A new method for in situ skin penetration analysis by confocal Raman microspectroscopy

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“Ein großer Teil der Sorgen besteht aus unbegründeter Furcht “
Jean-Paul Sartre

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Abbreviations

%	Percentage
°C	Celsius degree
µg/mL	Microgram per milliliter
µl	Microliter
µm	Micrometer
ATR	Attenuated total reflectance
a.u.	Arbitrary unit
AUC	Area under the curve
BE	Bioequivalence
BSA	Bovine serum albumin
C=O	Carbon oxygen double bond
C-C	Carbon-carbon bond
CCD	Charge-coupled device
CH_2	Methylene group
CH_3	Methyl group
cm	Centimeter
cm^{-1}	Per centimeters
CPE	Chemical penetration enhancer
CRM	Confocal Raman Microspectroscopy
et al.	Et alia, and others
FTIR	Fourier transform infrared spectroscopy
g	Gram
g/cm^2	Gram per square centimeter
g/cm^3	Gram per cubic centimeter
g/ml	Gram per milliliter
g/mm	Grooves per millimeter
h	Hour
HCl	Hydrochloric acid
HWN	High wavenumber
IR	Infrared spectroscopy
kg	Kilogram

MAD	Mean median deviation
min	Minute
min.	Minimum
mg	Milligramm
mm	Millimeter
mM	Millimole
MS	Mass spectroscopy
mW	Milliwatt
n	Number
NA	Numerical aperture
N-H	Nitrogen-hydrogen bond
nm	Nanometer
NMR	Nuclear magnetic resonance
O/W	Oil-in-water
OH	Hydroxyl group
PAMPA	Parallel artificial membrane permeability assay
PBS	Phosphate buffer saline
PCA	Principal component analysis
PCs	Principal components
PEG	Polyethylene glycol
pH	Negative log of activity of hydrogen ions
R^2	Correlation coefficients
ref.	Reference
rpm	Revolutions per minute
SB	Stratum basale
SC	Stratum corneum
SD	Standard deviations
SG	Stratum granulosum
SMD	Skin microdialysis
SNR	Signal-to-noise ratio
SS	Stratum spinosum
UV	Ultraviolet
Vis	Visible

Zusammenfassung

Hautpenetrationsuntersuchungen stellen eine Herausforderung dar, da die Möglichkeiten diese zu messen limitiert sind. Konventionelle Methoden sind häufig sehr arbeits- und ressourcen-intensiv, sowie unzureichend genau. Das Ziel dieser Arbeit war, ein Modell zur in situ, ex vivo Hautpenetrationsuntersuchung mittels konfokaler Raman Mikrospektroskopie (CRM) zu entwickeln. Im ersten Schritt wurde nach Vorbild einer Franz Diffusionszelle eine Penetrationszelle entwickelt, die direkt unter dem Raman Mikrospektroskop eingesetzt werden kann und kontinuierliche in situ Messungen ermöglicht.

Um die Leistungsfähigkeit des Modells zu evaluieren, wurde getestet, ob ein Einfluss durch Unterschiede in der Formulierungszusammensetzung auf die Hautpenetration des Wirkstoffes detektierbar ist. Der Zusatz von 1,2-Pentandiol zu einer wässrigen Koffein-Lösung führte zu einer deutlich erhöhten Koffein Hautpenetration. Weiterhin wurde die in-situ CRM-Messung zu Messungen konventionell inkubierter Haut verglichen, um eventuelle Einflüsse der Inkubation zu evaluieren. Beide Methoden führten zu vergleichbaren Ergebnissen, wobei die Zeitersparnis bei den in situ Messungen hervorzuheben ist. Zudem galt es die Untersuchungsergebnisse mit durch konventionelle Methoden untersuchten Daten zu vergleichen. Hierfür wurde die Hautpenetration von Retinol aus einer Emulsion mittels Klebebandabrisstechnik und CRM Messungen untersucht. Auch hier führten beide Methoden zu vergleichbaren Ergebnissen. Eine weitere Vergleichsuntersuchung wurde zur Hautpenetration von Koffein aus einem Hydrogel durchgeführt. Sowohl ex vivo als auch in vivo wurde mittels CRM und Klebebandabrisstechnik der Einfluss von 1,2-Pentandiol auf die Hautpenetration untersucht. Ebenso wurde frisch präparierte mit zuvor eingefrorener und aufgetauter Schweinehaut verglichen. Alle Methoden führten hier zu vergleichbaren Ergebnissen. Zusammenfassend bestätigen diese Ergebnisse das Potential, das CRM für Hautpenetrationsuntersuchungen hat. Im Vergleich zu konventionellen Methoden können zeit- und kosteneffizient verlässliche Hautpenetrationsprofile generiert werden, die

besonders im Hinblick auf die Formulierungsentwicklung oder -Optimierung interessant sind.

Summary

Skin penetration studies are a challenging topic, as the number of possible methods is limited. Conventional techniques are very resource- and time-consuming and lack in spatial resolution. The aim of this thesis was to develop a model for in situ, ex vivo skin penetration analysis by confocal Raman microspectroscopy (CRM). The first step was to develop an incubation cell, that can be fitted beneath a Raman microspectroscope to enable continuous in situ measurements.

To evaluate the model's effectiveness, the influence of the formulation composition on a drugs skin penetration was checked. The addition of 1,2-pentanediol on an aqueous caffeine solution led to a clear increase in caffeine skin penetration. Furthermore, a comparison between incubating and measuring in line and measuring after a conventional incubation step was done, to evaluate influences of the incubation step. Both methods produced comparable results, but with the in-situ measurements being way more time efficient. Also, results obtained by CRM measurements were compared to results obtained by conventional techniques. The skin penetration of retinol out of an emulsion was tested, using CRM and tape stripping. Both methods led to comparable results. Another comparative study was performed on skin penetration of caffeine out of a hydrogel. The influence of 1,2-pentanediol on caffeine skin penetration was tested, using CRM and tape stripping, ex vivo and in vivo. Also, freshly prepared porcine ear skin was compared to previously frozen and thawed porcine ear skin.

Generally, those results prove the big potential, CRM has in skin penetration studies. Compared to conventional techniques, reliable skin penetration profiles can be generated in a cost- and time-efficient way, which can be useful especially in terms of formulation-development and optimization.

List of publications

Publication 1

Richard Krombholz and Dominique Lunter, A New Method for In-Situ Skin Penetration Analysis by Confocal Raman Microscopy. Molecules. 2020. 25, 4222.
DOI: 10.3390/molecules25184222

Publication 2

Richard Krombholz, Yali Liu and Dominique Lunter, In-Line and Off-Line Monitoring of Skin Penetration Profiles Using Confocal Raman Spectroscopy. Pharmaceutics. 2021. 13, 67.
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Publication 3

Richard Krombholz, Stefanie Fressle and Dominique Lunter, Ex vivo – In vivo correlation of retinol stratum corneum penetration studies by confocal Raman microspectroscopy and tape stripping. International Journal of Cosmetic Science. 2022. 00, 1-10.
DOI: 10.1111/ics.12775

Publication 4

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Other publications

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Conceptualization, methodology, investigation, data curation, writing - original draft, visualization

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Conceptualization, methodology, writing - review and editing, resources, supervision, project administration, funding acquisition

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List of poster presentations

Poster presentation 1

13th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology (PBP), March 2022, Rotterdam, the Netherlands.

In-Situ Skin Penetration Analysis

by Confocal Raman Microscopy - A New Method

Richard Krombholz and Dominique Lunter

Poster presentation 2

Skin Forum 2022 Annual Meeting, June 2022, Malmö, Sweden

Ex vivo – in vivo correlation of Retinol skin penetration studies by confocal Raman microspectroscopy and tape stripping

Richard Krombholz and Dominique Lunter

1. Introduction

1.1 Background

Dermal preparations are among the most frequently prescribed and used drug delivery systems. The local treatment of numerous skin diseases is the most prominent usage of dermal drug preparations, protecting and emolliating the skin is a further field of action, especially in cosmetics. As drug delivery systems, also a transdermal drug delivery of systemically acting active ingredients, transdermal preparations play a major role in the treatment of multiple diseases. Although oral delivery systems are the preferred way for administrating a systemically acting drug, there are disadvantages, such as stability and solubility issues within the gastrointestinal fluids, or the first pass metabolism, which can be bypassed using dermal products [1].

In the approval process of an oral drug product, effectiveness-, and safety studies are mandatory. When it comes to generic drug formulations, proving bioequivalence (BE) of the generic to the original formulation is sufficient, as safety and effectiveness studies are available by the originator. For drugs with a systemic target, this can be achieved by established pharmacokinetic methods, usually by monitoring the plasma concentration of the active ingredient in a relatively small patient collective. In the case of topically applied drug formulations, there is an exception. When the target site is in the skin or the underlaying tissue, ideally no active ingredient should be detectable in the systemic circulation [2]. As monitoring the plasma concentration is not relevant and not possible, other ways of showing BE are required for topically applied drug delivery systems. Such a generic formulation must show the same efficacy and safety profiles as the original product in comparative clinical endpoint trials. As those are high in cost, as well as time consuming, the number of possible formulations to test has to be limited in advance, to avoid unnecessary expenses. This leads to a lack in generic competition, and shows, that non-clinical/in vitro approaches are required.

Ex vivo skin permeation and skin penetration methods provide pharmacokinetic data, which can be used to address the matter of proving BE between two

topically applied drug delivery systems [3]. But not only for proving BE, but also for the development and optimization of dermal products, good ex vivo models, providing reliable data are in need. As already mentioned, clinical endpoint trials are extremely high in cost and considering further ethical concerns, the final formulation should already be developed. Ex vivo skin penetration data can be useful in early stages of formulation development or while optimizing a dermal drug product, the impact of formulation composition on skin penetration properties is of high interest. Especially when trying to affect those properties by using skin penetration enhancers for example.

Unfortunately, the number of methods for investigating skin penetration of topically applied substances ex vivo is limited. Also, a lack of spatial resolution and a high labour intensely are disadvantages of conventional methods. Considered as gold standard, Franz diffusion cells are a useful tool, for incubating a suitable skin model with a dermal drug product. After this incubation step, the skin model can be analysed for drug content [4]. This analytical step is destructive for the most conventional methods, which results in a separate skin sample for each time point for every formulation to test. As to be discussed in the following chapters in more detail, confocal Raman microspectroscopy is a promising technique, to generate reliable, non-invasive ex vivo skin penetration data.

1.2. The skin

The skin with its large area of approximately 2 m^2 is the largest organ of the human body [5]. Its functions are various, particularly the barrier function is to mention, as the skin protects the body from external influences, hazards, mechanical impacts and controls the body temperature and the loss of water. An intact skin barrier is vital, still it is required to overcome that barrier when it comes to topically applied drug formulations, as the active ingredient must reach its target. Therefore, it is important to understand the structure of the skin and the stratum corneum (SC) properties [6].

1.2.1. Structure of the skin

The human skin consists of mainly three tissues, starting from the skin surface: the epidermis, dermis, and the hypodermis [7]. The epidermis can be further distinguished into stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS) and stratum basale (SB) [8]. The outermost layer, SC, forms the main barrier for external ingress of substances into the body. As it mostly contains cornified skin cells, so called corneocytes, from underlaying tissues, it is also called the horny layer. Corneocytes are terminally differentiated keratinocytes, which lose their cell organelles and cytoplasm in the process of cornification and consist mostly of keratin filaments. As they are embedded in a lamellar ordered lipid matrix, this structure is described best by a brick-and-mortar model. 10-15 layers of corneocytes, additionally linked by corneodesmosomes, form this highly effective barrier [9]. The mentioned keratinocytes are the main cell type of the viable epidermis, below the SC, comprising the SG, SS and SB. Those keratinocytes move upwards towards the SC in the process of proliferation. Furthermore, Langerhans cells, responsible for immune reactions, melanocytes, causing skin pigmentation and Merkel cells, containing touch receptors, are found in this part of the epidermis.

Below the epidermis, the dermis is providing elasticity and structure. It consists mostly of fibroblasts, macrophages, mast cells and matrix components, as collagen, elastin, causing strength and elasticity, as well as glycosaminoglycans and proteoglycans. Blood vessels, not only supplying the various cells, but also playing a role in wound healing and body thermoregulation, are found in the dermis, too. Also sweat glands, important in the regulation of the body temperature too, sebaceous glands and hair follicles are part of the dermis.

The hypodermis consists of fatty- and loose connective tissue, providing mechanical strength, energy storage and protects the body from external temperature influences. Nerve fibres and larger blood vessels are pervading the whole hypodermis [5].

1.2.2. Dermal absorption

The skin as our outermost barrier protects the body from external hazards. Simultaneously it is an important target for drug delivery, as well as a drug delivery route for systemic applications. Therefore, penetration into the skin, and permeation through the skin is desired to a certain extend. The main barrier for a topically applied drug to overcome is the SC, if the target is located in the viable epidermis or dermis, as well as for systemic acting drugs. After application, the drug is released from the formulation and distributes into the SC. Two major routes of penetration can be distinguished – the transepithelial and the appendageal route. The latter can be subdivided into the transglandular route, describing a pathway though the sweat glands, and the transfollicular route, via the hair follicles [10][11]. Although there is only a small share of these appendages on the skin, the transfollicular route is an interesting penetration pathway for larger or ionic molecules and plays a considerable role in the dermal absorption of active ingredients [10][7]. Within the transepithelial route, the trans- and the intercellular route can be distinguished, where the intercellular route is considered to be the predominant penetration route of topically applied substances [12]. Following the transcellular route, a drug must penetrate the hydrophilic interior of keratinocytes, as well as the surrounding SC lipids, these repeatedly alternations between hydrophilic and lipophilic regions explain why this route is subsidiary [12][13]. As the SC lipids, surrounding the corneocytes show amphiphilic characteristics, not only lipophilic molecules, but molecules with different polarities can also diffuse though this matrix, although the highly organized and tightly packed structure still forms an effective barrier. Therefore, a penetration route along the corneodesmosomes is the most likely pathway for hydrophilic molecules [13].

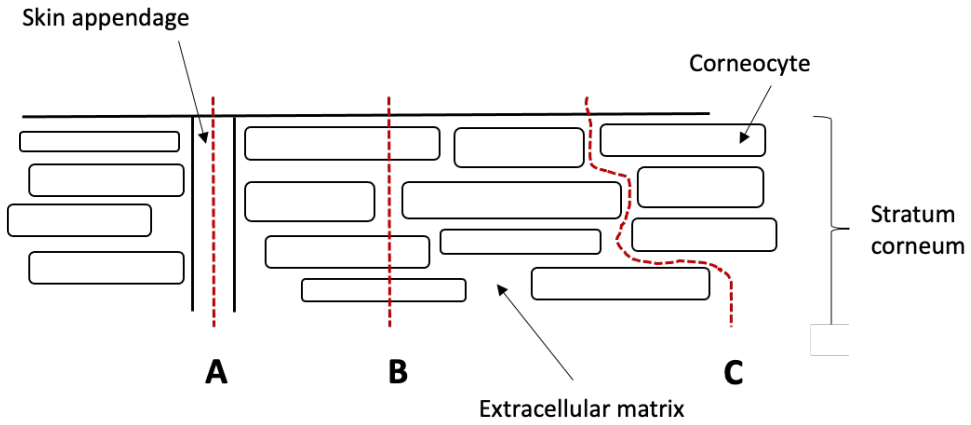

Figure 1: drug penetration pathways across the SC, showing the follicular route (A), the transcellular route (B) and the intercellular route (C).

After passing the SC, the viable epidermis and the dermis are the next structures a drug must penetrate, although the impact of these structures as barrier on drug penetration is considerably small, compared to the SC. When a drug diffuses from the viable epidermis into the dermis, the dense capillary network can adsorb the drug and lead to systemic effects, which is the aim for transdermal drug delivery systems. For a local treatment, this absorbance should be absent.

1.2.3. Skin penetration enhancers

The therapeutic success of topically applied formulation is often limited by poor skin penetration of the active ingredient. To increase skin penetration and hence increase the therapeutic efficacy of the formulation, various techniques have been developed. Physical approaches, such as electroporation, iontophoresis, or microneedles affect the skin barrier in a technically demanding way, which is not only laborious, but also affects the patient's compliance negatively [14][15][1]. Furthermore, vehicle-based approaches, such as micelles, microemulsions, or supersaturated solutions were made to increase skin penetration of an active ingredient [16][17]. Chemical penetration enhancers (CPEs) are the most popular way of increasing skin penetration during formulation development [18][19]. Also in this study, two different CPEs were used, 1,2-pentanediol and polyoxyethylene23-lauryl ether. There are various mechanisms improving drug penetration described for CPEs. For the intercellular penetration route, CPEs can

interfere with the lamellar lipid structure of the SC, resulting in a weakened skin barrier, due to a more fluid, disordered lipid state [20]. The penetration enhancing effect of 1,2-pentanediol, which was frequently used in this work, can be explained mainly this way. This structure can also be affected, by extraction of lipid components by CPEs, leading to an increased drug delivery [21]. This effect was used to improve the procaine-HCl skin penetration, by adding polyoxylene23-lauryl ether. An interaction of CPEs with the keratin filaments of corneocytes can improve the transcellular drug delivery [22][13]. Generally, the interaction of CPEs and SC is highly complex, which makes it impossible to demarcate a single mechanism for each respective CPE. Yet, it is important to understand their function, as a weakened skin barrier apparently has disadvantages too.

1.2.4. Ex vivo skin models

For ethical reasons, as well as for their high costs, in vivo skin penetration studies should be kept to a minimum. So, the objective of ex vivo skin models for skin penetration studies in general is to find the most realistic conditions, to obtain results, that can be transferred to in vivo conditions. When it comes to suitable ex vivo skin models, exercised human skin is ideal, as the structural properties remain the same [23][24][25]. In addition to ethical considerations, the availability of human skin is also limited, as it is mostly obtained from plastic surgery. This underlines the importance of alternative skin models. There are various skin surrogates developed and described, which can be classified into animal skin, artificial skin, and reconstructed skin [26]. Artificial- and reconstructed skin are designed to mimic physiological conditions. Although human derived keratinocytes are a major component, and they are commercially available, both lack in complexity compared to human skin. This makes them interesting for hazard analysis, regarding ex vivo skin penetration studies, other skin models are more suitable [26]. Animal skin models are prevalent, due to their broad availability and relatively low costs. When using animal skin, it is important to consider the highest possible similarity to human skin, regarding biochemical, histological, and physiological properties. Amongst the various animal skin models described, pig skin is

the most preferred. Rodent skin models, from which rat skin is considered to be the most suitable one, differ in many ways significantly from human skin. Despite showing a larger number of skin appendages, also the SC and dermis are distinctly thinner. Pig skin, especially porcine ear skin, but also skin from the flank show the highest similarities to human skin. Not only the thickness of the skin's respective layers, but also the SC's lipid composition, and the structure of hair follicles and sweat glands showing the same characteristics as human skin tissue. Similar penetration- and permeation properties have been shown for porcine ear skin in various studies [26][27]. Still there are differences, that have to be considered, most prominently human skin shows a higher barrier function than porcine ear skin [28].

In this project, porcine ear skin was used exclusively for all ex vivo experiments. Furthermore, the obtained ex vivo results were correlated to in vivo results on human skin. Also, the influence of freezing and thawing on porcine ear skin, regarding skin penetration of caffeine was tested, as for logistical reasons it is common to store skin samples frozen until the day of usage.

1.3. Skin penetration and permeation analysis

Following the rate and extend a drug penetrates into, or permeates through the skin, is an important challenge in the development of topically applied drug delivery systems. There are a few methods established so far, addressing this challenge, in vivo, and ex vivo. For testing those parameters ex vivo, Franz diffusion cells, are considered gold-standard [29][30]. As shown in Figure 2, diffusion cells in general consist of two components: an acceptor compartment and a donor compartment, separated by a membrane.

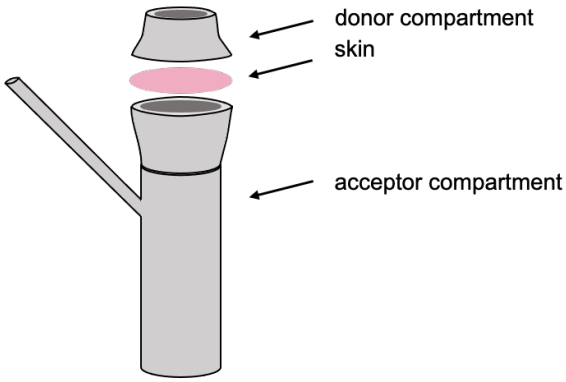

Figure 2: Franz diffusion cell.

The acceptor compartment is filled with a suitable medium, most prominently phosphate buffered saline (PBS). The sampling arm enables measuring the concentration of an active ingredient in the acceptor compartment. Another model for testing skin penetration, is the Saarbrucken penetration model, developed by Schäfer and Loth. Compared to a Franz diffusion cell, the Saarbrucken penetration model has no acceptor compartment, the skin is placed on a soaked filter disc [31]. This only allows skin penetration experiments, measuring skin permeation is not possible. Commercially available, there are also automatic diffusion cell systems, as for example the Phoenix robotic diffusion station (Teledyne Hanson Research, Chatsworth, USA), showing the same characteristic components as a regular Franz diffusion cell.

According to the design of the experiment, the membrane in a diffusion cell can be adapted for various purposes. When using synthetic membranes for example, Franz diffusion cells can also be used for measuring a formulation's in vitro release kinetics [32]. The various skin models mentioned in the previous chapter can all be used in Franz diffusion cells, where artificial and reconstructed skin surrogates, despite playing a minor role in skin penetration experiments, are being used for permeation analysis. As for the investigation of release kinetics, the experimental set up for testing skin permeation is the same when using Franz diffusion cells. The formulation to test is applied in the donor compartment on to the membrane, e.g., a suitable skin model, and samples of the acceptor compartment are collected at different time points and analysed for drug content. This

results in a concentration-time-profile of the drug in the acceptor medium. Afterwards, these profiles can be used, to calculate the flux of a drug through the skin. The parallel artificial membrane permeability assay (PAMPA) was developed as a time saving and cost-efficient way for testing permeation properties of topically applied drug delivery systems [33]. Based on a 96-well plate, using generally low volumes, determining a drugs permeation kinetics is highly efficient. A drawback is, that especially the low acceptor medium volumes can lead to a rapid increase in the drug concentration, which can affect the permeation properties. If the drug concentration in the acceptor compartment is close to its saturation solubility, the permeation decreases, leading to false low results. Furthermore, the artificial membranes used limit a transfer of the obtained results to in vivo conditions, as they do not represent the complexity of skin. Although there are modified diffusion cells available, enabling a continuous perfusion of the acceptor compartment, generally, Franz diffusion cells or PAMPA represent closed systems. Especially when following drug with limited water solubility, as most of the acceptor mediums in use are aqueous, this could be problematic. The analyst's concentration in the acceptor compartment should be below 10 % of its saturation solubility, to ensure sink conditions, otherwise the permeation flux is affected [34].

For determining the flux through the epidermis in vivo, skin microdialysis (SMD) is a promising tool [35]. For this minimally invasive technique, thin tubular dialysis membranes are inserted in the dermis or subcutis. The membranes are perfused with a suitable acceptor medium at low speed, which can be analysed for drug content subsequently [36]. Despite being minimally invasive, after inserting the membrane-tubes, the skin is in a diseased state, which could affect the results. If a drug's diffusion to the skin is limited, it can only be compensated by a low perfusion rate to a certain extent, as a certain concentration of the drug in the acceptor medium is crucial for quantification [36].

Skin penetration studies require a quantification of the drug within different skin layers, making the experimental set up more complex. As abovementioned, an incubation step in Franz diffusion cells is considered gold standard for ex vivo skin penetration studies [30]. For determining the amount of penetrated drug within the different SC layers, the skin has either to be segmented, for

extraction of the drug, or can be analysed by non-invasive techniques [37]. Conventionally, the skin sample is removed from the Franz diffusion cell, after the desired incubation time, and segmented for quantifying afterwards. After freezing the skin sample in liquid nitrogen, the segmentation step can be performed on a cryo-microtome. Fixed to a sample holder, by using a tissue embedding medium, the SC is cut into thin slices by a sharp blade [38]. The sectioned skin can be analysed for drug content in each respective slice after an extraction step. Depending on the analyte, a suitable solvent for extraction and a reliable quantification method must be chosen. Optical methods, such as fluorescence microscopy, Fourier transform infrared attenuated total reflection (FTIR-ATR) spectroscopy, or confocal Raman microspectroscopy can be used for quantifying a penetrated substance without the additional extraction step [39]. Cyanoacrylate biopsies is a further way of sectioning the epidermis. A small amount of cyanoacrylate glue is applied on the skin surface, followed by a glass slide, pressed down gently for a certain amount of time. By pulling the glass slide of in a fluent motion, the skin is segmented [40]. The samples obtained this way, can either be used for drug content analysis, or sectioned further.

The most prominent way of sectioning a skin sample for further investigations, is the tape stripping technique [41]. Adhesive tapes are applied to the skin sample after the incubation step, and analogous to the cyanoacrylate biopsy, by pressing gently and removing the tape afterwards in a single, fluid movement, the SC can be fully removed sequentially, layer by layer. This minimally invasive technique is also commonly used in in vivo studies. Further investigations of drug content within the skin removed by each respective tape can be either performed by optical methods, or by extracting the substance of interest and using chromatographical methods. For determining the thickness of the SC layers removed and hence the skin depth of the associated drug concentration measured, the mass of each tape before and after stripping is weighted. By this mass-difference, and the given diameter of the skin sample, as well as the SC density of 1 g/cm^3, the volume of the skin removed can be calculated [42]. This not only a time-consuming method, but also results can easily be affected by sebum or topically applied substances [4]. Other ways of determining the thickness of the SC layers removed by

each tape include determining the covering density of corneocytes on the tape strip by light microscopy, measuring the transepidermal water loss (TEWL), or using UV/vis spectrometry for example for measuring the protein content [4]. D-Squame scanning (Heiland electronics, Wetzlar, Germany) allows measuring the protein content directly on tape strips using infrared light. Given a respective SC depth for every tape strip and the amount of drug quantified within each tape strip, depth profiles of the drug can be displayed. As chromatographic techniques, after an extraction step, are the most common way of quantifying a drug after tape stripping, the absolute mass of the analyte within a tape strip is determined and hence can be displayed as a function of depth. As the amounts of cell layers removed differ with every tape, there are various possibilities of displaying the respective skin depth. In this work, the skin depth is displayed as the class-center of the SC removed. When comparing tape stripping results with other methods of skin penetration analysis, the amount of drug can be normalized on the application area for example.

As a skin sample is destroyed by this technique, every time point of every formulation to test results in a separate skin sample in the case of ex vivo studies. For in vivo studies, this limits the number of formulations and time points to test, as the skin area suitable for application is limited on every volunteer. Taking this into account and also considering the uncertainties in depth profiling, as well as the general time consumption of this techniques, destructive methods are not ideal, despite being considered gold standard. The abovementioned optical methods have the advantage, of non-destructive drug quantification. Especially confocal Raman microspectroscopy, which will be discussed in the following chapter, fulfils the requirements for non-destructive depth profiling [43].

1.4. Confocal Raman Microspectroscopy

Confocal Raman Microspectroscopy is a spectroscopic technique that relies on inelastic scattering of photons, the so called Stokes scattering, first described by the Indian physicist C.V. Raman in 1928 [44]. When a laser beam is focused on a sample, the largest fraction of the light is scattered elastically, which

means the wavelength of the scattered light is the same as the wavelength of the laser beam used. A small fraction of the incident light interacts with the molecules of the sample, causing the wavelength of the back-scattered light to differ from the primary light source. This inelastic scattering is caused by transitions of different vibrational and rotational states of the molecules. This can result in Raman scattering showing lower energy compared to the incident light, when the molecules of the sample take energy from the photons, called Stokes scattering. When the photons of the incident light take energy from the molecules, leading to Raman scattering with higher energy, it is called anti-Stokes scattering. These effects can be used to create a spectrum of the scattered light, representing the molecular vibrations and chemical structure of a sample.

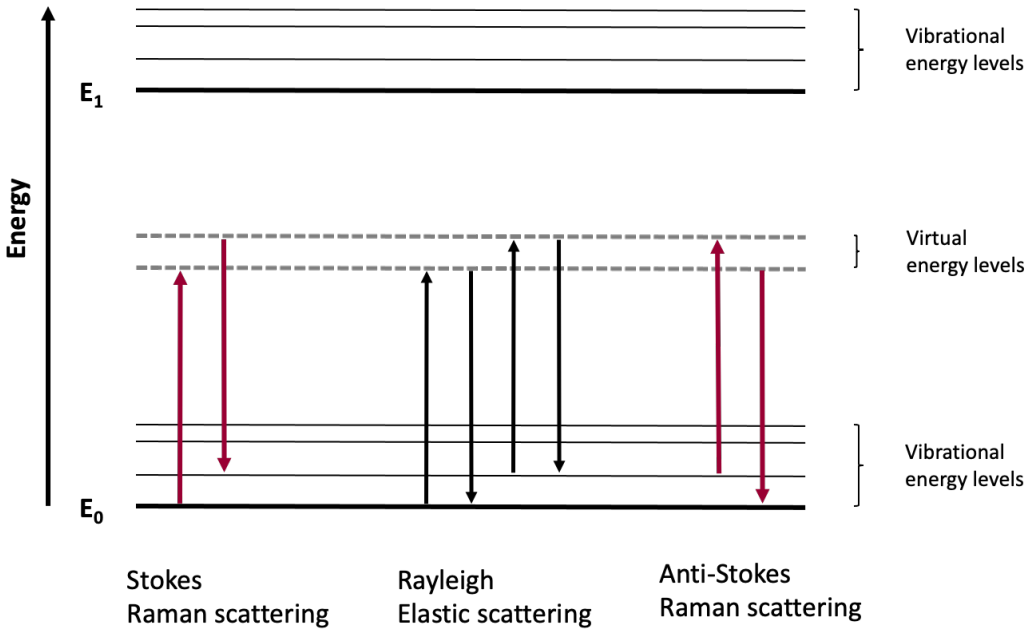

Figure 3: Jablonski diagram representation of Raman- and Rayleigh scattering.

By combining Raman spectroscopy with an optical microscope, confocal Raman microspectroscopy was created, enabling visual examinations with the addition of spectroscopic information. The laser beam can be focused by the microscope's objective on or into a specific point of the sample, a spatial pinhole collects the scattered light fraction and blocks out of focus light. This leads to a high spatial

resolution and enables depth profiling of samples, as modern CRM devices have motorized scan stages. This means, the position of the laser focus in the sample is known and can be moved at will, e.g., starting from the skin surface into the skin depth.

1.4.1. Confocal Raman Microspectroscopy for skin penetration analysis

Given the non-invasive, non-destructive, and label-free character of CRM, it is clear, that this technique got into the focus of skin research in the late 1990s [45]. First, the workgroup of Puppels used CRM to investigate the skins hydration status, which strongly promoted CRM as an interesting technique in various fields of skin- and dermal drug application research [46]. Over the last decades, CRM was used for analysing skin composition and structural properties, skin diagnosis, the comparison of different skin models, evaluating skin disruption and, of course skin penetration analysis [47][48][49][50]. Following the skin's hydration status by measuring characteristic water peaks and their variation in the skin, as pre-mentioned performed by the workgroup of Puppels, already showed the high potential of CRM as a technique for determining concentration profiles within the skin [46].

While IR-Spectroscopy is not suitable for direct depth profiling, as the signal gets lost when measuring in depth, most drugs must be linked to a marker to be detectable by Fluorescence-spectroscopic techniques. By labelling molecules, their physicochemical properties, and hence their penetration behaviour are affected. As barely any sample preparation is required for CRM measurements, and the high energy of the incident light allows measurements all through the SC, it is clear, that CRM was promoted as an interesting technique for measuring skin penetration over the last years. Many different approaches were made by various workgroups and helped CRM to even get into focus of the authorities, as it seems to be a powerful tool while addressing BE in topically applied generic drug products. The European Medicines Agency (EMA) released a draft guideline on quality and equivalence of topical products in 2018, mentioning CRM, as a technique for

providing supportive data, yet being not sufficiently established to provide pivotal equivalence [30]. Still, Franz diffusion cells and tape stripping being the only two methods proposed by this guideline. This makes clear, that although efforts have been made to establish CRM as an analytical method for determining skin penetration, there is still a need to investigate and develop it further.

Pudney et al. for example measured the delivery of trans-retinol depending on the solvent in vivo using CRM, showing the massive impact of the solvents penetration properties on dermal drug delivery [51]. Furthermore, monitoring chemically similar molecules is possible using CRM, as for example Zhang. et al have shown, imaging the prodrug to drug transformation of a 5-fluoruracil derivate in porcine ear skin [52]. Also, critical attempts were made addressing the experimental set up used and its impact on correct depth profiling with CRM [53][54]. Taking all these difficulties and critical parameters into account, still an inter-lab comparison between two widely used CRM devices showed comparable results, when measuring the impact of polyoxyethylene23-laryl ether on procaine HCl skin penetration ex vivo [55]. So far, the skin penetration of numerous drugs, including caffeine, flufenamic acid, hyaluronic acid, ibuprofen, lidocaine, triamcinolone acetonide, or penetration enhancers, such as glycerol, propylene glycol, ethanol or DMSO has been successfully investigated using CRM [56][57][23][58][59][60][61][62].

All the mentioned ex vivo studies, using CRM for quantitative depth profiling, still use Franz diffusion cells for the incubation step. This results in separate skin samples for every time point to test. As CRM allows continuous measurements of drug penetration, in-situ skin penetration analysis is possible. Therefore, we developed a special penetration cell, that can be fitted onto the motorized scan stage of a Raman microspectroscope. This penetration cell allows incubating a skin sample with a formulation to test, while measuring the penetration of a active ingredient in line. This reduces the skin samples needed to an absolute minimum, resulting in a less time- and labour-consuming way of generating reliable ex vivo skin penetration data. Correlating this in-line incubation/measurement technique, to results obtained by CRM measurements after conventional incubation in Franz diffusion cells were hence an important part of this project [63].

1.4.2. Data processing and drug quantification of Confocal Raman Microspectroscopy

Data processing and -analysis plays an important role in CRM measurements, not only due to the enormous amounts of data produced, but also, as the way of data processing affects the results obtained. As a part of this project, developing a suitable protocol for data analysis was mandatory. Cosmic rays, high-energy protons, and atomic nuclei can occur as very sharp peak in Raman spectra. Removing those without affecting the spectrum is the first step of data processing [40]. As, especially for the data produced in this project, a lot of spectral information is measured, the possibility of smoothing by multivariate statistical methods is given [64]. Especially principal component analysis (PCA) is a powerful tool for improving the signal to noise ratio, by concentrating the spectra on their main components, e.g., the peaks and, hence eliminating noise [65][66]. Furthermore, each recorded Raman spectrum shows a signal background, that should be subtracted, which can be done in various ways [64]. For all data used in this project, the baseline was corrected by a shape filter. Spectral data processed this way can be analysed further, for example regarding the distribution of different molecules.

There are various ways of determining the proportion of a certain substance out of the resulting Raman spectrum of several components. By using modern algorithms, a data set can be analysed for different components, the residual spectra consist of. Furthermore, reference spectra can be used to calculate their impact on the residual spectra. These ways of data processing have the advantages of taking every spectral characteristic into account. On the other hand, especially when working with reference spectra, this could bring in a bias, as the algorithm is pre-set on what to find. The elementary way of calculating a distribution, is by determining the area of a substance-specific peak, which of course is reliant on a peak, that does not interfere with other peaks. As all model drugs used in this project had the advantage of showing a prominent peak, not interfered by skin signals, calculating the peak area was used for determining depth profiles. This is possible, due to the linear dependency of the Raman scattering

and the concentration of a molecule. When measuring in depth, the problem of signal attenuation occurs, which means the linear dependency of Raman signal is affected by absorbance, reflectance, and scattering, resulting in a generally weakened signal. Therefore it is inevitable to compensate for this signal attenuation [67]. This can be done, by determining the depth profile of a skin-related peak, generated by a skin component, homogeneously distributed in the skin. By dividing the depth profile by its maximum value, for every depth measured, a fit coefficient for every depth step measured is obtained, with a value of 1 on the skin surface and lower values beyond. These coefficients can be used to compensate the depth dependent signal attenuation of other depth profiles [68]. For all depth profiles determined in this project, the C-C stretch vibration of the skin's aromatic amino acids, in the wavenumber range of 995-1018 cm^{-1}, also referred to as "ring breathing mode", was used to compensate for signal attenuation [67]. Furthermore, the half-maximum of this peak was used for determining the skin surface, as the half-maximum represents the transition from the objectives immersion medium to the skin, where half the laser focus is in the skin [69].

Skin penetration profiles of topically applied substances generated this way can give an insight into penetration kinetics and can be compared to penetration profiles after changing for example the formulation composition. Those depth profiles are only qualitative, semi-quantitative statements are only possible in relation to a reference formulation. Over the years, attempts were made, to directly quantify topically applied substances in the skin [70]. Especially a method developed by Caspers et al. makes direct drug quantification possible, by determining Raman intensity ratios of a material in a medium, e.g., a drug in the SC. As abovementioned, there is a linear dependency of Raman scattering and concentration. By determining different Raman signal intensity ratios of material and medium, respectively solvent, at different concentrations, a linear correlation, represented by a proportionality constant, between the mass concentrations and the Raman ratios can be found [71]. This can be expressed by equation 1

$$\frac{R_{material}}{R_{solvent}} = c_{material:solvent} \cdot \frac{m_{material}}{m_{solvent}} \qquad (1)$$

wherein R represents the Raman signal intensity, c is the proportionality constant, and m is the mass of the respective substance. For calculating the mass ratio, respectively the concentration, of a substance in the skin, the protein fraction of the skin, represented by the abovementioned ring-breathing mode of the aromatic amino acids can be used, to calculate the Raman signal ratio of substance to protein. The proportionality constant of protein to solvent can be determined by using bovine serum albumin (BSA) as a surrogate for skin-proteins, as their Raman signal intensities are comparable [68]. The mass fraction of protein in the stratum corneum is about 60 %. For non-water-soluble drugs, as it is the case for retinol, a second solvent is needed. By dissolving retinol in ethanol, and determining the proportionality constant of water and ethanol, the ratio of retinol to BSA can be calculated. Given these circumstances, equation 2 shows, how Raman signal intensity ratios can be translated into mass concentrations, using the proportionality constants of the measured calibration curves, exemplary for Retinol.

$$\frac{m_{material}}{m_{protein}} = c_{BSA:water} \cdot C_{retinol:ethanol}^{-1} \cdot c_{ethanol:water}^{-1} \cdot \frac{R_{retinol}}{R_{protein}} \qquad (2)$$

As for all quantification steps in this project, for the calibration curves, areas of characteristic, substance-related peaks, were determined using the trapezoidal rule. The area of the respective model drug peak can be assigned to a corresponding concentration in mg drug per cm^3 of skin afterwards.

1.5. References

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2. Objectives

The aim of this thesis was to develop a model for in situ skin penetration analysis by CRM. To enable CRM measurements of an active ingredient penetrating the skin while incubating, a suitable incubation cell had to be developed. Inspired by a conventional Franz diffusion cell, an electrical heatable penetration cell made of aluminium was built in cooperation with the mechanical workshop of the institute for pharmaceutical sciences. For precise depth profiling, the experimental set up of the Raman microspectroscope was evaluated and optimized, for high resolution depth profiling, a water immersion objective was used and its suitability for depth profiling was proven, as shown in Chapter 3. Also, creating a data analysis protocol was an important step, as previously discussed. Furthermore, the effectiveness of the model was tested, by comparing the caffeine skin penetration behaviour out of an aqueous solution containing 1,2-pentanediol as a penetration enhancer to a reference caffeine solution, the outcome of this study is also described in chapter 3.

Although CRM is used for quantitative skin penetration analysis already, incubating and measuring in-line are a novelty. Hence, showing that the in-line incubation leads to the same result as incubating the skin in Franz diffusion cells before analysis. This was tested by using a procaine-HCl hydrogel, already used in an inter-lab comparison between two commonly used CRM devices, to see if the in-situ skin penetration model detects the same penetration characteristics. Chapter 4 presents the findings of these investigations.

Of course, as Franz diffusion cells and tape stripping are considered gold standard for skin penetration analysis, a comparative study, measuring retinol skin penetration using both techniques, was performed. Also, by using retinol as a non-water-soluble, lipophilic model drug, the model`s suitability for different vehicles could be tested. For a meaningful comparison of both techniques, a calibration method by Caspers et. al. was adopted, enabling a quantification of retinol by CRM. The results of this study are discussed in chapter 5.

A good ex vivo model provides data, that can be transferred to in vivo conditions. Therefore, comparing ex vivo CRM results to in vivo skin penetration data

was an essential part of this project. Again, CRM and tape stripping were used to study the skin penetration of caffeine out of a hydrogel in vivo, and ex vivo. A second gel containing 1,2-pentanediol as a penetration enhancer was used to see if there are differences in caffeine skin penetration detectable. Furthermore, freshly prepared porcine ear skin, as well as frozen and thawed porcine ear skin of the same donor animal were used, to test the influence of freezing the skin, as it is common for storage, on caffeine skin penetration. As for retinol in Chapter 5, the same calibration model was adopted for caffeine, hence all results of all methods used could be displayed the same way. The results of this ex vivo in vivo comparison are shown in chapter 6.

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A New Method for In-Situ Skin Penetration Analysis by Confocal Raman Microscopy

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Abstract: In the development of dermal drug formulations and cosmetics, understanding the penetration properties of the active ingredients is crucial. Given that widespread methods, including tape stripping, lack in spatial resolution, while being time- and labour-intensive, Confocal Raman Microscopy is a promising alternative. In optimizing topically applied formulations, or the development of generic formulations, comparative in-situ measurements have a huge potential of saving time and resources. In this work, we show our approach to in-situ skin penetration analysis by confocal Raman Microscopy. To analyse feasibility of the approach, we used caffeine solutions as model vehicles and tested the effectiveness of 1,2-pentanediol as a penetration enhancer for delivery to the skin.

Keywords: confocal Raman spectroscopy (CRM); skin penetration; depth profiling

1. Introduction

The skin is the largest organ in the body and its functions are varied. Its provides an external barrier, protecting the body from external damage or water loss, as well as controls the body temperature and plays an important role in metabolism. Understanding how the barrier function works and in which rate and extend active ingredients penetrate the skin is extremely important [1,2] when it comes to topically applied drug dosage forms and cosmetics. The outermost layer, the stratum corneum (SC), provides this barrier function [3]. It is composed of keratin-filled corneocytes, embedded in a lipid-matrix and reaches a thickness of 15–20 µm [4]. The skin plays an important role in the penetration of topically applied substances because of its main function as a barrier.

To understand drug delivery of topically applied dosage forms, it is important to understand the rate and extent of the active ingredient penetrating into the SC, especially when it comes to optimizing formulations [5,6]. The number of analytical methods for measuring penetration properties of drugs are limited. Most commonly, the skin is incubated with the formulation to be tested, and segmented afterwards, either by removing the SC step by step with adhesive tape strips, or by cutting it using a cryo-microtome [7–9]. The amount of drugs in each layer can be determined by High-performance liquid chromatography (HPLC) subsequently. This procedure is very time consuming and labour intensive because every measuring point has to be incubated separately, due to the subsequent destruction of the skin sample. Optical methods have the advantage of being non-destructive, which allows continuous measurements [10]. Confocal Raman microscopy (CRM) has been described as a promising technique, especially because it does not rely on labels or dyes. For measuring deeper penetrations, spatially offset Raman spectroscopy (SORS) is described as an effective method to obtain stronger signals in even deeper sample layers, than in this study [11,12]. Direct drug quantification is possible because of the linear correlation of molecule concentration and Raman scattering [13]. So far, CRM has been used in many studies to acquire penetration profiles of several drugs in vivo and in vitro [14–17].

We focused on ex-vivo-experiments as in-vivo measurements face ethical difficulties and are not possible in early stages of formulation development. Most of the published studies describe a separate incubation step or a cross-sectioning of the skin before CRM measurements, while in-situ-measurements are still uncommon. In-situ measurements have a huge advantage of reducing the measuring time to the time of incubation, while providing real-time information. This means the amount of measuring points can also be increased.

The aim of this study was to develop a penetration cell, which enables the in situ measurement of drug penetration into the skin, in order to promote CRM as a standard analytical technique for drug depth profiling. Caffeine was used as a hydrophilic model drug, as it is commonly used in skin penetration as a reference drug [18]. To show the effectiveness of the model, a solution of caffeine in water was compared to a solution containing 1,2-pentanediol as a penetration enhancer.

2. Materials and Methods

2.1. Materials

Caffeine was obtained from Caesar & Loretz GmbH (Hilden, Germany). 1,2-Pentandiol was provided by BASF SE (Ludwigshafen, Germany), Parafilm was purchased from Bemis Company Inc. (Oshkosh, WI, USA). Sodium chloride, potassium chloride, disodium phosphate and monopotassium phosphate, as used for the phosphate buffered saline (PBS) pH 7.4 were all of European Pharmacopeia Grade. For the preparation of all solution ultra-pure water (Elga Maxima, High Wycombe, UK) was used. Porcine ear skins (German land race) were provided by a local butcher.

2.2. Preparation of Porcine Ear Skin

For the penetration experiments porcine ear skin was used because of its similarity regarding histology and morphology to human skin [19–21].

All ears used for the experiments were provided by a local butcher (Bio Metzgerei Griesshaber, Moessingen-Oeschingen, Germany). The porcine ears were picked up right after the animals were butchered. The Department of Pharmaceutical Technology is registered for the use of animal products at the District Office of Tuebingen (registration number: DE 08 416 1052 21) [22]. The fresh porcine ears were cleaned with isotonic saline and cotton swabs. Afterwards, the full-thickness skin was cut off the cartilage and, where necessary, cleaned again using cotton swabs and isotonic saline. The skin was cut into about 4 cm wide strips and fixed with pins onto an aluminium foil wrapped Styrofoam plate. A hair clipper (QC5115/15, Philips, The Netherlands) was used to trim hair on the skin sheets to approximately 0.5 mm. Then the skin was cut to a thickness of 1 mm using a Dermatom (GA 630, Aesculap AG & Co. KG, Tuttlingen, Germany) [23]. Circles of 35 cm diameter were cut out with a scalpel. Skin samples were wrapped in aluminium foil and were stored at −30 °C until the day of the experiment.

2.3. Incubation of Porcine Ear Skin in a Heatable Diffusion Cell

In order to incubate the porcine ear skin right under the Raman microscope to obtain in situ data, a heatable diffusion cell was developed, and custom made in cooperation with the mechanical workshop of the Institute for Pharmaceutical Sciences, Tuebingen. The incubation cell is inspired by Franz diffusion cells, which are widely used for ex vivo skin penetration studies [24]. A schematic of the cell is shown in Figure 1. It is composed of aluminium and contains an acceptor compartment with a total volume of 7 mL, a sample train, a grid to place the skin on and a donor chamber, which is placed above the skin and held in place by 6 screws. Two sealing rings ensure that the device is tightly sealed. To prevent water loss during incubation time, a piece of parafilm is tightened around the donor compartment and the objective of the Raman Microscope. The incubation cell is heated by two thermocouples with an external controlling device attached, which is set to 32.0 °C throughout the whole incubation time.

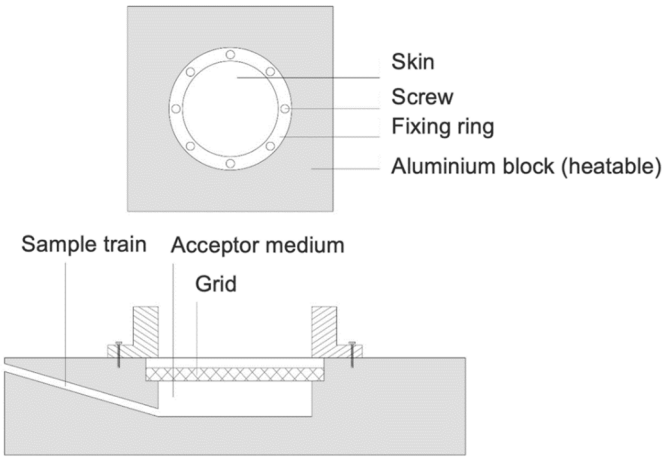

Figure 1. Penetration cell for in-situ CRM measurements.

For the measurement, the acceptor compartment was filled with 7.0 mL phosphate buffered saline (pH 7.4) and a skin sample was placed on the grid above, which was soaked in PBS. Before applying the sample solution, the skin was tempered for 30 min. After the equilibration time 3.0 mL of the sample solution were applied (infinite dose). Measurements were performed every hour, for a total incubation time of 6 h. All experiments were performed in triplicate on three different days.

2.4. Confocal Raman Microscopy (CRM)

For in-situ measuring the incubation cell was placed onto the scan table of an alpha 500 R confocal Raman microscope (WITec GmbH, Ulm, Germany), fixed by 4 pins on the bottom of the cell. The Raman microscope is equipped with a 532-nm excitation laser, a UHTS 300 spectrometer, a DV401-BV CCD detector and a 63× water immersion objective with numerical aperture of 1.0 (W "Plan-Apochromat" 63/1,0 M27, Carl Zeiss, Jena, Germany). To obtain a strong signal without damaging the skin, the laser intensity was set to 25 mW, using a pinhole size of 50 µm. The DV401-BV CCD detector was cooled to −60 °C and a spectral range from 501 cm^{-1} to 1635 cm^{-1} with the spectral centre of 1100 cm^{-1}, obtained by an optical grating (1800 g/mm, spectral centre: 1100 cm^{-1}). Two-dimensional image scans of 5 µm width and 25 µm in depth were performed, acquiring 10 spectra per line and 50 lines per vertical dimension, with an integration time of 1.5 s per spectra.

To ensure the suitability of the setup for depth profiling, the depth resolution was measured, by scanning into a silica plate and determining the full width at half maximum of the depth profile corresponding the 521 cm^{-1} band intensity [25]. Also, the thickness of a PET film was measured, in order to test whether valid depth profiles can be obtained.

2.5. Data Analysis

All recorded spectra were processed by cosmic ray removal and background subtraction using the software Project Plus 4 (WiTec GmbH, Ulm, Germany). Background subtraction was used in the "shape" option (size: 400). For noise reduction principal component analysis (PCA) was performed on all spectra also using the Project Plus 4 software. PCA is an eigenvector-based multivariate statistic method and is commonly used to simplify and structure extensive data sets. The first three principal components were selected for reconstructing a reduced spectrum, containing all essential information, discarding minor variations and noise [26]. Depth penetration profiles of caffeine were determined by calculating the area under the fitted curve (AUC) of the caffeine band at 556 cm^{-1}, arising from O=C–N deformation mode. This peak was selected, because it is not interfered with any signals of the skin. The calculation of the AUC was also performed on the Project Plus 4 software using trapezoidal method. The half-maximum of the aromatic amino acid peak at 1008 cm^{-1} was determined, to give the location of the skin surface [27]. All depth profiles were cropped to the skin surface, as the

measurement was started around 2 µm above the skin. The arithmetic mean of the aromatic amino acid peak at 1008 cm^{-1} (ring breathing mode) was used to normalize the caffeine signal as it signals attenuation in deeper skin regions and variations over time. Three depth profiles were calculated and extracted out of every image scan, so a total of 9 depth profiles for every solution was used to calculate the mean penetration profile of a specific formulation. To calculate the enhancement ratio, the area under the curve of the depth profiles were determined, using the trapezoidal method and the AUC of the solution with enhancer was divided by the AUC of the solution without enhancer. Representative spectra of the caffeine solution, the stratum corneum and stratum corneum incubated with caffeine are given in Figure 2.

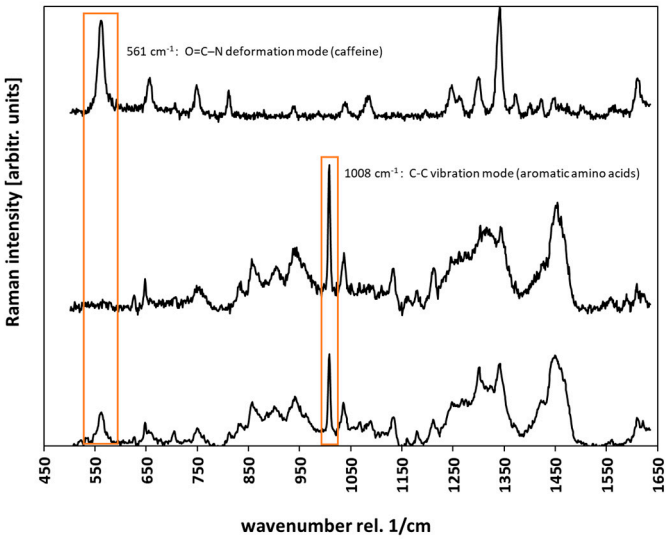

Figure 2. Raman spectra of a 2% caffeine solution (top), stratum corneum (middle) and stratum corneum incubated with caffeine (bottom). The peaks used for calculation of the depth profiles are marked orange.

3. Results and Discussion

Following the depth penetration of an API, confocal Raman microscopy has already been performed by several workgroups and has proved promising in replacing destructive methods, such as tape stripping or cryo-segmentation [28–30]. It is possible to gain a lot of information in a fraction of the time used for conventional methods. In order to obtain valid data, a suitable microscopic setup is crucial [31]. To validate our setups suitability for depth profiling, the depth resolution was calculated, using the 521 cm^{-1} band of a silica platelet. The depth resolution was found to be 1.86 µm. Furthermore, the thickness of a PET film was measured with the Raman setup and compared to its real thickness of 21.6 µm. The raman results show a thickness of 20.77 µm (standard deviation: ±0.155 µm), which shows an only marginal effect of underestimation of depth, compared to metallurgical objectives [25]. The difference between the real and the measured thickness is therefore 0.83 µm. As the SC is about 15–20 µm thick, which is comparable to the 21.6 µm PET film that was measured, an error of <1 µm across the whole thickness of the SC is deemed acceptable. This shows, that the setup is able to obtain valid depth profiles with a fairly high special resolution, compared to the 5 µm resolution of other instruments [13]. Figure 3 shows the results of in-situ measurements of caffeine penetration during 6 h of incubation with a 2% solution. The Raman intensity of the caffeine band at 556 cm^{-1} is displayed as a function of depth, beginning at the skin surface.

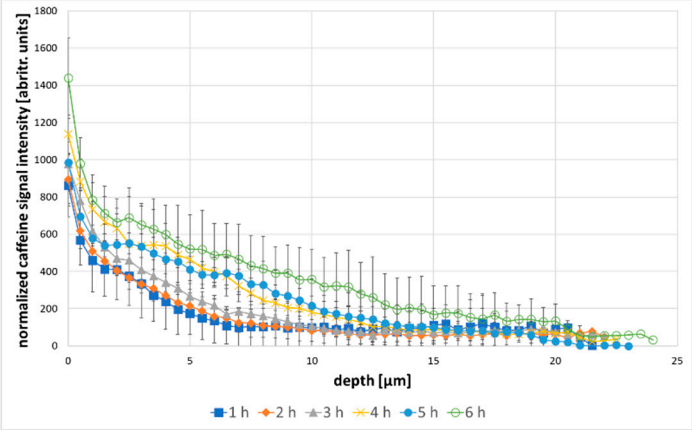

Figure 3. Caffeine depth profiles over 6 h incubation time with 2% caffeine, the error bars are showing the standard deviation.

The recorded depth profiles clearly show a steady penetration of caffeine into the skin over the incubation time of 6 h. The presence of caffeine in the SC was already measured after 1 h. For the following 5 h, a steady caffeine penetration was measured. In particular, within the first 10 µm a continuous increase of the caffeine signal over the incubation time was visible. Furthermore, penetration depth increased from 5 µm to 20 µm after 6 h. In fact, the determined penetration profiles coincide with the depth profiles measured by L. Franzen et al. for 12.5 mL/mg caffeine solutions, taking into account that the solutions used in this work are noticeably higher concentrated [32]. While, most of the drug was found in the SC within the first 5 h, which was also found by Laia Rubio et al. [33], after 6 h of incubation, caffein could be found even in 20 µm penetration depth.

The effect of 1,2-pentanediol on the caffeine penetration profile was tested to check the effectiveness of the model, in order to establish whether it is possible to detect differences induced by the penetration enhancer. Figure 4 shows the results of in-situ measurements of caffeine penetration during 6 h of incubation with 2% caffeine and 5% 1,2-pentanediol as a penetration enhancer [34].

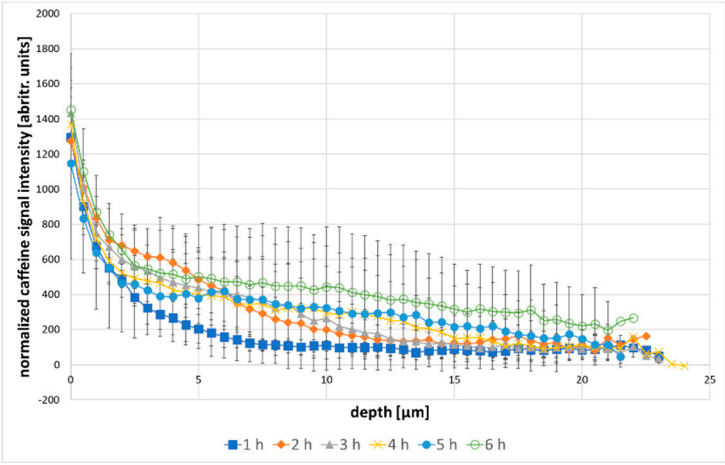

Figure 4. caffeine depth profiles over 6 h incubation time with 2% caffeine +5% 1,2-pentanediol, the error bars are showing the standard deviation.

Over the incubation time of 6 h, a steady increase of caffeine in the skin, as well as an increase in total penetration depth could be detected. Especially between the first and the second hour of incubation, the amount of caffeine in the skin rising was noticeable, while the depth profile between the second and the fifth hour mostly shifted to deeper skin regions. While, the caffeine amount in the upper layers remained the same, the penetration depth increased, which can be deduced by the

increasing caffeine signal in deeper skin layers. After 6 h of incubation, caffeine reached a penetration depth of over 20 µm. The amount of caffeine penetrating the skin is generally higher, when adding 1,2-pentanediol to the solution. After 2 h of incubation time, there was already a higher amount of drug in 10 µm depth (normalized caffeine signal: 200 abritr. units) compared to the solution without penetration enhancer (normalized caffeine signal: 82 abritr. units). Also, the penetration depth of caffeine was genuinely higher. After 1 h, the cumulative amount of drug within 10 µm of skin depth (4222 arbitr. units µm). is comparable to the amount of drug after 3 h of incubation time with the formulation without penetration enhancer (4243 arbitr. units µm).

Figure 5 show the normalized caffeine signal intensity of both solutions tested from the skin surface to 10 µm skin depth in 2.5 µm steps, as most of the differences can be observed within this depth. A shift of the caffeine penetration to deeper skin regions on Figure 5B, caused by the penetration enhancer, was clearly visible. After 2 h the penetration profile of the formulation with enhancer reached almost the same course as the formulation without enhancer after 4 h. Also, the caffeine signal at the skin surface was genuinely higher in the formulation with enhancer, reaching a mean of 1328 arbitrary units µm, in comparison to 1050 arbitrary units µm for the solution without penetration enhancer, as well as the overall amount of drug measured in 10 µm skin depth (273 arbitrary units µm vs. 173 arbitrary units µm). By the end of the incubation time of 6 h, the overall amount of caffeine delivered to the skin was still higher for the formulation containing 1,2-pentanediol, although the difference between both formulations was no long as distinct.

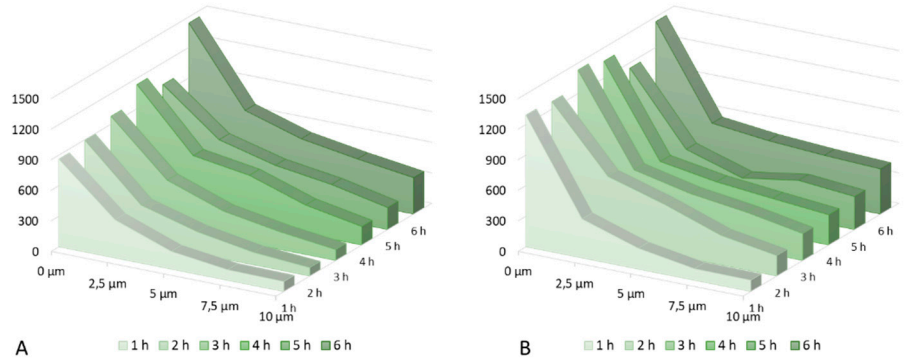

Figure 5. (**A**): Caffeine penetration over 6 h incubation time (2% caffeine solution); (**B**): Caffeine penetration over 6 h incubation time (2% caffeine solution + 1,2-pentanediol).

Table 1 shows the calculated enhancement ratios for every time. Enhancement ratios vary between 1.21 and 1.98. It is also shown, that after 2 h, the enhancement effect of 1,2-pentanediol is the clearest. After 3 h the values are decrease again, until the enhancement ratio after 6 h aligns with the ratio after the first hour. This shows that the method can give an insight into penetration kinetics of a topically applied drug.

Table 1. cumulative caffeine signal in the skin for every measurement point for the 2% caffeine solution (**A**) and the 2% caffeine solution +5% pentanediol (**B**), and the calculated enhancement ratio.

	AUC $_{caffeine}$ (A) [arbitr.units · µm]	**AUC $_{caffeine}$ (B) [arbitr.units · µm]**	**Enhancement Ratio**
1 h	3499	4222	**1.21**
2 h	3447	6841	**1.98**
3 h	4243	6541	**1.54**
4 h	5779	6816	**1.18**
5 h	5509	6962	**1.26**
6 h	7978	9636	**1.21**

4. Conclusions

In this work, we were demonstrated the effectiveness of a new model for in-situ drug penetration studies. In comparison to conventional methods, such as tape stripping or cryo-segmentation, CRM has the big advantages of being non-destructive and non-invasive. Also, the possibility of in-situ measurements brings further benefits to skin penetration studies, as it is less time consuming. The model we developed successfully shows that it is possible to detect significant differences between two formulations regarding the penetration depth and kinetics of a drug. Understanding the effects of penetration enhancers is important in the development of topically applied formulation, in pharmaceutics, as well as in cosmetics [35]. In particular, in comparative studies between two formulations, the developed method is a promising, time- and resources-saving alternative to the destructive conventional methods. This might be a huge advantage, for example, in the approval of generic formulations. Although, measuring absolute drug concentrations in the skin by CRM is still a challenge, the developed method shows the advantages and the high potential in penetration studies by CRM.

As this method is very promising, we will continue skin penetration studies to investigate further possibilities and limits. While, we showed the effectiveness of the model for caffeine as a hydrophilic model drug, further drugs, especially lipophilic drugs will be tested. Skin penetration studies of aqueous solutions are possible, as this work shows, so the next steps will be to test the model for in-situ-skin penetration studies with hydrogels and emulsions, as well as with oily solutions.

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In-Line and Off-Line Monitoring of Skin Penetration Profiles Using Confocal Raman Spectroscopy

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Abstract: Ex-vivo and in-vivo skin analysis has been extensively evaluated by confocal Raman spectroscopy (CRS). The off-line measurement with a CRS-suited skin-mounted device after Franz-cell incubations is the most popular choice. However, real-time monitoring of in-line measurement has clear advantages for obtaining dynamic and more timely results. In our study, a custom-built setup suitable for in-line measurements was implemented, which ensures constant skin incubation and in-situ skin detections. We aim to compare the differences between using in-line and off-line devices for monitoring skin drug penetrations. A well-assessed formulation gel with procaine-HCl as the active ingredient was used as reference. The PEG-23 lauryl ether was added to the formulation as a penetration enhancer to evaluate the enhancement effects of procaine on skin. After incubation times of 14, 20, and 24 h, skin penetration profiles were assessed. Comparable results between off-line and in-line measurements were obtained. Remarkable improvements in penetrated procaine amount and depth were observed. Based on the significant differences of their enhanced penetration amounts, fairly similar estimations were achieved from both methods. A slight difference of 14 h incubation between these two setups can still be found, which may be due to the different detection conditions and affected skin properties. Overall, in-line measurements could provide a more time- and labor-saving alternative for off-line measurements in ex-vivo study.

Keywords: in-line and off-line measurements; confocal Raman spectroscopy; procaine-HCl penetration; PEG-23 lauryl ether; penetration enhancement; skin

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1. Introduction

The skin is the largest organ of the human body and is considered as a crucial route for drug deliveries [1–3]. With the topical application of components, non-invasive and efficient techniques for monitoring their skin penetrations are strongly needed.

Nowadays, confocal Raman spectroscopy (CRS) as a method for skin penetration studies in-vivo and ex-vivo is on the rise [4–7]. Different methods have been described for obtaining depth profiles of topically applied actives using CRS [8,9]. In skin in-vivo studies, in-line measurements have been widely described for monitoring the distribution of actives or excipients on human skin [10,11]. However, the use of human skin faces a lot of difficulties concerning accessibility due to ethnic problems and poor convenience. Thus, excised substitute such as porcine skin has been commonly used as an appropriate model [12,13]. Meanwhile, ex-vivo experiments play a very important role in the development of semi-solid dosage forms, not only as drug delivery systems but also in cosmetics. While in such ex-vivo skin penetration studies, in-line measurement is fairly new and rarely performed yet due to the complexity of producing the Raman device-suited skin incubation cells, although it offers the great benefits of timesaving and real-time monitoring [14–16].

Franz diffusion cells are the most commonly used method for skin penetration studies. The formulation to test is applied on either the excised human skin or a suitable surrogate, fixed between two compartments, and tempered to the human skin temperature of 32 °C.

After a certain incubation time, the amount of drug in different skin layers or depths can be quantified. Either in a destructive way, by segmenting the skin, for example, with adhesive tape strips or by cryo-segmentation [17,18], followed by a quantification step, where high-performance liquid chromatography is the most popular method [19,20], or with a non-invasive method, as, for example, CRS [4,21,22]. Among other optical methods, as attenuated total reflectance infrared spectroscopy [23–25] or fluorescence microscopy [26,27], CRS has the advantage of high spatial resolution and chemical sensitivity, even when used for lateral depth scanning, making it an ideal technique for skin penetration studies. In-situ measurements have the further advantage of being even less time-consuming, as there is no separate incubation step required.

In our study, a suitable device for skin incubation and simultaneous CRS detection was developed and used for the in-line measurements. The device was custom-built in our department and has recently been described in [28]. The conventional stepwise CRS measurements with the stopping of the skin incubations in Franz cells were seen as the off-line method. The aim of this study was to evaluate differences in skin penetration profiles between off-line and in-line measurements. The procaine-HCl incorporated gel formulations were applied with and without penetration enhancer of PEG-23 lauryl ether (L23) on skin followed by the CRS detections. L23 contained an average number of 23 oxyethylene groups, has higher solubility in water and can be used in gel formulations. L23 was chosen as the penetration enhancer in our study as Shin et al., showed that it enhanced the penetration of local anesthetics. The calculated enhancement ratio has been observed with the incorporation of L23. The enhanced mechanism of this type of PEGylated emulsifiers might be induced by the disruption of stratum corneum lipid matrix and increased fluidity of lipid structures.

The formulations used in this study were chosen because their penetration rate and extent are already described [7], making them ideal for investigating the effect of the method used for skin penetration studies.

With comparisons of their penetration profiles, either the correlation of these two measurements can be established, proving their feasibility in use for future studies, or the respective details responsible for their differences can be addressed.

2. Materials and Methods

2.1. Materials

The model drug used was procaine-HCl, obtained from Ceasar & Loretz GmbH, (D-Hilden, Germany). PEG-23 lauryl ether was purchased from Croda GmbH, (Nettetal, Germany). Hydroxypropyl methylcellulose (HPMC) was from Shin Etsu Chemical Co. Ltd., (J-Tokyo, Japan). Polyoxyethylene polyoxypropylene copolymer (Poloxamer 407) was obtained from (BASF SE, D-Ludwigshafen, Germany). Parafilm® was from Bemis Company Inc., (Oshkosh, WI, USA). Sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, and potassium chloride were of European Pharmacopoeia grade. All aqueous solutions were prepared with ultra-pure water (Elga Maxima, High Wycombe, UK).

Porcine ear skins (German land race; age: 15 to 30 weeks; weight: 40 to 65 kg) were obtained from the Department of Experimental Medicine of the University Hospital Tuebingen. The live animals used were kept at the Department of Experimental Medicine and sacrificed in the course of the experiments, which were approved by the ethics committee of the University Hospital Tuebingen. The ears were obtained directly after the death of the animals. Prior to the study start, the Department of Pharmaceutical Technology was registered for the use of animal products at the District Office of Tuebingen (registration number: DE 08 416 1052 21).

2.2. Preparation of Gel Formulations

Procaine-HCl contained in a gel formulation was prepared to detect the procaine penetration on skin. The HPMC poloxamer gels were prepared according to the method

published by Shin et al. [29] and have also been described in our previous studies [5,7]. The formulation without penetration enhancer contained 1 g of procaine-HCl, 0.2 g of HPMC, and 2 g of poloxamer 407 in 6.8 g water. The formulation with enhancer additionally contained 0.5 g PEG-23 lauryl ether (L23). The water content was thus reduced to 6.3 g. The ingredients of the formulation are described in detail in Table 1.

Table 1. Composition of the formulation * used (g).

Formulation	Hydroxypropyl Methylcellulose	Poloxamer 407	L23	Procaine-HCl	Water
A	0.2	2.0	0	1.0	6.8
B	0.2	2.0	0.5	1.0	6.3

* The formulations were stored at 5 °C until the day of usage.

2.3. Preparation of Porcine Ear Skin

The donor pigs were 15 to 30 weeks old and weighed 40 to 65 kg. Fresh porcine ears were cleaned with isotonic saline. Full-thickness skin was removed from cartilage and gently cleaned from blood with cotton swabs and isotonic saline. The obtained postauricular skin sheets were then dried with soft tissue, wrapped with aluminum foil and stored in a freezer at −28 °C.

On the day of experiment, skin sheets were thawed to room temperature on PBS-soaked tissues, to prevent the skin from drying out, cut into strips of approximately 5 cm width and stretched onto a Styrofoam plate (wrapped with aluminum foil) with pins. Subsequently, skin hair was trimmed and the skin was dermatomed to a thickness of 1.0 mm (Dermatom GA 630, Aesculap AG & Co. KG, Tuttlingen, Germany). Then, the dermatomed skin used for Franz cell incubation was punched out for circles to a diameter of 25 mm whereas that used for in-situ incubation was cut out to a diameter of 35 mm. The procedures have been described in detail in previous publications [28,30].

2.4. Incubation of Dermatomed Porcine Ear Skin

Franz diffusion cell (Gauer Glas, Püttlingen, Germany), as a gold standard in ex-vivo dermal drug delivery and skin penetration and permeation experiments, was used as a benchmark here. The obtained data of drug penetrations can be compared with a recently established in-situ method [28] for detecting the drug penetration profiles by CRS (WITec GmbH, Ulm, Germany). After the Franz cell incubation for a certain time, skin samples were obtained and transferred to a specially made skin-placed device (mechanical workshop of the Institute for Pharmaceutical Sciences and the electronic workshop of the Institute of Chemistry, Tuebingen, Germany) for CRS measurements [5]. Thus, the incubation was stopped, which was deemed as the off-line method in this study. In contrast, dynamic measurements on the same skin samples for different incubation times can be carried out by using the custom-built in-line device.

For the incubation of skin samples in Franz-cells, 12 mL of degassed, prewarmed (32 °C) phosphate-buffered saline (PBS) was used as receptor fluid with a string speed of 500 rpm. Then, a skin sample was mounted and tightened with the donor compartment on top. Formulation prepared ahead with an amount of 0.25 g/cm^2 was then applied, with a piece of parafilm used as a cover to prevent water evaporation. The unjacketed Franz-cells were placed in a 32 °C water bath for the incubation step. After incubation for 14, 20 and 24 h separately, skin samples were removed from cells and each skin surface was gently washed and cleaned with isotonic saline and cotton swabs to remove the remaining samples and avoid erroneous measuring results [5]. Then, the actual application area (15 mm in diameter) was punched out, patted dry with cotton swabs and mounted onto the device for CRS measurements (Figure 1A). For a detailed description of the exact procedures including the Franz-cell incubation and aforementioned skin-mounted device, please see our previous publications [5,31].

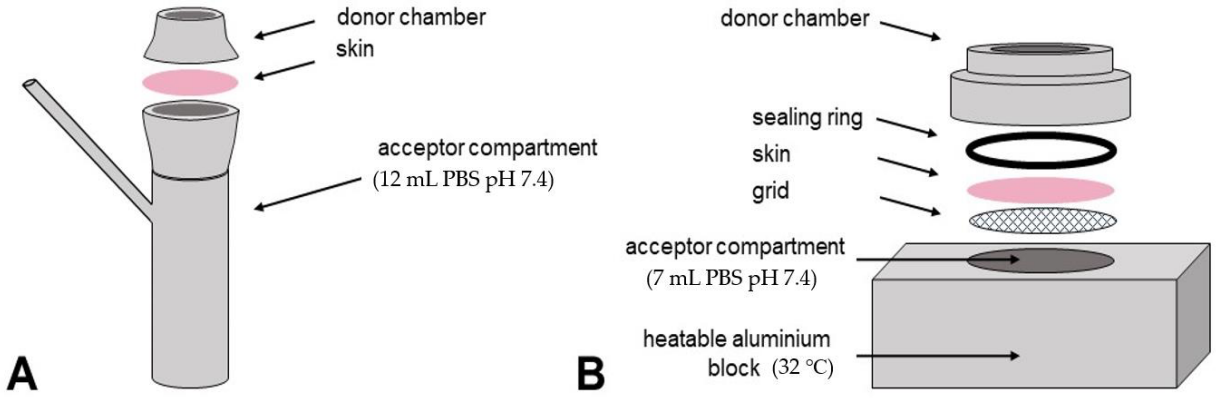

Figure 1. The schematic profiles of the off-line device (**A**) of Franz diffusion cell for skin incubation and in-line device (**B**) of skin incubation cells used.

The set-up of the custom-built device for in-line measurements is shown in Figure 1. For the in-line skin incubation and CRS measurements, 7.0 mL of PBS was filled in the acceptor compartment and a skin sample was placed on the grid above, which was soaked in PBS previously to displace all air in the grid. After an equilibration time of 30 min to temper the skin and the device (32 °C), 2.0 mL of the formulation to test was applied (0.25 g/cm^2). Parafilm was tightened between the objective of the Raman Microscope and the donor chamber to prevent any water loss. The schematic view of the custom-built in-line device is shown in Figure 1B. A detailed description of it can be found in our previous publication [28].

2.5. Confocal Raman Spectroscopy Analysis of Skin Penetrations

In-line and off-line devices used on CRS for detecting skin drug penetration profiles were compared. For both skin-placed devices, the skin spectra were acquired with an alpha 500R confocal Raman microscope (WITec GmbH, Ulm, Germany), which was equipped with a 532 nm excitation laser, UHTS 300 spectrometer and DV401-BV CCD camera. The optical grating was 1800 g/mm for recording the spectra in the range of 700 to 1800 cm^{-1}. Laser power was adjusted to 25 mW for the off-line skin measurements and to 32 mW for the in-line measurements by the optical power meter (PM100D, Thorlabs GmbH, Dachau, Germany). Simultaneously, 63 × 1.0NA water immersion objective (W Plan-Apochromat, Carl Zeiss, Jena, Germany) was utilized, which also enabled the dynamic measurements of in-line device by dipping the immersing objective in the formulation gel.

In off-line analysis, a line scan was implemented in order to collect the skin spectra from depth, with the laser spot recording from 10 µm above the skin down to 40 µm inside the skin with a step size of 1 µm. The exposure time was 2 s for one measurement with 2 accumulations for each spectrum. Spectra were collected from three randomly selected spots on three skin samples, resulting in nine measurements. The skin pieces for in-line and off-line CRS measurements were from two different donors. Their comparison of penetration profiles was accomplished by analyzing the relative penetration enhancements from different incubation times. Furthermore, in the off-line spectral analysis, line scans were performed for quick collections of depth skin signals due to the non-stopping penetration behaviors inside the skin samples after taking them out of the incubation cells. Thus, the spatial resolution recorded by the line scan would appear to have a relatively lower resolution (Figure 2).

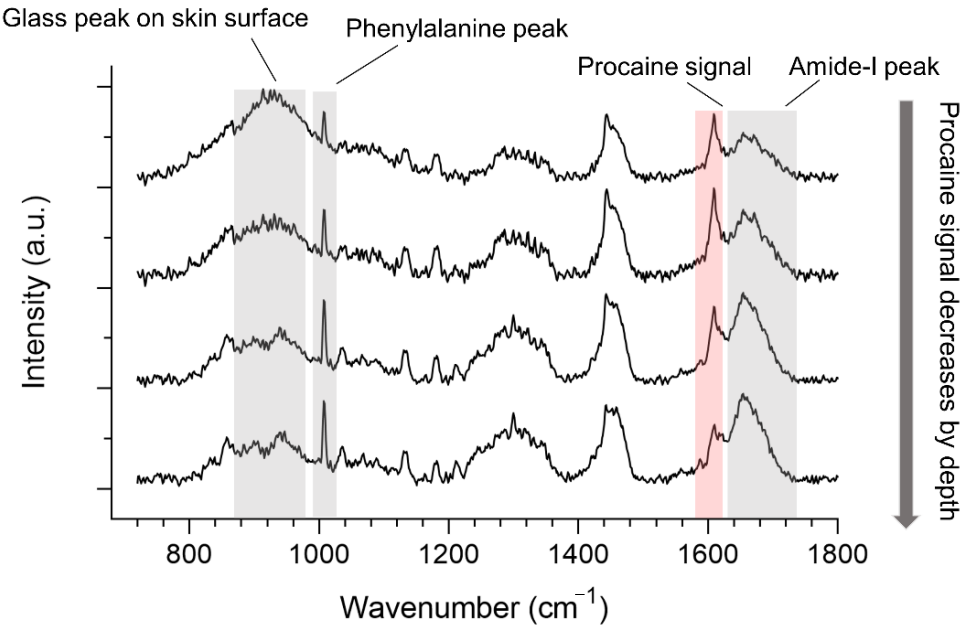

Figure 2. Typical spectra in off-line skin analysis of procaine signal decreasing by depth with crucial spectral features highlighted in colors.

For in-line measurements, two-dimensional image scans of 5 µm width and 25 µm depth were performed, acquiring 10 spectra per line and 50 lines per vertical dimension, using an exposure time of 1.5 s per spectra. Measurements were performed after 14, 20 and 24 h, focusing always on the same position, chosen randomly at the beginning. Each formulation was tested on three pieces of skin of the same donor-animal on three different days. Three depth profiles out of every two-dimensional scan were extracted, resulting in 9 depth profiles per formulation ($n \geq 9$).

The skin surface can be determined from different signal profiles by depth. The half maximum of the signal profile of the skin (amide-I, phenylalanine peak) and glass peak signals are both suitable for use. The skin surface in in-line measurements was determined as the position with the half maximal intensity of the phenylalanine signal. For off-line measurements, the phenylalanine signal will be affected and covered by the glass signal near the skin surface. Therefore, the skin surface here was determined as the position of the intersection of the amide-I and glass signals (Figure 2).

2.6. Spectral Data Analysis

The initial processing step of Raman spectra included the spectral cosmic ray removal, smoothing as well as background subtraction, which were performed using the WITec Project Software (Project 5 plus, WITec GmbH, Ulm, Germany). Referring to the smoothing process, a Savitzky-Golay (SG) filter was applied with a third polynomial order and nine smoothing points. For the background subtraction, an automatic polynomial function was fitted to the spectrum and subtracted. Furthermore, the area under the curve (AUC) in this study is the integrated area under a specified peak of the spectrum and could be calculated using a trapezoidal method on WITec Project Software.

2.7. Characterization of Penetration Profiles

Based on the spectral information, peak areas of the procaine peak and the amide-I peak were calculated using off-line measurements. The amide-I peak signal has been proved to be stable from different donors and within one donor and can be successfully used to normalize the procaine peak to account for the depth attenuation effects [30]. Thus, the relative procaine penetration in skin can be calculated by the ratio of AUC of the procaine and amide-I peaks.

Regarding the in-line measurements, depth penetration profiles were determined by calculating the AUC of the procaine peak and normalized to the arithmetic mean of the aromatic amino acid peak at 1008 cm^{-1}, as this peak does not interfere with the spectra of procaine and because no coverslip was used, this peak does not overlap with any glass-signal (Figure 3), as with the off-line-measurements.

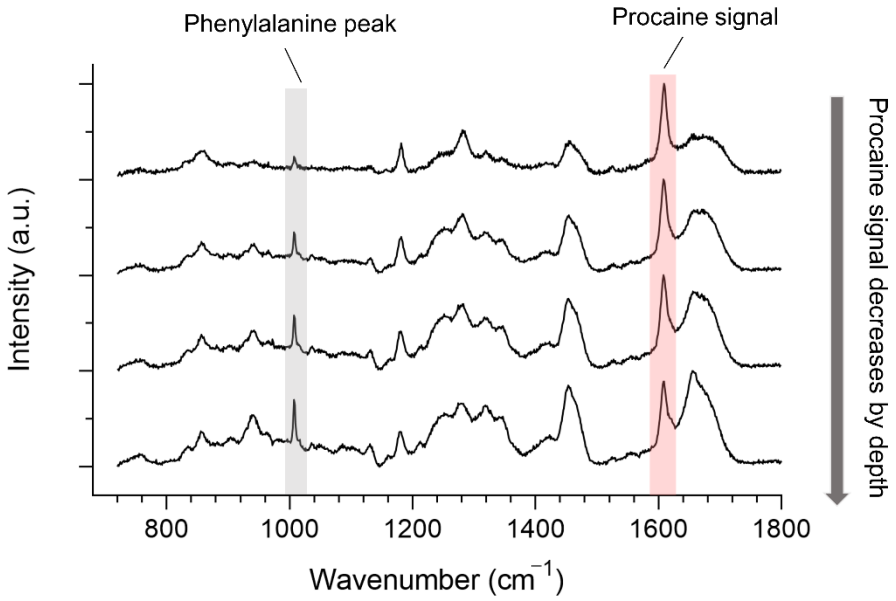

Figure 3. Typical spectra in in-line skin analysis of procaine signal decreasing by depth with crucial spectral features, which are highlighted in color.

2.8. Statistical Data Analysis

Spectra were obtained from repeated measurements ($n \geq 9$). The graphs were shown with mean values ± standard deviations (mean ± SD). Statistical differences were determined using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK), which were employed using GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, CA, USA). Diagrams and statistical differences were ultimately generated. Significant differences were marked with a different number of asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results and Discussion

3.1. Off-Line Monitoring of Drug Penetration

The drug penetration of procaine-HCl out of two different hydrogel formulations was investigated from different time points, in order to display the effect of L23 as a penetration enhancer and to compare the obtained results to in-line measurements performed in parallel.

Figure 4 shows the penetration profiles at different incubation times with and without the addition of L23 as the penetration enhancer.

Figure 4A shows the procaine penetrations after 14 h incubation within formulation gels. The depth detections reached more than 25 µm of the skin. The Raman intensity of the procaine band at 1612 cm^{-1} is displayed as a function of depth, where 0 is assigned to the surface of the skin. The red triangles represent the penetration profile of procaine out of the formulation containing L23 as a penetration enhancer, while the green circles show the penetration profile of the reference formulation without penetration enhancer. It is clear that the procaine signal was detected from the skin surface to around 6 µm of the skin with no enhancers added. However, procaine signals deeper in the skin of nearly 8–9 µm can still be found with the effect of adding L23 in formulations. This is in line with our previous studies [6,32]. Higher procaine signals were observed at the upper layer of

the skin (within 4 µm). Thus, it becomes obvious that the addition of L23 enhanced the depth of drug penetration. These enhancement effects can be noticed, although in a shorter incubation time of 14 h.

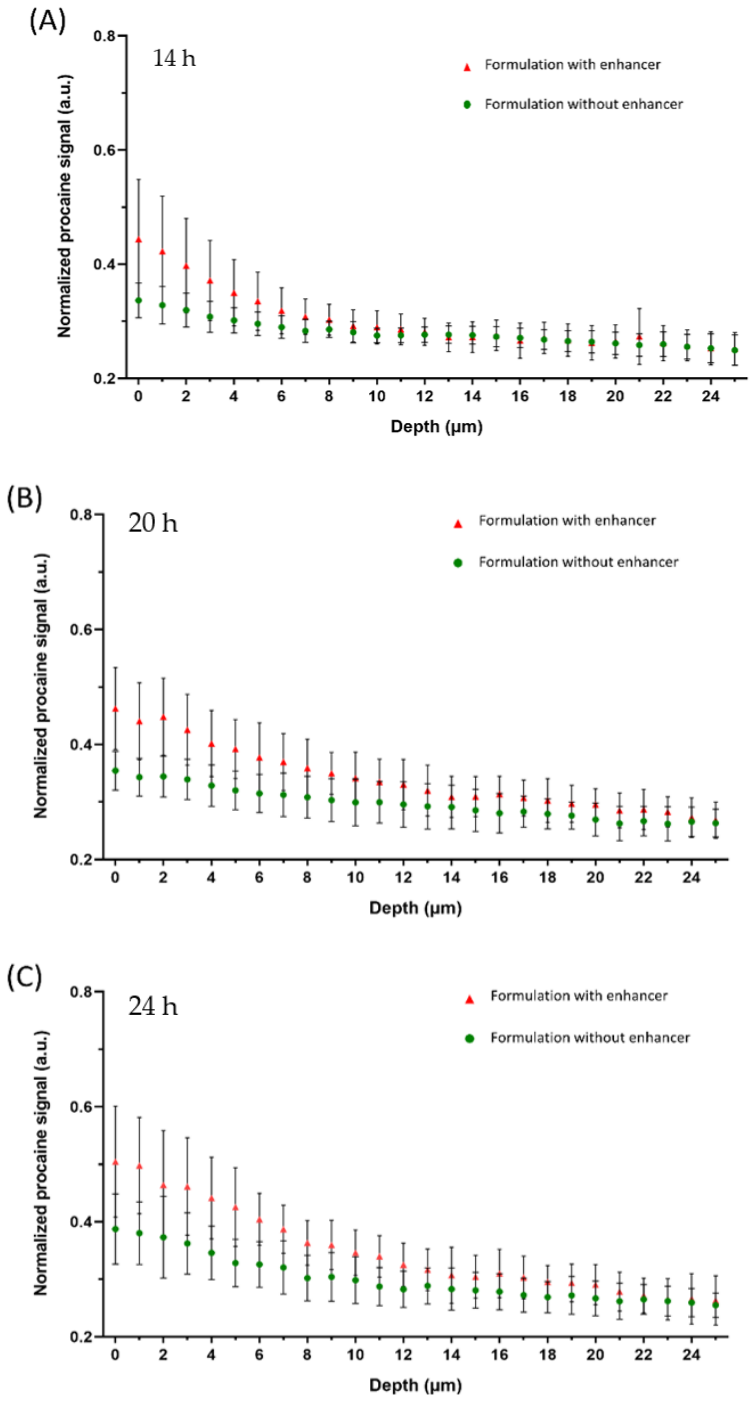

Figure 4. Penetration profiles of procaine HCl in porcine skin after the skin incubation time of 14 h (**A**), 20 h (**B**) and 24 h (**C**) with the measurements of the off-line device. Two procaine-HCl containing formulations were compared: formulation with enhancer (red triangles) and without enhancer (green circles). Mean ± SD ($n \geq 9$).

Extending the skin incubation time to 20 h (Figure 4B), a higher amount of procaine in the skin could be detected for both formulations and the enhancement effect is even clearer. In detail, the reference formulation shows a higher penetrated amount of procaine in the upper layer of the skin compared with 14 h incubation. Meanwhile, the penetration depth

presented a slight increase as well, to around 10 µm. Comparatively, in the formulation with the addition of L23, it can be better noticed that the enhancer strongly improved the procaine penetration to around 20 µm. The difference between the two formulations was more pronounced with 20 h skin incubations.

At the 24 h time point, Figure 4C shows a very similar tendency with 20 h incubation. However, the gap in their penetration profiles is more distinct than at 20 h. It can be noticed that the reference represented a similar penetration depth of procaine compared with 20 h incubation, with a detectable procaine signal to approximately 11 µm. While the penetrated amount is noticeably higher, especially within the first 5 µm of the skin. Likewise, the L23 added formulation indicated similar tendencies. The penetration depth of procaine with the enhancement effect of L23 was not more pronounced than after 20 h. A similar depth was remarked to be approximately 20 µm as the result of 20 h incubations.

Overall, time-dependent penetration properties were observed in the comparisons of two formulations. With the increase of incubation time, drug penetration amount and depth both showed apparent improvement. However, the maximum penetration depth was reached after around 20 h incubations. The penetrated drug amount constantly increased.

3.2. In-Line Monitoring Drug Penetration

Figure 5 shows the results of in-situ measurements of procaine-HCl penetration into porcine ear skin over a total incubation time of 24 h.

For both formulations, after 14 h, procaine could be found at a skin depth of more than 20 µm (Figure 5A), although most of the drug was detected within the first 10 µm of the skin. As expected, the penetration profile of the formulation containing the penetration enhancer shows a higher procaine signal within the first 7 µm of the stratum corneum.

After 20 h incubation time, the enhancement effect of L23 is clearly visible and the penetration profiles of both formulations show the same characteristics as the profiles obtained by off-line measurements (Figure 5B). Still, the highest amount of procaine was found within the first 10 µm of the stratum corneum, for both formulations. While the procaine signal of the reference formulation decreased after 5 µm, the profile of the formulation with penetration enhancer shows a flatter course, with a distinct procaine signal even below a skin depth of 20 µm. The enhancement effect was even more pronounced after 24 h. As Figure 5C shows, the amount of procaine within the stratum corneum was significantly higher after incubating with L23 as penetration enhancer. Still, the largest amount of procaine was found within a skin depth of 10 µm for both formulations, although the decrease in procaine signal was more pronounced for the reference formulation. Below a skin depth of 20 µm, procaine was detected for both formulations—the signal in both cases was considerably stronger compared to the previous time points.

3.3. In-Line and Off-Line Comparisons

Monitoring the in-situ depth penetration of a drug by CRS from an aqueous solution has already been performed by our group [28]. Following the depth penetration of procaine-HCl from a semisolid formulation in-situ constituted a completely new challenge. While an aqueous solution serves as a perfect immersion medium for the objective used, a hydrogel-layer between objective and skin could be used as an immersion medium too, but leads to a certain signal attenuation, which could, fortunately, be compensated by higher laser intensity. To obtain an evaluable signal, an absolute absence of air-bubbles in the hydrogel-layer is absolutely crucial. Because the formulations used in these experiments were fluid at low temperatures, a hydrogel-layer free of air inclusions could be achieved by applying the cold formulation using a pipette.

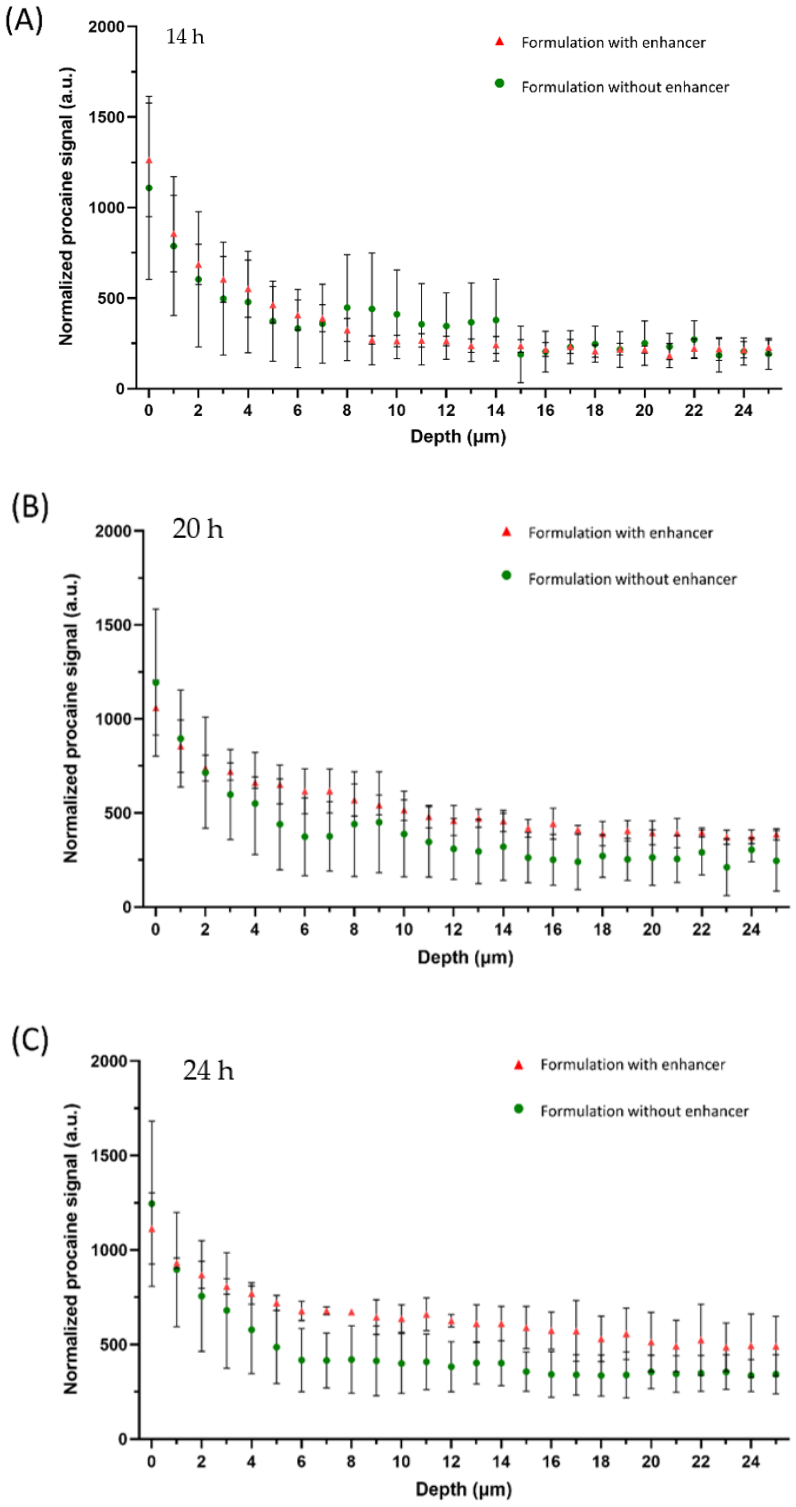

Figure 5. Penetration profiles of procaine HCl in porcine skin after the skin incubation time of 14 h (**A**), 20 h (**B**) and 24 h (**C**) with the measurements of the in-line device. Two procaine-HCl containing formulations were compared: formulation with enhancer (red triangles) and without enhancer (green circles). Mean ± SD ($n \geq 9$).

For off-line measurements, the relative amount of procaine penetration was obtained with a procaine spectral signal normalized by the amide-I signal. Although the advantages of using the phenylalanine peak for normalization and relative quantification have been well discussed [1,33], its overlap with the glass peak signal in the off-line detections pre-

cludes its use here. In this case, the normalized ratio would be still higher than 0 even without the contribution of the procaine signal, since the procaine signal was partly overlapped with the amide-I peak. Thus, by calculating the normalized ratio, with a constant value of around 0.27, as shown in Figure 4, would be the drug penetration baseline that cannot detect any procaine signals.

Despite the 14 h penetration profiles for the reference formulation, both methods lead to comparable results. Especially the depth profiles of the formulation containing the penetration enhancer show a very similar course at each time point. After 14 h, procaine was mostly located within the first 10 µm of the stratum corneum. At a skin depth below 10 µm, both formulations show the same characteristics as the course flattens. However, there was still a drug signal at 20 µm skin depth. Correlated penetration properties were also described by L. Binder et al. [5] in their interlab comparability study. As the mentioned study compared two different CRS-devices, the effect of the measurement setup used has been already described. Likewise, by calculating the AUC of varied penetration profiles, the enhancement effects can be properly compared by analyzing their significant differences.

As shown in Figure 6, off-line measurements show evident enhancement effects at three incubation time points comparing two different formulations. The reason why the in-situ measurements show no significant difference between reference formulation and the formulation with penetration enhancer at the 14 h time point could be explained by the hydration state of the skin in the experimental setup, which is due to the fact that in-situ measurements were more affected by swelling of the skin during long incubation times, while skins incubated in conventional Franz-diffusion cells were patted dry before being analyzed.

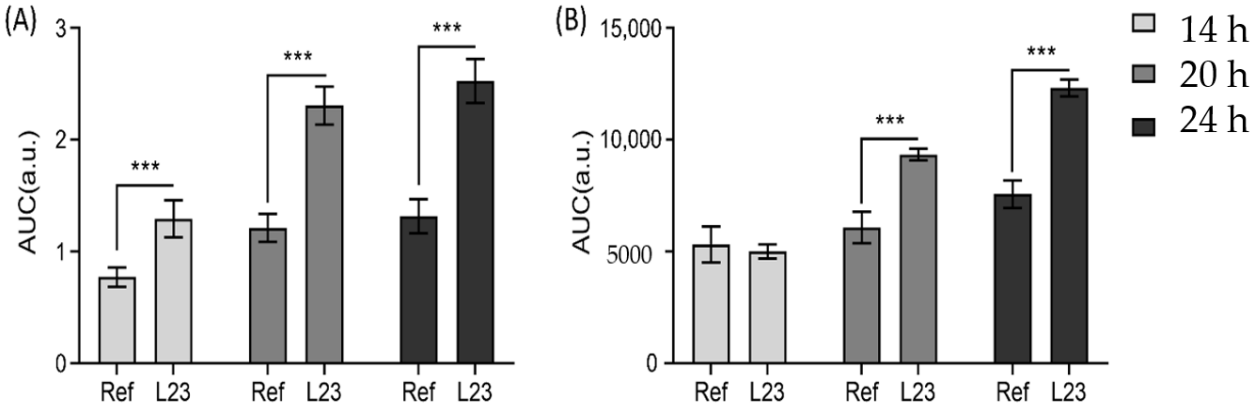

Figure 6. Area under the curve (AUC) comparison of off-line (**A**) and in-line (**B**) measurements between formulation with enhancers (L23) and formulation without enhancers (Ref) at different time points (14, 20 and 24 h). *** $p < 0.001$. Mean ± SD ($n \geq 9$).

Taken into account that the formulation with penetration enhancer shows a noticeably smaller standard deviation than the reference formulation, a different hydration state of the skin could be the reason for the similarity of both penetration profiles. As L23 affects the water uptake of the skin, after 14 h the stratum corneum may become saturated, whereas the skin incubated with the reference formulation still shows signs of swelling, leading to higher variability in the procaine signal. Furthermore, the effect of temperature should be considered. Although the skin in both methods was incubated at 32 °C, this temperature is kept constant for the whole in-line drug penetration monitoring process, while the off-line measurements were performed at room temperature by stopping the incubation process. This condition could lead to differences in penetration profiles especially while working with saturated solutions, that could crystalize during measurements at room temperature while being soluble at 32 °C [34,35].

Another big difference that should be taken into account is the impact of the donor animals used. As the skin samples from one donor allow for a limited number of measurements, two different donors were used in respective in-line and off-line studies, which may also lead to differences. Different donors may respond differently to the penetration enhancer [32].

After 20 h incubation time, the enhancement effect was visible in both methods and again, the penetration profiles and depths were well correlated. For both methods, a shift of the depth profiles to deeper skin regions in comparison to the 14 h time point was observed—the curves of the hydrogels with penetration enhancer show a steady decrease in signal until a skin depth of 15 µm. For the subsequent course, there is only a minimal decrease in signal.

While the off-line measurements show an enhancement effect mostly for the first 15 µm of the skin, a strong effect in deeper skin regions is visible for the in-situ measurements, especially after 24 h incubations, where the formulation with penetration enhancer shows a stronger shift of signal to deeper skin regions. The penetration profile flattens after a skin depth of 5 µm, showing an almost constant course with only a small signal decrease from 6 to 25 µm, while the off-line measurements show a constant signal decrease until a skin depth of 14 µm. As Figure 6 shows, the enhancement effect of L23 is more pronounced in the off-line measurements, but still both experimental setups lead to similar results. The differences in the enhancement effect are caused as the in-line measurements show a stronger procaine penetration out of the reference formulation.

4. Conclusions

In this study, we compared the results on the skin penetration of procaine-HCl obtained by two different methods, in-line and off-line measurements. Although the spectral information in both cases was recorded using the same Raman microscope, differences in skin penetration profiles were expected to vary because of the different experimental setups, in particular the incubation step and the different donor animals used. Our results show that both methods demonstrate the expected and already described influences of L23 as a penetration enhancer for procaine-HCl embedded formulations.

Furthermore, the data evaluation in both methods differs, as the phenylalanine peak used for signal-normalization in the in-line measurements was not available in the off-line measurements because a glass coverslip was needed in order to use the same objective for both experimental setups. As the penetration profiles of the formulation containing the penetration enhancer show, the different data evaluation methods led to highly comparable results.

While in-line measurements have the big advantage of being less labor-intensive and time-consuming, there are also some effects on the obtained depth profiles, such as hydration state of the skin, for example, that must be considered if using such an experimental setup. Whereas off-line measurements are more laborious, consume more resources and are, therefore, more expensive, the actual measurement could be repeated more often, as it has no need to be on time, as the in-line measurements. Because various methods and experimental setups regarding skin penetration studies are described and discussed, comparative studies are very important. As our study shows, both methods obtain comparable results.

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ORIGINAL ARTICLE

Ex vivo—In vivo correlation of retinol stratum corneum penetration studies by confocal Raman microspectroscopy and tape stripping

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Abstract

Skin penetration studies of topically applied drugs are a challenging topic in the development of semisolid formulations. The most commonly used methods can be differentiated by their character into ex vivo/in vivo, invasive/non-invasive and offline/in-line measurements. In this study, we compare ex vivo tape stripping, an invasive technique, which is often used, to confocal Raman microspectroscopy (CRM), ex and in vivo, to establish a correlation between those methods. Retinol was used as a model drug, applied in an oil-in-water emulsion, to compare the skin penetration profiles obtained by the different methods.

KEYWORDS

confocal Raman, spectroscopy, delivery, dermatology, retinol, skin barrier, skin penetration, spectroscopy

Résumé

Les études de pénétration cutanée des médicaments appliqués par voie topique constituent un véritable défi dans le développement de formulations semi-solides. Les méthodes les plus couramment utilisées peuvent être différenciées selon que leurs mesures ont lieu ex vivo/in vivo, leur caractère invasif/non invasif, ou encore selon qu'elles ont lieu en ligne/hors ligne. Dans cette étude, nous comparons le tape stripping ex vivo, une technique invasive et souvent utilisée, à la microspectroscopie confocale Raman (confocal Raman microspectroscopy, CRM), ex et in vivo, pour établir une corrélation entre ces méthodes. Le rétinol a été utilisé comme médicament modèle et appliqué dans une émulsion huile dans l'eau afin de comparer les profils de pénétration cutanée obtenus à l'aide des différentes méthodes.

INTRODUCTION

In the development of topically applied dosage forms, for the treatment of local and for systemic diseases, as well as in cosmetic research, knowing the rate and extend of ingredients penetrating the skin is essential. Monitoring drug delivery into the skin is challenging, as the number of possible analytical methods is limited. Especially in early stages of formulation development, where there is a high number of possible formulations, in vivo studies are not

possible, the additional ethical difficulties of in vivo studies show clearly, that good and reliable ex vivo models for testing skin penetration are crucial. Despite the financial and ethical burden, clinical trials in the process of approval of a generic formulation require the demonstration of bioequivalence (BE) of the generic to the reference listed drug [1]. While in the development of orally applied generic drug products, the assessment of BE is possible by established pharmacokinetic methods, showing BE of two topically applied dosage forms proves to be difficult, due to a limitation of methods accepted [2,3,4]. To address BE in dermatologic products ex vivo, Franz diffusion cells are still considered as gold standard. For this method, exercised human skin or a suitable surrogate is incubated in a Franz diffusion cell with the formulation to test and segmented afterwards, for example by tape stripping [5], to quantify the amount of active ingredient in the different stratum corneum layers. This is not only labour-intense but also very time-consuming, as every time point for every test formulation needs a separate Franz diffusion cell, followed by segmentation and extraction steps. When comparing multiple formulations at various time points, this leads to a big expense. Optical methods on the other hand, have the advantage of allowing continuous measurements, as they are non-destructive [6]. Confocal Raman microspectroscopy (CRM) has been described as a promising, non-invasive technique, for in and ex vivo skin penetration studies [7,8,9]. The non-invasive character and the fact, that many active ingredients are Raman-active and therefore can be detected without using any linkers or markers, as it is the case for example when using fluorescence microscopy, make CRM suitable for skin depth profiling. The intensity of the scattered light detected after focusing the laser beam in the sample is linearly dependent of the concentration of the substance, which enables a correlation of substance concentration and Raman signal intensity [10]. To obtain penetration profiles of an active ingredient, lateral scanning of a cross section of the skin can be performed or focusing the laser sequentially to different depths of the skin (depth scan).

In previous studies we already showed, that in situ skin penetration measurements by CRM are possible [11] and compared the impact of in-line and offline CRM measurements [12], were we tested the differences of measuring skin penetration with the formulation kept on top of the skin (in-line) and after a separate incubation step in Franz cells (offline). The purpose of this study is to find a correlation between ex vivo tape stripping, ex vivo, in situ CRM and in vivo CRM for measuring the skin penetration of retinol. Pig skin was used for the ex vivo experiments, as it is widely used for skin penetration studies, while the in vivo experiments were performed on human volunteers. Retinol was used as a lipophilic model drug, applied in an oil-in-water emulsion, as it is commonly used in cosmetic formulations, for its multiple benefits as, for example positive effects on skin ageing [13,14]. Furthermore, skin penetration of Retinol, when dissolved in medium-chain triglycerides, as it is the case for the emulsion used in this study, is already described in literature [15,16], which makes it ideal for a comparative study like this.

MATERIALS AND METHODS

Materials

Retinol (BioXtra), Methanol and Ethanol (HPLC gradienxt grade) were purchased from Sigma-Aldrich Corporation (St. Louis).

Medium-chain triglycerides (MCT; Myritol 318, BASF).

Hydroxypropyl methyl cellulose (HPMC 2208; Metolose 90 SH 100 SR, Shin Etsu Chemical Co. Ltd).

Sodium chloride, potassium chloride, disodium phosphate and monopotassium phosphate used for the preparation of phosphate-buffered saline (PBS) pH 7.4 and isotonic saline were all of European Pharmacopoeia Grade. Parafilm was obtained from Bemis Company Inc. and the tape strips (D-squame tapes Ø 2.2 cm) from CuDerm Corporation. All solutions used in this study were prepared with ultra-pure water (Elga Maxima). A local butcher provided porcine ear skin.

Preparation of the retinol emulsion

Retinol was formulated as a HPMC-stabilized emulsion according to Wollenweber et al. [17]. To this end HPMC was dispersed in hot water (80°C) being magnetically stirred and afterwards stored at 8°C overnight until a clear solution was obtained. Retinol was dissolved in medium-chain triglycerides before the HPMC solution was added, and the mixture was homogenized for 5 min (9500 rpm) using an ultra turrax T25 (IKA Werke GmbH & Co. KG).

Preparation of porcine ear skin

Postauricular porcine ear skin was used for all ex vivo studies, due to its similarity in histology and morphology to the human skin [18,19]. The pig ears (German land race, 15–30 weeks old, 40–65 Kg) used for all experiments in this study were obtained from a local butcher (Bio Metzgerei Griesshaber) and were picked up directly after the animals were butchered. The Department of Pharmaceutical Technology is registered for the use of animal products at the District Office of Tuebingen (registration number: DE

084161052 21) [20]. After the ears were cleaned, using cotton swabs and isotonic saline, the postauricular skin was removed from the cartilage, cut into 4 cm strips and pinned on aluminium foil wrapped Styrofoam bars. The skin hair was trimmed to approximately 0.5 mm and the full-thickness skin was cut to a thickness of 1.0 mm using a Dermatom (GA 630, Aesculap AG & Co. KG). As described in previous publications, the dermatomed skin was punched out to circles with a diameter of 25 mm, used for Franz cell incubation studies, whereas the skin used for in situ incubation was cut out to a diameter of 35 mm [11,12,21].

The obtained skin discs were wrapped in aluminium foil and stored at −28°C until the day of usage.

Incubation of porcine ear skin

For the conventional skin penetration experiments, Franz diffusion cells (Gauer Glas) were used. As the receptor fluid 12 ml of degassed, prewarmed (32°C) phosphate-buffered saline (PBS) were used with a string speed of 500 rpm. The porcine ear skin was placed on top and mounted with the donor compartment, before the diffusion cells were placed in a 32°C water bath for an equilibration time of 30 min. Retinol emulsion was applied using a plexiglass cylinder resulting in an amount of 0.163 mg/cm^2 retinol per skin area. To prevent water evaporation during the incubation time, all Franz diffusion cells were covered with parafilm. After the incubation time of either 1.5, 3 or 4.5 h, the skin samples were removed and the remaining formulation was gently washed off, using cotton swabs and isotonic saline. Afterwards, the actual application area of 1.5 mm diameter was punched out and carefully patted dry for tape stripping.

The skin used for in situ measurements was incubated in a custom-built device shown in Figure 1 and described in detail in our previous publication [11]. The receptor compartment was filled with 7.0 ml of degassed, prewarmed (32°C) phosphate-buffered saline (PBS) and a skin sample was placed on the grid above, ensuring the grid was completely soaked in PBS. As with the Franz diffusion cells, the donor compartment was mounted on top and the skin was tempered at 32°C for 30 min, before the formulation was applied. To this end, a plexiglass cylinder was used, leading to an amount of 0.163 mg/cm^2 of retinol per skin area. As the measurements were performed using a water immersion objective, a quartz cover slip was placed on top of the formulation, onto which a drop of water (immersion fluid) was placed, so the immersion medium did not dilute the formulation. The incubation cell was fixed underneath the Raman microscope, with the objective protruding into the donor compartment. To prevent water evaporation during the incubation time, parafilm was tightened between the incubation cell and the objective.

All experiments were performed in triplicate on separate days.

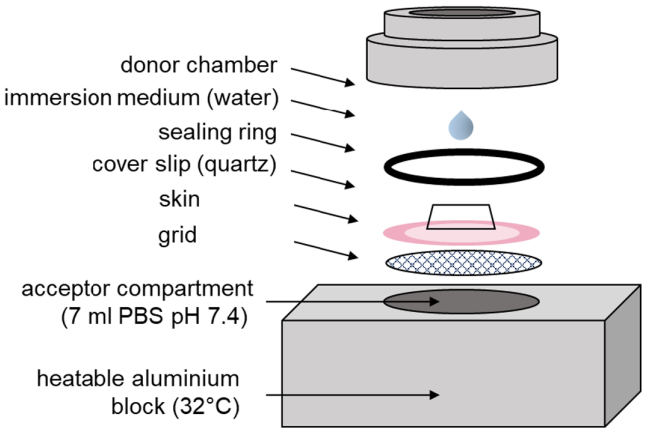

FIGURE 1 Incubation cell for in situ CRM measurements

Tape stripping

Tape stripping was performed as described by Ilić [22], segmenting each skin sample by the use of 12 adhesive tapes. For the skin removal, a tape was pressed down with a constant pressure of 140 g/cm^2 on top of the skin for 10 s and stripped off afterwards. The orientation of the following tapes was changed by 90° clockwise respectively. The mass of the skin removed was determined by weighting each individual tape before and 30 min after stripping, using a high precision analytical balance (Mettler Toledo XPE205 Delta Range; Mettler). Out of the mass of the skin removed, the stripped area (1.77 cm^2) and the density of the SC (1 g/cm^3) [23], the corresponding thickness of the skin removed by each tape was calculated. After weighing, each tape was placed in a separate microcentrifuge tube (2 ml safe lock tube ambra, Eppendorf AG). For the extraction of retinol, 2.0 ml of ethanol were added to each tube, sonicating them for 30 min subsequently (Sonorex Super RK 510/H, Bandelin electronic GmbH & Co. KG, Berlin, Germany), before centrifuging at 13 400 rpm for 15 min (MiniSpin Centrifuge, Eppendorf AG).

Retinol quantification by HPLC

Retinol was quantified using the CBM-20A HPLC system (Shimadzu Europa GmbH), equipped with an UV detector. The HPLC column EC 125/4 Nucleosil 100–5 C18 (Macherey-Nagel GmbH & Co. KG) was used along with the HPLC precolumn EC 4/3 Universal RP

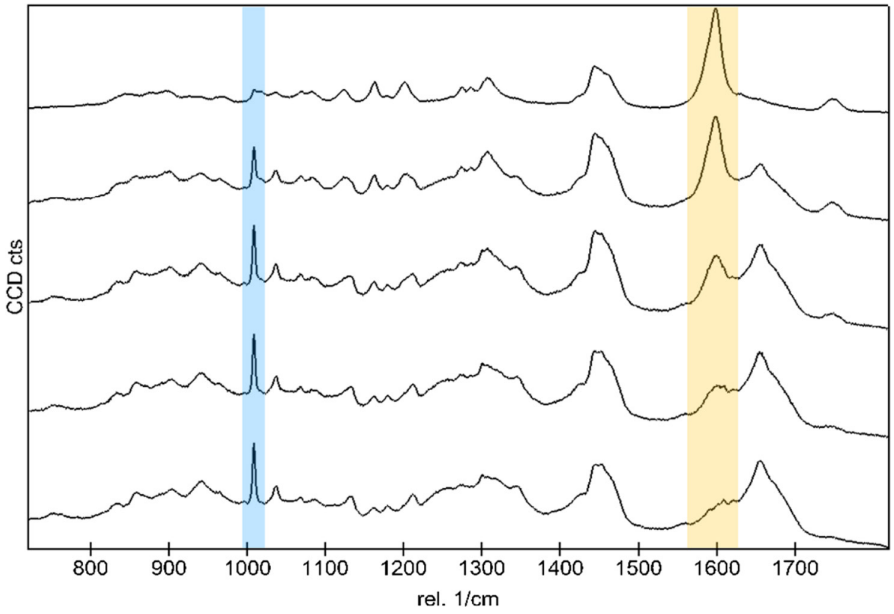

FIGURE 2 Raman spectra of the stratum corneum incubating with the 0.25% retinol emulsion, starting above the skin surface

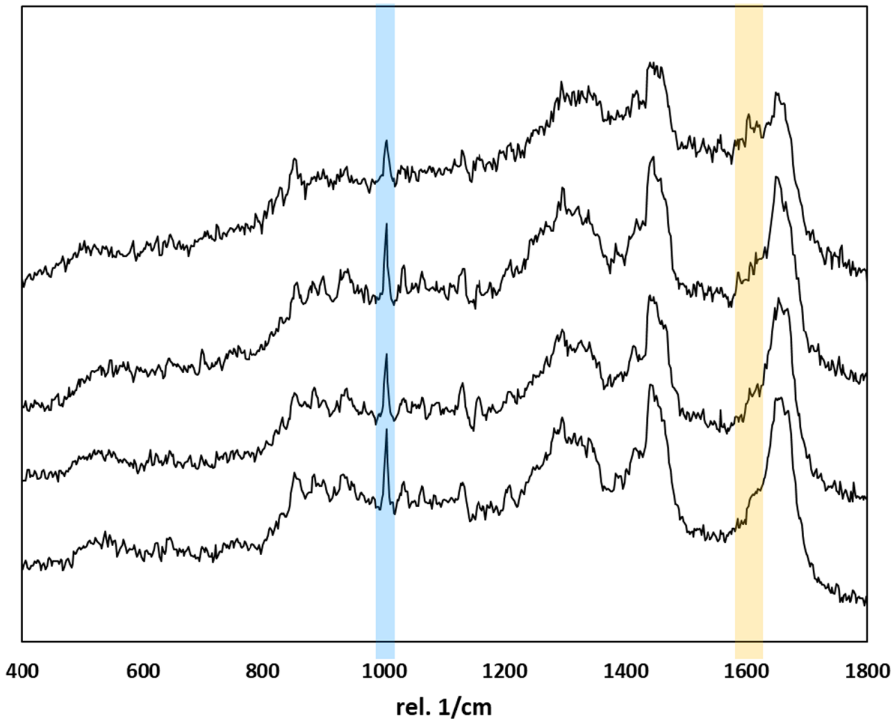

FIGURE 3 Raman spectra of the stratum corneum after incubation with the 0.25% retinol emulsion, showing the skin surface on top

(Macherey-Nagel GmbH& Co. KG). The column-oven temperature was set to 25°C. The mobile phase consisted of 90% methanol and 10% water with a flow rate of 1.0 ml/min. Aliquots of 20 µl were injected, the UV absorbance was measured at 325 nm. The calibration ranged from 0.01 to 10 µg/ml retinol with a limit of detection at 0.0009 µg/ml and a limit of quantification at 0.0027 µg/ml ($R^2 > 0.96$).

Confocal Raman microspectroscopy (CRM) ex vivo/in situ

For the in situ measurements, an alpha 500 R confocal Raman microscope (WITec GmbH), equipped with a 532-nm excitation laser, set to 30 mW by an optical power metre (PM100D, Thorlabs GmbH), a UHTS 300

spectrometer, a DV401-BV CCD detector and a 63× water immersion objective with numerical aperture of 1.0 (W 'Plan-Apochromat' 63/1,0 M27; Carl Zeiss) was used. Spectra in the range of 700–1800 cm^{-1} were obtained by an optical grating (1800 g/mm, spectral centre, 1300 cm^{-1}). Two-dimensional image scans of 5 μm width and 25 μm depth were recorded, acquiring a total of 10 spectra per line and 50 lines per vertical dimension, with an integration time of 1.5 s per spectra [11,12].

All recorded spectra were processed by cosmic ray removal and background subtraction, in the 'shape' option (size: 400), using the software Project Plus 5 (WiTec GmbH, Ulm, Germany). For the background subtraction, a rounded shape is approached pixel by pixel to the spectrum from below [24]. Principal component analysis (PCA) was performed on all spectra for noise reduction [25]. Depth penetration profiles were calculated by determing the area under the fitted curve (AUC) of the retinol band at 1594 cm^{-1} (C=C stretching mode), marked yellow in Figure 2, using the trapezoidal method. Furthermore, the AUC of the aromatic amino acid peak at 1008 cm^{-1} (ring breathing mode), marked blue in Figure 2, was calculated and used to normalize the retinol signal, as it comes to signal attenuation within deeper skin regions.

The half maximum of the phenylalanine signal was also used to determine the skin surface [26], as the measurements were started above the skin. Out of every two-dimensional image scan, three individual depth profiles were extracted, so for every incubation time point a total of nine depth profiles was used to calculate the mean penetration profile.

Retinol quantification by CRM

To quantify the amount of retinol detected by CRM within the skin, the method by Caspers et al. was used [27]. Therefore solutions of retinol in ethanol were prepared with mass ratios of 0.01, 0.5, 1, 5 and 10 mg/g (retinol: ethanol). Furthermore, solutions of ethanol and water with mass ratios of 10, 20, 30, 40 and 50 mg/g (ethanol: water), as well as solutions of BSA in water with mass ratios of 20, 40, 60, 80 and 100 mg/g (BSA:water) were prepared. The Raman signal intensity ratios of material and solvent were calculated for every solution. The Raman signal intensity ratios is plotted as a function of their corresponding mass ratios and the proportionality constant c (material to solvent) was calculated as the slope of the linear regression (coefficient of determination for all regressions $R^2 > 0.99$). Equation (1) shows the correlation of the proportionality constants and the Raman signal ratio, out of which the retinol/protein mass ratio can be calculated.

$$\frac{m_{\text{retinol}}}{m_{\text{protein}}} = c^{-1}_{\text{BSA:protein}} \cdot c_{\text{BSA:water}} \cdot c^{-1}_{\text{ethanol:water}} \cdot c^{-1}_{\text{retinol:ethanol}} \cdot \frac{R_{\text{retinol}}}{R_{\text{protein}}} \quad (1)$$

Confocal Raman microspectroscopy (CRM) in vivo

The in vivo study was performed in three healthy volunteers (male and female, BMI < 30, skin types I-III according to Fitzpatrick, no use of topical medicines within the last 14 days, no Tattoos in the study site) after the written informed consent had been obtained from each volunteer. This non-medical study on healthy human subjects was executed according to the principle requirements of the Declaration of Helsinki and according to the main principles of Good Clinical Practice (GCP).

Study was performed in controlled climate conditions (22 ± 2°C and at 50% ± 7.5% relative humidity). After 30 min of acclimatization, measurements were started in another air-conditioned examination room with the same temperature and relative humidity.

Three test areas for three different measurement time points (1.5, 3 and 4.5 h) were assigned to the left volar forearm of each subject. 250 μl of the retinol emulsion was applied by using an extra-large Finn chamber (Epitest Ltd Oy) without filter disc. Three measurements were performed 1.5, 3 and 4.5 h after applying the retinol emulsion. Before starting the measurements, the test area was cleaned with a water moistened paper towel. For in vivo CRM measurements the 'gene2-SCA Ultimate' by RiverD International B. V., Rotterdam, Netherlands was used. The device has two built-in wave class 3B lasers. The red light laser operates at a wavelength of 671 nm and is configured to measure at high wavenumbers (HWN) between 2500 and 3800 cm^{-1}. The second laser, a near-infrared laser, operates at a wavelength of 785 nm and measures in the 'fingerprint region' with wavenumbers from 400 to 1800 cm^{-1}. A pinhole of 50 μm in diameter was used and fingerprint profiles recorded of the outermost layers of the epidermis starting on the skin surface at 0 μm and going down to 28 ± 5 μm deep into the skin with steps of 4 μm. Repeated measurements were taken from a skin region in the centre of each test area on a subarea of 500 × 500 μm. Eight to ten profiles were acquired with an integration time of 5 s. Figure 3 shows Raman spectra of the stratum corneum after incubation with the retinol emulsion, starting with the skin surface on top.

Data evaluation and statistics

Data were found not to be distributed normally and different numbers of samples/measurements were obtained

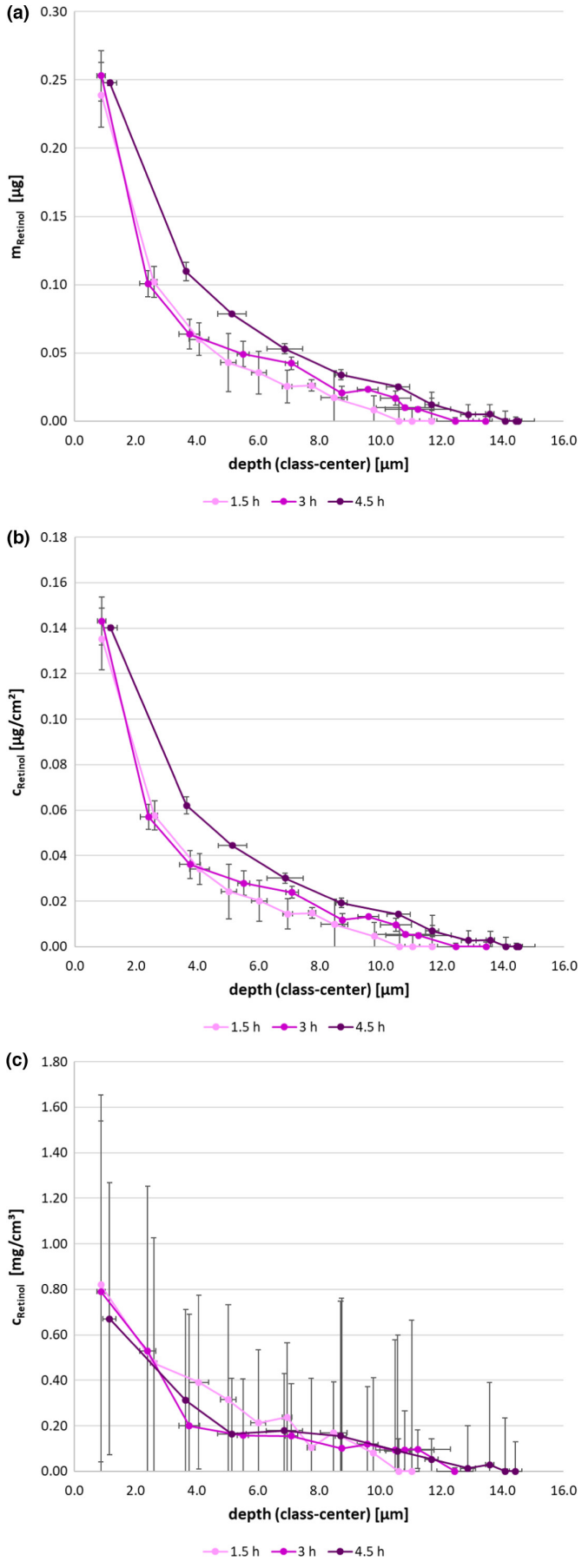

FIGURE 4 Skin penetration profiles of retinol over 4.5 h incubation time with 0.25% retinol emulsion, the error bars are showing the median absolute deviation. (a: Amount of retinol per class-centre of skin removed; b: Amount of retinol/skin area per class-centre of skin removed; c: Amount of retinol/skin volume per class-centre of skin removed)

from the three methods. To compensate for this, median ± median absolute deviation of the median were calculated and are shown in diagrams.

RESULTS

Ex vivo tape stripping

Figure 4 shows the depth penetration profiles of retinol during a total incubation time of 4.5 h obtained by HPLC analysis of skin samples segmented via tape stripping. As for each experiment, 12 tapes were used, each depth profile consists of 12 data points. The amount of retinol found within each tape can be displayed as the median (Figure 4a). For the corresponding skin depth, the class-centre of the skin layers removed by the equivalent tapes is shown, as the amount of skin removed by each tape and therefore the skin depth varies strongly with every tape. In tape stripping experiments, the absolute amount of drug removed by each tape strip is obtained. Penetration profiles showing amount versus depth are displayed in Figure 3. For better comparability to the CRM results, the depth profiles are also displayed as a function of the retinol amount per skin area (µg/cm^2) over depth as different areas were used in Franz cell experiments and CRM in-line measurements due to technical reasons (Figure 4b). As CRM measures the amount of a substance inside a volume (the laser focal volume) it gives the concentration of retinol inside the skin. Thus, results from tape stripping are also shown as a function of the retinol concentration in the skin (mg/cm^3) over depth (Figure 4c).

The highest amounts/concentrations are reached within the first 5 µm of the stratum corneum and the total penetration depth is below 15 µm. The depth profiles show a slight increase of the retinol concentration in the skin over the incubation time. As expected, the highest amounts of retinol within the skin are found after 4.5 h of incubation time.

The plots showing amount/area versus depth give the same curve progressions as the plot showing amount versus depth while absolute values are decreased by a factor of two. Which was expected as the detected amount was only normalized from an area of

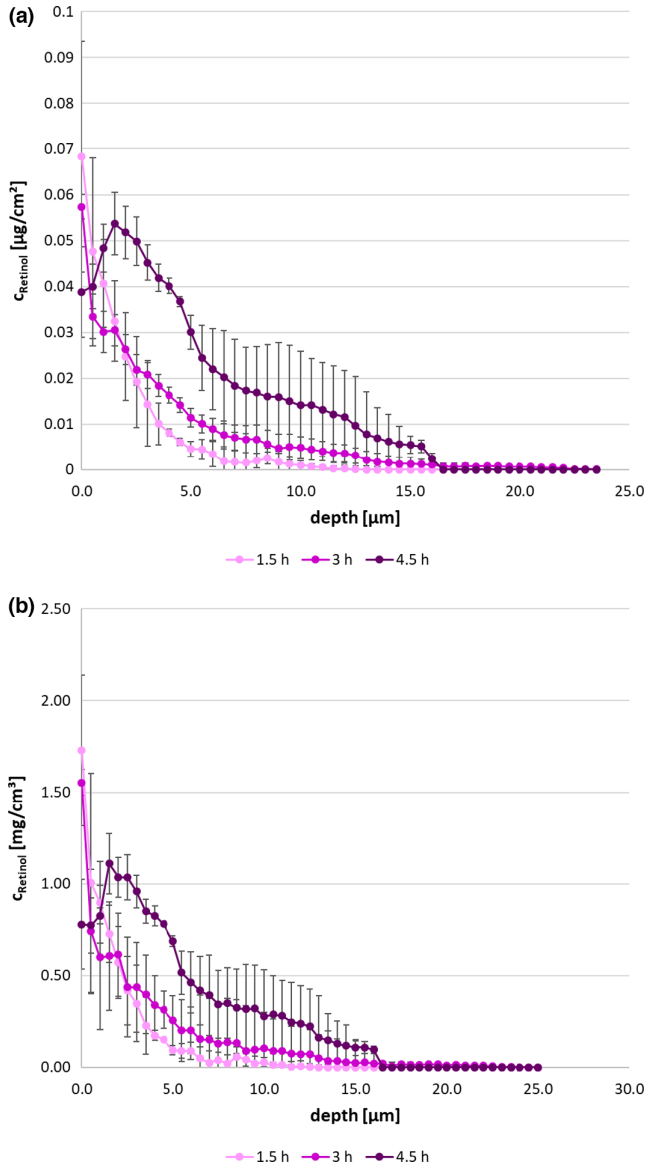

FIGURE 5 Skin penetration profiles of retinol over 4.5 h incubation time with 0.25% retinol emulsion, the error bars are showing the median absolute deviation. (a: Amount of retinol/skin area per skin depth; b: Amount of retinol/skin volume per skin depth)

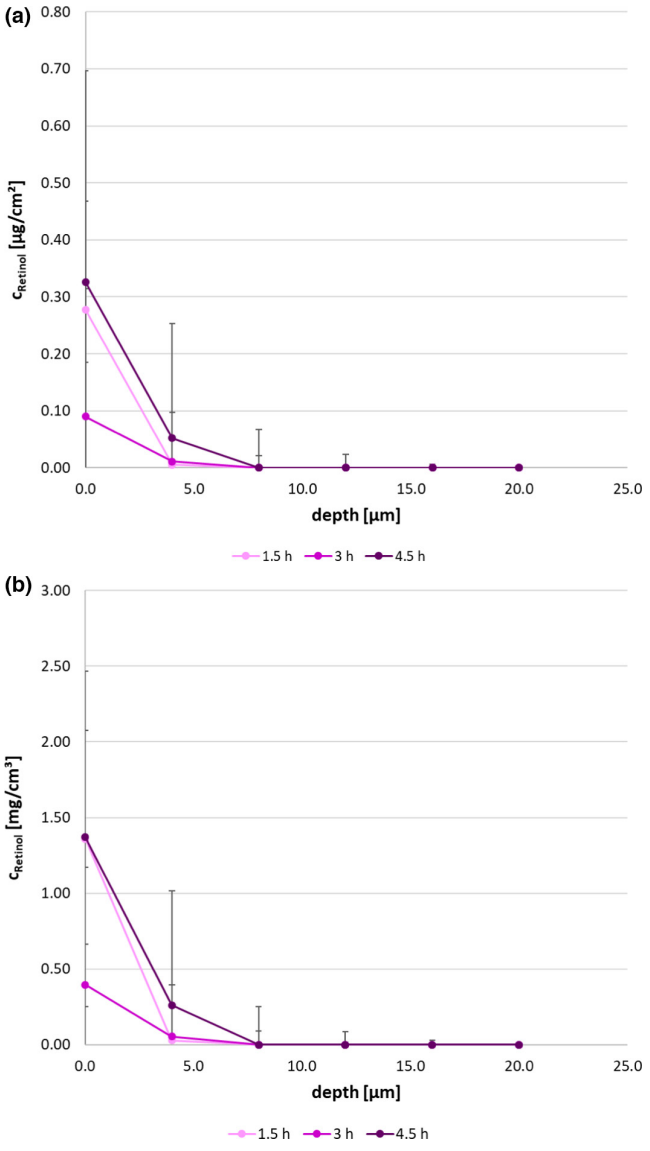

FIGURE 6 Skin penetration profiles of retinol over 4.5 h incubation time with 0.25% retinol emulsion, the error bars are showing the median absolute deviation. (a: Amount of retinol/skin area per skin depth; b: Amount of retinol/skin volume per skin depth)

2 cm^2 (penetration area in the Franz cells) to an area of 1 cm^2. When displayed as retinol amount per skin volume, shown in Figure 4b, the concentrations range between 0 and 0.82 mg/cm^3.

Ex vivo/in situ CRM

Figure 5 shows the results of in situ CRM measurements of retinol skin penetration during 4.5 h of incubation with the emulsion described. Starting at the skin surface, the amount of retinol per skin area (A), as well as the amount of retinol per skin volume (B) is displayed as a function of depth.

Again, the curve progressions are the same, but absolute values deviate due to the fact that the actual penetration area of 7.1 cm^2 was normalized to 1 cm^2 for better comparison with tape stripping results. After 1.5 h of incubation time retinol can be found almost exclusively within the first 5 µm of skin depth, between 5 and 10 µm the retinol concentration is very low and below 10 µm no retinol can be detected. The retinol concentration within the skin increases as expected after 3 h of incubation time, even in 10 µm skin depth

it is clearly detectable, while the highest concentrations can still be found within the first 5 µm. After 4.5 h, a further shift towards deeper skin regions is visible, but there is no further increase in total penetration depth, compared with the 3 h depth profile. Only negligible retinol concentrations are found below 16 µm of skin depth. While the first two time points show only concentrations below 0.2 mg/cm^3 below 5 µm skin depth, the retinol concentration in the skin is remarkably higher for the 4.5 h time point, showing concentrations above 0.2 mg/cm^3 until 10 µm of skin depth.

In vivo CRM

Figure 6 shows the results of in vivo CRM measurements of retinol skin penetration during 4.5 h of incubation with the emulsion described. Starting at the skin surface, the amount of retinol per skin area (A), as well as the amount of retinol per skin volume (B) is displayed as a function of depth. This is again for better comparison with ex vivo measurements which were performed with different penetration areas.

After 1.5 h of incubation time most of the retinol can be found within the first 10 µm of skin depth, decreasing further to a maximum penetration depth of 16 µm. The amount of retinol in the skin decreases after 3 h of incubation time, the vast amount can be detected almost exclusively within the first 10 µm of skin. As several measurements in the application area of all three subjects show no retinol concentration at all, the median is considerably smaller than compared with the other two time points. After 4.5 h the amount of retinol found in the skin increases again to values similar to those found after 1.5 h.

DISCUSSION

The penetration of retinol from an oil-in-water emulsion was analysed over 4.5 h by ex vivo tape stripping, ex vivo/in situ CRM and in-vivo CRM. All methods showed that retinol penetrates the skin, but penetration depth (10–15 µm) as well as extent of penetration are low. This was expected as retinol shows a high logP. The oil phase of the emulsion and the SC lipids both exhibit high lipophilicity and thus partitioning from the oil phase of the emulsion to the lipophilic SC is not favoured. This finding complies with previous research e.g. by Caspers & Puppels [16] (*J Contr Rel* 2009) who found penetration depths of 10 µm when retinol penetrated from medium-chain triglycerides (which is the same oil as the oil phase in the present emulsion). On the skin surface, concentrations between 0.5 and 2.5 µg/cm^3 were obtained with the three methods and over the three time points. As comparison was performed using pig skin ex vivo and human skin in vivo as well as utilizing skin from different donors ex vivo, the deviations are within a range that can typically be seen in skin penetration research. This concentration is still well below values found for retinol penetration after application in a hydrophilic vehicle (10 µg/cm^3 after 1 h from propylene glycol/ethanol) from which retinol is known to penetrate very well into the skin. Thus, the values obtained in our research are assumed to be in a valid range. Some deviations in the curve progressions appear and are worth to be discussed.

In tape stripping experiments, the penetration plots of the three time points are almost convergent with a slight increase after 4.5 h. In ex vivo CRM measurements, the concentration at the skin surface decreases over time while the concentration in deeper skin layers increases. This may be a result of retinol depletion from the skin surface as a finite amount of formulation was used. Also in ex vivo CRM, an occluding effect due to the cover slip used to separate skin and formulation from immersion medium and objective may have led to an increased hydration over time, thus increasing the volume of the SC and as a result decreasing the concentration. As the SC is most dry at its surface, such hydrating effect may have led to most pronounced effects on the SC surface, thereby reducing retinol concentrations at the skin surface. A factor that may have affected retinol quantification in ex-vivo tape stripping is its instability against oxidation [28]. Out of the three methods, tape stripping is the one in which sample preparation takes the longest. Although the highest efforts have been taken to perform experiments as quickly as possible, some amount of retinol may have been degraded during sample processing. As CRM measures the skin directly, no sample processing is needed, and degradation effects will have had negligible influence.

While the depth penetration profiles measured ex vivo show retinol concentrations between 0.25 and 0.50 µg/cm^2 between 5 and 10 µm of skin depth, in vivo the concentrations in these skin regions are noticeably lower. On the other hand, the amounts of retinol detected on the skin surface are considerably higher for every time point than compared with the ex vivo studies. As explained above, in ex vivo CRM measurements, the skin may have been more hydrated due to occlusion by the cover slip. In in-vivo CRM measurements, the skin was only placed onto the measuring window for a short time so that higher hydration levels will not have been an issue in in-vivo experiments. In tape stripping experiments, the skin was first 'dried' (i.e. exposed to air) for 30 min before tape stripping as preliminary experiments had shown that when

stripping the SC directly after removal from the Franz cells, weighing the tapes was impossible due to evaporation of water from them. Exposing the skin samples to air for 30 min before weighing them, solved this issue as SC hydration decreased to normal/equilibrium values. This may explain the differences in penetration profile curve progressions between ex vivo and in vivo experiments. Also, despite being a suitable skin surrogate, there are still differences in skin barrier function between human skin and porcine ear skin, which could lead to differences in the skin penetration profiles [29].

In vivo experiments showed the highest standard deviations among the three methods used. This is due to inter-individual variability, which is taken more into account, as there were three subjects participating in the in vivo study whereas ex vivo all experiments were carried out on skin from the same donor (one for ex vivo CRM, one for ex vivo tape stripping). The finding that retinol amount penetrated decreases after 3 h but increases again after 4.5 h is quite peculiar. It should be noted that a large number of '0' values was obtained after 3 h which is assumed to be the reason for the detected decrease in the penetrated amount. It is assumed that this is an artefact and due to the fact that different sample areas were used for the three incubation times. It seems like that by chance areas with lower penetration were selected for the 3 h time point.

On the skin surface after 4.5 h, the retinol concentration of 1.37 mg/cm^3 or 0.83 µg/cm^2 is considerably higher, compared with the results obtained by both ex vivo methods.

CONCLUSION

The aim of this study was to compare the skin penetration profiles of retinol obtained by two different ex vivo methods to the profiles obtained by a small in vivo study and find a correlation. Furthermore, a calibration method described by Caspers et al. [27] was performed on the set up used for the ex vivo CRM measurements, to be able to compare not only the characteristics of a skin penetration profile but also have the corresponding concentration of retinol at each measuring point. This is a further improvement to the in situ skin penetration method we developed [11,12].

As in vivo skin penetration studies are not only laborious and expensive but also face ethical difficulties, ex vivo methods are indispensable. In this study, we were able to show, that all three methods deliver comparable skin penetration profiles of retinol, when applied as an emulsion. Previous studies already showed that the rate and extend of retinol skin penetration depends on the solvent. When dissolved in medium chain triglycerides, as in the emulsion used in this study, only little skin penetration occurs [15]. As expected, this was the case in our present study as well, all three methods showed presence of retinol especially within the first 10 µm of skin depth and a total penetration depth of approximately 15 µm. The concentration range of retinol in the skin is also comparable within all three methods, whereby the way the data is displayed is important when comparing different methods. As the depth profiles in this study show, depending on the method used, differences in depth profiles occur when the concentration of the active ingredient is displayed either normalized to the skin area (cm^2) or on the skin volume (cm^3).

In the early stages of formulation development, in vivo studies are not possible and face ethical difficulties in general. While tape stripping, as a destructive method for skin penetration studies is very time- and resource-consuming, this study emphasizes again that CRM is an excellent technique for ex vivo skin penetration studies.

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CONFLICT OF INTEREST

None.

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ex vivo – in vivo comparison of drug penetration analysis by confocal Raman microspectroscopy and tape stripping

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Abstract

When it comes to skin penetration analysis of a topically applied formulation, the number of suitable methods is limited, and they often lack in spatial resolution. In vivo studies are pivotal, especially in the approval of a new product, but high costs and ethical difficulties are limiting factors. For that reason, good ex vivo models for testing skin penetration are crucial. In this study, caffeine was used as a hydrophilic model drug, applied as a 2 % (w/w) hydrogel, to compare different techniques for skin penetration analysis. Confocal Raman microspectroscopy (CRM) and tape stripping with subsequent HPLC analysis were used to quantify caffeine. Experiments were performed ex vivo as well as in vivo. Furthermore, the effect of 5 % (w/w) 1,2-pentanediol on caffeine skin penetration was tested, to compare those methods regarding their effectiveness in detecting differences between both formulations.

Keywords

drug delivery; Dermatology; skin penetration; skin barrier; caffeine; confocal Raman spectroscopy

1. Introduction

Skin penetration analysis of topically applied drug delivery systems is still a challenging subject, as the number of suitable analytical methods is limited. As it is indispensable to know at which rate and to which extend active ingredients penetrate the skin, when it comes to formulation development the demand for new models for analysing skin penetration is high. Furthermore, there is a new approach in the approval of generic semisolid formulations, by establishing bioequivalence (BE) (1)(2)(3). As for most of the topically applied formulations, the target is in the skin, addressing BE through monitoring the drug concentration in blood or plasma, as it is the case for oral products, is not suitable (4). In vivo studies of clinical efficacy are still the gold standard, even in approval of generic products, but due to their high costs, ethical burdens, and inter-individual variability, especially in early stages of formulation development, they are not practicable. The ideal ex-vivo model for skin penetration analysis,

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that gives reliable information about the drug penetration properties of a topically applied dosage form in vivo, is still to be developed. Franz diffusion cells, where exercised human skin or a suitable surrogate, most commonly postauricular pig skin (5)(6), is placed between an acceptor- and a donor compartment, are state of the art. After the incubation step in Franz diffusion cells, the skin can be analysed for drug content. Conventionally this is done, by segmenting the skin using for example tape stripping or a cryomicrotome (7)(8). Optical methods, above all confocal Raman microspectroscopy (CRM), show huge benefits as many active ingredients are Raman-active and hence can be detected, without any linkers or markers. , Further, their non-invasive, non-destructive character makes the skin segmentation redundant, allowing continuous measurements and therefore save time and resources (9).
As many different approaches are being made, to address the challenges of skin penetration studies, especially regarding BE of two formulations, the aim of this study was, to compare different techniques, confocal Raman microspecctroscpoy and tape stripping. Their effectiveness to monitor skin penetration was investigated by comparing caffeine skin penetration out of two different hydrogels. In one of them, 1,2-pentanediol was used as an penetration enhancer (10), to see if both methods are capable of detecting differences in caffeine skin penetration, when changing formulation composition (11). Tape stripping with subsequent HPLC analysis, as a well-established but destructive method, and CRM, as a novel approach, were used to analyse caffeine skin penetration in vivo, as well as ex vivo. Furthermore, ex vivo CRM measurements were performed both on fresh and on frozen and thawed porcine ear skin, as for logistic reasons, porcine ear skin is usually stored at -28 °C until usage. Topically applied caffeine as a cosmetic ingredient is well tolerated and no risk to health, it is used as a hydrophilic reference substance in dermal risk assessment studies (12) and was furthermore used in this study, as it is Raman-active and its penetration characteristics are well described (13)(14).

2. Materials & Methods

2.1 Materials

Caffeine and Carbomer were obtained from Caesar & Loretz GmbH (Hilden, Germany) and 1,2-pentanediol was provided by BASF SE (Ludwigshafen, Germany). Sodium chloride, potassium chloride, disodium phosphate and monopotassium phosphate, used for the preparation of phosphate buffered saline (PBS) pH 7.4 and isotonic saline were all of European Pharmacopeia Grade. Ethanol and Methanol were obtained from Sigma-Aldrich Corporation (HPLC gradient grade, St. Louis, USA). Parafilm was purchased from Bemis Company Inc. (Oshkosh, WI, USA), finn chambers from Epitest Ltd (Hyryla, Finland) and the tape strips (D-squame tapes Ø 2.2 cm) from CuDerm Corporation (Dallas, USA). All solutions used in this study were prepared with ultra-pure water (Elga Maxima, High Wycombe, UK). Porcine ear skin was provided by a local butcher.

2.2 Preparation of caffeine gels

Table 1 shows the composition of the caffeine gels used in this study. Caffeine gels were prepared by a lab mixer (Unguator, Gako International GmbH, München, Germany), using the gel-program (15).

Table 1: composition of the caffeine gels used in this study (% w/w).

	Gel 1 [%]	Gel 2 [%]
Caffeine	2.0	2.0
1,2-pentanediol	-	5.0
Carbomer	0.5	0.5
NaOH-solution (5 %)	3.0	3.0
Water	94.5	89.5

2.3 Preparation of porcine ear skin

For all ex-vivo skin penetration studies, porcine ear skin was used, due to its well described similarity in histology and morphology to human skin (5)(6)(16).
The porcine ears were obtained from a local butcher (Bio Metzgerei Griesshaber, Moessingen-Oeschingen, Germany) on the day of the animal's death and immediately prepared. The Department of Pharmaceutical Technology is registered for the use of animal products at the District Office of Tuebingen (registration number: DE 08 416 1052 21). The full-thickness skin was cut off the cartilage, after cleaning the ears gently with isotonic saline and cotton swabs. After cutting the skin into about 4 cm wide strips, those were fixed with pins onto a Styrofoam block, which was wrapped in aluminium foil. The hair on the skin sheets was trimmed to approximately 0.5 mm, using a hair clipper (QC5115/15, Philips, The Netherlands), before the skin was cut to a thickness of 1 mm using a Dermatom (GA 630, Aesculap AG & Co. KG, Tuttlingen, Germany). Circles of 35 mm diameter were cut out for the in-situ CRM incubation experiments and circles of 25 mm were punched out for the Franz-diffusion cell incubation experiments. Skin samples were used within 24 h after preparation for the fresh-skin measurements or stored at -28 °C wrapped in aluminium foil until the day of usage.

2.4 Incubation of porcine ear skin

Franz diffusion cells (Gauer Glas, Püttlingen, Germany) were used for incubating the porcine ear skin samples, which were segmented and quantified after the incubation step. For the in-situ CRM measurements, porcine ear skin was incubated in a custom-built in-line device (mechanical workshop of the Institute for Pharmaceutical Sciences and the electronic workshop of the Institute of Chemistry, Tuebingen, Germany) (11).

For the incubation of skin samples in Franz diffusion cells, 12 ml of degassed and prewarmed (32 °C) PBS were used as receptor medium, which was constantly stirred at 500 rpm. Skin samples were mounted and tightened with the donor compartment on top. A schematic presentation is given in Figure 1 A. Before applying the gel to test, Franz diffusion cells were tempered to 32 °C in a water bath for 30 min. Then, 173 mg of each caffeine gel were applied (98.8 µg/cm²) using a finn chamber (Epitest Ltd Oy, Finland) without filter disk, and the cell was covered with a piece of parafilm to prevent water evaporation. After incubating the skin for 1, 2 and 3 h separately, the skin samples were removed and gently cleaned with a cotton swab, to remove excess formulation. The actual application area of 15 mm was punched out and equilibrated at ambient conditions for 20 min (17).

For the in-situ CRM measurements, a recently established method was used (11)(18). As shown in Figure 1 B, 7 ml of PBS were used as a receptor medium, and a skin sample was placed on the grid above the acceptor compartment, ensuring the grid was properly soaked in PBS, to prevent any air inclusion between the skin and the acceptor medium. As with the Franz diffusion cell, the donor compartment was placed on top, holding the skin in place.

Throughout the incubation time, the temperature of the incubation cell was held constantly at 32.0 °C by two thermocouples with an external controlling device attached. After an equilibration time of 30 min, 694 mg of each caffeine gel to test were applied (98.8 µg/cm²) and a piece of parafilm was tightened between the donor chamber and the objective of the Raman microscope to prevent water evaporation. The total incubation time was 3 h, measurements were performed after one, two and three hours. All experiments were performed in triplicate on separate days.

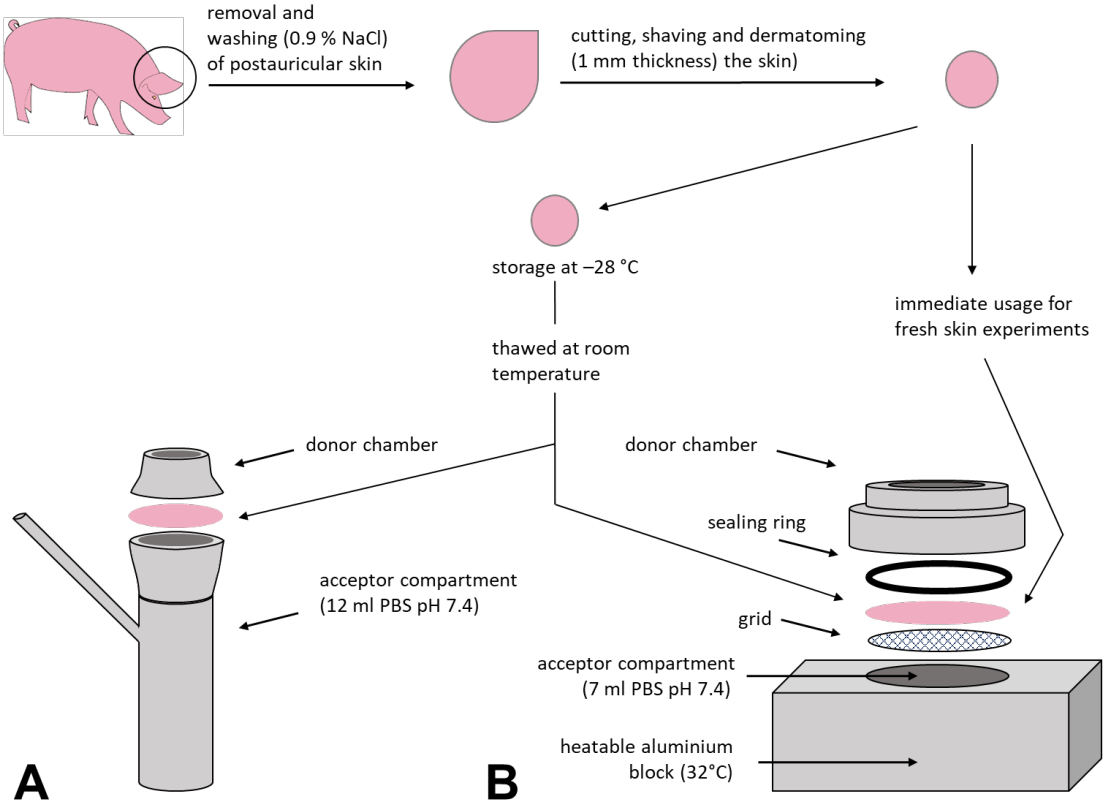

Figure 1: preparation and incubation of porcine ear skin for ex vivo experiments, showing a Franz diffusion cell (A) and the custom-built CRM incubation cell (B)

2.5 Tape stripping ex vivo

Tape stripping was performed according the method described by T. Ilić et al (19), using 12 adhesive tapes per skin sample. Each tape was weighted before and immediately after stripping, to determine the mass of the skin removed using a high-precision analytical balance (Mettler Toledo XPE205 Delta Range, Mettler, Columbus, USA). As the stripped area (1.5 cm) and the density of the SC (1 g/cm^3) are known, the thickness of the removed skin per tape can be converted out of the difference in weight of each corresponding tape strip. Skin depth is displayed as the class-center of the calculated thickness of the stratum corneum removed. For the actual stripping process, the tape is placed on top of the skin and pressed down constantly (140 g/cm^2) for 10 s. Each skin sample was stripped by 12 tapes, changing the orientation of each tape by 90° clockwise. The tapes were placed in separate centrifuge tubes (15 ml cellstar, Greiner Bio-One GmbH, Frickenhausen, Germany) afterwards and caffeine was extracted using 2.0 ml of ethanol. The tubes were sonicated (Sonorex Super RK 510/H, Bandelin

electronic GmbH & Co. KG, Berlin, Germany) for 30 min and centrifuged at 4000 rpm for 10 min (Megafuge 1.0 R, Heraeus Holding GmbH, Hanau, Germany). Then, 1.0 ml of each obtained supernatant was analysed for caffeine content.

2.6 Tape stripping in vivo

The in vivo study was performed in five healthy volunteers (female, BMI<30, skin types I-III according to Fitzpatrick, no use of topical medicines within the last 14d, no tattoos in the study site) after the written informed consent had been obtained from each volunteer. This non-medical study on healthy human subjects was executed according to the principal requirements of the declaration of Helsinki and according to the main principles of Good Clinical Practice (GCP) and with approval of the local ethics committee. 250 µg of each caffeine gel were applied (98.8 µg/cm²) by using an extra-large Finn chamber (Epitest Ltd Oy, Finland) without filter disk on the ventral surface of each forearm. After each time point, residual formulation was gently removed with a cotton swab. Tape stripping was performed according to the method described by T. Ilić et al (19), using 12 adhesive tapes per time point of each formulation. Each tape was weighted before and immediately after stripping, to determine the mass of the skin removed using an analytical balance (Sartorius BP210D, Sartorius AG, Goettingen, Germany). As the stripped area (1.5 cm) and the density of the SC (1 g/cm^3) are known, the thickness of the removed skin per tape can be converted out of the difference in weight of each corresponding tape strip. For the actual stripping process, the tape is placed on top of the skin and pressed down constantly (140 g/cm^2) for 10 s. Each skin sample was stripped by 12 tapes, changing the orientation of each tape by 90° clockwise. The tapes were placed in separate glass centrifuge tubes afterwards and caffeine was extracted using 2.0 ml of ethanol. The tubes were sonicated for 30 min and 1.0 ml of each obtained supernatant was analysed for caffeine content. Every incubation time was repeated five times for each formulation.

2.7 Caffeine quantification by HPLC

Caffeine samples obtained by ex-vivo tape stripping were quantified using the CBM-20A HPLC system (Shimadzu Europa GmbH, D Duisburg), equipped with an UV detector. The HPLC column EC 125/4 Nucleosil 100-5 C18 (Macherey-Nagel GmbH & Co. KG, Dueren, Germany) was used. The column-oven temperature was set to 35 °C. The mobile phase consisted of 80 % water and 20 % acetonitrile with a flow rate of 0.8 ml/min. Aliquots of 10 µl were injected, the UV absorbance was measured at 273 nm.

Caffeine samples obtained by in-vivo tape stripping were quantified using a Dionex Ultimate 3000 HPLC system (Thermo scientific, USA). The mobile phase was a mixture of 35 % methanol and 65 % water. The chromatographic column used was Zorbax Eclipse Plus C18, 4.6x150 mm, 5 µm (Agilent, Santa Clara, CA, USA), column temperature was set to 40 °C and autosampler temperature to 10 °C. The flow rate of the mobile phase was 1 ml/min. UV detection was performed on 275 nm.

2.8 Confocal Raman Microspectroscopy (CRM) ex vivo

For the in-situ measurements, the incubation cell was placed on the scan table of an alpha 500 R confocal Raman microscope (WITec GmbH, Ulm, Germany). To ensure that the exact same position of the skin could be tracked over the whole incubation time, the

incubation cell was held in place by four pins on the bottom of the device. The Raman microscope is equipped with a 532-nm excitation laser, a UHTS 300 spectrometer, a DV401-BV CCD detector and a 63× water immersion objective with numerical aperture of 1.0 (W "Plan-Apochromat" 63/1,0 M27, Carl Zeiss, Jena, Germany), which was placed into the donor compartment of the incubation cell, using the gel to test as immersion medium. The laser intensity was set to 25 mW, using an optical power meter (PM100D, Thorlabs GmbH, Dachau, Germany), which results in a strong signal without leading to thermal damage of the skin.

The DV401-BV CCD detector was cooled to −60 °C and a spectral range from 501 cm^{-1} to 1635 cm^{-1}, obtained by an optical grating (1800 g/mm, spectral centre: 1100 cm^{-1}) was recorded. Two-dimensional image scans of 5 µm width and 25 µm depth, with an integration time of 1.5 s per spectra were performed, acquiring 10 spectra per line and 50 lines per vertical dimension. All recorded spectra were processed using the software Project Plus 5 (WiTec GmbH, Ulm, Germany), performing a cosmic ray removal and a background subtraction in the "shape" option (size: 400). Also, principal component analysis (PCA) was performed on all spectra for noise reduction (20). For the determination of depth penetration profiles, the area under the fitted curve (AUC) of the caffeine band at 556 cm^{-1} (O=C-N deformation mode) was calculated, as this peak is selective for caffeine and not interfered with any signals of the skin. For the determination of the skin surface, as well as for the normalization of the caffeine signal the aromatic amino acid peak at 1008 cm^{-1} (ring breathing mode) was used. As the measurements were started above the skin surface, all depth profiles were cropped to the skin surface, which was defined as the half maximum of the aromatic amino acid peak (14). For normalizing the caffeine signal, the arithmetic mean of this peak at the skin surface was used, as it comes to signal attenuation in deeper skin regions and signal variations over the incubation time. Out of every two-dimensional scan, three depth profiles were extracted, which leads to a total of 9 depth profiles for each formulation, which were used to calculate the mean penetration profile of the corresponding formulation. For the calculation of enhancement ratios, the areas under the curves of the mean depth profiles were determined using the trapezoidal method. The AUC corresponding to the formulation with penetration enhancer was divided by the AUC of the reference formulation, resulting in the enhancement ratio. Representative spectra of the stratum corneum incubated with caffeine are given in Figure 2.

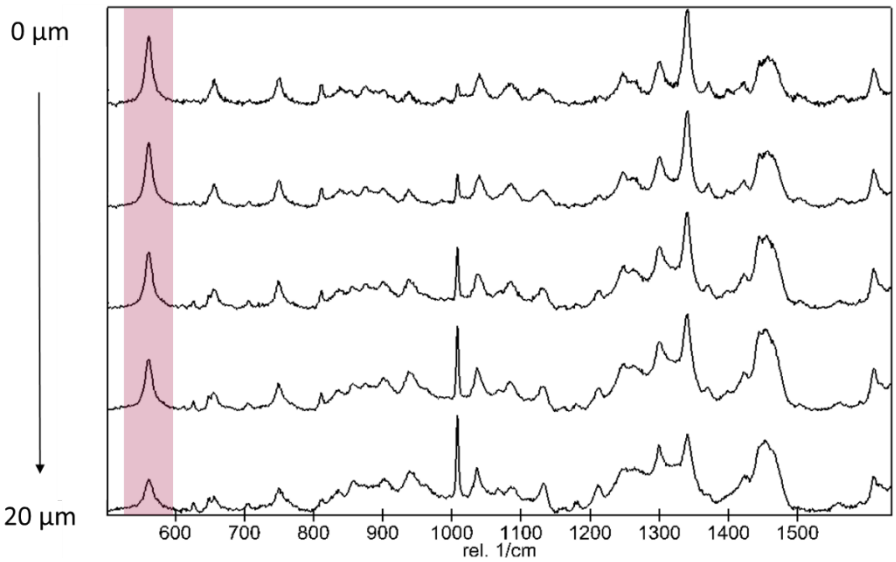

Figure 2: Raman spectra of the stratum corneum incubated with the 2 % caffeine hydrogel, starting above the skin surface and gradually moving into the skin sample(532 nm excitation laser).

To quantify the amount of caffeine in the skin, the method by Caspers et al. was adapted, as already shown for retinol in a previous publication (21)(22). The total amounts of caffeine were determined by calculating the area under the penetration profile.

2.9 Confocal Raman Microspectroscopy (CRM) in vivo

The in vivo study was performed in three healthy volunteers (male and female, BMI<30, skin types I-III according to Fitzpatrick, no use of topical medicines within the last 14d, no tattoos in the study site) after the written informed consent had been obtained from each volunteer. This non-medical study on healthy human subjects was executed according to the principal requirements of the declaration of Helsinki and according to the main principles of Good Clinical Practice (GCP).

Study was performed in controlled climate conditions (22 ± 2 °C and at 50 ± 7.5 % relative humidity). After 30 minutes of acclimatization, measurements were started in another air-conditioned examination room with the same temperature and relative humidity.

Three test areas for three different measurement time points (1.5, 3 and 4.5 hours) were assigned to the left volar forearm of each subject. 250 µg of each caffeine gel were applied by using an extra-large Finn chamber (Epitest Ltd Oy, Finland) without filter disk. Three measurements were performed 1, 2 and 3 hours after applying each caffeine gel. Before starting the measurements, the test area was cleaned with a water moistened paper towel. For in vivo CRM measurements the "gene2-SCA Ultimate" by RiverD International B. V., Rotterdam, Netherlands was used. The device has two built-in wave class 3B lasers. The red light laser operates at a wavelength of 671 nm and is configured to measure at high wavenumbers (HWN) between 2500 and 3800 cm^{-1}. The second laser, a near-infrared laser, operates at a wavelength of 785 nm and measures in the ''fingerprint region'' with wavenumbers from 400 to 1800 cm^{-1}. A pinhole of 50 µm in diameter was used and fingerprint profiles were recorded starting above the skin surface and going down to 28 +/-5 µm deep into the SC with steps of 4 µm. Repeated measurements were taken from a skin region in the center of each test area on a subarea of 500 x 500 µm. Eight to ten profiles were acquired with

an integration time of 5 seconds per spectrum (22). Representative spectra of the skin incubated with caffeine are given in Figure 3.

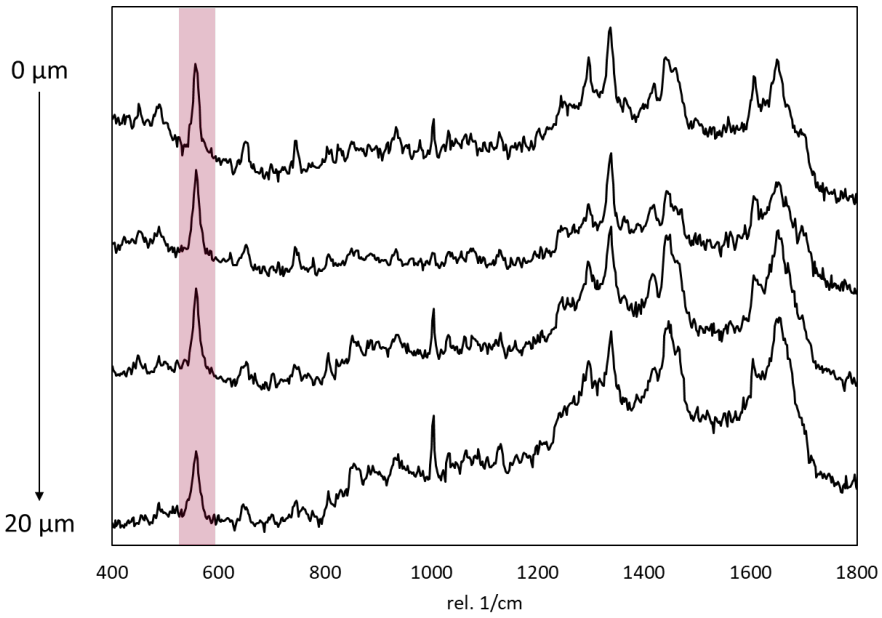

Figure 3: Raman spectra of the stratum corneum incubated with the 2 % caffeine hydrogel, starting above the skin surface surface and gradually moving into the skin sample (785 nm excitation laser).

2.10 Data evaluation and statistics

Data was found not to be distributed normally and different numbers of samples/measurements were obtained from the four methods. To compensate for this, median +/- median absolute deviation of the median were calculated and are displayed in all diagrams. No further statistical analysis was performed.

3. Results

3.1 Ex vivo tape stripping

Figure 4 shows the results of caffeine skin penetration obtained by ex vivo tape stripping after a total incubation time of 3 h with gel 1 (A) and gel 2 (B).

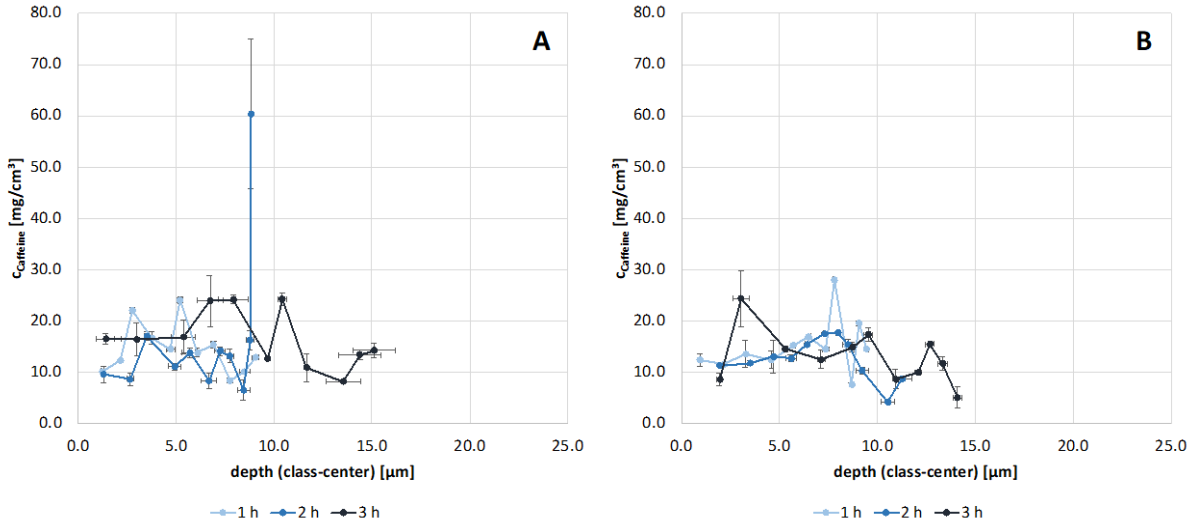

Figure 4: skin penetration profiles of caffeine over 3 h incubation time with 2.0 % caffeine hydrogel (A) and 2 % caffeine hydrogel + 5 % 1,2-pentanediol (B) (% w/w); the error bars are showing the median absolute deviation. n = 5

As the skin is segmented by 12 tapes, this leads to 12 data points for each depth profile. The corresponding skin depth is displayed as the class-centre of the skin layers removed by each tape, as the amount of skin removed and therefore the calculated skin depth varies for every tape used. As the amount of caffeine within every tape is quantified (µg), displaying the amount of caffeine as a function of depth is common with tape stripping results; also, the amount of caffeine per skin area could be displayed as a function of depth (Supplementary material figure S1 and S2). For better comparability to the results obtained by CRM, here the depth profiles show the amount of caffeine per skin volume (concentration; mg/cm^3) as a function of skin depth. In CRM measurements, the amount of drug is detected inside a volume (the laser focal volume) giving a concentration; therefore, the caffeine concentration in the skin was also calculated for all tape stripping results.

After incubating with gel 1 for 1 h, caffeine can be detected within the first 10 µm. As only little skin was removed by the tape strips, all 12 data points are located within the first 10 µm of skin depth. This is also the case after 2 h of incubation time, only the last data point shows a significant increase, indicating a concentration of over 60 mg/cm^3. That means, the last tape extracted showed only a small mass-difference, which leads to this high concentration value, as the displayed caffeine concentration was calculated from the extracted amount of caffeine and the stratum corneum volume, determined by the mass-difference of the tape before and after stripping. With other ways of data display (supplementary material), this effect is negligible. After 3 h of incubation time, the total penetration depth increases, caffeine can be detected even below 15 µm of skin depth. With the addition of 5 % (w/w) 1,2-pentanediol, caffeine can be detected below 10 µm after 2 h of incubation time, while the course of the 1 h time point has similar penetration properties, as after incubating with gel 1. Despite the penetration enhancer, no caffeine can be detected below 15 µm after incubating for 3 h.

3.2 In vivo tape stripping

Figure 5 shows the results of caffeine skin penetration obtained by in vivo tape stripping after a total incubation time of 3 h with gel 1 (A) and gel 2 (B).

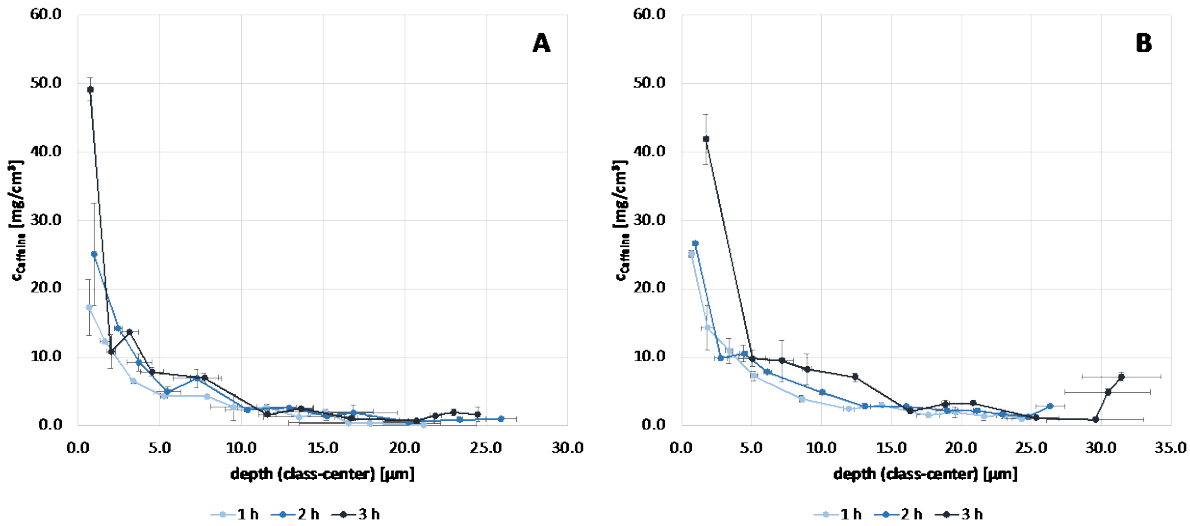

Figure 5: skin penetration profiles of caffeine over 3 h incubation time with 2.0 % caffeine hydrogel (A) and 2 % caffeine hydrogel + 5 % 1,2-pentanediol (B) (% w/w); the error bars are showing the median absolute deviation. n = 5

For better comparability to the results obtained by CRM, the depth profiles again show the concentartion of caffeine as a function of skin depth. As the amount of caffeine within every tape is quantified (µg), displaying the amount of caffeine as a function of depth is common with tape stripping results; also, the amount of caffeine per skin area could be displayed as a function of depth (Supplementary material figure S3 and S4).

After incubation with gel 1 for one hour, caffeine can be detected especially within the first 10 µm of skin depth, between 10 and 25 µm of skin depth, the caffeine concentrations are neglectable. After 2 h the caffeine concentration increases on the skin surface, showing significantly higher amounts of caffeine within the first 10 µm of skin depth, but no change in total skin penetration. Also, after 3 h of incubation time, only a slight increase of caffeine below 15 µm of skin depth is detected, while the concentration on the skin surface is remarkably higher, than compared to the previous time points. The addition of 5 % (w/w) 1,2-pentanediol to the caffeine hydrogel leads to a clear difference in the skin penetration profile. As Figure 5 B shows, caffeine skin penetration is generally increased for all three time points. After incubating for one hour, caffeine is still found especially within the upper 10 µm of skin, the concentrations detected between 10 and 25 µm skin depth are higher, than after incubating with gel 1. Over the following two hours of incubation time, a further shift of the penetration profiles towards deeper skin regions can be detected. While longer incubation times with gel 1 lead to differences in caffeine concentrations mostly between 0 to 10 µm of skin depth, the 2 h time point of gel 2 shows an increase in caffeine concentration at lower skin depths (below 10 µm). After 3 h, the shift towards deeper skin regions is even more pronounced.

3.3 Ex vivo CRM

Figure 6 shows the results of ex-vivo CRM measurements of caffeine skin penetration during 3 h of incubation time with the two caffeine gels described (A: gel 1, B: gel 2) on freshly prepared porcine ear skin. Starting at the skin surface, the amount of caffeine per skin volume is displayed as a function of depth.

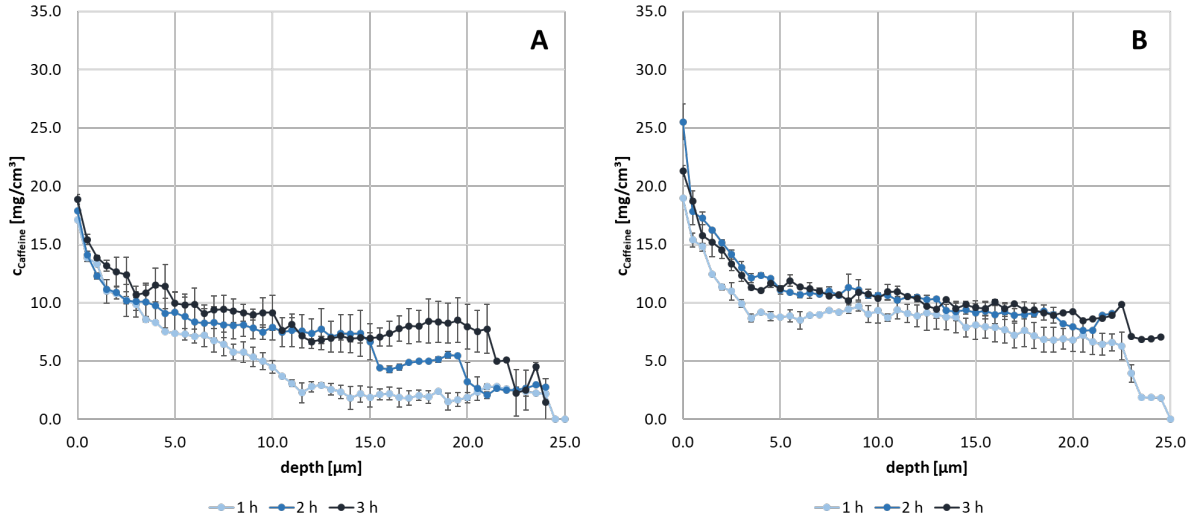

Figure 6: skin penetration profiles of caffeine over 3 h incubation time with 2.0 % caffeine hydrogel (A) and 2 % caffeine hydrogel + 5 % 1,2-pentanediol (B) (% w/w); the error bars are showing the median absolute deviation. n = 3

The amount of caffeine per skin area could be displayed as a function of depth (Supplementary material figure S5). Over the incubation time of 3 h with gel 1, a steady increase of the caffeine concentration in the skin over time is visible. After the first hour, most of the caffeine is located within the first 10 µm of skin depth, reaching a total penetration depth of approximately 15 µm. This total penetration depth also increases steadily over the following two hours of incubation time. Especially between 15 and 20 µm of skin depth, there is a significant increase in caffeine concentration over time. The addition of 5 % (w/w) 1,2-pentanediol as a penetration enhancer leads to an increased caffeine concentration within the skin for all three time points. After 1 h, the penetration profile shows similar properties than the penetration profile, after incubating with gel 1 for 3 h, where caffeine can be found even below 20 µm of skin depth. The increased caffeine concentrations in deeper SC depths are reached earlier compared to incubation with gel 1.

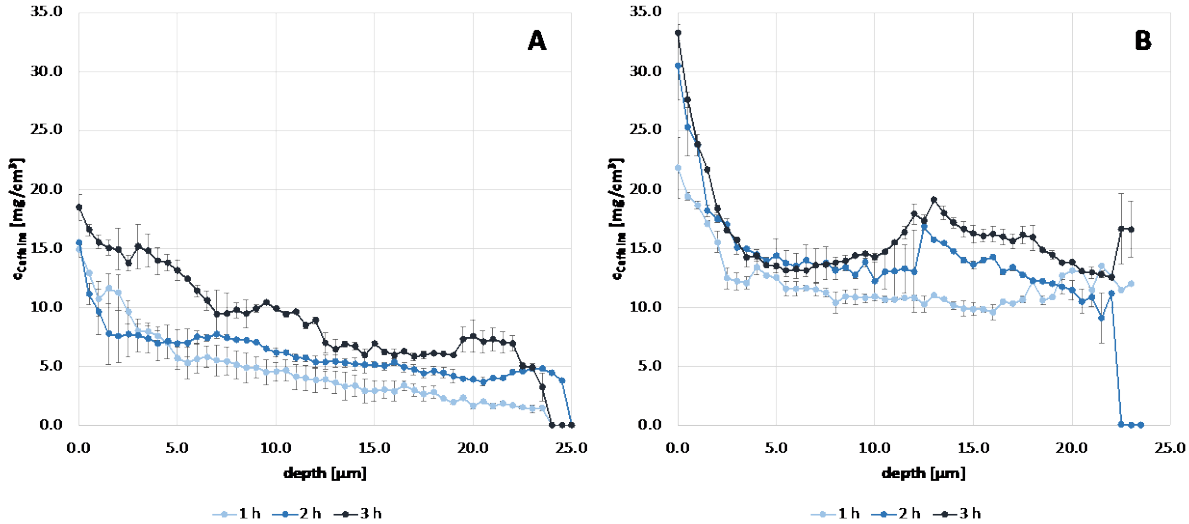

Figure 7: skin penetration profiles of caffeine over 3 h incubation time with 2.0 % caffeine hydrogel (A) and 2 % caffeine hydrogel + 5 % 1,2-pentanediol (B) (% w/w); the error bars are showing the median absolute deviation. n = 3

Figure 7 shows the results of ex-vivo CRM measurements of caffeine skin penetration during 3 h of incubation time with the two caffeine gels described (A: gel 1, B: gel 2) on frozen and thawed porcine ear skin. Starting at the skin surface, the concentration of caffeine is displayed

as a function of depth. The amount of caffeine per skin area could be displayed as a function of depth (Supplementary material figure S6). After incubating the skin with gel 1, the penetration profiles of all three time points show the same characteristics as for the fresh porcine ear skin. While most of the caffeine is located within the first 10 µm of skin after 1 h, the depth profiles flatten after further incubation time, again a steady increase especially between 15 and 20 µm skin depth is clearly visible. The penetration enhancing effect of 1,2-pentanediol on the other hand seems to be more distinct on frozen porcine ear skin. Already after 1 h of incubating with gel 2, the overall caffeine concentration in the skin is genuinely higher, than compared to gel 1, showing caffeine concentrations above 10 mg/cm^3 even below 20 µm. While there is not much change within the first 10 µm of skin depth over time, the increase in caffeine concentration is particularly visible between 15 and 20 µm.

3.4 In vivo CRM

Figure 8 shows the results of in-vivo CRM measurements of caffeine skin penetration during 3 h of incubation time with the two caffeine gels described (A: gel 1, B: gel 2). Starting at the skin surface, the concentration of caffeine is displayed as a function of depth.

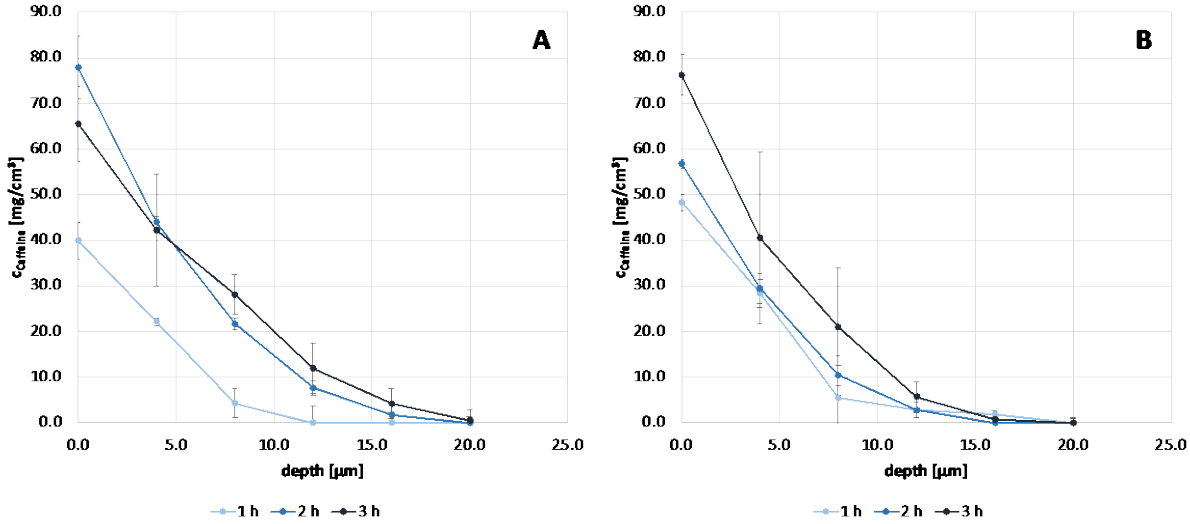

Figure 8: skin penetration profiles of caffeine over 3 h incubation time with 2.0 % caffeine hydrogel (A) and 2 % caffeine hydrogel + 5 % 1,2-pentanediol (B) (% w/w); the error bars are showing the median absolute deviation. n = 3

The amount of caffeine per skin area could be displayed as a function of depth (Supplementary material figure S7). After 1 h of incubating with gel 1, caffeine can be detected within the first 12 µm of skin depth. The 2 h time point already shows higher caffeine concentrations within the skin, increasing only slightly further after 3 h. Total penetration depth is below 20 µm, despite constantly increasing, too. The effect of 1,2-pentanediol on caffeine skin penetration can be seen after 1 h of incubating with gel 2. Within the first 16 µm of skin depth caffeine can be detected. This is 4 µm deeper than without penetration enhancer. After 2 h of incubation, caffeine concentrations within the skin increase a little, showing a bigger increase after 3 h of incubation time. Again, caffeine penetrates to a total depth of approximately 20 µm.

3.5 total amounts of caffeine

Table 2 shows the total amounts of caffeine within the SC, normalized to the application area, as the area of skin incubated is different within all four methods.

Table 2: total amount of caffeine per skin area (median). (CRM: n = 3; tape stripping: n = 5).

		Gel 1	Gel 2	
		$c_{caffeine}$ [µg/cm²]	$c_{caffeine}$ [µg/cm²]	ER
In vivo CRM	1 h	18.58	25.09	1.35
	2 h	45.69	28.45	0.62
	3 h	47.98	42.45	0.88
Ex vivo CRM (fresh skin)	1 h	11.76	20.71	1.76
	2 h	16.92	24.21	1.43
	3 h	20.58	26.12	1.27
Ex vivo CRM (frozen skin)	1 h	11.28	27.89	2.47
	2 h	14.87	31.77	2.14
	3 h	21.98	37.00	1.68
In vivo tape stripping	1 h	7.17	10.89	1.52
	2 h	10.65	13.27	1.25
	3 h	12.35	20.92	1.69
Ex vivo tape stripping	1 h	13.20	13.86	1.05
	2 h	10.41	13.85	1.33
	3 h	24.93	18.60	0.75

Enhancement ratios are calculated by dividing the amount of caffeine within the SC after treatment with gel 2 by the amount of caffeine within the SC after treatment with gel 1 (reference).

The total amounts of caffeine within the SC range from 7.17 µg/cm², after incubating for one hour with gel 1 (in vivo tape stripping) to 47.98 µg/cm² after incubating for 3 h with gel 1 (in vivo CRM). The enhancement ratios range from 0.62 (in vivo CRM, 2 h) to 2.47 (ex vivo CRM, frozen skin, 1 h). All methods show the highest enhancement ratio after 1 h, ranging from 1.35 (in vivo CRM) to 2.47 (ex vivo CRM, frozen skin), except for ex vivo tape stripping, where the effect of 1,2-pentanediol is most pronounced after 2 h and in vivo tape stripping, where the enhancement ratio is the highest after 3 h. For all methods, except for ex vivo tape stripping, the amount of caffeine within the SC increases over time. After 2 h of incubation time, for both gels, the ex vivo tape stripping results indicate a slight decrease.

4. Discussion

The skin penetration of caffeine from two 2 % (w/w) hydrogels was analysed over 3 h by ex- and in-vivo tape stripping, ex-vivo/in-situ CRM on frozen and on fresh porcine ear skin, as well as by in-vivo CRM. All methods lead to caffeine skin penetration profiles with similar characteristics and show total caffeine amounts within the SC in the same magnitude. Differences, especially within the in-vivo results can be attributed to inter-individual variability of the subjects.

Depth profiles obtained by ex vivo tape stripping differ from the other methods, as the total penetration depth determined is remarkably lower. This can be explained by the fact, that porcine ear skin is exposed to ambient conditions for 20 min after the incubation step in Franz diffusion cells, to prevent mass loss due to water evaporation while weighting the tapes. As the SC hydration state of the skin samples decrease to normal/equilibrium values, the stripped mass decreases and as a result the determined total penetration depth is reduced. Furthermore, the amount of skin removed by adhesive tape is affected by the donor animal,

as sometimes the applied pressure is not sufficient to remove the total SC. Caffeine may be present in deeper layers but will not be detected as it is not removed by the 12 tape strips. This leads to ex vivo tape stripping showing generally the lowest caffeine concentrations within the skin, ranging from 13.20 to 24.93 µg/cm^2 after incubating with gel 1. Regarding the penetration enhancing effect of 1,2-pentanediole on caffeine skin penetration, ex vivo tape stripping and in vivo CRM only show a positive effect for one time point. As the ex vivo tape stripping results only show a penetration of caffeine to the upper 15 µm of skin depth, not all caffeine within the skin might be detected, leading to false enhancement ratios, as the other methods show differences in penetration profiles between the two gels used especially below 15 µm of skin depth. This supposedly contrary effect of 1,2-pentanediol on caffeine skin penetration also measured by in vivo CRM for the latter two time points can be explained by inter-individual variability, as only three subjects were participating in this study, while five subjects were part of the in vivo tape stripping experiments and all ex vivo experiments were carried out on the skin from one donor animal, respectively. Porcine ear skin is widely used as a skin surrogate in ex vivo studies, still there are differences in skin barrier function, that must be taken into account when comparing ex vivo porcine to in vivo human results (23). Despite that, penetration curves show the same characteristics – especially when comparing the ex vivo to the in vivo CRM results, this becomes clear. After incubating with gel 1, the course of the 2 h and 3 h time point is similar, showing a bigger increase in caffeine skin concentration between 1 h and 2 h than compared to the 3 h value. After incubating with gel 2, the 1 h and 2 h depth profiles show a bigger similarity, while the 3 h time point shows a stronger increase in caffeine skin concentration. This is shown not only by the in vivo results (tape stripping and CRM) but also by ex vivo CRM, on fresh, as well as on frozen porcine ear skin. Using frozen instead of fresh porcine ear skin leads to a more pronounced effect of 1,2-pentanediol on caffeine skin penetration, while the total amount of caffeine within the skin after incubating with gel 1 is not affected. For all three time points, 1,2-pentanediol leads to remarkably higher caffeine concentrations in beforehand frozen porcine ear skin. Previous studies already discussed the impact of freezing on skin permeability, showing increased permeability for drugs from aqueous vehicles (24)(25). In our case, freezing of the skin only had an impact on caffeine skin penetration in presence of 1,2-pentanediol.

5. Conclusion

The aim of is study was to compare different methods of skin penetration analysis, two different ex-vivo methods and two in-vivo studies, by investigating the effect of 1,2-pentanediol on caffeine skin penetration, when applied as a 2 % (w/w) hydrogel. In our previous work we already found a positive effect of 1,2-pentanediol on caffeine skin penetration out of aqueous solutions and showed the potential and advantages of ex vivo/in situ CRM measurements for skin penetration analysis (11)(18)(22). With the additional calibration step, according to a method described by Caspers et al. (26) the obtained skin penetration profiles can be expressed as concentrations (as opposed to arbitrary units) and directly compared to other methods as for example tape stripping like we did in this study. Caffeine was used as a hydrophilic model drug, as it is used in cosmetics and well described as a Raman-active reference drug (27)(15)(28). The depth profiles of caffeine skin penetration of both techniques used in this study, CRM, and tape stripping, both in- and ex vivo all showed similar courses and caffeine concentrations in the same range could be measured. All methods were suitable for analysing caffeine skin penetration, leading to comparable results, differing mostly in their time- and labour-consumption, spatial resolution, and regarding the in vivo

studies in ethical aspects. As expected, a positive effect of 1,2-pentanediol on caffeine skin penetration could be shown. This positive effect seems to be more pronounced on beforehand frozen porcine ear skin, as the comparison of fresh and frozen skin of the same donor animal showed.

Especially in terms of formulation development, knowing rate and extend of drug penetration, as well as effects of penetration enhancers on penetration properties is crucial. As the results show, the amount of caffeine penetrating the SC can be substantially affected by the addition of 5 % (w/w) 1,2-pentanediol to the formulation. In this study we furthermore were able to show the advantages of ex vivo/in-situ CRM measurements for analysing and understanding skin penetration of active ingredients. When it comes to show BE of a generic topically applied formulation, skin penetration studies by CRM has a huge potential, as it is a time-efficient and resource-saving technique, providing reliable real-time information.

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8. Conflicts of Interest:

The authors declare no conflict of interest.

9. Author contributions:

Conceptualization, R.K. and D.L.; Data curation, R.K and S.F.; Funding acquisition, D.L. and S.S.; Investigation, R.K, S.F., I.N., I.P. and M.C.S.; Methodology, R.K, S.F., I.N., I.P., M.C.S., S.S., and D.L.; Project administration, D.L.; Supervision D.J.L.; Writing—original draft, R.K.; Writing—review & editing, D.L. All authors have read and agreed to the published version of the manuscript.

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