ENNIATIN-CONTAINING MEDICINES FOR TOPICAL USE: QUALITY-BY-DESIGN RISK ASSESSMENT OF COMPOSITION VARIABILITY

Apr. Sven DETROYER

Master of Science in Industrial Pharmacy

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SUMMARY

Due to the very recent recognition and understanding of enniatins as mycotoxins, there is currently no information regarding their mucosal penetration capabilities despite their use in topical medicines.

The global aim of this project was to determine to what extent enniatins (fusafungine) in topical medicines (Locabiotal®) permeate through the oral mucosa. More specifically, the influence of variability in excipient composition on the mucosal permeability was determined by ex vivo in vitro transmucosal experiments.

As there was no readily available information concerning the quantitative composition of Locabiotal®, we have developed and validated a GC-FID assay method for the pharmacokinetic-relevant topical excipients ethanol and isopropylmyristate. In addition, we have also validated a UHPLC-MS/MS method for the determination of fusafungine. Afterwards, it was established that the batch-to-batch composition of isopropyl myristate and ethanol in Locabiotal® varied over the range of 91.6 ± 6.1% (V/V) and 1.7 ± 0.1% (V/V), respectively. Furthermore, we have observed that the fusafungine content level of each Locabiotal® batch was approximately 80% label claim, which was attributed to a possible ion suppression effect of the Locabiotal® matrix.

Next, using an ex vivo in vitro Franz diffusion cell set-up mounted with porcine buccal mucosae, we have concluded that ENNs permeate the porcine buccal mucosa when administered in 1 – 10% (V/V) EtOH in IPM formulations. Moreover, it was also established that, within the experimentally determined batch-to-batch compositional variability of isopropyl myristate and ethanol, no significant effects on the transmucosal behavior of fusafungin were observed, under the investigated in vitro experimental conditions.
Writing this thesis was quite an undertaking that could not have been accomplished without the help of a great deal of people, so I would like to express my gratitude to everybody who has aided me during this time.

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1 INTRODUCTION

1.1 EMERGING MYCOTOXINS: ENNIATINS AND BEAUVERICIN

1.1.1 Overview

Cyclic depsipeptides are a large group of natural occurring, structurally diverse cyclic peptides, characterized by the presence of at least one intra-molecular ester (depsi) bond instead of a peptide bond. They are claimed to possess a diverse range of biological activities such as antitumoral, antibiotic, immunosuppressive, antifungal and anti-inflammatory properties, eliciting interest regarding their therapeutic possibilities.

Enniatins (and the structurally related beauvericin) are of special interest because of their recent recognition and, more importantly, recent understanding of their role as mycotoxins (i.e. secondary metabolites, produced by filamentous fungi under certain environmental conditions, characterized by their toxicity for vertebrates and other animal groups in low concentrations). As a consequence of the limited data available, their presence is currently neither regulated nor routinely determined, although the frequency and health impact of these secondary metabolites are increasingly proven important. Hence, they are being referred to as “emerging” mycotoxins.

1.1.2 Occurrence

Beauvericin (BEA) was first isolated from a culture of the fungus Beauverina bassiana by Hamill et al. in 1969, whereas enniatins (ENNs) were first found in cultures of Fusarium orthoceras var. enniatinum by Gäumann et al. in 1947. The biosynthesis of these emerging mycotoxins is, however, not confined to these fungi only. Various fungal species such as Alternaria, Fusarium, Halosarpheia and Verticillium are known manufacturers of ENNs, whereas BEA is found in Paecilomyces fumosoroseus as well as in Fusarium and Beauveria species.

As these mycotoxin-producing fungal species are widely spread, grow on a vast amount of substrates and under a wide range of environmental conditions, human and animal exposure to these emerging mycotoxins cannot be disregarded. Within the scope of food safety, samples were collected from several Northwest European and Mediterranean
Introduction

countries to investigate their presence in different sorts of grains. The European Food Safety Authority (EFSA) concluded that these mycotoxins were found in all grains investigated, serving as an example for the extensive dispersion of above mentioned fungi.

1.1.3 Chemistry

ENNs A-G and BEA are cyclohexadepsipeptides (CHDPs) comprising of altering D-α-hydroxy-isovaryl and amino acid units. BEA contains N-methyl-phenylalanine elements, whereas ENNs A and B consist of N-methyl-valine and/or -isoleucine parts. Furthermore, ENNs C-G contain a mixture of N-methylated leucine, isoleucine and valine amino acids.

Figure 1.1: Structure of BEA and most common ENNs

All other ENNs (H-O) consist of various hydroxylated groups and amino acids, nevertheless they show high structural similarity with BEA and other ENNs by positioning the isovaryl-group on the amino acid element.

Due to N-methylation of the amino acids the labile hydrogen atom on the nitrogen atom is absent, which mediates two effects: (i) the disability to form stabilizing intramolecular hydrogen bonds, resulting in an increased flexibility and (ii) the non-ionic character of these fore mentioned mycotoxins. However, several polar groups contain free electron pairs through which they can behave as nucleophiles and form ion-dipole interactions with cations, hence their ionophoric properties.

A direct consequence of their flexibility is the ability to adjust their conformation to the exposed, i.e. polar or apolar, environment. Despite their amphiphilic properties, these
Introduction

CHDPs are considered fairly lipophilic due to the presence of apolar groups on the outer surface\textsuperscript{15}.

1.1.4 Biological activity

1.1.4.1 Overview

Both BEA and ENNs bear potential favorable pharmacological properties. A specific ENN mixture, fusafungine, is currently commercialized as a topically administered antibiotic for upper respiratory tract infections (1.3). BEA, on the other hand, was already renowned in traditional Chinese medicine for its anticonvulsant, antineoplastic and anticholesteremic actions\textsuperscript{3}.

Because of their recent discovery as mycotoxins, data regarding the toxicity, both in vivo and in vitro, are very limited\textsuperscript{3}. Many researchers, however, do agree on the primary mechanism of toxicity, i.e. the ionophoric activity which mediates changes in cell membrane permeability and/or disruption of the ion homeostasis\textsuperscript{3, 16}. \textit{In vitro} studies reported these emerging mycotoxins having antimicrobial, insecticidal, phytotoxic and cytotoxic properties on various organisms, tissues and cell lines. \textit{In vivo} experiments, on the other hand, are scarcer and exhibit less distinct toxic effects\textsuperscript{3}.

The toxicity of these mycotoxins should not only be additionally assessed in light of their possible therapeutic use, but, more importantly, also for the accidental dietary, respiratory, dermal and other exposure to such toxic fungal metabolites\textsuperscript{2}. For example, they are commonly found in various sorts of grains (e.g. wheat, barley, maize, etc.), through which they could enter the animal and/or human food chain\textsuperscript{3}. This is certainly the case for underdeveloped nations where food is poorly processed and stored, where malnutrition is an issue and where few regulatory agencies supervise the food safety. However, other populations are plausible subjects for mycotoxicoses as well, in lesser extent by food contamination, but more likely to occur due to the presence of high levels of molds (e.g. moldy buildings)\textsuperscript{2}.

1.1.4.2 Ionophoric properties

Thanks to their ionophoric (i.e. the ability to transport small ions across lipid barriers) properties, these pore-shaped compounds are able to mediate changes in membrane
Introduction

permeability and disruptions of ionic gradients. These ionophores form various sorts of complexes with cations. In addition to the more stable 1:1 complex, several other configurations such as sandwich complexes, with two (2:1) or three (3:2) ionophore molecules, and dimeric (2:2) complexes exist as well. Considering the abundance of ionophore molecules, the 2:1 sandwich complex is favored. The mechanism of action is considered to be twofold, i.e. via cation carrier and/or cation-selective channel. The latter principle is explained by the sandwich complex (2:1) as it penetrates into the phospholipid membrane, probably due to interaction with lecithin, forming a transmembrane spanning pore and allowing the cations to diffuse along their electrochemical gradient across the membrane. Cation carriers, on the other hand, bind cations and then undergo a series of conformational changes in order to transport the bound cation across the membrane.

1.1.4.3 Antimicrobial activity

A wide array of human, animal and plant pathogenic bacteria (both Gram-positive and Gram-negative) was found susceptible to the effects of BEA and ENNs, e.g. *Mycobacterium* spp., *Staphylococcus* spp., *Bacillus* spp., etc. The antibacterial mode of action is yet to be discovered, although involvement of the ionophoric activity has been suggested. Moreover, several studies reported the chemosensitizing properties of BEA as an additional factor as it inhibits the active efflux of other antibiotics (e.g. tetracyclines) by membrane transport proteins. Recently, in vitro antiviral activity has been demonstrated against the human immunodeficiency virus (HIV) for BEA and, to a lesser degree, for ENNs through inhibition of HIV-1 integrase.

1.1.4.4 Cytotoxic activity

The main mechanism of cytotoxicity for BEA (not confirmed for ENNs) was suggested to require two steps. First, BEA would increase the intracellular calcium (Ca\(^{2+}\)) concentration by exerting its ionophoric action, ultimately resulting in the impairment of the cell membrane after which BEA is able to penetrate the cell. Next, BEA molecules enter the nucleus and form complexes with DNA. Meanwhile, through yet to be discovered mechanisms, BEA causes various receptor-independent events (e.g. DNA damage) and the presence of cytosolic mitochondrial cytochrome c which in turn mediate the activation of caspase-3. Through an additional Ca\(^{2+}\) release from the endoplasmatic reticulum, this protein of the cysteine-dependent enzyme family activates calcium-dependent
endo nucleases, which in turn facilitates the fragmentation of damaged DNA segments (i.e.

apoptosis).26-28

Recently, one study underlined the importance of the mitochondria in the apoptosis
mechanism for BEA and ENNs. Under normal circumstances, BEA and ENNs strongly bind
intracellular K⁺, thus obtaining a net positive charge after which they will be drawn to the
negatively charged mitochondrial matrix through electrostatic attraction. Due to the K⁺
influx, a decrease in the mitochondrial membrane potential is achieved, which in turn
uncouples the oxidative phosphorylation and inhibits the respiration. In addition, these
ionophoric toxins mediate a K⁺ efflux from the cytosol to the extracellular space as well,
thereby depleting the cytoplasm of potassium and disrupting the cell’s homeostasis. Also,
swelling of the mitochondria has been reported due to the osmosis of water. Moreover,
mitochondria regulate the cellular homeostasis of calcium by pumping the excess of Ca²⁺ out
of the cytosol into their matrix. When exceeding its capacity (i.e. calcium retention capacity),
mitochondrial permeability transition pores (MPTPs) open to discharge the Ca²⁺ excess (and
cytochrome c) into the cytosol. It is established that ENNs and BEA decrease the
mitochondrion’s calcium retention capacity, and therefore potentiating the calcium-
dependent apoptosis mechanism (cf. previous paragraph). Also, oxidative stress has been
correlated with increased MPTP sensitivity to calcium in the mitochondrial matrix, which
therefore contributes to the cytotoxicity as well29. Thus, despite the absence of a K⁺ influx, a
Ca²⁺ influx can mediate a depolarization of the mitochondrial membrane potential as well,
that is through a direct MPTP-mediated approach.

Still, other mechanisms were observed as well, depending on the cell line used in the
cytotoxicity assays3. Not only normal cell lines were affected, but various cancer cell lines
were susceptible to their cytotoxic effects also, justifying their potential as
chemotherapeutic3. Though, its brightest future may lie in the combination with other
chemotherapeutics, as BEA inhibits the multidrug transport protein in human cancer cells30.

1.1.4.5 Enzyme inhibition

Furthermore, ENNs and BEA showed inhibitory activity (IC₅₀ in low micromolar range)
against acyl-CoA:cholesterol acyltransferase (ACAT)31. This membrane-bound enzyme is
found in a variety of cells and tissues, catalyzing the esterification of cellular cholesterol. Due
to the decrease in polarity, a shift of cholesterol from the phospholipid bilayers to cytoplasmic lipid droplets is established, making ACAT a crucial element in the cellular cholesterol storage regulation\textsuperscript{32}.

Over the last two decades, the scientific community has attained a better understanding of the metabolic pathways underlying atherosclerosis, through which ACAT was identified as a promising target for antiatherosclerotic therapy. However, up till now, its potential falls short of expectations as several ACAT inhibitors failed in phase III clinical trials due to non-favorable clinical outcomes\textsuperscript{36}.

1.2 MUCOSAL PENETRATION

Locabiotal\textsuperscript{\textregistered}, the ENN-containing medicine of interest, is administered via the oral and nasal routes, after which the major disposition of enniatins occurs respectively at the oropharyngeal and nasal mucosae\textsuperscript{33,34}. Due to operational difficulties with oropharyngeal mucosae, ENN permeation will be experimentally assessed using the structurally similar oral (buccal) mucosae\textsuperscript{35,36}.

1.2.1 Oral mucosa

1.2.1.1 Structure

The oral mucosa acts as the lining of the oral cavity and includes the buccal, sublingual, gingival, palatal, and labial mucosae. Like the skin, its primary role is to preserve the underlying structures against mechanical stress and xenobiotics\textsuperscript{37}.

Underneath a mucus layer, the oral mucosa comprises an outermost layer of stratified squamous epithelium, which is separated from the lamina propria underneath by means of a basement membrane, and subsequently followed by a submucosa as innermost layer (\textbf{Figure 1.2}). Due to the presence of an active basal cell layer, the epithelial stratum (approximately 40 cell layers) possesses well-defined cell layers of varying degrees of differentiation. The epithelium starts at the basal layer, followed by the prickle cell layer (basal cuboidal cells), then several differentiating intermediate layers and ultimately bigger, flatter superficial cells at the surface of the epithelium\textsuperscript{38-40}. The lamina propria comprises collagen fibers, connective tissue, blood vessels, and smooth muscle\textsuperscript{40}. A connective tissue layer, \textit{i.e.} the submucosa, lies below the lamina propria and attaches the lamina propria to
the underlying bone or muscle structure. This layer is of variable thickness and density, but usually comprises various glands, blood and lymph vessels, nerves, and adipose tissue\textsuperscript{41}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image}
\caption{Schematic representation of the buccal layer composition (500-600 µm)}\textsuperscript{42}
\end{figure}

1.2.1.2 Mucus

The squamous epithelium is covered with a so-called mucus layer, which plays a role in the adhesion as well as the lubrication among cells\textsuperscript{43}. In the oral cavity, mucus is secreted as a part of saliva by major and minor salivary glands\textsuperscript{44}. At physiological pH, the mucus network bears a negative charge and attaches to the epithelial cell surface as a cohesive gel structure, thereby protecting the corresponding epithelia against mechanical, chemical and microbial insults\textsuperscript{39,43}. Saliva itself, however, provides a certain degree of protection too, as it continuously flows across the oral cavity, thereby limiting the substance’s contact time with the mucus layer\textsuperscript{40}.

Water (± 95%), glycoproteins and lipids (0.5 – 5%), mineral salts (0.5 – 1%), and free proteins (± 1%) are the main constituents of mucus\textsuperscript{37}. These macromolecular glycoproteins, known as mucins, consist of a large peptide backbone with oligosaccharide side chains and are indispensable in their function as protectors of the epithelial surface. The peptide core contains high amounts of serine, threonine, alanine, glycine and proline amino acids to which sugar residues are linked through glycosidic bonds. In general, N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose and sialic acid represent the major fraction of sugar residues found in mucin\textsuperscript{45}. About 25% of the polypeptide backbone lacks the presence of sugars, i.e. “naked” protein region, which is involved in the cross-linking of mucin molecules through disulfide bonds and, by extent, in the formation of its aggregated structure.
Nonetheless, physical entanglement and other interactions, e.g. hydrogen bonds, electrostatic and hydrophobic interactions contribute to its network as well.46, 47. Owing to their great water retention, viscous or even gel-like structures are established48.

1.2.1.3 Permeability

The permeability of the oral mucosa in general is intermediate between that of the epidermis and that of the intestinal mucosa, it is estimated to be 4 - 4000 times greater than the skin's permeability49, 50. This wide range of variability can be explained by the deviating structures of the skin and oral mucosae at different anatomical regions, e.g. thickness, keratinization, and other parameters51. Not keratinization per se, but the different composition of the membrane coating granules in keratinized versus non-keratinized cells is assumed to impact the permeability in a significant manner. During differentiation, the contents of these granules are released into the intercellular spaces by exocytosis. The intercellular matrix of keratinized epithelia disposes short stacks of neutral lipid lamellae, e.g. different types of apolar ceramides, which have been associated with the barrier function (i.e. water impermeable). In contrast, the non-keratinized epithelia contain less neutral, but more polar and amorphous lipids (e.g. cholesterol sulfate and glucosyl ceramides), which water is able to penetrate better52-57. Considering these factors, it is believed that the permeability decreases in the order of sublingual, buccal (both non-keratinized) and palatal (keratinized) mucosae39.

Even though the viscoelastic properties of mucus are found to be of the utmost importance for its physiological functions, it is not expected to influence drug diffusion across the gelatinous layer in a direct manner. In fact, the diffusion of a substance through mucus varies according to the size of the substance, the mesh size of the mucous layer, and potential interactions between the compound and the mucus45. In general, it can be assumed that diffusion of small, neutral molecules across the mucous barrier should not encounter any limitations, since even larger substances (e.g. antibodies) were reported as being transported relatively unimpeded across the same barrier58. The gel-structure as physical barrier seems to be of inferior importance in comparison to its potential interactions with penetrating drug compounds45.
In addition to the mucus layer’s mechanical barrier, the presence of a metabolic barrier in the oral cavity has been established as well. This barrier comprises oxidases, reductases, lipoxigenases, cyclo-oxygenases, phosphatases, carbohydrases, nucleases, esterases, and peptidases, most of which originate in the buccal epithelial cells. Phosphatases and carbohydrases, and lipoxigenases and cyclo-oxygenases are exceptions to the rule as they are respectively found in the saliva and inflammatory cells.\textsuperscript{59}

Permeability studies, using large molecular weight tracers, proclaim the surface cell layers of the epithelium to be the main barrier (due to presence of intercellular lipids) of mucosal permeation. Although the outer epithelial layers are generally considered to be the rate-limiting step in mucosal penetration, minor resistance to permeation may be offered by the basement membrane as well.\textsuperscript{39, 51}

\section*{1.2.2 Drug absorption}

\subsection*{1.2.2.1 Overview}

The buccal and sublingual mucosae are of main interest as they cover 60\% of the total oral mucosa surface area (200 cm\textsuperscript{2}) and possess a relative high permeability. Extensive mucosal vascularization (and high rate of blood flow) partially compensates for their limited surface area, as the diffusing substances reach the systemic circulation with great ease through capillaries and venous drainage (in sink conditions).\textsuperscript{39} Thus, due to the direct drainage of blood into the internal jugular vein, the gastrointestinal tract and first-pass metabolism in the liver are by-passed.\textsuperscript{60}

Still, in order to successfully reach the systemic circulation, previously discussed barrier mechanisms of the mucosa must be overcome. In that regard, several variables will influence the absorption efficiency, e.g. drug concentration and degree of ionization, vehicle, mucosal contact time, venous drainage, pH of the absorption site, and drug size and lipid solubility.

It is necessary to point out that this dissertation will only elaborate on the absorption of low-molecular weight substances, given the nature of our research question.
1.2.2.2 Pathways

Drugs diffusing from one side of an oral mucosa to the other will take the path of least resistance. This path is determined by the physicochemical properties of the diffusing drug, on the one hand, and the structure and organization of the mucosae, on the other hand. Due to the presence of the relatively hydrophilic intercellular matrix (1.2.1.3) and the lipophilic nature of the plasma membranes, the majority of the scientific community accepted the hypothesis for the existence of two major routes for drug penetration across the non-keratinized buccal and sublingual mucosae. According to this theory, drugs with high lipid solubility would be carried across the plasma membranes of the epithelial cells by means of partitioning and diffusion, whereas carrier proteins and/or lipoproteins would transport the lipophilic substances across the aqueous cytoplasm (transcellular route). In contrast, hydrophilic substances would be transported along the intercellular, hydrophilic matrix by means of passive diffusion (paracellular route). Despite the fairly hydrophilic nature of the intercellular area, water soluble drugs could experience significant resistance during penetration of the mucosa owing to the presence of tight junctions. However, in contrast to intestinal epithelium, their presence in the oral mucosa was reported as rare. Since there is no evidence for carrier-mediated transport mechanisms of lipophilic drugs within the buccal mucosa, their migration through the aqueous cytoplasm of epithelial cells and, by extent, the whole transcellular route, seems doubtful under normal circumstances.

Thus, a more appropriate classification for the orotransmucosal drug penetration mechanisms was suggested, using polarity as its determinant. Two pathways were reported for the transport of drugs across the oral mucosa, i.e. the polar and the non-polar route. Lipophilic drugs prefer the non-polar route as they move across the mucosa by partitioning into the lipid bilayer of the plasma membrane or into the lipids of the intercellular matrix. In contrast, the polar route mediates the passage of more hydrophilic drugs, as they penetrate through the aqueous channels in the intercellular spaces of the epithelium. Thus, under normal circumstances, it is believed that all components are transported along the intercellular, relatively hydrophilic matrix by means of passive diffusion, except for the highly lipophilic substances which may become associated with the lipophilic cell membranes.
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1.2.3 Chemical penetration enhancers

1.2.3.1 Overview

Due to the barrier function and limited surface area of the oral mucosa, various penetration enhancement strategies have been investigated in order to convey therapeutically significant doses of drugs to the systemic circulation. Several approaches have been studied, e.g. chemical penetration enhancers (CPEs), prodrugs, physical methods, etc. This paragraph will focus on CPEs, because of their relevance for the quality-by-design (QbD) risk assessment of Locabiotal®.

A chemical penetration enhancer is defined as a substance added to a pharmaceutical formulation to mediate an increase in membrane permeability or absorption rate of the drug of interest, without causing damage to the membrane. As passive diffusion was suggested as the primary mechanism of penetration across the buccal mucosa, penetration enhancement can be achieved by capitalizing on the variables in Fick's first law of diffusion (Eq. 1.1).

\[ J_{ss} = \frac{DK}{h} C_p \]  

(Eq. 1.1)
where: $J_{ss}$ steady state flux (mol cm$^{-2}$ min$^{-1}$)
$D$ diffusion coefficient of the drug of interest in the buccal mucosa (cm$^2$ min$^{-1}$)
$K$ partition coefficient between the buccal mucosa and the donor chamber solution
$h$ length of the diffusion route (cm)
$C_D$ concentration of the drug of interest in the donor compartment (mol cm$^{-3}$)

Thus, CPEs could improve the penetration of compounds by (i) enhancing their permeability through the buccal mucosa by means of increasing the diffusivity through the tissue and/or the partitioning into the tissue and (ii) increasing the concentration of the permeant at the mucosal surface$^{60}$.

1.2.3.2 Mechanisms

Overall, four main mechanisms for buccal mucosa penetration enhancement have been suggested in literature$^{60}$:

(i) Increasing the drug partitioning in the buccal mucosa ($K$),
(ii) Increasing the retention time of drugs at the buccal mucosa surface (bio adhesion) ($C_D$),
(iii) Interaction with squamous epithelial protein domains ($D$), and
(iv) Extraction of intercellular lipids ($D$).

This list, however, does not exclude other possible mechanisms for enhancing buccal drug delivery. Its potential as an alternative site for systemic drug delivery has only been thoroughly investigated over the two last decades. The mechanisms of skin chemical penetration enhancement, on the other hand, have already been studied extensively and have been mostly elucidated. Seen the structurally differences, it has been often erroneously assumed that skin penetration enhancers that only act on the disruption of intercellular lipid organization enhances buccal penetration as well. More research needs to be performed in order to clarify other possible mechanisms for buccal penetration enhancement$^{60}$.
Introduction

1.3 LOCABIOTAL®

Locabiotal® (Servier) is a topically administered antibiotic of fungal origin claiming to possess bacteriostatic properties against frequently present pathogenic Gram-positive cocci in the upper respiratory tract, i.e. *S. aureus*, *S. epidermis*, *S. pneumoniae*, *S. pyogenes*, *S. agalactiae*, and antifungal activity against *Candida albicans*. Noteworthy, resistant bacterial strains, in particular meticillin-resistant strains of *Staphylococcus aureus* (MRSA), were found susceptible to the effects of Locabiotal® as well. The antibiotic spectrum has been defined by a Minimum Inhibitory Concentration (MIC) lower than 40 µg/ml.

The API of Locabiotal®, fusafungine, is extracted from the fungus *Fusarium laetarium WR strain 437*, and comprises an enniatin mixture of 43% ENN B, 31% ENN B1, 15% ENN A1, 4% ENN D, 3% ENN A, 3% ENN E and 1% ENN C or F. In addition to its antibiotic activity, a local anti-inflammatory effect via diverse mechanisms has been established.

Both effects contribute to the improvement of upper respiratory tract infections and inflammations such as sinusitis, rhinitis, pharyngitis, tonsillitis, laryngitis, pharyngitis and tracheitis.

The Summary of Product Characteristics (SmPC) lists the qualitative excipient composition of Locabiotal® as follows: saccharin, ethanol, flavor composition 14869 (isoamyl acetate, anise alcohol, pure ethanol, extracts of star anise – caraway – clove – coriander – tarragon – Chinese mint – “petit grain Paraguay” – reddish brown pepper – rosemary, ethylvanillin, eucalyptol, piperonal, isopropyl myristate, propylene glycol, vanilla resin, vanillin) and isopropyl myristate quantum satis pro 5 mL.

Three excipients in its formulation are well known CPEs, i.e. ethanol, isopropyl myristate and propylene glycol. Nonetheless, since Locabiotal® aims to provide a local antibacterial and anti-inflammatory effect by precipitation of fusafungine on the mucosae, these excipients were most likely formulated for purposes other than their penetration enhancing effects (e.g. drug solubility in formulation).

The penetration enhancing effects of ethanol in the oral cavity were demonstrated by Squier, et al. for carcinogens in tobacco, later these effects have been confirmed for hydrophilic components on ventral tongue and porcine buccal mucosae. As their permeability was enhanced, ethanol was suggested to affect the intercellular lipids in the
Introduction

epithelium\textsuperscript{70, 71}. Moreover, the effects of ethanol have also been investigated for the apolar route of the skin. Several studies suggested that ethanol solvates the polar head regions of the lipid bilayer, thereby disrupting the interactions of the polar groups and the nearby apolar chains. This effect would ultimately result in an increase of the interfacial surface of the bilayer, which in turn promotes the diffusivity and partitioning of lipophilic permeants towards the lipid bilayer\textsuperscript{71}. Furthermore, high concentrations of ethanol have been correlated with the extraction of intercellular lipids in mouse skin and human buccal epithelia, after which the lipid domains were reorganized\textsuperscript{72, 73}. Also, the penetration enhancing effects of ethanol could be partially contributed to its ability to act on protein domains (e.g. denaturation, conformational changes, etc.), for example keratin\textsuperscript{74, 75}. In galenics, ethanol is also renowned as co-solvent for its solubility enhancing effect which indirectly enhances the permeability of solutes (cf. Fick’s law, Eq. 1.1)\textsuperscript{76, 77}. Lastly, solutes could be transported across the mucosae through a cotransport mechanism in the presence of ethanol\textsuperscript{78}. Although these mechanisms were more thoroughly investigated in transdermal experiments, many of them could apply for mucosal penetration as well.

Isopropyl myristate (IPM) has been reported to interfere with stratum corneum (SC) lipids by anchoring its isopropyl group into the polar regions of the lipid bilayer\textsuperscript{79}. Moreover, the incorporation of IPM in the SC lipid matrix, the extraction of several SC lipids and the perturbation of the multilamellar lipid assembly was established as well\textsuperscript{80}. Considering the lack of lamellar ordered lipid structures in the non-keratinized oral mucosa, it is plausible to assume that IPM only has a minor influence (extraction of SC lipids) regarding the penetration of solutes across non-keratinized oral mucosa. Nonetheless, recent findings has suggested that IPM could still play a role as penetration enhancer due to its ability to modify the drug’s solubility\textsuperscript{81}.

Propylene glycol (PG) has been widely used as co-solvent and transdermal penetration enhancer. Although its mechanism of penetration enhancement is not fully understood, several theories seem plausible. Most evidently, some researchers believe that its main penetration enhancement mechanism relies on its ability to function as a co-solvent, in combination with a carrier-solvent effect\textsuperscript{82, 83}. Nevertheless, a dehydration effect on the skin has been observed as well, which damages the epithelial layers, which in turn compromises its barrier function\textsuperscript{84}. Several studies also established the incorporation of PG
Introduction

into the hydrophilic regions of the packed lipids, creating a gap in the lamellar phase\(^{85, 86}\). Ultimately, there is no reason to believe that PG does not affect the penetration of solutes across the oral non-keratinized mucosae in a similar manner.
2 OBJECTIVE

The global aim of this project is to determine to what extent enniatins (fusafungine) in topical medicines permeate through the oral mucosa. More specifically, the influence of variability in excipient composition on the mucosal permeability will be determined by ex vivo in vitro transmucosal experiments.

The more specific thesis objectives are:

I. API and major excipient variability

Currently, there is no readily available information concerning the quantitative composition of Locabiotal®. Therefore, quality control analytics will be performed in order to assess the batch-to-batch consistency of fusafungine and its pharmacokinetic-relevant topical excipients.

II. Transmucosal experiments with different formulations

Due to the very recent understanding and recognition of enniatins as mycotoxins, there is no information regarding their mucosal penetration capabilities. In spite of the SmPC’s statement that fusafungine only acts locally, DruQuaR recently demonstrated significant transdermal absorption of enniatins. Therefore, the transmucosal permeation will be quantitatively explored. Moreover, the influence of the batch-to-batch compositional variability on the transmucosal permeation of fusafungine will be assessed. This study is following quality-by- design principles, where the patient is central in the experimental design.
3 MATERIALS AND METHODS

3.1 PART I: EXCIPIENT VARIABILITY

3.1.1 Chemicals and reagents

Isopropyl myristate was bought from Fluka (St. Louis, Missouri, USA). Ethanol (absolute) and acetone (HPLC grade) were acquired from Fisher Scientific (Waltham, Massachusetts, USA). Dimethylacetamide was purchased from Janssen (Beerse, Belgium). Locabiotal® samples (produced by Servier, Surenes, France) from five different batches were obtained through community pharmacies in Belgium.

3.1.2 GC-FID method description

Gas chromatography (GC) with flame ionization detection (FID) was used for the assay of isopropyl myristate (IPM) and ethanol (EtOH) in Locabiotal®. The PerkinElmer Clarus 600 gas chromatograph is equipped with a liquid autosampler and a programmable split/splitless capillary injector, and is operated by TotalChrom V6.3.2 software (Waltham, Massachusetts, USA). Both components were determined using the SE-54 (5%-phenyl)(1%-vinyl)-methylpolysiloxane capillary column (30m x 0.32mm x 0.25µm) from Agilent (Santa Clara, California, USA). Two independent methods, summarized in Table 3.1, were used for the assay of IPM and EtOH in Locabiotal®.

Table 3.1: Overview GC-FID method parameters for IPM and EtOH assay

<table>
<thead>
<tr>
<th></th>
<th>IPM</th>
<th>EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Agilent SE-54 (30m x 0.32mm x 0.25µm)</td>
<td></td>
</tr>
<tr>
<td>Carrier gas</td>
<td>Nitrogen gas</td>
<td></td>
</tr>
<tr>
<td>N₂ linear velocity</td>
<td>12.0 cm/s</td>
<td></td>
</tr>
<tr>
<td>Hydrogen for detector</td>
<td>40 mL/min</td>
<td></td>
</tr>
<tr>
<td>Air for detector</td>
<td>400 mL/min</td>
<td></td>
</tr>
<tr>
<td>Injection volume</td>
<td>1 µL</td>
<td></td>
</tr>
<tr>
<td>Split ratio</td>
<td>50:1</td>
<td></td>
</tr>
<tr>
<td>Injection temperature</td>
<td>250 °C</td>
<td></td>
</tr>
<tr>
<td>Detector temperature</td>
<td>250 °C</td>
<td></td>
</tr>
<tr>
<td>Oven temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 – 8 min</td>
<td>223 °C</td>
<td>0 – 7.5 min</td>
</tr>
<tr>
<td>7.5 – 13 min</td>
<td></td>
<td>100 °C (30°C / min)</td>
</tr>
<tr>
<td>13 – 20 min</td>
<td></td>
<td>265 °C</td>
</tr>
<tr>
<td>Retention time</td>
<td>7.7 min</td>
<td>4.2 min</td>
</tr>
<tr>
<td>Test concentration</td>
<td>100 ppm (V/V)</td>
<td>1‰ (V/V)</td>
</tr>
</tbody>
</table>
3.1.3 GC-FID method validation

The method validation is based on the ICH Q2 (R1) guideline.

3.1.3.1 Linearity of the equipment response

A linear relationship over the range of the analytical procedure was first assessed by visual inspection of the plot of signals as a function of the analyte concentration. Then, an appropriate statistical test (lack-of-fit F-test) was performed on the linear regression model, calculated by the method of least squares, to ensure its statistical significance. The correlation coefficient, y-intercept, slope of the regression line, residual sum of squares and lack-of-fit F test were submitted. A plot of the data was included as well.

Linearity was investigated for five concentration levels (I) with three replicates (J). The linearity was checked using 50, 75, 100, 125 and 150 ppm (V/V) IPM in acetone standard solutions and 0.50, 0.75, 1.00, 1.25 and 1.50‰ (V/V) EtOH in dimethylacetamide (DMAc) standard solutions. The summation of the three squared differences i.e. residual error sum of squares (SSr), pure experimental error sum of squares (SSe) and lack-of-fit error sum of squares (SSlof) were calculated according to Eq. 3.1 – 3.3. The degrees of freedom (DF) corresponding to these parameters were calculated as follows: $DF_r = [(I \cdot J) – 2]$, $DF_e = [(I \cdot J) – I]$ and $DF_{lof} = (I – 2)$.

\[
\begin{align*}
SS_r &= \sum_{i=1}^{I} \sum_{j=1}^{J} (y_{ij} - \hat{y}_i)^2 \\
SS_e &= \sum_{i=1}^{I} \sum_{j=1}^{J} (y_{ij} - \bar{y}_i)^2 \\
SS_{lof} &= SS_r - SS_e = \sum_{i=1}^{I} (\bar{y}_i - \hat{y}_i)^2
\end{align*}
\]

where:
- $y_{ij}$ experimental response
- $\hat{y}_i$ estimated response using the calibration curve
- $\bar{y}_i$ average response at every concentration level

The F-value was then calculated according to Eq. 3.4 and compared against the critical F-value at the 95% confidence level for I-2 degrees of freedom and IJ-J degrees of freedom in the nominator and denominator, respectively. When experimental F-value < tabulated F-value, the assumption of linearity between the peak area and concentration is confirmed as the null hypothesis ($H_0$: no significant lack of fit) could not be rejected.
Materials and Methods

\[ F_{I-2/IJ-I} = \frac{\sigma_{\text{lof}}}{\sigma_{\varepsilon}} = \frac{SS_{\text{lof}}/(IJ-I)}{SS_{\varepsilon}/(I-2)} \]

(Eq. 3.4)

where: \( \sigma_{\text{lof}} \) experimental variance
\( \sigma_{\varepsilon} \) lack-of-fit variance

3.1.3.2 Range

The range was derived from linearity studies and was established by confirming that the analytical procedure provided an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. The evaluated range for IPM was 75 – 125 ppm (V/V), while for EtOH this was 0.75 – 1.25‰ (V/V).

3.1.3.3 Accuracy

Accuracy was assessed using 9 determinations over 3 concentration levels covering the specified range. Accuracy was reported as the mean percent recovery (bias) by the assay of independent QC samples 75, 100 and 125 ppm (V/V) IPM and 0.75, 1.00 and 1.25‰ (V/V) EtOH (Eq. 3.5)\(^{12} \). The experimental concentration was calculated using the previously constructed calibration curve for IPM and EtOH, respectively. The acceptance criterion for bias was set at 3%\(^{89} \).

\[
\text{Bias (\%)} = \frac{\text{theoretical concentration} - \text{experimental concentration}}{\text{theoretical concentration}} \times 100\% \quad \text{(Eq. 3.5)}
\]

3.1.3.4 Repeatability

The injection repeatability was assessed for both components by determination of the relative standard deviation (coefficient of variation) on the data obtained by the accuracy validation procedure (3.1.3.3). The acceptance criterion for repeatability was set at 2%\(^{89} \).

3.1.3.5 Limit of detection and quantification

Both limits were assessed using the signal-to-noise approach over 3 concentration levels (in duplicate) covering the LOD (3 S/N) and LOQ (10 S/N) ranges. Calibration curves (ratio S/N as a function of concentration) were constructed and the concentration levels
corresponding to 3 and 10 times the S/N ratio were interpolated as respectively LOD and LOQ. The S/N ratios were calculated using the algorithm provided by TotalChrom.

The LOD and LOQ for the IPM method were determined with respectively 1, 2 and 5 ppm (V/V) IPM and 5, 10 and 20 ppm (V/V) IPM standard solutions. The LOD for EtOH was evaluated over 2.5, 5 and 10 ppm (V/V) EtOH range, while its LOQ was assessed using 10, 15, 20 ppm (V/V) EtOH samples.

The interpolated results were subsequently validated by the independent analysis of a suitable number of samples (3) at the determined LOD and LOQ.

3.1.3.6 Specificity
A suitable identification test was performed to discriminate between compounds of closely related structures. The specificity for EtOH was investigated with methanol (MeOH) via visual comparison of standard chromatograms.

3.1.3.7 Robustness
To ensure that the validity of the analytical methods over the time interval from method validation to quality control analysis of Locabiotal® was maintained, two system suitability parameters (i.e. response factor and retention time) of the analytes (i.e. IPM and EtOH) were established at suitable time intervals (i.e. beginning of method validation and beginning of QC assay of Locabiotal®).

3.1.4 Quality control: Locabiotal
Both IPM and EtOH content and their respective batch-to-batch variability in Locabiotal® were evaluated for five different batches, using the previously validated GC-FID methods (Error! Reference source not found.).

3.1.4.1 Experimental design
Reference solutions 50, 75, 100, 125 and 150 ppm IPM (V/V) in acetone and a blank solution (acetone) were injected at the start of the assay. After the first blank injection, three replicates of each batch sample were analyzed in a blocked design. Afterwards, the same reference solutions and blank solution were injected.
3.1.4.2 Preparation of test solutions

The test solutions were prepared as follows: Locabiotal® samples were first shaken for 15s to ensure homogenization after which the required quantities of each batch sample were sprayed into separate beakers.

Next, for IPM assay, 110 µL of each batch sample was transferred into separate 10.0 mL volumetric flasks which were filled to volume with acetone and shaken 20 times for homogenization (test solution 1: ± 1% V/V IPM). Then, 100 µL of each test solution 1 was added to separate 10.0 mL volumetric flasks which were subsequently filled to volume with acetone and shaken 20 times for homogenization (test solution 2: ± 100 ppm V/V IPM). This procedure was repeated in threefold in order to acquire three replicates per batch sample. An aliquot was taken of each test solution 2 and transferred to separate GC vials which were capped afterwards.

For the determination of EtOH, 500 µL of each batch sample was added to separate 10.0 mL volumetric flasks. The samples were subsequently diluted to volume with DMAc (± 1‰ V/V EtOH) and homogenized by shaking the volumetric flasks 20 times. This procedure was repeated in threefold in order to acquire three replicates per batch sample. An aliquot of each test solution was brought to separate GC vials after which crimp caps were applied to the vials.

The concentration IPM and EtOH in the test solutions was determined by means of the respective IPM and EtOH calibration curves (reference solutions).

3.1.4.3 Content

IPM and EtOH content in each Locabiotal® sample was evaluated in regard to their pre-set acceptance content limits at batch release (90 - 110% L.C.) (4.1.2). The overall mean IPM and EtOH concentration, obtained from 5 batches, was considered as target value (i.e. 100% L.C.) for IPM and EtOH, respectively.
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3.1.4.4 Batch-to-batch variability

One-way analysis of variances (ANOVA), homogeneity of variances (Levene’s test) and post hoc tests were performed on IPM and EtOH concentrations in the different batch samples at the 95% confidence level to assess the batch-to-batch composition variability of IPM and EtOH in Locabiotal®. The post hoc Bonferroni test was only justified after significant ANOVA results, when equal variances in concentration among the investigated samples were present. When the assumption of equal variances was violated, the post hoc Games-Howell test (for unequal variances) was applied.

Following hypotheses were set:

**Analysis of variances**

- **\( H_0 \):** No significant difference in mean IPM (EtOH) concentration among samples 1 – 5
- **\( H_A \):** Significant difference in mean IPM (EtOH) concentration among samples 1 – 5

**Levene’s test**

- **\( H_0 \):** No significant difference in variance of IPM (EtOH) concentration among samples 1 – 5
- **\( H_A \):** Significant difference in variances of IPM (EtOH) concentration among samples 1 – 5

**Post hoc Bonferroni/Games-Howell**

- **\( H_0 \):** No significant difference in mean IPM (EtOH) concentration between samples x and y
- **\( H_A \):** Significant difference in mean IPM (EtOH) concentration between samples x and y

3.2 PART II: API VARIABILITY

3.2.1 Chemicals and reagents

Beauvericin (BEA) and the enniatin (ENN) mixture were purchased from BioAustralis (Smithfield NSW, Australia) and Cfm Oscar Tropitzsch (Marktredwitz, Germany), respectively. The ENN composition (m/m) was previously determined by the DruQuaR group: 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. Sigma-Aldrich (St. Louis, Missouri, USA) supplied dimethyl sulfoxide (DMSO), while UHPLC grade ACN was acquired from Fisher Scientific (Waltham, Massachusetts, USA). UHPLC-MS grade acetonitrile (ACN), formic acid (FA) and isopropanol, used for preparation of the mobile phase, were purchased from Biosolve (Valkenswaard, The Netherlands). Ultrapure water (18.2 MΩ × cm) was produced by a Sartorius Arium pro VF TOC water purification system (Göttingen, Germany). Locabiotal® samples (produced by Servier,
Materials and Methods

Suresnes, France) from five different batches were obtained through community pharmacies in Belgium.

3.2.2 UHPLC-MS/MS method description

DruQuaR previously developed a sensitive, specific and high-throughput ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) bioanalytical method for the quantitative and simultaneous determination of cyclic depsipeptides BEA and ENNs (A, A1, B, B1, D, E, C/F) in \textit{in vitro} Franz diffusion cell samples\textsuperscript{12}, which was adopted for the assay of fusafungine in Locabiotal\textsuperscript{®}. The UHPLC-MS/MS platform combined an Acquity UHPLC with a Xevo TQ-S MS detector (Waters, Milford, Massachusetts, USA). Chromatographic separation was achieved using an Acquity UHPLC CSH C\textsubscript{18} column (1.7 µm, 100 × 2.1 mm, 130Å), attached to an Acquity UHPLC VanGuard pre-column (1.7 µm, 5 × 2.1 mm, 130Å), which were thermostated at 45°C (Waters, Milford, Massachusetts, USA). The samples were placed in an autosampler and thermostated at 25°C. An injection volume of 10 µL was programmed and the needle was pre- and post-washed with 10/10/80 (V/V/V) DMSO/isopropanol/ACN. The run time was set at 4.5 min with an isocratic flow rate of 0.6 mL/min, using 70/30 (V/V) ACN/H\textsubscript{2}O with 0.1% FA and 0.1% isopropanol as mobile phase. The mass spectrometer was operated in the positive electrospray ionization mode (ESI\textsuperscript{+}), with a capillary voltage of 3.50 kV and cone voltage of 50 V. Source and desolvation temperatures were established at 150°C and 600°C, respectively, while cone and desolvation gas flows were set to 150 and 1000 L/h, respectively. Data were acquired using Masslynx software (V4.1 SCN 843, Waters, Milford, Massachusetts, USA). Acquisition was performed in the multiple reaction monitoring (MRM) mode. The selected precursor and product ions, with the corresponding applied collision energies between brackets, are stated. The selected precursor ion for ENN B was \textit{m/z} 639.91 with two selected product ions at \textit{m/z} 196.08 (25 V) and \textit{m/z} 527.26 (22 V), for ENN D and B1 the precursor ion was \textit{m/z} 653.99 and \textit{m/z} 196.09 (23 V) and \textit{m/z} 541.05 (21 V) were the product ions. For ENN E and A1, \textit{m/z} 668.07 was the precursor ion and \textit{m/z} 209.99 (24 V) and \textit{m/z} 555.29 (21 V) were its product ions. ENNs A and C or F have a precursor ion of \textit{m/z} 682.47 with product ions \textit{m/z} 209.93 (26 V) and \textit{m/z} 555.01 (23 V). Lastly, BEA has a precursor ion at \textit{m/z} 783.94, with \textit{m/z} 255.04 (24 V) and \textit{m/z} 623.23 (23 V) as its product ions. For structure isomers (ENN D-B1, ENN E-A1 and ENN C/F-A) identical transitions were acquired.
3.2.3 UHPLC-MS/MS method validation

The UHPLC-MS² method (3.2.2) was validated for the assay of fusafungine in Locabiotal® based on the ICH Q2 (R1) guideline for linearity, accuracy and repeatability. The limits of detection and quantification were adopted from the bioanalytical method validation.

3.2.3.1 Linearity of the equipment response

A linear relationship over the range of the analytical procedure was first assessed by visual inspection of the plot of signals as a function of the analyte concentration. Then, an appropriate statistical test (lack-of-fit F-test) was performed on the linear regression line, calculated by the method of least squares, to ensure its statistical significance (3.1.3.1). The correlation coefficient, y-intercept, slope of the regression line, residual sum of squares and lack-of-fit F test were submitted. A plot of the data was included as well.

Linearity was investigated for five concentration levels with three replicates. The linearity was evaluated for each individual ENN in the mixture by construction of a linear regression line by the method of least squares using 1, 5, 10, 50, and 100 ng/mL ENN mixture in 70:30 ACN/H₂O (V/V) standard solutions.

3.2.3.2 Range

The range was derived from linearity studies and was established by confirming that the analytical procedure provided an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. The range was evaluated from 1 to 100 ng/mL ENN mixture.

3.2.3.3 Accuracy

Accuracy was assessed using 9 determinations over 3 concentration levels covering the specified range. Accuracy was reported as the mean percent recovery (bias) by the assay of independent QC samples 1, 5, 10, 50 and 100 ng/mL ENN mixture in 70:30 ACN/H₂O (V/V). The experimental concentration of the individual ENNs, needed for the calculation of the bias (Eq. 3.5), was determined by means of the corresponding calibration curve provided in the linearity experiment. The acceptance criterion for bias was set at 3%.
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3.2.3.4 Repeatability

The injection repeatability was assessed for both components by determination of the relative standard deviation (coefficient of variation) on the data obtained by the accuracy validation procedure (3.2.3.3). The acceptance criterion for repeatability was set at 10%.

3.2.4 Quality control: Locabiotal®

Fusafungine content and its batch-to-batch variability in Locabiotal® was evaluated for five different batches, using the previously validated UHPLC-MS² method (3.2.2). The label claim of fusafungine in Locabiotal® is 10 mg/mL.

3.2.4.1 Experimental design

Reference solutions 1, 5, 10, 50 and 100 ng/mL ENN mixture in 70:30 ACN/H₂O (V/V) and a blank solution (70:30 ACN/H₂O V/V) were injected at the start of the assay. After the first blank injection, three replicates of each batch sample were analyzed in a blocked design. Afterwards, the same reference solutions and blank were injected.

3.2.4.2 Preparation of test solutions

The test solutions were prepared as follows: Locabiotal® samples were first shaken for 15s to ensure homogenization after which approximately 400 µL of each batch sample was sprayed into separate beakers.

Next, 100 µL of each sample was transferred into separate 20.0 mL volumetric flasks which were filled to volume with 70:30 ACN/H₂O (V/V) and shaken 20 times for homogenization (test solution 1: ± 50 µg/mL FF). Then, 1.0 mL of each test solution 1 was added to separate 25.0 mL volumetric flasks and was diluted to volume with 70:30 ACN/H₂O (V/V) (test solution 2: ± 2 µg/mL FF). Finally, after homogenization, 625 µL of each test solution 2 was transferred to separate 25.0 mL volumetric flasks, diluted to volume with 70:30 ACN/H₂O (V/V) and homogenized (test solution 3: ± 50 ng/mL FF). This procedure was repeated in threefold in order to acquire three replicates of each batch sample. An aliquot was taken from each test solution 3 and transferred to separate HPLC vials (Waters, Milford, Massachusetts, USA) which were capped afterwards.
Materials and Methods

The concentration of the individual ENNs (B, D, B1, E, A1, C/F and A) in the test solutions was determined using their respective calibration curves (reference solutions).

3.2.4.3 Content

The fusafungine content in each Locabiotal® sample was evaluated in regard to the API content acceptance limits at batch release (95 – 105% L.C.)[^1]. The fusafungine content of each sample was determined using the calibration curve of the ENNs total peak area of the reference solutions. Fusafungine content was expressed as percentage label claim, according to Eq. 3.6:

\[
L.C. (\%) = \frac{C_{FF, batch \ sample}}{C_{FF, label \ claim}} \times 100\% \quad (Eq. 3.6)
\]

where:  
\( C_{FF, batch \ sample} \) concentration fusafungine in batch sample x (mg/mL)  
\( C_{FF, label \ claim} \) label claim concentration (10 mg/mL)

To determine the compliance of each batch sample to the specification limits, a one-sample two-sided t-test was performed to evaluate the statistical difference between the mean label claim (%) per batch sample and the acceptance limits (upper and lower) at the 95% confidence level. The null hypothesis (\( H_0: \) no significant difference between the mean label claim per batch sample (%) and the acceptance limits) was rejected if \( p < 0.05 \).

The relative enniatin distribution of fusafungine was also determined for each sample. The fraction (m/m) of every individual enniatin in fusafungine was calculated using Eq. 3.7 below.

\[
f_{ENN} = \frac{Area_{ENN}}{Area_{total \, ENN}} \quad (Eq. 3.7)
\]

where:  
\( Area_{ENN} \) peak area individual enniatin (B, D, B1, E, A1, C/F or A) in batch sample x  
\( Area_{total \, ENN} \) total peak area of enniatins in batch sample x
**Materials and Methods**

The direct use of ENN peak areas in the calculations is justified as no significant differences in ionization and fragmentation are expected among the structurally similar enniatins.

### 3.2.4.4 Batch-to-batch variability

One-way analysis of variances (ANOVA), homogeneity of variances (Levene’s test) and post hoc tests were performed on (i) the fusafungine concentration and (ii) its relative enniatin distribution in the different samples at the 95% confidence level to assess the batch-to-batch composition variability of fusafungine and its individual enniatins in Locabiotol®. The post hoc Bonferroni test was only justified after significant ANOVA results, when equal variances in concentration among the investigated samples were present. When the assumption of equal variances was violated, the post hoc Games-Howell test (for unequal variances) was applied.

Following null hypotheses ($H_0$) were set:

**Analysis of variances**

(i) $H_0$: No significant difference in mean FF concentration among samples 1 – 5  
(ii) $H_0$: No significant difference in mean ENN (B, D, B1, E, A1, C/F and A) fraction among samples 1 – 5

**Levene’s test**

(i) $H_0$: No significant difference in variance of FF concentration among samples 1 – 5  
(ii) $H_0$: No significant difference in variance of ENN (B, D, B1, E, A1, C/F and A) fraction among samples 1 – 5

**Post hoc Bonferroni/Games-Howell**

(i) $H_0$: No significant difference in mean FF concentration between samples x and y  
(ii) $H_0$: No significant difference in mean ENN (B, D, B1, E, A1, C/F and A) fraction between samples x and y

### 3.3 PART III: QUALITY BY DESIGN RISK ASSESSMENT

#### 3.3.1 Chemicals and reagents

The same beauvericin and the enniatin mixture reference materials were used as previously stated in section 3.2.1. Sigma-Aldrich (St. Louis, Missouri, USA) supplied GC grade dimethyl sulfoxide and 0.01 M phosphate buffered saline (PBS), while isopropyl myristate was bought from Fluka (St. Louis, Missouri, USA). Absolute ethanol and UHPLC grade
Materials and Methods

Acetonitrile was acquired from Fisher Scientific (Waltham, Massachusetts, USA). UHPLC-MS grade acetonitrile, formic acid and isopropanol, used for preparation of the mobile phase, were purchased from Biosolve (Valkenswaard, The Netherlands). Ultrapure water (18.2 MΩ × cm) was produced by a Sartorius Arium pro VF TOC water purification system (Göttingen, Germany). Hydroxypropyl-β-cyclodextrin (HPBCD) (pharma grade) was acquired from Cerestar (Mechelen, Belgium).

3.3.2 Method description

Enniatins (A, A1, B, B1, D, E, C/F) and beauvericin (as internal standard to the enniatins) in Franz diffusion cell (FDC) samples were quantitatively determined by the bioanalytical UHPLC-MS/MS method described in section 3.2.2.

![Figure 3.1: Standard type static Franz diffusion cell](image)

A static FDC set-up (Figure 3.1) with a receptor compartment of ± 5 mL and an available diffusion area of 0.64 cm² (Logan Instruments Corp., New Jersey, USA) was used for evaluating the penetration behaviour of enniatins across porcine buccal mucosae. The mucosal samples were harvested from freshly slaughtered pigs (Porc Meat, Zele, Belgium). After surgical extraction, the mucosae were cleaned with 0.01 M PBS pH 7.4, wrapped in aluminium foil and stored at -20 °C. A constant amount of enniatin mixture was dissolved in four different formulations of varying EtOH to IPM quantities (1, 3, 5 and 10 V/V% EtOH in IPM) to evaluate the potential mucosal penetration enhancing effects of these Locabiotal® excipients on enniatins. In the final stage of Locabiotal® formulation, IPM is added to volume and therefore its content variability is assumed mainly to be dependent on the content
variability of the other formulated components. This FDC experiment simulated the same conditions by adding IPM “as needed” (i.e. to volume) which simplified its design to a univariate approach, namely the content (V/V) ratio ethanol to isopropyl myristate. The experiments were replicated six times for each condition, each time using a different mucosa donor (n = 24).

3.3.3 Start-up of FDC experiment

Just before the start of the experiment, the full-thickness mucosa samples were thawed, dried and mounted on a template and subsequently dermatomed at 0.64 mm with an electrical powered dermatome (Integra Life Sciences, New Jersey, USA). The thickness of the mucosae was measured twice by means of a micrometer (Mitutoyo, Tokyo, Japan) after which the mucosae were visually inspected for pin holes and subsequently sandwiched, epithelial side facing upwards, between the donor and acceptor compartment of the FDC. Sink conditions were simulated by adding 1% HPBCD to the 0.01M PBS (pH 7) receptor fluid (RF) and by continuous magnetic stirring (600 rpm). Once prepared, the FDCs were filled with RF and the heating block and magnetic stirrer bar were turned on for equilibration before the start of the experiment. Next, 500 µL PBS was added to the dose compartment after which the integrity of the mucosae was checked using an automatic microprocessor-controlled LCR impedance bridge (Tinsley, Croydon, UK). The system suitability cut-off value for mucosal integrity was set at an impedance value of 10 kΩ, mucosa samples below this cut-off level were replaced by new pieces with satisfying integrity. Using cotton wool, the previously added PBS solution was removed from the donor compartment. While maintaining a constant enniatin mixture concentration of 1 mg/mL, dose solutions were prepared with 1, 3, 5 and 10% (V/V) EtOH in IPM. These dose solutions (400 µL) were topically applied to the epithelial side of the mucosa, after which the donor compartments were sealed with a parafilm and the receptor fluid (37 ± 1°C) was accurately brought to volume.

The applied concentration of enniatins was experimentally determined in duplicate: 100 µL of each of the remaining dose solutions was 1:10 000 diluted with 70:30 ACN/H₂O (V/V) and spiked with beauvericin as internal standard (IS). An aliquot of each solution was transferred to separate HPLC vials (Waters, Milford, Massachusetts, USA) for UHPLC-MS² analysis.
3.3.4 Sampling during FDC experiment

Aliquots (200 µL) of the receptor fluid were drawn via the sampling port at specified time intervals (0.5h, 1h, 2h, 3h, 4h, 6h and 8h) after which the RF was immediately replenished with 200 µL fresh receptor fluid solution. The RF samples were subsequently 1:2 diluted in LoBind Eppendorf tubes (Eppendorf, Hamburg, Germany) with ACN, containing IS beauvericin, and aliquots were taken for UHPLC-MS² analysis.

3.3.5 End of FDC experiment

The mucosal penetration of the investigated mycotoxins was immediately stopped after collecting the last receptor fluid sample at the 8h interval by putting a small piece of cotton wool in the donor compartment, which absorbed the remaining donor solution and was used to construct the mass balance. The cotton wool swabs were collected per FDC in volumetric flasks and incubated overnight at 40 °C, while shaking (150 rpm) in a mixture of 70:30 ACN/H₂O (V/V). Afterwards, the swabs were 1:10 000 diluted with 70:30 ACN/H₂O (V/V) and spiked with beauvericin. Aliquots were transferred to separate HPLC vials for UHPLC-MS² analysis.

Next, the exposed mucosal area (i.e. 0.64 cm²) of each FDC was cut out with a scalpel, transferred to separate LoBind Eppendorf tubes and extracted overnight at 25°C, while shaking (750 rpm) in a mixture of 95/5 (V/V) ACN/H₂O. The solution was then centrifuged for 10 min at 20 000 rpm and an aliquot was drawn from the supernatant for UHPLC-MS² analysis.

3.3.6 Quantification

The FDC samples (i.e. donor start, RF, donor end and mucosa) were quantitatively determined in a blocked design, using the bioanalytical UHPLC-MS² method (3.3.2). Reference solutions 1, 5, 10, 50, 100, 500 and 1000 ng/mL ENN mixture in 70:30 ACN/H₂O (V/V) were prepared and analyzed at the start and end of each sample block. Calibration curves of the individual enniatins were constructed for each sample block and were subjected to linear regression analysis (F-test).
3.3.7 Kinetic evaluation

3.3.7.1 Kinetic parameters

The mucosal permeation parameters were calculated for each individual enniatin under 1, 3, 5 and 10% (V/V) EtOH conditions. Using the RF samples, flux curves (i.e. cumulative amount individual enniatin permeated as a function of time) were constructed for each FDC. Outliers were detected with the Grubb's outlier test and accordingly rejected from the curve. Linear regression was performed on the curves using the best fitting steady-state data (mainly between 2h – 8h). Linear regression analysis (F-test) was performed on the linear steady-state part of the curve.

The linear regression model was subsequently used for calculation of the transmucosal kinetic parameters. The secondary permeation parameters steady-state flux ($J_{ss}$), lag time ($t_{lag}$) and cumulative quantity ($Q$) were calculated with Eq. 6.1 - 6.3. The apparent primary permeation parameters permeability coefficient $K_p$, diffusion coefficient $D_m$ and partition coefficient $K_m$ were derived from the secondary permeation parameters, according to Eq. 6.4 - 6.6. Equations are enclosed in Attachment 1.

Since the transmucosal permeability was assessed in sextuplicate for each formulation condition, each kinetic parameter was examined for outliers using the Grubb's outlier test, and accordingly rejected from the experiment. Using ANOVA, differences in kinetic parameters between conditions 1, 3, 5 and 10% (V/V) EtOH were investigated at the 95% confidence level. When a significant difference was observed, Levene's test for homogeneity of variances and a post hoc test were performed for further data evaluation. The assumption of equal variances was correct when Levene's test provided for $p$ values > 0.05, which consequently allowed the use of the Bonferroni post hoc test. In the case of unequal variances ($p < 0.05$), the Games-Howell post hoc test was conducted.$^{90}$

3.3.7.2 Cumulative curve

For each individual enniatin, the influence of the different EtOH to IPM quantities was also investigated using the mean flux curves (i.e. mean cumulative amounts of the corresponding enniatin at each time interval). The calculated cumulative amounts were subjected to the same statistical treatments as was suggested for the kinetic parameters (3.3.7.1). The results were plotted in a chart with the standard error of the mean (SEM).
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3.3.7.3 Mucosal distribution

At the end of the experiment, i.e. 8h exposure time, the distribution of enniatins in the FDC cell was evaluated for each formulation. The ENN concentration in the donor compartment, the mucosa and the RF was plotted for each enniatin separately.

For assessment of the potential influence of the formulation in ENN mucosal distribution, the mucosal concentrations needed to be normalized since (minor) differences in actual applied concentration were experimentally established for each of the dose solutions. Assuming a linear relationship, the mucosal concentrations were normalized to estimate the mucosal concentration after application of 1 mg/mL ENN dose solution (Eq. 3.8).

\[
C_{\text{normalised mucosa}} = C_{\text{mucosa}} \cdot \frac{1 \text{ mg/mL}}{C_{\text{dose solution applied}}}
\]  

(Eq. 3.8)

where:
- \(C_{\text{normalised mucosa}}\) normalised ENN mucosal concentration (mg/mL)
- \(C_{\text{mucosa}}\) experimentally determined ENN mucosal concentration (mg/mL)
- \(C_{\text{dose solution applied}}\) experimentally determined ENN applied dose concentration (mg/mL)
4 RESULTS AND DISCUSSION

4.1 PART I: EXCIPIENT VARIABILITY

4.1.1 GC-FID method validation

Validation demonstrates, by evaluation of specific validation characteristics, that the proposed gas chromatographic method, when used correctly, is suitable for the quantification of Locabional® excipients IPM and EtOH.

4.1.1.1 IPM

The target test concentration of IPM was set to 100 ppm (V/V) in acetone after preliminary experiments concluded acceptable method repeatability was achieved at this concentration.

The linearity of the IPM method was evaluated over the 50 – 150 ppm (V/V) range, using five concentration levels: 50, 75, 100, 125 and 150 ppm (V/V). Linear regression using the method of least squares provided for a correlation coefficient $R^2$ of 0.995. In literature, the assumption of linearity is often wrongfully evaluated by inspection of the correlation coefficient $R^2$, which supplies insufficient statistical evidence for linearity. The Analytical Methods Committee suggests the use of the (lack-of-fit) F-test as reliable approach to assess linearity. The lack-of-fit F-statistic was determined at 1.34. At the 95% confidence level, this calculated F-value was lower than the tabulated value of 3.71, therefore the null hypothesis (i.e. no significant lack of fit for the linear regression line) could not be rejected ($p > 0.32$) which confirms the assumption of linearity between the peak area and the concentration IPM in the 50 – 150 ppm (V/V) range.

The establishment of accuracy and precision was investigated over the range of 75 to 125 percent test concentration (i.e. 75 to 125 ppm V/V IPM). The accuracy and injection repeatability were assessed using 75, 100 and 125 ppm (V/V) IPM in acetone QC samples. In the specified range of 75 – 125 ppm (V/V) IPM, the bias (Eq. 3.5) and injection repeatability (%RSD) ranged from -1.68 to 0.59% and from 1.86 to 4.15%, respectively. Acceptance criteria for bias and repeatability were previously set at 3% and 2%, respectively. It was concluded that the 75 – 125 ppm (V/V) IPM linear range passed the criteron for bias,
Results and Discussion

however, the repeatability values were slightly higher than the specification limits given by the Office of Regulatory Affairs in their guidelines for human drug analytical methods\(^9\), but were considered sufficient for our purposes.

The limit of detection and quantification were respectively found at 3.0 ± 0.9 (mean ± SEM, n = 3) and 12.2 ± 2.2 ppm (mean ± SEM, n = 3) (V/V) IPM.

Robustness was assessed using response factor and retention time as system suitability tests (SSTs). These tests were performed at the beginning of the validation procedure and at the beginning of the quality control analysis of Locabiotol\(^*\) samples. Retention time of IPM remained unchanged (\(t_{R} = 7.73\) min), whereas the response factor was in agreement with the injection repeatability (2.3%).

4.1.1.2 EtOH

The linearity was determined at the 0.5 – 1.5% (V/V) EtOH range. Linear regression using the method of least squares provided for a correlation coefficient R\(^2\) of 0.9986. Next, the linearity was checked using a lack-of-fit F-test \((H_{0}:\text{linearity between concentration EtOH and area})\) which resulted in an F-value of 0.981. At the 95% confidence level the critical tabulated F-value is 3.71. Since experimental F-statistic < tabulated F-value, the null hypothesis could not be rejected which provided for significant statistical evidence the assumption of linearity between peak area and concentration of EtOH, ranging from 0.5 to 1.5%, was correct.

Subsequently, the accuracy and injection repeatability were assessed by injecting three replicates of reference standards 0.75, 1.00 and 1.25% (V/V) EtOH in DMAC. In the specified range of 0.75 – 1.25% (V/V) EtOH, the bias (Eq. 3.5) and injection repeatability (%RSD) ranged respectively from 0.28 to 2.92% and from 1.52 to 5.28%. Adopting the same acceptance criteria as specified in section 4.1.1.1, the method passed the criterion for bias, but not for repeatability. Although the latter parameter was out of specification (OoS), it was considered sufficient for our purposes.

The limit of detection and quantification were respectively found at 4.2 ± 1.2 (mean ± SEM, n=3) and 16.2 ± 3.0 ppm (mean ± SEM, n=3) (V/V) EtOH.
Results and Discussion

Specificity of the EtOH method was assessed using methanol (MeOH) as compound with closely related structure. As discrimination between EtOH and MeOH was achieved by visual examination of standard chromatograms, the method was found suitable regarding its specificity.

Robustness was assessed using response factor and retention time as system suitability tests (SSTs). These tests were performed at the beginning of the validation procedure and at the beginning of the quality control analysis of Locabiotal® samples. Retention time of EtOH remained unchanged ($t_R = 4.21$ min), whereas the response factor was in agreement with the injection repeatability (3.4%).

4.1.2 Quality control: Locabiotal®

According to the European Medicines Agency (EMA) guideline on 'Specification and control tests on the finished product', excipients which affect the biological availability of active substances must be subjected to a quantitative determination in each batch. As there is reason to believe that both IPM and EtOH influence the bioavailability of fusafungine, we have adopted the excipient acceptance limits specified for preservatives (90 – 110% L.C.)

The principle of QbD analytics has been applied considering our main objective and the limited resources available. Only an estimation of the batch-to-batch composition variability was required for our purposes. However, in light of quality control for batch release, the developed analytical methods did not meet the required specifications and therefore need further optimization.

4.1.2.1 IPM

The concentration of IPM was experimentally determined in 5 different batches of Locabiotal® (with three replicates) of which the results are summarized in Table 4.1 below.

Table 4.1: IPM concentration in each sample (triplicate)

<table>
<thead>
<tr>
<th>Batch</th>
<th>$X_1$</th>
<th>$X_2$</th>
<th>$X_3$</th>
<th>Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>83.30</td>
<td>95.52</td>
<td>99.72</td>
<td>92.85</td>
<td>4.93</td>
</tr>
<tr>
<td>2</td>
<td>97.28</td>
<td>84.90</td>
<td>86.68</td>
<td>89.62</td>
<td>3.86</td>
</tr>
<tr>
<td>3</td>
<td>87.21</td>
<td>97.66</td>
<td>82.63</td>
<td>89.17</td>
<td>4.45</td>
</tr>
<tr>
<td>4</td>
<td>100.97</td>
<td>105.01</td>
<td>97.90</td>
<td>101.29</td>
<td>2.06</td>
</tr>
<tr>
<td>5</td>
<td>87.75</td>
<td>87.29</td>
<td>80.12</td>
<td>85.05</td>
<td>2.47</td>
</tr>
</tbody>
</table>


Results and Discussion

The overall average IPM concentration of Locabiotal® was experimentally determined at 91.60 ± 2.02% (V/V) (mean ± SEM). Assuming this overall average value as the target value, i.e. 100% L.C. IPM, its 90 – 110% variability is equivalent to 82.4 – 100.0% (V/V) IPM. Except for batch 4, all batches comply with the 10% pre-set composition variability limit. This overall value of 91.60 ± 2.02% (V/V) (mean ± SEM) will be taken into consideration for the transmucosal experiment.

A significant difference in IPM concentration among the different samples was observed at the 90% confidence level ($p_{\text{ANOVA}} = 0.094$). The assumption of equal variances in IPM concentration among samples 1 – 5 was justified ($p > 0.30$), which in turn allowed the use of the post hoc Bonferroni test. After reviewing the $p$-values of the post hoc Bonferroni test, the difference in IPM concentration was not found significant ($p > 0.10$). Compared to the post hoc Bonferroni test, ANOVA is a more sensitive statistical test as it identifies significant differences for combinations of means as well as differences between pairs of means. As we are only interested in the latter, the more conservative Bonferroni test was considered more appropriate. In conclusion, no statistical significant differences in IPM concentration were found between batches 1 – 5 ($p > 0.10$).

4.1.2.2 EtOH

The EtOH concentration was experimentally determined in 5 different batches of Locabiotal® (with three replicates) of which the results are summarized in Table 4.2 below.

| Table 4.2: EtOH concentration (% V/V) in five batch samples of Locabiotal® (triplicate) |
|---------------------------------------------|-----------|-----------|-----------|-----------|-----------|
| Concentration EtOH (% V/V)                | X₁        | X₂        | X₃        | Mean      | SEM       |
| Batch 1                                   | 1.68      | 1.44      | 1.43      | 1.51      | 0.08      |
| Batch 2                                   | 1.90      | 1.73      | 1.64      | 1.75      | 0.07      |
| Batch 3                                   | 1.81      | 1.63      | 1.63      | 1.69      | 0.06      |
| Batch 4                                   | 1.74      | 1.72      | 1.64      | 1.70      | 0.03      |
| Batch 5                                   | 1.71      | 1.68      | 1.64      | 1.68      | 0.02      |

The overall average EtOH concentration of Locabiotal® was experimentally determined at 1.67 ± 0.03% (V/V) (mean ± SEM). Assuming this overall average value as the target value, i.e. 100% L.C. EtOH, its 90 – 110% variability is equivalent to 1.50 – 1.84% (V/V) EtOH. Therefore, all batches comply with the 10% pre-set composition variability limit. This
Results and Discussion

Overall value of $1.67 \pm 0.03$ (mean ± SEM) will be taken into consideration for the transmucosal experiment.

In addition, no statistical significant differences in EtOH concentration were observed among the different samples ($p_{\text{ANOVA}} > 0.10$).

4.2 PART II: API VARIABILITY

4.2.1 UHPLC-MS/MS method validation

The UHPLC-MS/MS method adopted for quantitative fusafungine determination in Locabiotal® was previously developed and validated (in-house verification) for bioanalytical purposes\textsuperscript{12}. As changes in procedure (i.e. the switch from bioanalysis to analysis) require revalidation, analytical validation characteristics accuracy, repeatability and linearity were reassessed for analysis of ENNs in Locabiotal\textsuperscript{87}. The acceptance limits for these validation characteristics were adopted from Office of Regulatory Affairs (guidelines for human drug analytical methods) and European Directorate for the Quality of Medicines (qualification of mass spectrometers)\textsuperscript{89, 91}.

Calibration curves of the individual enniatins were constructed using the method of least squares on 5, 10, 50 and 100 ng/mL ENN mixture reference samples for the assessment of linearity. The 1 ng/mL ENN mixture standard was rejected from the calibration curves due to high injection repeatability variability (RSD: 28 – 77%), since most enniatins approached or dropped below their respective LOQ (35 – 70 pg/mL)\textsuperscript{12}. The linearity was evaluated within each individual component’s specified concentration interval, calculated using the enniatin composition provided in section 3.2.1. Acceptable correlation coefficients were obtained for all compounds ($R^2 > 0.995$)\textsuperscript{91}. Next, the linearity was checked by performing a lack-of-fit F-test ($H_0$: linearity between concentration of the individual ENNs and peak area) of which the results are rendered in Table 4.3 below. At the 95% confidence level with $DF_e = 4$ and $DF_{lof} = 2$, the experimental F-values were compared to the critical F value of 6.94. Since each of test values was lower than the critical value, the linear regression model constructed between the peak area and concentration enniatin was found to be a good fit for each of the individual enniatins in the mixture at their respective specified concentration intervals.
Results and Discussion

Table 4.3: Investigated concentration interval, determination coefficients and lack-of-fit (LoF) F values of the individual ENN compounds

<table>
<thead>
<tr>
<th>ENN</th>
<th>Concentration interval (ng/mL)</th>
<th>R²</th>
<th>LoF F values</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>2.08 - 41.61</td>
<td>0.9996</td>
<td>0.194</td>
</tr>
<tr>
<td>D</td>
<td>0.17 - 3.42</td>
<td>1</td>
<td>0.008</td>
</tr>
<tr>
<td>B1</td>
<td>1.63 - 32.68</td>
<td>1</td>
<td>0.011</td>
</tr>
<tr>
<td>E</td>
<td>0.09 - 1.71</td>
<td>0.9998</td>
<td>0.060</td>
</tr>
<tr>
<td>A1</td>
<td>0.67 - 13.30</td>
<td>0.9999</td>
<td>0.057</td>
</tr>
<tr>
<td>C/F</td>
<td>0.02 - 0.38</td>
<td>1</td>
<td>0.009</td>
</tr>
<tr>
<td>A</td>
<td>0.09 - 1.71</td>
<td>0.9983</td>
<td>0.197</td>
</tr>
</tbody>
</table>

Acceptance criteria for accuracy (bias) and repeatability were set respectively at 3.0% and 10.0%\(^9,14\). At the target test concentration (50 ng/mL), the method passed the criterion for injection repeatability (1.61 – 3.28%), but not for bias (-4.49 – 2.24%). Although the latter parameter was OoS, it was considered sufficient for our purposes.

The LODs of the individual ENNs have been adopted from the bioanalytical method validation\(^12\). As a consequence of their structural similarity, it was expected that all compounds have similar experimentally determined detection limits: 17 pg/ml for ENN B, 14 pg/ml for ENN D and ENN B1, 15 pg/ml for ENN E, ENN A1 and ENN A and 10 pg/ml for ENN C/F.

4.2.2 Quality control: Locabiotal

4.2.2.1 Label claim

The concentration of the individual ENNs (B, D, B1, E, A1, C/F and A) was determined in five Locabiotal\(^*\) batches. The results are tabulated for each batch sample in Attachment 2.

The fusafungine content in each Locabiotal\(^*\) sample was evaluated in regard to the API content acceptance limits at batch release (95 – 105% L.C.). Figure 4.1 depicts the recoveries (%) of the fusafungine label claim (10 mg/mL) for each batch sample in triplicate.

At 95% confidence level, none of batches met the lower acceptance limit of 95% L.C. Therefore, none of the examined batches passed the specification test for finished products regarding API content acceptability. The consistency by which the fusafungine concentration in each sample lies about 20% below label claim could be rationalized by an ion suppression effect of the Locabiotal\(^*\) matrix.
Moreover, it was established that samples 1 – 5 do not significantly differ to one another in terms of label claim (%) \((p > 0.10)\).

![Figure 4.1: Evaluation label claim (%) fusafungine in different batches of Locabiotal®. The target label claim (100%) is indicated by the green line, while the pre-set specification limits (95-105%) are denoted by the red lines.](image)

### 4.2.2.2 Relative enniatin distribution

The relative distribution of the different ENNs in fusafungine was determined in 5 different batches of Locabiotal®. The results are summarized in Table 4.4 and graphically represented per batch in Figure 4.2.

**Table 4.4: Relative ENN distribution (%) of FF, evaluated over 5 batches of Locabiotal®**

<table>
<thead>
<tr>
<th></th>
<th>ENN B</th>
<th>ENN D</th>
<th>ENN B1</th>
<th>ENN E</th>
<th>ENN A1</th>
<th>ENN C/F</th>
<th>ENN A</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>41.73</td>
<td>3.66</td>
<td>33.80</td>
<td>2.52</td>
<td>15.07</td>
<td>0.71</td>
<td>2.50</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td>0.13</td>
<td>0.04</td>
<td>0.08</td>
<td>0.02</td>
<td>0.14</td>
<td>0.01</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Results and Discussion

ENN B

ENN D

ENN B1
Results and Discussion

ENN E

ENN A1

ENN C/F
Results and Discussion

Figure 4.2: Relative ENN distribution (%) of fusafungine, investigated for five batches Locabiotal®

One-way ANOVA, homogeneity of variances and post hoc tests were performed on the relative ENN composition of fusafungine in order to evaluate the variability in relative distribution of the individual compounds between batches 1 to 5. It was established for ENN B, D, B1, E and C/F that samples 1 – 5 do not significantly differ to one another in terms of relative distribution at the 95% confidence level. Nonetheless, significant differences (p < 0.05) in relative distribution were found for ENN A1 between samples 1 and 5, and, for ENN A between samples 4 and 1/2 (Table 4.5).

Table 4.5: p-values of post hoc ANOVA analysis

<table>
<thead>
<tr>
<th>ENN A1</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>-</td>
<td>0.225</td>
<td>0.309</td>
<td>0.781</td>
<td><strong>0.007</strong></td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.225</td>
<td>-</td>
<td>1.000</td>
<td>1.000</td>
<td>0.563</td>
</tr>
<tr>
<td>Sample 3</td>
<td>0.309</td>
<td>1.000</td>
<td>-</td>
<td>1.000</td>
<td>0.413</td>
</tr>
<tr>
<td>Sample 4</td>
<td>0.781</td>
<td>1.000</td>
<td>1.000</td>
<td>-</td>
<td>0.161</td>
</tr>
<tr>
<td>Sample 5</td>
<td><strong>0.007</strong></td>
<td>0.563</td>
<td>0.413</td>
<td>0.161</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ENN A</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>-</td>
<td>1.000</td>
<td>0.403</td>
<td><strong>0.026</strong></td>
<td>0.828</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1.000</td>
<td>-</td>
<td>0.718</td>
<td><strong>0.045</strong></td>
<td>1.000</td>
</tr>
<tr>
<td>Sample 3</td>
<td>0.403</td>
<td>0.718</td>
<td>-</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Sample 4</td>
<td><strong>0.026</strong></td>
<td><strong>0.045</strong></td>
<td>1.000</td>
<td>-</td>
<td>0.669</td>
</tr>
<tr>
<td>Sample 5</td>
<td>0.828</td>
<td>1.000</td>
<td>1.000</td>
<td>0.669</td>
<td>-</td>
</tr>
</tbody>
</table>

However, as it concerns a distribution, an overall significant difference in ENN composition between batch samples can only be concluded when all enniatins differ significantly to one another. This is however not observed in the experiment, thus, it is
plausible to assume (with 95% confidence) that the fermentation process of fusafungine is not subjected to significant batch-to-batch variability regarding its relative ENN composition, nonetheless, this could not be demonstrated for ENN A and A1 at the 95% confidence level.

**4.3 PART III: QUALITY BY DESIGN RISK ASSESSMENT**

Quality by Design (QbD) is a science and quality risk-based approach to pharmaceutical development and manufacturing that aims to ensure the product quality (i.e. its predefined characteristics) through identification, explanation and management of all sources of variability. In the third part of this thesis, we evaluated the influence of the experimentally determined batch-to-batch composition variability of major Locabiotal® excipients IPM and EtOH on the transmucosal permeability of its API, fusafungine.

It has been established that the cyclic hexadepsipeptide mycotoxins ENNs permeate the porcine buccal mucosa when administered in 1–10% (V/V) EtOH in IPM formulations, assumingly via passive diffusion along the intercellular lipids or along the bilayer plasma membrane. The transmucosal kinetic parameters, and kinetic evaluation in general, could however not be assessed for ENN A and C/F as the concentration of these compounds in the receptor fluid samples was below their respective LOQs 50 and 33 pg/mL. All other enniatins followed the uni-directional steady-state principle, as shown in the flux curves where the mean cumulative amount ± SEM in the RF as a function of time was plotted for each individual enniatin at conditions 1, 3, 5 and 10% (V/V) EtOH. After 8 hours, only 0.006 – 0.048% (1% EtOH), 0.006 – 0.035% (3% EtOH), 0.008 – 0.053% (5% EtOH) and 0.008 – 0.053% (10% EtOH) of the applied dose was cumulatively found in the receptor fluid.

The transmucosal permeability parameters steady-state flux $J_{ss}$, lag time $t_{lag}$, cumulative quantity at 8h $Q_{8h}$, permeability coefficient $K_p$, diffusion coefficient $D_m$ and partition coefficient $K_m$ were calculated for each individual enniatin at conditions 1, 3, 5 and 10% (V/V) EtOH and are represented in Attachment 3. The data shows that the steady-state apparent permeability coefficients ($K_p$) and cumulative quantity at 8h ($Q_{8h}$) of the individual enniatins were inversely related to their log P values (ENN A1 $\approx$ ENN E > ENN B1 $\approx$ ENN D > ENN B), valid at each level of ethanol investigated. The enhanced permeability of the more hydrophilic compounds is possibly explained by their increased partitioning out of the dose solution into the mucosa, which was confirmed by increased $K_m$ values.
Results and Discussion

ENN B

ENN D

ENN B1

ENN E
Results and Discussion

Figure 4.3: Flux curves of ENN B, D, B1, E and A1. Mean ± SEM cumulative amount permeated ENN in the RF plotted as a function of time (sampling times: 0.5, 1, 2, 3, 4, 6 and 8h), using different formulation compositions: 1% EtOH, 3% EtOH, 5% EtOH, 10% EtOH.

Since the diffusion coefficient $D_m$ and partition coefficient $K_m$ are only estimated parameters, the permeability coefficient $K_p$ is the main transmucosal kinetic parameter of interest. In addition, its value is fairly constant over a range of concentrations and therefore the $K_p$ can be used for concentrations other than the one used in the experiment. This is particularly interesting for extrapolation to Locabiotal® which possesses a label claim of 10 mg/mL fusafungine, in contrast to the 1 mg/mL ENN mixture applied to the mucosae in this experiment. For each investigated formulation, the permeability coefficients $K_p$ of the individual enniatins are shown in Table 4.6.

No significant differences in the transmucosal permeability coefficient $K_p$, evaluated for each individual ENN, were observed between 1 and 10% EtOH (V/V) in IPM ($p > 0.05$) (Table 4.7). As a first approach, using one-way ANOVA, these results were confirmed by assessment of the cumulative amount enniatin permeated as a function of time. At each time interval, the cumulative amount of each enniatin permeated in the RF was statistically equal among the different investigated ethanol quantities ($p > 0.10$). However, a mixed-model ANOVA is more suitable, due to the within-subject time variable. Moreover, no
Results and Discussion

Statistical significant differences in normalized ENN mucosal concentration was found between the various formulations, evaluated for each individual compound (p > 0.05) (Table 4.8).

Table 4.6: Permeability coefficients Kp (cm/h) of investigated ENNs at different %EtOH formulations

<table>
<thead>
<tr>
<th></th>
<th>ENN B</th>
<th>ENN D</th>
<th>ENN B1</th>
<th>ENN E</th>
<th>ENN A1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% EtOH</td>
<td>Mean</td>
<td>4.36E-05</td>
<td>3.04E-05</td>
<td>1.62E-05</td>
<td>1.20E-05</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>4.34E-06</td>
<td>3.90E-06</td>
<td>2.33E-06</td>
<td>2.22E-06</td>
</tr>
<tr>
<td>3% EtOH</td>
<td>Mean</td>
<td>3.25E-05</td>
<td>2.14E-05</td>
<td>1.06E-05</td>
<td>7.95E-06</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>4.25E-03</td>
<td>3.31E-06</td>
<td>1.90E-06</td>
<td>1.44E-06</td>
</tr>
<tr>
<td>5% EtOH</td>
<td>Mean</td>
<td>4.75E-05</td>
<td>2.95E-05</td>
<td>1.79E-05</td>
<td>1.36E-05</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>3.95E-06</td>
<td>2.54E-06</td>
<td>2.18E-06</td>
<td>2.10E-06</td>
</tr>
<tr>
<td>10% EtOH</td>
<td>Mean</td>
<td>4.68E-05</td>
<td>3.47E-05</td>
<td>1.87E-05</td>
<td>1.45E-05</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>5.12E-06</td>
<td>4.77E-06</td>
<td>3.23E-06</td>
<td>2.96E-06</td>
</tr>
</tbody>
</table>

Table 4.7: p-values ANOVA: differences in mean permeability parameters between different investigated conditions (1, 3, 5 and 10% V/V EtOH)

<table>
<thead>
<tr>
<th></th>
<th>ENN B</th>
<th>ENN D</th>
<th>ENN B1</th>
<th>ENN E</th>
<th>ENN A1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jss</td>
<td>0.177</td>
<td>0.163</td>
<td>0.136</td>
<td>0.242</td>
<td>0.602</td>
</tr>
<tr>
<td>Q8h</td>
<td>0.114</td>
<td>0.172</td>
<td>0.338</td>
<td>0.618</td>
<td>0.703</td>
</tr>
<tr>
<td>Kp</td>
<td>0.092</td>
<td>0.118</td>
<td>0.117</td>
<td>0.208</td>
<td>0.602</td>
</tr>
<tr>
<td>tlag</td>
<td>0.099</td>
<td>0.299</td>
<td>0.295</td>
<td>0.105</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.8: Mucosal concentrations (mg/mL) of investigated ENNs at different %EtOH formulations after 8h. Normalized for 1 mg/mL applied ENN concentration.

<table>
<thead>
<tr>
<th></th>
<th>ENN B</th>
<th>ENN D</th>
<th>ENN B1</th>
<th>ENN E</th>
<th>ENN A1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% EtOH</td>
<td>Mean</td>
<td>1.63E-02</td>
<td>5.16E-02</td>
<td>4.71E-03</td>
<td>6.18E-03</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>2.48E-03</td>
<td>1.85E-02</td>
<td>1.08E-03</td>
<td>1.33E-03</td>
</tr>
<tr>
<td>3% EtOH</td>
<td>Mean</td>
<td>1.52E-02</td>
<td>4.48E-02</td>
<td>7.65E-03</td>
<td>6.10E-03</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>2.21E-03</td>
<td>1.41E-02</td>
<td>1.54E-03</td>
<td>1.09E-03</td>
</tr>
<tr>
<td>5% EtOH</td>
<td>Mean</td>
<td>2.81E-02</td>
<td>8.20E-02</td>
<td>1.62E-02</td>
<td>1.31E-02</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>4.58E-03</td>
<td>2.50E-02</td>
<td>3.54E-03</td>
<td>2.86E-03</td>
</tr>
<tr>
<td>10% EtOH</td>
<td>Mean</td>
<td>2.44E-02</td>
<td>7.36E-02</td>
<td>9.41E-03</td>
<td>1.07E-02</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>2.66E-03</td>
<td>1.81E-02</td>
<td>2.52E-03</td>
<td>1.69E-03</td>
</tr>
</tbody>
</table>

Biologically speaking, when considering the maximal variability observed in Figure 4.3 and 80 – 125% limits for bioequivalence provided by EMA\textsuperscript{96}, no significant biological differences are found between the different formulations.

Considering all results, it was concluded that the excipient variability, investigated for the range 1 – 10% (V/V) EtOH in IPM, has no significant effect on the transmucosal penetration behavior of ENNs, under our experimental conditions. As the experimentally determined EtOH batch-to-batch composition variability of 1.67 ± 0.06% (V/V) EtOH (95%
Results and Discussion

confidence level) lies within the investigated range, it can be therefore concluded that EtOH and IPM excipient variability of Locabiotal® has no significant effects on the transmucosal behavior of fusafungine, under the investigated in vitro experimental conditions.

However, as stated in section 1.2.1, the human in vivo situation has additional barrier functions which were not included in the investigated in vitro FDC model. A major barrier to transmucosal penetration is the continuous secretion of saliva in the oral cavity, which significantly reduces the retention time of substances and to extent the concentration gradient over the mucosal layer. Moreover, the oral cavity possesses a metabolic barrier which mediates the enzymatic degradation of a wide variety of substrates. In particular interest are peptidases and esterases as catalyzers of the cleavage of peptide and ester bonds in cyclic hexadepsipeptide enniatins. As stated previously, the mucus layer as physical barrier seems to be of inferior importance in comparison to its potential interactions with penetrating drug compounds. The barrier function of the mucus layer is particularly important for positive charged substances at physiological pH due to interactions with the negative charged sugar residues of the mucin glycoproteins, present in the mucus layer. Considering these factors, the mucus layer would only play a minor role as barrier to the fusafungine transmucosal penetration. However, the mucus layer could still be important as it consists of 95% H₂O. DruQuaR has previously established that the lipophilic enniatins are practically insoluble in water (S_{aq} = ± 0.3 mg/mL), and therefore probably would precipitate on the mucosae, as stated in the SmPC.

Nonetheless, assuming above mentioned barrier functions and considering the ex vivo in vitro differences with the in vivo situation, it is plausible to assume that amounts of fusafungine, however small, could penetrate across the mucosae and reach the systemic circulation. To confirm these suspicions, a small clinical trial should be conducted with Locabiotal® administration according to the SmPC recommendations and with timely collected blood samples, which should be analyzed with a high sensitive bioanalytical method (e.g. UHPLC-MS²).
5 CONCLUSION AND PERSPECTIVES

The global aim of this project was to determine to what extent enniatins (fusafungine) in topical medicines (Locabiotal®) permeate through the oral mucosa. More specifically, the influence of variability in excipient composition on the mucosal permeability was determined by ex vivo in vitro transmucosal experiments.

As there was no readily available information concerning the quantitative composition of Locabiotal®, we have developed and validated a GC-FID assay method for the pharmacokinetic-relevant topical excipients ethanol and isopropylmyristate. In addition, we have also validated a UHPLC-MS/MS method for the determination of fusafungine. Afterwards, it was established that the batch-to-batch composition of isopropyl myristate and ethanol in Locabiotal® varied over the range of 91.6 ± 6.1% (V/V) and 1.7 ± 0.1% (V/V), respectively. Furthermore, we have observed that the fusafungine content level of each Locabiotal® batch was approximately 80% label claim, which was attributed to a possible ion suppression effect of the Locabiotal® matrix.

Next, using an ex vivo in vitro Franz diffusion cell set-up mounted with porcine buccal mucosae, we have concluded that ENNs permeate the porcine buccal mucosa when administered in 1 – 10% (V/V) EtOH in IPM formulations. Moreover, it was also established that, within the experimentally determined batch-to-batch compositional variability of isopropyl myristate and ethanol, no significant effects on the transmucosal behavior of fusafungin were observed, under the investigated in vitro experimental conditions.

The human in vivo situation, however, has additional barrier functions which were not included in the investigated in vitro FDC model. Nonetheless, assuming these barrier functions and considering the ex vivo in vitro differences with the in vivo situation, it is plausible to assume that amounts of fusafungine, however small, could penetrate across the mucosae and reach the systemic circulation. To confirm these suspicions, a small clinical trial should be conducted with Locabiotal® administration according to the SmPC recommendations and with timely collected blood samples, which should be analyzed with a high sensitive bioanalytical method (e.g. UHPLC-MS²).
6 ATTACHMENTS

**Attachment 1:** Calculation of permeability coefficients (2 pages)

**Attachment 2:** Overview determined concentrations of individual ENNs and total ENN mixture (= FF) in five investigated Locabiotal® batches (1 page)

**Attachment 3:** Transmucosal permeability parameters for each individual ENN with conditions 1, 3, 5, 10% (V/V) EtOH (1 page)

**Attachment 4:** Concentration of the individual enniatins (log scaled) per compartment, calculated for 1, 3, 5 and 10% (V/V) EtOH (2 pages)
**Attachment 1:** Calculation of permeability coefficients (2 pages)

\[ J_{ss} = \frac{a}{A} \]  
(Eq. 6.1)

where:  
- \( J_{ss} \) steady-state flux \( \left( \frac{ng}{cm^2h} \right) \)
- \( a \) slope of the linear steady-state part of the curve \( \left( \frac{ng}{h} \right) \)
- \( A \) exposed mucosal area \( (cm^2) \)

\[ t_{lag} = -\frac{b}{a} \]  
(Eq. 6.2)

where:  
- \( t_{lag} \) lag time \( (h) \)
- \( b \) intersect axis cumulative permeated amount enniatin \( (ng) \)
- \( a \) slope of the linear steady-state part of the curve \( \left( \frac{ng}{h} \right) \)

\[ Q_{8h} = \frac{m_{RF}}{m_{applied}} \cdot 100\% \]  
(Eq. 6.3)

where:  
- \( Q_{8h} \) cumulative quantity at time interval 8h \( \left( \frac{m}{m\%} \right) \)
- \( m_{RF} \) cumulative amount enniatin permeated at time \( t \) \( (ng) \)
- \( m_{applied} \) amount enniatin applied to the mucosa \( (\frac{ng}{h}) \)

\[ K_p = \frac{J_{ss}}{C_v} \]  
(Eq. 6.4)

where:  
- \( K_p \) permeability coefficient \( \left( \frac{cm}{h} \right) \)
- \( J_{ss} \) steady-state flux \( \left( \frac{ng}{cm^2h} \right) \)
- \( C_v \) concentration enniatin applied to the mucosa \( \left( \frac{ng}{cm} \right) \)
where: 

\( D_m \) diffusion coefficient \((\frac{cm}{h})\) 

\( d \) mucosa thickness \((cm)\) 

\( t_{\text{lag}} \) lag time \((h)\) 

\( K_m = \frac{K_p \cdot d}{D_m} \) \hspace{1cm} (Eq. 6.6) 

where: 

\( K_m \) partition coefficient mucosa/dose-vehicle \((\frac{cm}{h})\) 

\( K_p \) permeability coefficient \((\frac{ng}{cm^2 \cdot h})\) 

\( C_v \) concentration enniatin applied to the mucosa \((\frac{ng}{cm})\)
Overview determined concentrations of individual ENNs and total ENN mixture (= FF) in five investigated Locabiotal® batches (1 page)

<table>
<thead>
<tr>
<th></th>
<th>Sample 1</th>
<th></th>
<th>Sample 2</th>
<th></th>
<th>Sample 3</th>
<th></th>
<th>Sample 4</th>
<th></th>
<th>Sample 5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration (ng/mL)</strong></td>
<td><strong>Mean (ng/mL)</strong></td>
<td><strong>Concentration (ng/mL)</strong></td>
<td><strong>Mean (ng/mL)</strong></td>
<td><strong>Concentration (ng/mL)</strong></td>
<td><strong>Mean (ng/mL)</strong></td>
<td><strong>Concentration (ng/mL)</strong></td>
<td><strong>Mean (ng/mL)</strong></td>
<td><strong>Concentration (ng/mL)</strong></td>
<td><strong>Mean (ng/mL)</strong></td>
</tr>
<tr>
<td>ENN E</td>
<td>0.778</td>
<td>0.907</td>
<td>1.234</td>
<td>0.973</td>
<td>0.957</td>
<td>1.012</td>
<td>1.004</td>
<td>0.991</td>
<td>0.962</td>
</tr>
<tr>
<td>ENN C/F</td>
<td>0.209</td>
<td>0.249</td>
<td>0.321</td>
<td>0.259</td>
<td>0.269</td>
<td>0.291</td>
<td>0.297</td>
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<td>ENN A</td>
<td>1.118</td>
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<td>0.965</td>
<td>1.135</td>
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<td>1.122</td>
<td>1.048</td>
<td>1.109</td>
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<tr>
<td>FF</td>
<td>35.863</td>
<td>42.118</td>
<td>35.876</td>
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<td>41.004</td>
<td>41.688</td>
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### Attachment 3: Transmucosal permeability parameters for each individual ENN with conditions 1, 3, 5, 10% (V/V) EtOH (2 pages)

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<tr>
<th>ENN B</th>
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<td>JSs</td>
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<td>SEM</td>
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<table>
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<tbody>
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<td>JSs</td>
<td>Q8h</td>
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<tr>
<td>mean</td>
<td>21.24</td>
</tr>
<tr>
<td>SEM</td>
<td>1.76</td>
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</table>

<table>
<thead>
<tr>
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<th>ENN B1</th>
</tr>
</thead>
<tbody>
<tr>
<td>JSs</td>
<td>Q8h</td>
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<tr>
<td>mean</td>
<td>19.43</td>
</tr>
<tr>
<td>SEM</td>
<td>2.13</td>
</tr>
</tbody>
</table>

- $J_{ss}$: Apparent steady state flux ($\mu$g/cm²/s)
- $Q_{8h}$: Apparent steady state flux ($\mu$g/cm²/s)
- $K_p$: Permeability coefficient ($\mu$g/cm²/s·mm Hg⁻¹)
- $t_{lag}$: Lag time (h)
- $D_m$: Membrane thickness (μm)
- $K_m$: Membrane permeability coefficient ($\mu$g/cm²/s·mm Hg⁻¹)

- **1% EtOH**
- **3% EtOH**
- **5% EtOH**
- **10% EtOH**
<table>
<thead>
<tr>
<th></th>
<th>Jss</th>
<th>Qrh</th>
<th>Kp</th>
<th>tiag</th>
<th>Dm</th>
<th>Km</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1% EtOH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>0.68</td>
<td>6.02E-03</td>
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<td>-</td>
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<td>SEM</td>
<td>0.12</td>
<td>8.74E-04</td>
<td>8.48E-07</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>3% EtOH</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>mean</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>SEM</td>
<td>0.18</td>
<td>1.74E-03</td>
<td>1.20E-06</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>5% EtOH</strong></td>
<td></td>
<td></td>
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<tr>
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<td>6.64E-06</td>
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<td>-</td>
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<tr>
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<td>1.59E-03</td>
<td>1.31E-06</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>10% EtOH</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>0.71</td>
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<td>6.16E-06</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SEM</td>
<td>0.12</td>
<td>1.15E-03</td>
<td>1.02E-06</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Attachment 4: Concentration of the individual enniatins (log scaled) per compartment, calculated for 1, 3, 5 and 10% (V/V) EtOH (2 pages)

ENN B

ENN D
**ENN B1**

- **1% EtOH**
  - Dose applied: $3.3 \times 10^5$
  - Dose left: $3.4 \times 10^3$
  - Mucosa: $1.9 \times 10^4$
  - RF: $4.9$

- **3% EtOH**
  - Dose applied: $3.3 \times 10^5$
  - Dose left: $2.5 \times 10^3$
  - Mucosa: $3.4$
  - RF: $3.4$

- **5% EtOH**
  - Dose applied: $3.5 \times 10^5$
  - Dose left: $5.4 \times 10^3$
  - Mucosa: $5.2$
  - RF: $4.7$

- **10% EtOH**
  - Dose applied: $3.0 \times 10^5$
  - Dose left: $2.9 \times 10^3$
  - Mucosa: $3.2 \times 10^3$
  - RF: $4.7$

**ENN E**

- **1% EtOH**
  - Dose applied: $1.6 \times 10^4$
  - Dose left: $1.8 \times 10^3$
  - Mucosa: $1.1 \times 10^2$
  - RF: $0.2$

- **3% EtOH**
  - Dose applied: $1.6 \times 10^4$
  - Dose left: $1.7 \times 10^3$
  - Mucosa: $1.0 \times 10^2$
  - RF: $0.1$

- **5% EtOH**
  - Dose applied: $1.7 \times 10^4$
  - Dose left: $1.8 \times 10^3$
  - Mucosa: $2.3 \times 10^2$
  - RF: $0.2$

- **10% EtOH**
  - Dose applied: $1.4 \times 10^4$
  - Dose left: $1.5 \times 10^3$
  - Mucosa: $1.6 \times 10^2$
  - RF: $0.2$
ENN A1

Concentration (ng/mL; LOG scaled)

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Dose applied</th>
<th>Dose left</th>
<th>Mucosa</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% EtOH</td>
<td>1.2E+05</td>
<td>1.4E+05</td>
<td>6.3E+02</td>
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</tr>
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<td>1.3E+05</td>
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<td>1.3E+05</td>
<td>1.7E+03</td>
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</tr>
<tr>
<td>10% EtOH</td>
<td>1.1E+05</td>
<td>1.1E+05</td>
<td>1.2E+03</td>
<td></td>
</tr>
</tbody>
</table>

Concentrations are given in ng/mL and are logarithmically scaled.
7 REFERENCES


References


References

33. Summary of Product Characteristics (SmPC) of Locabiotal (24 SEPT 2014).
34. Summary of Product Characteristics (SmPC) of Locabiosol (04 NOV 2014).
References

References


79. Brinkmann I, Muller-Goymann CC. (2003). Role of isopropyl myristate, isopropyl alcohol and a combination of both in hydrocortisone permeation across the human stratum corneum. Skin pharmacology and applied skin physiology, 16(6), 393-404.

80. Engelbrecht TN, Deme B, Dobner B et al. (2012). Study of the influence of the penetration enhancer isopropyl myristate on the nanostructure of stratum corneum lipid
References


87. ICH Topic Q2 (R1): Validation of Analytical Procedures: Text and Methodology (CPMP/ICH/381/95).


89. ORA LABORATORY PROCEDURE: Food and Drug Administration. APPENDIX 1 - ORA Validation and Verification Guidance for Human Drug Analytical Methods (ORALAB.5.4.5), August 2014.


91. European Directorate for the Quality of Medicines and Health Care (EDQM), Qualification of equipment–Annex 7: qualification of mass spectrometers, 2011, pp. 11.


References


96. EMA guideline on the investigation of bioequivalence. DOC REF CPMP/EWP/QWP/1401/98 Rev (Jan 2010).