  F: (+) APCI corona SIM MS [236.2–238.2, 1144.00–1148.00].

#### Automated Multiple Development (AMD)-HPTLC

Using the AMD-HPTLC system Camag (all components from Camag, Muttenz, Switzerland) dCER was quantified. Before sample application, the HPTLC Silicagel F254 GLP glass plates 20  $\times$  10 cm (Merck, Darmstadt, Germany) were kept for 2 h in isopropanol and further dried in a drying oven VO500 (Mettmert GmbH, Schwabach, Germany) for 30 min at 100 °C. Samples were applied with TLC Sampler 4 autosampler using 25  $\mu\text{L}$  syringe with dosage speed of 10  $\mu\text{L s}^{-1}$ . Sixteen samples per plate, with the start line 8 mm from the bottom, were applied. Calibration was done using a 100  $\mu\text{g mL}^{-1}$  dCER solution in different amounts (in range from 0.5 to 20  $\mu\text{L}$ ). The final calibration concentrations were: 0.05, 0.4, 0.8, 1.2, 1.6, 2.0  $\mu\text{g}$  of dCER per spot. The quality controls (15, 35, 45, 70  $\mu\text{g mL}^{-1}$ ) were applied in amount of 20  $\mu\text{L spot}^{-1}$ . This correlates to 0.3, 0.7, 0.9



**Fig. 2** Optimized gradient steps used for HPTLC. Percentage of the solvents used in AMD

and 1.4  $\mu\text{g}$  of dCER per spot. For development 18-step gradient (Fig. 2) was performed with AMD-2 system. The drying time after each gradient step was 1.5 min. After final drying, plates were derivatized by applying two times into an aqueous solution of 10%  $\text{CuSO}_4$  for 10 s. Then the plates were dried for 20 min at 150  $^\circ\text{C}$ . A densitometric evaluation of plates was done using the TLC Scanner 3 Densitometer at 546 nm wavelength (slit dimensions  $4.0 \times 0.2$  mm at a scan speed 20  $\text{mm s}^{-1}$ , data resolution 25  $\mu\text{m}$  per step).

### Validation

**Calibration curve** Six calibration concentration levels were chosen in the next range of freshly spiked samples: 0.1, 0.5, 1.0, 1.5, 2.0, 2.5  $\mu\text{g mL}^{-1}$  of dCER and 1.5  $\mu\text{g mL}^{-1}$  of CBZ (IS) for HPLC/APCI-MS (see Fig. 3) and 0.05, 0.4, 0.8, 1.2, 1.6, 2.0  $\mu\text{g spot}^{-1}$  of dCER for AMD-HPTLC. In case of HPLC/MS method validation, the blank sample without analyte and IS and a sample with IS only were measured additionally. Each calibration standard for HPLC/APCI-MS method validation was analyzed in replicate (5 times each).

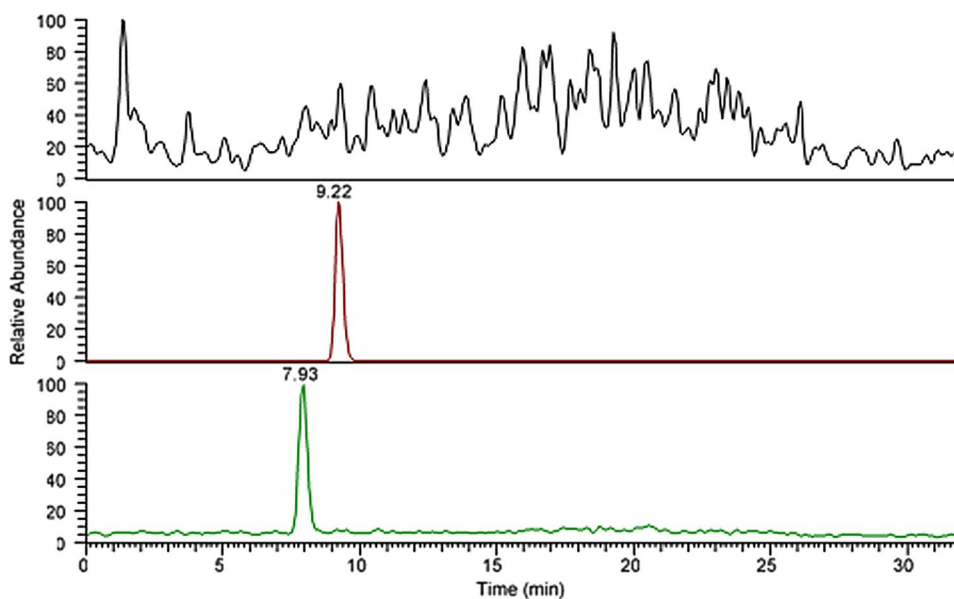
Calibration curves were plotted and further determined using linear regression analysis. The calibration parameters (slope and intercept), 3 calibration curves, as well as the back calculated concentrations of the calibration standards together with mean accuracy values were reported.

**Carry-over** During the validation of the HPLC/APCI-MS method, the carry-over was tested by injecting a blank sample [pure solvent chloroform/methanol 9:1 (v/v)] in three repeats after high concentration samples (calibration standard with 2.5  $\mu\text{g mL}^{-1}$  of dCER and 1.5  $\mu\text{g mL}^{-1}$  of CBZ (IS)). In total the carry-over was studied during five runs on five different days.

**Limit of detection (LOD) and limit of quantification (LOQ)** LOQ and LOD were measured as described in [18]. The same standard concentrations, as for calibration studies, were used for calculation of LOQ and LOD of HPLC/APCI-MS and AMD-HPTLC methods. The error probability was set to 5%.

**Precision and accuracy** The samples were spiked independently from the calibration standards and analyzed using calibration curve and the obtained concentrations were

**Fig. 3** Chromatogram of the optimal CBZ concentration ( $1.5 \mu\text{g mL}^{-1}$ ) which was used as internal standard



compared with the nominal value. The accuracy was reported as the percentage of the nominal value. It was evaluated for the values of the samples obtained within a single run (within-run accuracy). Fresh calibration was performed each day. Four different concentrations levels that cover the calibration curve range (at 5 repeated measurements for each) were used: the lower LOQ (LLOQ), within three times the LLOQ, around 50% of the calibration curve range (medium range) and at least 75% of the upper calibration curve range. In case of HPLC/APCI-MS method validation they correlate to 0.4, 0.9, 1.2,  $1.8 \mu\text{g mL}^{-1}$  of dCER and  $1.5 \mu\text{g mL}^{-1}$  for CBZ (IS), for AMD-HPTLC—0.05, 0.4, 0.8, 1.2, 1.6,  $2.0 \mu\text{g}$  of dCER per spot. Measurements were conducted in five independent runs on five different days for HPLC/APCI-MS and in three independent runs on two different days for AMD-HPTLC. The same runs and data as for demonstrating of accuracy were used for precision studies. The accuracy and precision were demonstrated as percentage of recovery and relative standard deviation (RSD), respectively.

*Matrix effect and selectivity (for HPLC/APCI-MS)*  
The matrix effect and selectivity were investigated for the HPLC/MS method using a blank matrix from full thickness skin of an individual donor. More skin material for matrix effect investigation was unavailable. The blank matrix from skin extract was prepared as follows: full thickness human skin  $1.2 \times 1.2$  mm with previously removed subcutaneous fat, was extracted in 5 mL of chloroform/methanol 9:1 (v/v) at room temperature overnight. Afterwards, the skin piece was removed and the obtained extract was filtered through a lipophilic filter PERFECT-FLOW®PTFE  $0.45 \mu\text{m}$ , 25 mm (WICOM Germany GmbH, Heppenheim, Germany). The obtained extract was further diluted 1/10 with chloroform/methanol 9:1 (v/v) and spiked with CBZ (IS) and dCER.

For the matrix effect the determination occurred at a low level of concentration (maximum 3 times LLOQ)— $0.8 \mu\text{g mL}^{-1}$  as well as a high level of concentration (close to the ULOQ)— $2.2 \mu\text{g mL}^{-1}$  of dCER. The IS (CBZ) in concentration  $1.5 \mu\text{g mL}^{-1}$  was added to each sample. Each concentration was measured 5 times within a single run. As well the same concentrations in pure solvent chloroform/methanol 9:1 (v/v) were prepared and measured.

The matrix factor (MF) and IS normalized MF were calculated using the following equations:

$$\text{MF}_{\text{IS}} = \frac{\text{Peak area of IS with matrix}}{\text{Peak area of IS without matrix}} \quad (1)$$

$$\text{MF}_{\text{dCER}} = \frac{\text{Peak area of dCER with matrix}}{\text{Peak area of dCER without matrix}} \quad (2)$$

$$\text{MF}_{\text{normalized}} = \frac{\text{MF}_{\text{dCER}}}{\text{MF}_{\text{IS}}} \quad (3)$$

For the selectivity studies the obtained diluted extract was spiked with IS  $1.5 \mu\text{g mL}^{-1}$  and dCER  $1.0 \mu\text{g mL}^{-1}$ . The obtained chromatograms were analyzed.

## Results and Discussion

### HPLC/APCI-MS

#### *Method Development and Optimization*

The quantitative method for dCER was developed on HPLC-system as described in “HPLC/APCI-MS”. Both RP (on 6 different HPLC columns) and NP (on 2 different

HPLC columns) methods were tested with various mobile phase compositions.

For RP tested mobile phases consist of variable proportional combinations of methanol and water, with percentage of water from 0 to 10% v/v. As organic modifiers to RP mobile phase chloroform in concentration 1 and 5% v/v, isopropanol and butanol in concentration 5% v/v were applied. For NP different compositions of *n*-heptane, isopropyl, ethanol and chloroform were investigated. Formic acid and ammonium formate aqueous solution were tested as additives to the mobile phases.

Both gradient and isocratic modes were used during method development. Starting from long gradient the method was switched to an optimized isocratic mode. The optimal solvent composition was chosen according to the dCER optimal elution parameters, defined in the long gradient mode. It was found that dCER tends to precipitate in RP solvents that in result made a reliable quantification of dCER via RP methods impossible. Thereof the NP method, with the mobile phase consists of solvent A [*n*-heptane/isopropanol 95:5 (v/v)] and solvent B [*n*-heptane/isopropanol/ethanol 50:25:25 (v/v/v)], was chosen as the optimal. The usage of additives was rejected, as formic acid was not compatible with chosen ionization mode, while ammonium formate tends to crystallize on a column. The following gradient elution mode was developed and finally optimized: 0 min 100% A; 15 min 25% A, 75% B; 16 min 100% B; 20 min 100% B; 21 min 100% A. The best performance was reached with the not endcapped Si-based NP column without pre-column (2.1 × 150 mm, particle size 5 μm and pore size 80 Å).

The methods were also optimized regarding the sample solvent [methanol, chloroform or chloroform/methanol 9:1 (v/v)]. In all cases, single ion monitoring (SIM) mode for dCER and IS was applied. The chloroform/methanol 9:1 (v/v) was chosen as the best sample solvent, based on dCER solubility properties, stability of the working solutions and chromatographic performance. The CBZ was chosen as the most suitable internal standard as it gives sharp peak (retention time (RT) 7.93 min) close to the dCER peak (RT 9.22 min) with proper chromatographic separation (Fig. 2). The optimal concentration of CBZ was tested with addition of 1.0 μg mL<sup>-1</sup> dCER (approximate middle of the calibration concentrations). Between 1.0, 1.5 and 2.0 μg mL<sup>-1</sup>, 1.5 μg mL<sup>-1</sup> was chosen as the most optimal concentration of CBZ. At this concentration no chromatographic overlapping was observed simultaneously with sufficient correlation between dCER and CBZ peak areas.

The (+) APCI ionization mode was chosen as the most suitable as it allows ionization of both target substance and internal standard, and has a smaller fluctuation between obtained peak areas for dCER (1.5 μg mL<sup>-1</sup>) in series of three repeats (RSD = 5.1%) The (-) ESI mode shows

higher peak area of dCER, but at the same time it was not possible to detect the IS—CBZ. Most probably it did not ionize under applied conditions. As well as a disadvantage of this ionization mode could be mentioned the next: high RSD of the obtained peak areas for dCER (1.5 μg mL<sup>-1</sup>) in three repeats (RSD = 36.0%). This ionization mode cannot tolerate high flow rates; optimal maximum is around 0.4 mL min<sup>-1</sup> that could be a limiting factor for further method optimization. Furthermore, ionization temperature and voltage parameters were also optimized and a special layout for validation of spectra was developed.

The increase of the flow rate from 0.4 to 0.5 and 0.6 mL min<sup>-1</sup> did not result in a sufficient change of the retention time of the substances (just approximate 2 min less), that could allow a shortening of the gradient. On the other hand, the increase of the flow rate has affected the peak area of the analytes. Thus, the flow rate of 0.4 mL min<sup>-1</sup> was chosen as optimum. The flow rate of 0.6 mL min<sup>-1</sup> was still applied after the elution of target substances during the column washing step with the strongest mobile phase composition and the back equilibration of the column to the starting parameters. Between all tested, the injection volume of 10 μL provided the highest peak area of dCER in combination with optimal peak area of the internal standard carbamazepine. As well an optimum S/N ratio was reached.

The temperature (30 °C) was chosen as the most suitable because the column temperature (40 °C) did not show any significant improvement of the method.

### Validation

The method used for quantification was according to the recommendations described in the Guideline on bioanalytical method validation, European Medicine Agency, 2012 [18].

**Linearity** The linearity of the calibration curves was tested on three different days by six concentrations (0.1–0.5–1.0–1.5–2.0–2.5 μg mL<sup>-1</sup>) with five repeats for each. The obtained slope, intercept and coefficient of determination (*R*<sup>2</sup>) were within the required range (Table 1).

**Lower (L)LOD and Lower (L)OQ** The LLOD and LLOQ were calculated with the DIN test. The data were extrapolated from three calibration curves. Calibration concentrations of dCER were: 0.1–0.5–1.0–1.5–2.0–2.5 μg mL<sup>-1</sup>, CBZ 1.5 μg mL<sup>-1</sup>. Each concentration was measured in five repeats. The LLOD is 0.167 μg mL<sup>-1</sup> while LLOQ is 0.334 μg mL<sup>-1</sup>.

**Precision and accuracy** The precision was investigated on 4 concentrations of dCER: 0.4 μg mL<sup>-1</sup> (close to LLOQ), 0.9 μg mL<sup>-1</sup> (around 3 times LLOQ), 1.2 μg mL<sup>-1</sup> (medium—approximately 50% of calibration curve range), 1.8 μg mL<sup>-1</sup> (at least 75% of the upper calibration curve range); CBZ 1.5 μg mL<sup>-1</sup>. Each concentration was measured in five repeats on five different days. The obtained

**Table 1** Determination of dCER by HPLC/MS: linearity study

| (A) Calibration curve parameters                               |   |              |                 |
|--|---|--------------|-----------------|
| Date of analysis   | Slope   | y-intercept  | $R^2$           |
| 19.11.14   | 4.094   | +0.433       | 0.9936          |
| 22.11.14   | 3.927   | -0.096       | 0.9967          |
| 23.11.14   | 3.803   | -0.484       | 0.9967          |
| (B) Mean of back calculated concentrations and accuracy values |   |              |                 |
| Nominal conc. ( $\mu\text{g mL}^{-1}$ )                        | Back calculated conc. ( $\mu\text{g mL}^{-1}$ ) | Accuracy (%) | RSD (%) $N = 3$ |
| 0.1  | 0.08  | 79.65        |                 |
| 0.5  | 0.49  | 97.11        | 6.88            |
| 1.0  | 0.99  | 98.71        | 10.62           |
| 1.5  | 1.55  | 103.41       | 4.42            |
| 2.0  | 2.09  | 104.59       | 0.74            |
| 2.5  | 2.40  | 96.16        | 3.38            |

**Table 2** Determination of dCER by HPLC/MS: accuracy and precision studies

| Conc. ( $\mu\text{g L}^{-1}$ ) | Back calculated conc. $\pm$ SD ( $\mu\text{g mL}^{-1}$ ) ( $n = 5$ ) | Within-run precision ( $n = 5$ )<br>RSD | Within-run accuracy ( $n = 5$ )<br>Recovery (%) |
|--------------------------------|--|---|---|
| 0.4                            | 0.36 $\pm$ 0.08  | 5.36                                    | 88.94   |
| 0.9                            | 0.90 $\pm$ 0.11  | 3.90                                    | 100.25  |
| 1.2                            | 1.18 $\pm$ 0.18  | 5.75                                    | 98.68   |
| 1.8                            | 1.63 $\pm$ 0.28  | 5.72                                    | 90.74   |

precision values were in the required range ( $\leq 15$  and  $\leq 20\%$  for LLOQ, see Table 2).

The accuracy was investigated with the same set of dCER concentrations (0.4–0.9–1.2–1.8  $\mu\text{g mL}^{-1}$ ), CBZ 1.5  $\mu\text{g mL}^{-1}$ . The recovery was in the required range, according to the normative documentation ( $\leq 15$  and  $\leq 20\%$  at LLOQ, see Table 2).

**Matrix effect** The matrix effect was investigated in presence of a complex matrix from a skin extract (skin extraction methodology was described in “Materials and methods”). The matrix effect was tested using the skin matrix obtained from one individual donor.

The matrix was spiked with dCER in concentrations of 0.8 and 2.2  $\mu\text{g mL}^{-1}$  [maximum 3 times LLOQ and closer to Uper (U)LOQ], and CBZ (IS) 1.5  $\mu\text{g mL}^{-1}$ . In parallel, the same concentrations were measured in a pure solvent [chloroform/methanol 9:1 (v/v)]. The matrix factors (MF) of dCER and IS were calculated, as well as the matrix effect (in %). The MF for lower concentration was close to the requirements of the Guideline (should be not higher than 15%), while for a higher concentration the MF was very insignificant (Table 3). Overall matrix effect is close to the accepted range and determine the method as suitable for the quantification of dCER in presence of a skin matrix, for instance for penetration studies.

**Selectivity** No sufficient change in the peak shape, peak area and the retention time of dCER and CBZ (IS) was observed in presence of skin matrix (see Fig. 4). Thus, it shows that the developed method is selective with no other peaks detected within the retention window of dCER and CBZ in SIM mode.

## AMD-HPTLC

### Method Development and Optimization

A procedure for quantification of dCER by means of AMD-HPTLC method with 18-step gradient has been developed (see Fig. 2). The first 12 steps were performed using mixtures of chloroform, ethanol and acetone, thereafter followed by 2 isocratic steps with chloroform. These steps allowed the separation of dCER and various endogenous CER classes (present in lipid mix). For the separation of further lipids, that could be present in skin matrix, three additional steps were required with a mixture containing *n*-hexane and ethylacetate followed by an isocratic *n*-hexane step. Before each step the plates were automatically dried for 90 s in vacuum and then conditioned in an acetic acid atmosphere obtained by bubbling air through a 4 M acetic acid solution in order to focus the bands and to achieve a

**Table 3** Matrix effect

| Conc. ( $\mu\text{g mL}^{-1}$ ) | MF <sub>dCER</sub> | MF <sub>IS</sub> | MF <sub>normalized</sub> (%) |
|---------------------------------|--------------------|------------------|------------------------------|
| 0.8                             | 1.156446           | 0.994234         | 16.32                        |
| 2.2                             | 0.55063            | 0.557359         | -1.21                        |

better resolution. This gradient enables separation and quantification of dCER with simultaneous base line separation of the CERs [AP]; [AS]; [NP]; [EOP]; [NS]; [EOS].

The different concentrations of dCER were tested. The dCER chromatographic performance was analyzed in comparison to standard lipidmix, containing CERs [AP]; [AS]; [NP]; [EOP]; [NS]; [EOS] (Fig. 5). The sharp peaks of dCER were observed and no co-elution with standard

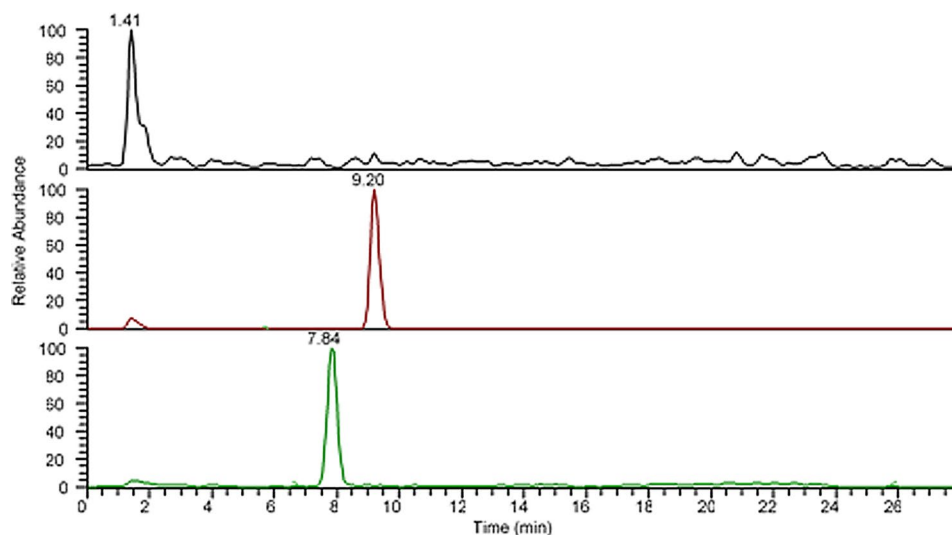
CERs, that are present in major amounts in skin extracts, did occur. This could serve as proof of suitability of the method for the quantification of dCER in presence of skin matrix components.

#### Validation

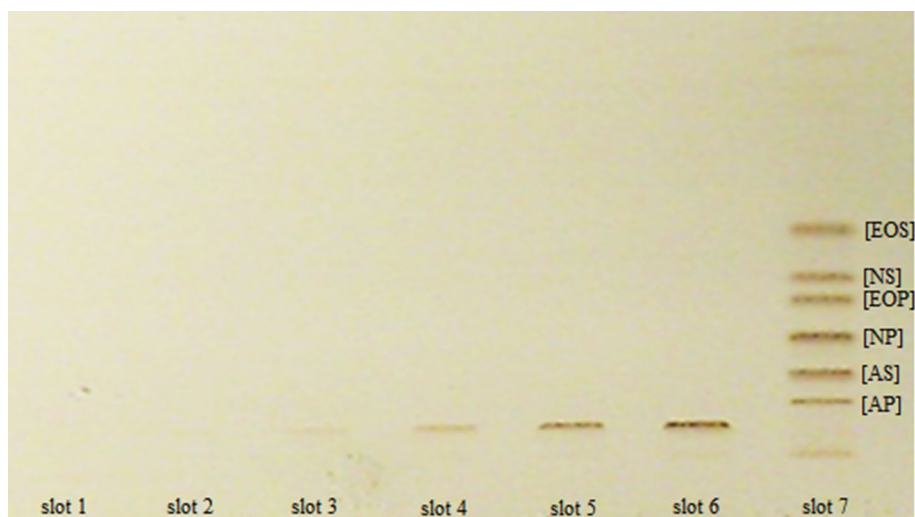
The recommendations of the Guideline on bioanalytical method validation, European Medicine Agency, 2012, were taken into consideration [18].

**Linearity** The linearity was tested by performing 3 calibrations on 2 different days (Table 4). Calibration concentrations: 0.05–0.4–0.8–1.2–1.6–2.0  $\mu\text{g}$  of dCER per spot. The obtained slope, intercept and coefficient of determination ( $R^2$ ) were in the required range.

**Fig. 4** Chromatogram obtained from spiked matrix sample. *Top* blank, *Middle* IS (CBZ), *Bottom* dCER



**Fig. 5** HPTLC chromatogram of dCER and lipid mix: dimeric ceramide 0.05, 0.1, 0.2, 0.5, 1.0, 2.0  $\mu\text{g}$  (slots 1–6). Lipid mix (CER [AP]; [AS]; [NP]; [EOP]; [NS]; [EOS]) 1  $\mu\text{g}$  (slot 7)





**Table 4** Determination of dCER by HPTLC: linearity study

| (A) Calibration curve parameters                               |   |              |                 |
|--|---|--------------|-----------------|
| Date of the analysis   | Slope   | y-intercept  | $R^2$           |
| 24.11.14   | 11,318.589                                      | -748.885     | 0.9908          |
| 26.11.14 (1)   | 10,203.335                                      | -524.413     | 0.9948          |
| 26.11.14 (2)   | 12,936.887                                      | -446.22      | 0.9926          |
| (B) Mean of back calculated concentrations and accuracy values |   |              |                 |
| Nominal conc. ( $\mu\text{g spot}^{-1}$ )                      | Back calculated conc. ( $\mu\text{g mL}^{-1}$ ) | Accuracy (%) | RSD (%) $N = 3$ |
| 0.05   | 0.05  | 101.37       | 31.27           |
| 0.40   | 0.32  | 81.08        | 5.81            |
| 0.80   | 0.81  | 101.26       | 3.24            |
| 1.20   | 1.30  | 108.03       | 4.85            |
| 1.60   | 1.68  | 104.76       | 3.98            |
| 2.00   | 1.89  | 94.61        | 1.17            |

**Table 5** HPTLC accuracy and precision studies

| Conc. ( $\mu\text{g spot}^{-1}$ ) | Back calculated conc. $\pm$ SD ( $\mu\text{g spot}^{-1}$ ) ( $n = 3$ ) | Within-run precision ( $n = 3$ )<br>RSD | Within-run accuracy ( $n = 3$ )<br>Recovery (%) |
|-----------------------------------|--|---|---|
| 0.3                               | $0.28 \pm 0.17$  | 18.21                                   | 94.27   |
| 0.7                               | $0.67 \pm 0.08$  | 13.72                                   | 95.83   |
| 0.9                               | $0.98 \pm 0.11$  | 5.24                                    | 108.79  |
| 1.4                               | $1.55 \pm 0.09$  | 7.27                                    | 110.52  |

**Table 6** Comparison of the HPLC/MS and of the HPTLC method for the dCER quantification

| Validation parameters  | HPLC/MS                     | AMD-HPTLC   |
|------------------------|-----------------------------|---|
| Linearity              | >0.99                       | >0.99   |
| LLOD                   | $0.167 \mu\text{g mL}^{-1}$ | $13.05 \mu\text{g mL}^{-1}$ ( $0.261 \mu\text{g spot}^{-1}$ ) |
| LLOQ                   | $0.334 \mu\text{g mL}^{-1}$ | $22.80 \mu\text{g mL}^{-1}$ ( $0.456 \mu\text{g spot}^{-1}$ ) |
| Precision <sup>a</sup> | RSD (%)                     | RSD (%)   |
| LLOQ                   | 5.36                        | 18.21   |
| Low QC                 | 3.90                        | 13.72   |
| Middle QC              | 5.75                        | 5.24  |
| High QC                | 5.72                        | 7.27  |
| Accuracy <sup>a</sup>  | Recovery (%)                | Recovery (%)  |
| LLOQ                   | 88.94                       | 94.27   |
| Low QC                 | 100.25                      | 95.83   |
| Middle QC              | 98.68                       | 108.79  |
| High QC                | 90.74                       | 110.52  |
| Matrix effect          | $\leq 15\%$ <sup>a</sup>    | N/A   |
| $\leq 3$ times LLOQ    | 16.32                       | -   |
| Closer to ULOQ         | -1.21                       | -   |

<sup>a</sup> Requirements of EMA guideline for precision and accuracy:  $\leq 15$  and  $\leq 20\%$  for LLOQ

**LLOD and LLOQ** The LLOD and LLOQ were calculated as described in [18]. The data were extrapolated from three calibration curves, measured on two different days. Calibration concentrations of dCER: 0.05–0.4–0.8–1.2–1.6–2.0  $\mu\text{g}$  per spot. The LLOD is 0.261  $\mu\text{g spot}^{-1}$  that represents concentration 13.05  $\mu\text{g mL}^{-1}$ , while LLOQ is 0.456  $\mu\text{g spot}^{-1}$  that represents a concentration of 22.8  $\mu\text{g mL}^{-1}$ .

**Precision and accuracy** The precision was investigated on four concentrations of dCER: 0.3  $\mu\text{g spot}^{-1}$  (15  $\mu\text{g mL}^{-1}$ , close to LLOQ), 0.7  $\mu\text{g spot}^{-1}$  (35  $\mu\text{g mL}^{-1}$ ), 0.9  $\mu\text{g spot}^{-1}$  (45  $\mu\text{g mL}^{-1}$ ), 1.4  $\mu\text{g spot}^{-1}$  (70  $\mu\text{g mL}^{-1}$ ). Each concentration was measured in three repeats on three different days. The obtained precision values were in the required range ( $\leq 15$  and  $\leq 20\%$  for LLOQ), see Table 5.

The accuracy was investigated with the same set of dCER quality control concentrations (0.3–0.7–0.9–1.4  $\mu\text{g spot}^{-1}$ ). The recovery was in the required range according to the normative documentation, range ( $\leq 15$  and  $\leq 20\%$  for LLOQ), see Table 5.

## Conclusion

A novel dimeric ceramide (dCER) was synthesized in order to stabilize the SC barrier. Sensitive, accurate, repeatable, robust and selective AMD-HPTLC and LC/APCI-MS methods were developed and validated for the identification and quantification of exogenous dCER in SC and deeper layers of the skin. The methods should also enable to study the permeability profile of dCER from various pharmaceutical dosage forms and cosmetic preparations. The concept of the method can be extrapolated for quantification of other CERs and SC lipids in the skin. In comparison to the AMD-HPTLC method, the HPLC/MS method is proposing a higher sensitivity.

Overall two methods have been developed and successfully applied for the quantification of dCER. Both methods are complimentary due to their different specifications (Table 6). With the application of both methods it is possible to cover a wide range of situations, where quantification of dCER is necessary, starting from substance research and drug formulation steps to quality control in manufacturing process and skin penetration studies.

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## Compliance with ethical standards

The experiments were accepted by the Ethical Committee of the Martin Luther University Halle-Wittenberg. The number of the permission is K16-15.

**Conflict of interest** The authors have no conflicts of interest to disclose.

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