Journal of Pharmaceutical and Biomedical Analysis 60 (2012) 7–13

Contents lists available at SciVerse ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development and validation of LC/ESI-MS method for the detection and quantification of exogenous ceramide NP in stratum corneum and other layers of the skin

Fitsum F. Sahle [a], Stefan Lange [b], Bodo Dobner [b], Johannes Wohlrab [c], Reinhard H.H. Neubert [a,*]

[a] Department of Pharmaceutical Technology and Biopharmaceutics, Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck Str. 4, D-06120 Halle (Saale), Germany
[b] Department of Pharmaceutical Chemistry and Clinical Pharmacy, Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck Str. 4, D-06120 Halle (Saale), Germany
[c] Department of Dermatology and Venereology, Medical Faculty, Martin Luther University Halle-Wittenberg, Ernst-Grube Str. 40, D-06120 Halle (Saale), Germany

ARTICLE INFO

Article history:
Received 25 July 2011
Received in revised form 27 October 2011
Accepted 28 October 2011
Available online 6 November 2011

Keywords:
Ceramide
Deuterated ceramide NP
Stratum corneum
Skin
LC/ESI-MS

ABSTRACT

Ceramides (CERs) are integral parts of the intercellular lipid lamellae of the stratum corneum (SC), which provide the barrier function of the skin. Administration of CERs deep into the SC may help to restore the barrier function in affected or aged skin. However, quantification of the amount of CER penetrated into the target site needs a selective and sensitive analytical method. Therefore, an LC/ESI-MS method was developed and validated for the detection and quantification of exogenous CER [NP] in the SC as well as other skin layers. The strategy involved synthesis of ceramide [NP]-D3-18 (deuterated CER [NP]) to distinguish it from the endogenous CER [NP] in MS on weight basis. The method was linear over 10–800 ng/ml and sensitive with a limit of detection (LOD) and limit of quantification (LOQ) of 3 and 10 ng/ml, respectively. It was also accurate with within-run and between-run percentage recoveries of 97.1–103.2 and 99.0–104.9, respectively. The within-run and between-run relative standard deviations (RSDs) were 0.9–5.4% and 2.1–7.4%, respectively, suggesting the method is precise. The method was highly selective and the matrix effect was too minimal with matrix factor (MF) mean and RSD values of 1.002 and 4.57%, respectively.

1. Introduction

Ceramides (CERs) are sphingolipids that contain a sphingosine (S), phytosphingosine (P), 6-hydroxysphingosine (H) or dihydrosphingosine (DS) moiety attached through an amide linkage to a nonhydroxy (N), α-hydroxy (A) or ester-linked ω-hydroxy (EO) long chain free fatty acid (FFA) [1–4]. "Ceramide XY" stands as the current rational nomenclature of CERs, where X and Y represent the type of the FFA moiety and the sphingoid base, respectively. Accordingly, CER [NP] is a sphingolipid that comprises a nonhydroxy acyl group linked with phytosphingosine moiety through an amide bond [1,3]. In addition, to date 11 CER classes, all the above possible sphingoid base and acyl group combinations except "CER EODS", are identified in human stratum corneum (SC) and each CER class further contains several types of CERs, which differ in their acyl group chain length and/or the type of esterified FFA in ω-esterified CERs [3,4].

CERs are integral parts of the intercellular lipid lamellae of the SC, which is responsible for the barrier function of the mammalian skin [1,5,6]. Many skin disease conditions, such as psoriasis [7], atopic dermatitis [8–10] and irritant/allergic contact dermatitis [8] are associated with depletion or disturbance of the level of CERs in the SC. Hence, administration of appropriate CER (s) into the lipid lamellae of the SC might help to prevent and treat such skin conditions and to that end there are many skin preparations on the market that contain CERs.

Some analytical methods were developed for the identification and quantification of CERs in the SC, which include LC/ESI-MS [11,12], LC/APCI-MS [13–15], AMD-HPTLC [16–18], LC-MS/MS [19,20] and HPTLC combined with LC/ESI-MS [21]. However, none of these methods can be used to discriminately identify and quantify the amount of exogenous CERs permeated into the SC, which is necessary for *ex vivo* or *in vivo* bioavailability studies that involve the skin.

Abbreviations: CER, ceramide; EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydrochinolin; FFA, free fatty acid; HAC, acetic acid; LOD, limit of detection; LOQ, limit of quantification; MF, matrix effect; SC, stratum corneum; SIM, selected ion monitoring; S/N, signal to noise ratio; RSD, relative standard deviation; THF, tetrahydrofuran.

* Corresponding author. Tel.: +49 345 5525000; fax: +49 345 5527292.
E-mail address: reinhard.neubert@pharmazie.uni-halle.de (R.H.H. Neubert).

0731-7085/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2011.10.032

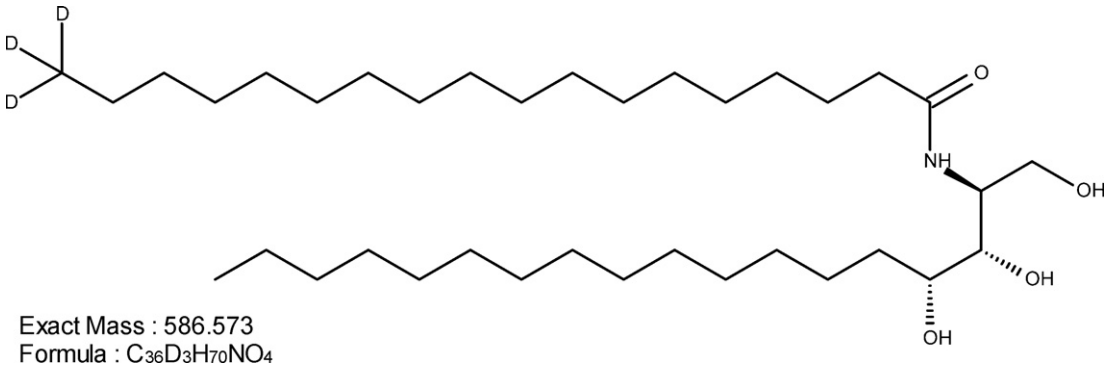

Fig. 1. Chemical structure of ceramide [NP]-D3-18 (deuterated CER [NP]).

LC/MS technique is a highly sensitive and selective technique that can be used for identification and quantification of minute quantities of analyte in biological fluids [22]. However, despite its sensitivity and specificity, it is not possible to differentiate between exogenous and endogenous compounds that have same chemical structures and molecular masses using LC/MS. Nonetheless, deuterated organic compounds can be detected using mass spectrometry with great sensitivity and are widely used to study the movement of drugs in the body [23].

Besides, quantification of compounds using LC/MS demands adequate degree of substance separation. Especially in ESI-MS lack of adequate degree of separation of compounds in a biological fluid might lead to a significant degree of ion suppression, which is referred as "matrix effect", and the reproducibility and accuracy of the method could be highly compromised [22,24]. Thus, analysis of biological fluids, such as SC extract, needs development of appropriate HPLC method for maximum separation of the substance to be quantified from the rest of the matrix.

Therefore, the aim of this work was to synthesize a deuterated CER NP (Fig. 1), which can be distinguished from the endogenous CER [NP] using a mass spectrometer and develop a sensitive and selective LC/ESI-MS method which helps to quantify trace amounts of the exogenous CER [NP] in SC and other skin layers.

2. Materials and methods

2.1. Materials

Octadecanoic-18,18,18-D3 acid was purchased from Dr. Ehrenstorfer GmbH, Augsburg, Germany. 2-Ethoxy-1-ethoxycarbonyl-1,2-dihydrochinolin (EEDQ) was obtained from Fluka, Buchs, Switzerland. Phytosphingosine (1,3,4-Octadecanetriol, 2-amino-, (2S, 3S, 4R)-) was generously donated by Evonik Goldschmidt GmbH, Essen, Germany. Chloroform and silica gel 60, 0.06–0.200 mm, were obtained from Merck KGaA, Darmstadt, Germany. HPLC grade tetrahydrofuran (THF) was supplied by Sigma-Aldrich, Steinheim, Germany. Acetic acid (HAC) was from Grüssing GmbH, Filsum, Germany. Hexane was purchased from NeoLab Migge GmbH, Heidelberg, Germany. HPLC grade methanol was purchased from VWR International, Darmstadt, Germany. Absolute ethanol was supplied by Bundesmonopolverwaltung für Branntwein, Offenbach, Germany. Double distilled water was used throughout the experiment. A human thigh skin was kindly donated by the Department of Dermatology and Venereology of the Faculty of Medicine, Martin Luther University Halle-Wittenberg after approval by the independent ethics committee of the Faculty.

2.2. Methods

2.2.1. Synthesis of deuterated CER [NP]

For the synthesis of the deuterated CER [NP] 0.98 mmol 18,18,18-D3-octadecanoic acid, 1.46 mmol EEDQ [25], and 0.98 mmol phytosphingosine were dissolved in 30 ml ethanol and stirred continuously for 24 h at 55 °C. Additional amount of EEDQ, equivalent to 1 mmol, was further added to the reacting mixture and was allowed to react for additional 12 h. TLC was used to control the reaction process and following completion of the reaction process, the reacting mixture was concentrated to 1/5 of its original volume and 52.5 ml chloroform-methanol (20:1, v/v) was added to the mixture. Subsequently, the mixture was transferred into a separatory funnel and 100 ml of 3% HCl (36% v/v) aqueous solution was added and the mixture was thoroughly shaken. The formed emulsion was broken through filtration and the filter was rinsed twice with two 25 ml portions of warm chloroform. Finally, the two layers were allowed to equilibrate in a separatory funnel and the organic layer was removed, dried for 24 h over sodium sulfate, filtered and the organic solvent was evaporated leaving the synthesized deuterated CER [NP]. Chloroform was dried by refluxing over P_2O_5 for 2 h before use.

2.2.2. Column chromatography

Following its synthesis, the deuterated CER [NP] was purified using a column chromatographic technique. The column was filled with silica gel at a stationary phase to substance ratio of 100:1 (m/m) and was conditioned using chloroform. Then the substance to be purified was dissolved in chloroform and was slowly poured into the column. Increasing percentage of methanol in chloroform (200 ml 0%, 1%, 2%, 3%, 4% and 5% methanol in chloroform) was used as a mobile phase and about 30 ml chromatographic fractions were collected. The collected fractions were monitored using TLC technique. Chloroform was refluxed over P_2O_5 for 2 h before use.

2.2.3. Thin layer chromatography

TLC development was carried out by applying samples and reference substances on a TLC plate (TLC silica gel 60 F_{254} (5 cm × 10 cm), Merck KGaA, Darmstadt, Germany) using capillary tubes. The reference substance was dissolved in chloroform before application. The TLC plate was developed in a saturated chromatographic chamber containing chloroform-methanol (9:1, v/v) until the solvent front reached to the end of the TLC plate. Finally, the plate was removed from the chamber, dried and sprayed with aqueous solution of bromothymol blue as an indicator, which formed a yellowish molecular complex that turned blue when fumigated with ammonia. Whenever detection of the florescent active EEDQ was necessary, the TLC plate was observed under UV-lump before spraying it with the bromothymol blue solution. The Rf values of