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UHPLC-MS/MS method for the determination of the cyclic depsipeptide mycotoxins beauvericin and enniatins in *in vitro* transdermal experiments

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ABSTRACT

Currently, dermal exposure data of cyclic depsipeptide mycotoxins beauvericin and enniatins are completely absent with a lack of local skin and systemic kinetics, despite their widespread skin contact and intrinsic hazard. Therefore a sensitive and specific bioanalytical high-throughput UHPLC-MS/MS method was developed for the quantitative and simultaneous determination of cyclic depsipeptide mycotoxins beauvericin and enniatins (A, A1, B, B1, D, E, C/F) in human skin Franz diffusion cell samples. The limits of detection ranged between 10 and 17 pg/ml, while the total run time was only 4.5 min. There was no significant effect of endogenous skin compounds on the mycotoxin MS signal observed, and the accuracy (0.68–24.68% bias) and precision (0.57–10.70% RSD) were considered acceptable for our purposes. Moreover, it was demonstrated that these cyclic depsipeptides are stable for at least 7 days when formulated in different organic or aqueous mixtures. Finally, adsorption to glass did occur: at least 50% ethanol or acetonitrile is required to prevent significant adsorption effects, which could be as high as 45%.

1. Introduction

Studying the local pharmacokinetics of molecules through human skin is not only important within the pharmaceutical industry but also in the field of environmental toxicology. The skin, being the largest organ, is considered as a route of administration for topically applied medicines [1,2], but is also important in the dermal risk assessment of hazardous compounds, such as mycotoxins [3]. Both *in vivo* and *in vitro* methods can be used to measure the skin absorption. Laboratory animals (such as guinea pigs, rats, mice and pigs), readily available, provide indeed a reproducible, physiologically and metabolically intact test system to investigate the skin absorption of all kinds of compounds (*e.g.* pharmaceuticals, cosmetics, hazardous chemicals). However, these have also their limitations, *i.a.* inter-species variability, with often a higher permeability than for human skin. Therefore, human skin studies remain the "gold standard" by which all methods for measuring percutaneous absorption should be judged. However, given the extreme toxicity of some chemicals, such as mycotoxins, it is ethically unacceptable to use living human beings in the transdermal studies [3,4]. *In vitro* Franz diffusion cell (FDC) methods are currently the ideal alternative, since (i) it is possible to maintain the barrier properties of the stratum corneum in excised skin, (ii) there is good evidence that the obtained *in vitro* data are predictive for *in vivo* percutaneous absorption (*in vitro in vivo* correlation) and (iii) there are standardisation recommendations, guidelines and protocols on how to execute these diffusion cell studies available, proposed by both regulatory entities and committees of interested parties [4,5].

Cyclic depsipeptides are a large group of naturally occurring bioactive peptides. Some of these are secondary fungal metabolites, which are toxic to humans and animals, such as the emerging mycotoxins beauvericin (BEA) and enniatins (ENNs) [6–18]. Currently, dermal exposure data of these compounds are completely absent with a lack of local skin and systemic kinetics, despite their widespread skin contact and intrinsic hazard. BEA and ENNs are well-known *Fusarium* cyclic hexadepsipeptides, but they are also produced by other fungi such as *Beauveria* and *Paecilomysces* and *Alternaria*, *Halosarpheia* and *Verticillium* species, respectively [19–25]. These compounds, possessing cation-complexing

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ionophoric and lipophilic properties, which act as inhibitors of acyl-CoA: cholesterol acyltransferase, exert cytotoxic effects in various cell lines [6–9,11–15,17,18] and have different effects on the immune system [26–30]. Moreover, it was also demonstrated that BEA is genotoxic [6,10].

UHPLC-MS/MS analysis of these cyclic depsipeptides has only been reported in a few studies, all aiming for a multi-mycotoxin determination in food. Considering the abundance (up to 191 compounds) and diversity of the investigated mycotoxins, these methods have relatively long run times, *e.g.* up to 21 min [31–33]. Quantitative transdermal kinetics are characterised by multiple skin donors with sufficient replicates and time points, which result in a large amount of different samples, *i.e.* dose solutions, skin extraction samples and receptor fluid samples. Hence, there is a need for a sensitive, selective and rapid high-throughput method for analysis of these frequently low concentrated samples, obtained in each FDC experiment. During these FDC experiments, the analytes are also exposed to elevated temperatures for significant amounts of time, *i.e.* ±32 °C (mimicking the human skin temperature) during 24 h, indicating the importance of a stability study under these conditions. During analytical processes, adsorption of peptides, which is believed to be mostly due to non-covalent interactions and depending upon the experimental conditions, cannot only lead to significant loss of the analyte, but also to increased analytical variability [34,35]. The adsorption of these lipophilic cyclic depsipeptide analytes to the FDC glass wall, of which the quality differs from analytical volumetric glassware, was not yet investigated.

The goal of this study was to develop a sensitive, selective and rapid high-throughput bioanalytical method to quantitatively determine the cyclic depsipeptide mycotoxins beauvericin and enniatins (A, A1, B, B1, D, E, F or C) in different FDC samples, using Ultra High Performance Liquid Chromatography combined with electrospray ionisation (ESI) tandem Mass Spectrometry (UHPLC-MS/MS). In addition, stability and adsorption to glass under our *in vitro* test conditions were investigated as well.

2. Materials and methods

2.1. Chemicals and reagents

Mycotoxins beauvericin (BEA) and enniatin B (ENN B) were supplied by BioAustralis (Smithfield NSW, Australia), while the enniatin mixture (ENNs) was obtained from Cfm Oscar Tropitzsch (Marktredwitz, Germany). No formal ENN composition was supplied by the manufacturer, therefore the composition was experimentally determined by our group (Appendix A, Supplementary data): 43.8% ENN B, 34.4% ENN B1, 14.0% ENN A1, 3.6% ENN D, 1.8% ENN A, 1.8% ENN E and 0.4% ENN C or F. ULC-MS grade acetonitrile (ACN), formic acid (FA) and 2-propanol, used for preparation of the mobile phase, were purchased from Biosolve (Valkenswaard, The Netherlands). Ultrapure water (H_2O) was produced by an Arium pro VF TOC water purification system (Sartorius, Göttingen, Germany), resulting in ultrapure water of 18.2 MΩ × cm quality. Sigma–Aldrich (St. Louis, MO, USA) supplied 0.01 M phosphate buffered saline (PBS) and dimethyl sulfoxide (DMSO). Ethanol (EtOH), used for the dose solutions, was purchased from Merck (Darmstadt, Germany) and UHPLC grade ACN was bought from Fisher Scientific (Waltham, MA, USA). Pharma grade hydroxypropyl-β-cyclodextrin (HPBCD) was supplied by Cerestar (Mechelen, Belgium). This was used as a solubilising modifier to the receptor fluid (PBS), in order to guarantee sink conditions of the hydrophobic cyclic depsipeptide mycotoxins throughout the experiment [36].

2.2. Analytical method

2.2.1. Preparation of standard solutions

A separate stock solution of 100 μg/ml in ACN was prepared for BEA and the ENN mixture. ENN B (pure) was used as an internal standard (IS) for the determination of BEA, while BEA was used for the different enniatins present in the enniatin mixture. For each internal standard, a stock solution of 10 μg/ml in ACN was prepared and stored at −80 °C. For all experiments, except for the stability and adsorption tests, an internal standard was added to each sample, with a final IS concentration of 20 ng/ml. From these four stock solutions (BEA 100 μg/ml in ACN, ENNs 100 μg/ml in ACN, ENN B 10 μg/ml in ACN and BEA 10 μg/ml in ACN), the standard solutions were prepared by dilution in ACN–H_2O (70:30, v/v).

2.2.2. In vitro human skin Franz diffusion cell protocol

Briefly, the set-up consists of static Franz diffusion cells with a receptor compartment of 5 ml and an available diffusion area of 0.64 cm^2 (Logan Instruments Corp., New Jersey, USA). Human skin from the abdominal region is collected from patients who had undergone cosmetic reduction surgery, with informed consent and confidentiality procedures in place (University Hospital, Ghent, Belgium). Immediately after surgical removal, the skin is cleaned with 0.01 M PBS pH 7.4 and the subcutaneous fat is removed (OECD, 2004). The skin samples are wrapped in aluminium foil and stored at −20 °C for no longer than 6 months. Just before the start of the experiments, the full-thickness skin is thawed, mounted on a template and dermatomed using an electrical powered dermatome (Integra Life Sciences, New Jersey, USA). The skin samples are visually inspected for skin damage and are then sandwiched between the donor and acceptor chambers, with the epidermis facing upwards, making sure all air under the skin is removed. The whole assembly is fixed on a magnetic stirrer and the receptor fluid was continuously stirred using a Teflon coated magnetic stirring bar (600 rpm) to ensure sink conditions. Before starting the experiments, skin integrity is checked by measuring the skin impedance using an automatic micro-processor controlled LCR impedance bridge (Tinsley, Croydon, UK). Skin pieces with an impedance value <10 kΩ, a validated system-suitability cut-off, are discarded and replaced by a new piece [37]. The dose solutions are topically applied to the epidermal surface of the skin. Then, the donor chamber is covered with parafilm and the temperature of the receptor compartment is kept at 32 ± 1 °C. Samples (200 μl) are drawn at regular time intervals from the sampling port and are immediately replaced by 200 μl fresh receptor solution (the analytically determined assay values in the FDC samples are correspondingly corrected for these replenishments). At the end of the experiment (*i.e.* after 24 h), the skin surfaces are swabbed with cotton wool to remove the remaining donor solution and then epidermis and dermis are separated. These samples are analysed as well and are used to construct a mass balance.

2.2.3. Ultra high performance liquid chromatography

The chromatography platform consisted of an Acquity UHPLC equipped with a temperature controlled autosampler tray and column oven, thermostated at 25 °C (±5 °C) and 45 °C (±5 °C), respectively (Waters, Milford, MA, USA). Chromatographic separation was achieved on an Acquity UHPLC charged surface hybrid (CSH) C_{18} column (1.7 μm, 100 mm × 2.1 mm, 130 Å), attached to an Acquity UHPLC VanGuard pre-column (1.7 μm, 5 mm × 2.1 mm, 130 Å), both obtained from Waters. The injection volume was 10 μl and the needle wash consisted of DMSO-2-propanol-ACN (10:10:80, v/v/v). The isocratic flow rate was set to 0.6 ml/min, using ACN–H_2O (70:30, v/v) containing 0.1% FA and 0.1% 2-propanol as mobile phase. The run time was 4.5 min, of which the