How Confocal Is Confocal Raman Microspectroscopy on the Skin? Impact of Microscope Configuration and Sample Preparation on Penetration Depth Profiles

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Key Words

Confocal Raman microscopy · Depth profiling · Skin penetration

Abstract

Background/Aims: The aim of the study was to elucidate the effect of sample preparation and microscope configuration on the results of confocal Raman microspectroscopic evaluation of the penetration of a pharmaceutical active into the skin (depth profiling). **Methods:** Pig ear skin and a hydrophilic formulation containing procaine HCl were used as a model system. The formulation was either left on the skin during the measurement, or was wiped off or washed off prior to the analysis. The microscope configuration was varied with respect to objectives and pinholes used. **Results:** Sample preparation and microscope configuration had a tremendous effect on the results of depth profiling. Regarding sample preparation, the best results could be observed when the formulation was washed off the skin prior to the analysis. Concerning microscope configuration, the use of a 40 × 0.6 numerical aperture (NA) objective in combination with a 25-μm pinhole or a 100 × 1.25 NA objective in combination with a 50-μm pinhole was found to be advantageous. **Conclusion:** Complete removal of the sample from the skin

before the analysis was found to be crucial. A thorough analysis of the suitability of the chosen microscope configuration should be performed before acquiring concentration depth profiles.

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Introduction

Confocal Raman microspectroscopy (CRM) is increasingly used to determine penetration profiles of xenobiotics in the skin due to the fact that it is a non-destructive method and only minimal sample preparation is needed [1–12]. Furthermore, it can also be performed in a non-invasive manner and therefore enables in vivo measurements. In the course of the acquisition of penetration profiles of the skin, the experimenter takes advantage of the ability of CRM to give spatially resolved information on the chemical composition of a sample. The principle of CRM measurements is that a laser beam is focused on a sample which can either scatter the light elastically (without alteration of the wavelength) or inelastically (with alteration of the wavelength). The inelastic scattering leads to the generation of a spectrum which is determined by the chemical nature of the sampled sub-

Table 1. Examples of microscope configurations and sample preparation methods

Objective magnification	Objective numerical aperture	Pinhole, μm	Laser wave-length, nm	Laser power, mW	Amount of applied formulation	Removal of formulation	Ref. No.
100×	1.25	not specified	785	20	2 $\mu\text{g}/\text{cm}^2$	left on	1
Not specified	not specified	not specified	785	4	2 $\mu\text{l}/\text{cm}^2$	not specified	2
Not specified	not specified	not specified	671	not specified	5 $\mu\text{l}/\text{cm}^2$	‘wiped gently’	3
100×	1.25	not specified	532	<30	20 $\mu\text{l}/\text{cm}^2$	‘removed’	4
‘Immersion oil’	not specified	not specified	671 and 785	not specified	4.4 $\mu\text{l}/\text{cm}^2$	‘no washing and no dabbing’	5
50×	0.9	not specified	785	not specified	85 or 100 $\mu\text{l}/\text{cm}^2$	‘gentle washing with purified water’	6
Not specified	not specified	not specified	671 and 785	<20 and <30	4.4 $\mu\text{l}/\text{cm}^2$	‘no washing and no dabbing’	7
‘Immersion oil’	not specified	25	671 and 785	<20	95 $\mu\text{l}/\text{cm}^2$	‘gently wiped with tissue’	8
50×	0.42	200	514.5	10	1.9 $\mu\text{g}/\text{cm}^2$	‘absorbed by two filter papers’	9
Not specified	not specified	not specified	785	not specified	1 mg/cm^2	not specified	10
100×	0.9	150	785	50	20 μl	‘cleaned with a dry cotton swab’	11
Not specified	not specified	not specified	785	4	2 $\mu\text{l}/\text{cm}^2$	not specified	12
50×	0.8	100	785	50	stratum corneum sheets immersed in saturated solution of drug	‘blotted dry between two filter papers’	13
100×, immersion oil	not specified	not specified	785	7	240 $\mu\text{l}/\text{cm}^2$	‘cleaned with wet cotton swabs’	14

stance. Mixtures of substances give mixed spectra within which the substances contribute to the spectrum according to their concentration in the mixture. Thus, the concentration of a substance within a mixture can be determined by its contribution to the spectrum of the mixture. The (relative) concentration of a substance within a sample (e.g. a pharmaceutical active within the skin) can thereby be determined. The confocal configuration is obtained such that the scattered light is guided through a pinhole before entering the spectrometer. Thus, light from out-of-focus regions is rejected and only rays from the focal plane can contribute to the spectrum. As a result, concentration depth profiles can be generated by scanning a skin sample from the stratum corneum surface to deeper layers.

The increasing use of CRM to establish skin penetration profiles has led to an increasing variety of microscope configurations and sample preparation methods used therein (table 1) [3, 4, 13–24].

Concerning microscope configuration, researchers used lasers of different wavelengths, different objectives and different pinholes. Sample preparation varies from no sample preparation at all (leaving the formulation on) to wiping the formulation off with tissue up to gentle washing of the application site. These differences may impact the results of CRM depth profiling, and this conse-

quently gives rise to the question of how much results obtained with different approaches are comparable.

As with every optical method, there are some points that may impact the outcome of a measurement and therefore need to be taken into account at an early stage of the experiment. Concerning the resolution of a confocal Raman microscope, 3 factors need to be considered: (1) the laser wavelength and (2) the objective numerical aperture (NA), which determine the size of the laser focus volume, and (3) the diameter of the pinhole through which the scattered light is guided back into the spectrometer and which thereby determines the degree of confocality. Laser wavelength and NA determine the size of the laser focal volume and thereby the volume from which the spectrum is recorded. Higher laser wavelengths lead to increased focal volumes and decreased resolution. Generally, the resolution increases with increasing NA as well as with decreasing pinhole diameter. This assumption does hold true when focusing on the surface of a sample. However, if the laser beam is focused more deeply into a sample – as is done in depth profiling of the skin – there are more points to consider. The following section will give a brief overview of the principles by which the objective and pinhole influence the resolution in depth profiling of the skin. The effect of laser wavelength on depth profiling of the skin has already been

studied by Tfaily et al. [2]. For a more detailed elaboration on the subject, the interested reader is referred to the studies of Everall [25–28] and Tfayli et al. [29]. This study is focused on the impact on depth resolution, although the outlined principles may also hold true for lateral resolution. First of all, one must take into account that any beam focused into a sample with a different refractive index than that of the immersion medium will be subject to refraction. The laser intensity distribution will be shifted and distorted. Thus, when using metallurgical objectives, especially those with high NA, one will always focus more deeply into the sample than the sample displacement from the objective would indicate, and the focus volume broadens. As a result, the penetration depth of an active in the skin will be underestimated, and the depth resolution decreases with increasing depth. The use of an immersion objective may reduce the discontinuity in the refractive index and may thereby improve depth resolution and accuracy of the acquired depth profile [25, 30].

In confocal microspectroscopy, a pinhole is used to suppress light from out-of-focus regions and to guide only light from the focus region into the spectrometer. Therefore, the smaller the pinhole diameter, the smaller the region from which light may pass through it and the higher the depth resolution. Still, it is difficult to obtain pure spectra from the focus region as light from layers which are directly within the beam path (above or beyond the focal plane) will not be suppressed completely from passing the pinhole. Spectra from deeper layers within a sample will therefore always suffer from spectral contamination of the surrounding material [25]. With regard to the analysis of the skin penetration of pharmaceutical actives, this may result in the erroneous detection of the active in deeper skin layers due to part of the active being located in superficial layers or on the skin surface. Furthermore, taking into account the broadening of the focal volume with increasing depth and the pinhole rejecting light from out-of-focus regions, it becomes evident that the signal intensity must decrease with increasing depth. As a result, the signal-to-noise ratio will decrease (similar noise level while decreasing signal intensity), and therefore the accuracy of the determined concentrations must decrease with increasing depth [25].

These principal effects have already been studied and could be regarded as well-known [25–30]. Still, most studies used thin polymer films, and only few were taken out on skin samples. A systematic review of the impact of microscope configuration on the results of CRM penetration experiments on the skin is still lacking. Furthermore, to date neither has any study examined the impact of sam-

ple preparation on skin penetration profiles nor have mutual effects of microscope configuration and sample preparation been investigated.

The aim of this study was therefore to clarify the impact of (1) the microscope configuration (objective and pinhole) and (2) the sample preparation on the results of depth profiling of the skin. Mathematical corrections of the skin penetration profiles which would correct the bias due to refraction effects [29] are not within the scope of this investigation.

A hydrophilic gel containing procaine HCl was used as a model formulation. The penetration of procaine HCl from this gel into the skin and CRM investigations thereof have already been reported elsewhere [31, 32].

Materials and Methods

Materials

The assay materials used were procaine HCl (Caesar & Loretz GmbH, Hilden, Germany), hydroxypropyl methylcellulose (HPMC, Shin Etsu Chemical Co. Ltd., Tokyo, Japan), polyoxyethylene polyoxypropylene copolymer (Poloxamer 407, BASF SE, Ludwigshafen, Germany), polyoxyethylene-23-lauryl ether (POE-23-lauryl ether, Croda GmbH, Nettetal-Kaldenkirchen, Germany), propylene glycol (BASF SE) and tissue-embedding medium NEG 50 (Thermo Scientific Inc., Langenselbold, Germany). Disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride and ethanol were European Pharmacopoeia grade. Pig ears were supplied by the Department of Experimental Medicine at the University of Tübingen.

Methods

Preparation of Procaine HCl-Containing Formulations

HPMC-poloxamer gels were prepared according to the method published by Shin et al. [32]. First, 7 g of procaine HCl were dissolved in water. Thereafter, 0.2 g of HPMC and 2 g of poloxamer 407 were dispersed in the solution by a magnetic stirrer. The preparation was stored at 4–8°C overnight to ensure complete swelling of the polymers and was kept refrigerated until the day of the experiment.

Preparation of Dermatomed Pig Ear Skin

Fresh pig ears were washed with isotonic saline. Postauricular skin was excised. Skin samples were cleaned of blood with isotonic saline and cotton swabs, patted dry with tissue, wrapped in aluminium foil and stored at –30°C. On the day of the experiment, the skin was thawed at room temperature, cut into strips of approximately 3 cm in width and fixed to a block of Styrofoam with pins. The hair was trimmed to approximately 0.5 mm with electric clippers. Subsequently, the skin was dermatomed to a thickness of 1 mm (Dermatom GA 630, Aesculap AG & Co. KG).

Incubation of Pig Ear Skin in Franz Diffusion Cells

Incubation of pig ear skin with procaine HCl-containing formulations was carried out using modified Franz diffusion cells (Gauer Glas, Püttlingen, Germany) with a receptor volume of 12

Fig. 1. Devices used for confocal Raman measurements. **a** Closed device (assembled). **b** Closed device (disassembled). **c** Open device (assembled).

Table 2. Instrument settings for confocal Raman measurements

Objective magnification	Objective numerical aperture	Pinhole size, μm	Laser power, mW	Acquisition time, s	Device used
10×	0.25	50	39.6	2	open
40×	0.6	50	21.6	2	open
40×	0.6	25	21.6	8	closed
100×	0.9	50	10.0	4	open
100×	1.25	50	5.0	8	closed

ml. Phosphate-buffered saline, pH 7.4 (PBS, European Pharmacopoeia grade 8.0), was used as receptor fluid. Degassed, prewarmed (32°C) receptor medium was filled into Franz diffusion cells. Subsequently, the cells were fitted with dermatomed pig ear skin (thickness: 1 mm, diameter: 25 mm) and donor compartments. The cells were mounted in a water bath heated to 32°C . After a brief equilibration period of 30 min, 0.5 g of the formulation was applied to each skin sample and spread uniformly across the surface. The cells were capped with Parafilm to prevent the evaporation of water. The receptor medium was stirred at 500 rpm. After 14 h, the skin samples were removed from the Franz diffusion cells, and the remaining formulation was removed using PBS and cotton swabs. The actual permeation area (2 cm^2) was punched out and was mounted onto the devices used for the CRM investigation (for a description of the devices used for confocal Raman measurements, see the section Sample Preparation for CRM Analysis below).

Sample Preparation for CRM Analysis

To investigate the impact of sample preparation on the results of CRM penetration experiments, 3 different types of sample preparation were performed: (1) a finite dose of the procaine HCl-containing formulation was left on the skin during the CRM analysis, (2) the formulation was removed from the skin by a dry cotton swab or (3) it was washed off with a cotton swab and PBS. Untreated skin and skin that had been incubated with an infinite dose of the formulation in Franz diffusion cells served as controls.

In the case of untreated skin, the skin was prepared as explained in the section Preparation of Dermatomed Pig Ear Skin. Circular skin samples of 2 cm^2 were punched out and transferred to the device used for the CRM analysis (the devices used for confocal Raman measurements will be described later in this section).

In the case of incubated skin, the samples were prepared as described in the section Incubation of Pig Ear Skin in Franz Diffusion Cells and subsequently transferred to the device for CRM analysis.

To simulate the impact of the sample preparation, a finite dose (approx. 2 mg/cm^2) of the formulation was applied to and spread uniformly across the surface of the skin samples. The formulation was allowed to dry for 15 min. Then, the formulation was either left on the skin, removed with a dry cotton swab or removed using a cotton swab and PBS and patted dry with another cotton swab. Subsequently, the skin was transferred to the device for CRM analysis. Preliminary experiments (data not shown) had shown that no traces of procaine could be detected in the skin even if this finite dose of the procaine gel was left on the skin for up to 5 h and then washed off completely. Thus, no procaine signal was to be expected in these samples.

The two different devices which were used for the CRM depth profiling are depicted in figure 1a–c. They were custom built and inspired by the devices used by Ashtikar et al. [4] and Bonnist et al. [13]. The choice of device depended on the analysis time (closed device for longer duration) and whether the objective was or could be corrected for use with a coverslip (table 2).

The open device (fig. 1c) consisted of an aluminium block with a cylindrical cavity of 1.5 cm diameter and 1 mm depth. Into this cavity first a piece of tissue soaked with PBS was laid on top of which the skin sample was placed. The gap between the skin and the aluminium block was filled with white soft paraffin and covered with a piece of aluminium foil to prevent the skin sample from drying out during the CRM analysis. The aluminium foil contained a hole to allow for CRM measurements.

The closed device (fig. 1a, b) was made of Teflon and consisted of 3 parts that allowed the skin sample to be tightly fixed between a coverslip and the back of the device. It thereby prevented drying of the sample during the analysis. The upper and middle parts of the device contained a hole through which the Raman signals could be detected. First, a coverslip was fixed between the upper and the middle parts of the device such that it completely covered

Fig. 2. Typical spectra of procaine HCl (top curve), skin incubated with procaine HCl-containing formulation (middle) and untreated skin (bottom). **a** Fingerprint region; the box highlights the characteristic peak of procaine which was used for depth profiling. **b** High wave number region; the box highlights the keratin peak which was used for depth profiling.

the hole. Subsequently, the skin was laid onto the coverslip with the stratum corneum facing towards the coverslip. A piece of tissue soaked with PBS was laid onto the dermis side and a cylindrical piece of polyethylene foil was laid on top of it. Finally, the third part of the device was attached, and thereby the skin was pressed gently against the coverslip.

CRM Analysis of the Skin Samples

Spectra of the skin samples were acquired using an alpha 500R confocal Raman microscope (WiTec GmbH, Ulm, Germany) equipped with a 532-nm excitation laser, UHTS 300 spectrometer, a 1,800 g/mm grating and DV401-BV CCD camera. Spectra were recorded in the fingerprint region between 719 and 1,820.5 cm^{-1} and in the region of higher wave numbers between 2,776 and

3,581.6 cm^{-1} . Typical spectra of procaine HCl, the skin and skin incubated with the procaine HCl-containing formulation are shown in figure 2. Spectral resolution was 1 cm^{-1} . In-depth profiling spectra were recorded moving from above the skin surface into the depth of the skin with a step size of 1 μm . The skin surface was determined as the position with the highest intensity of the keratin signal (2,920–2,960 cm^{-1}). The spectrum that gave the highest keratin signal and the following 19 spectra (fingerprint and higher wavelength region) were used to calculate the penetration profiles of procaine in the skin. For each type of sample preparation, spectra were recorded in 3 randomly selected spots on 3 skin samples, resulting in 9 measurements.

To investigate the influence of the microscope configuration, a wide range of objectives (10 $\times$ 0.25 NA, 40 $\times$ 0.6 NA, 100 $\times$ 0.9 NA

and 100×1.25 NA) and 2 different pinhole sizes (25 and 50 μm) were used (table 2). The laser power and integration time were adjusted to different values depending on the objective and pinhole used. Laser powers and integration times are given in table 2. This was done to (a) avert sample degradation due to the laser light and (b) obtain similar signal-to-noise ratios, which were determined as the ratio between the area under the curve of the ring-breathing mode ($1,000\text{--}1,010\text{ cm}^{-1}$) and that between 720 and 820 cm^{-1} (in this region no specific peaks appear) from spectra of untreated skin. In all experiments 2 spectra were acquired at each depth, and the average of these spectra was used for further calculations.

Calculation of Penetration Profiles

To calculate the skin penetration profiles, all spectra were processed by cosmic ray removal and baseline correction with the software Project Plus 4 (WiTec GmbH). Cosmic ray removal was performed by comparing each pixel (i.e. each CCD count value at each wave number) to its adjacent pixels. If the difference between the pixels exceeded a certain value, the pixel was identified as a cosmic ray [33]. Baseline correction was carried out using the 'shape' option in the software dialogue. Here, the background is subtracted using a rounded shape which is approached to the spectrum from below, pixel by pixel of the spectrum (WiTec Project Plus 4 user manual). Then, the spectra were exported, and the peak areas of the procaine peak [$1,600\text{--}1,625\text{ cm}^{-1}$; $\nu(\text{NH}) \delta(\text{C}=\text{C})$ vibration] and that of the keratin peak ($2,920\text{--}2,960\text{ cm}^{-1}$; CH-stretching mode) were calculated by Matlab (The MathWorks Inc.) as the areas under the curves using the trapezoidal method. (A narrower spectral range was used for the calculation of the keratin peak area compared to other groups [5, 15, 19, 24] as preliminary experiments had shown that doing so prevented interference of the formulation signal with the keratin signal, especially in case of the samples with the formulation left on.) For a given microscope configuration, all experiments were carried out on skin samples from the same donor. As the overall intensity of the collected spectra differed between and within the skin samples, all spectra were normalized to the geometric mean of the keratin signal at the skin surface. In this way, the inter- and intrasample variations of the overall intensity of the spectra cannot bias the values of the procaine signals. To account for the signal attenuation which occurs by moving the laser into the depth of a sample, the procaine peak areas were normalized to the keratin signal [5]. From these corrected procaine peak areas, the geometric mean $\pm$ standard deviation were plotted against the skin depth to obtain the penetration profile of procaine in the skin.

Statistical Analysis

Data were obtained from repeated measurements ($n = 9$). Data treatment was carried out using Matlab (The MathWorks Inc.). The geometric mean $\pm$ standard deviation was used throughout this analysis as the data were found to approximately follow log-normal distribution. To obtain the values of the geometric mean and standard deviation, the normalized procaine signals were log-transformed and the arithmetic mean, standard deviation as well as the values of mean $+$ standard deviation and mean $-$ standard deviation of the log-transformed normalized procaine signals were calculated. Then, the data were transformed back to linear space to yield the geometric mean as well as the values of geometric mean $+$ standard deviation and geometric mean $-$ standard deviation. In penetration plots, geometric means $\pm$ standard deviation were plotted against the penetration depth in the skin.

Results

The aim of this study was to elucidate the influence of sample preparation and microscope configuration on the results of ex vivo skin penetration experiments by CRM. Procaine HCl in a hydrophilic gel was used as a model formulation. The permeation and penetration characteristics of procaine HCl from this gel into the skin have already been found to be approximately $10\text{ }\mu\text{m}$. The investigations are discussed in detail elsewhere [31].

To clarify the impact of the microscope configuration (objective and pinhole) and the sample preparation on the results of depth profiling of the skin, 3 different sample preparation methods and 5 different microscope configurations were used. Samples were prepared by either leaving on a finite dose of the formulation ('finite dose samples'), wiping it off with a cotton swab ('wiped-off samples') or washing it off with PBS and cotton swabs ('washed samples'). Untreated samples served as a negative control, and skin samples incubated with an infinite amount of the procaine gel were used as a positive control. The impact of the microscope configuration was evaluated using 4 different objectives (10×0.25 NA, 40×0.6 NA, 100×0.9 NA and 100×1.25 NA) as well as 2 different pinholes (50 and 25 μm). The results are given in figure 3a–e.

All figures show that a small peak at $1,600\text{--}1,625\text{ cm}^{-1}$ can be detected in the untreated samples. This is attributed to a small endogenous peak of the skin at $1,600\text{--}1,625\text{ cm}^{-1}$ [31]. Furthermore, the diagrams show that procaine penetrated the skin after 14 h of incubation with an infinite dose. Apart from this, it can be seen that if a finite dose of the formulation is left on the skin, procaine is detected not only on the surface or upper layers of the skin, but also in its depth. Accordingly, the detected amount in the skin is higher than or similar to the amount found in incubated samples. Additionally, the amount detected in the finite dose samples does not decrease to the value detected in the untreated skin, not even at a depth of $20\text{ }\mu\text{m}$. The wiped-off samples show a similar behaviour. There, the procaine amount detected is again similar or higher than the one in the incubated samples. Again, the procaine signal does not reach the level of the untreated samples at any depth. In the washed samples a very low value of the normalized procaine is detected in the skin with either set-up. The value is similar to the one found in untreated samples. The results obtained with the different microscope configurations will be discussed in more detail in the following section.

Fig. 3. Depth profiles of procaine HCl in pig skin acquired with different microscope configurations. AU = Arbitrary units. Comparison of different sample preparation methods: $\square$ = leaving-on of a finite dose; $\diamond$ = formulation wiped off dry; ∇ = formulation washed off with PBS; $\star$ = after incubation with an infinite dose; $\circ$ = untreated skin; $n = 9$; upper part: geometric mean $\pm$ standard

deviation; lower part: geometric mean. **a** 10×0.25 NA objective and $50\text{-}\mu\text{m}$ pinhole. **b** 40×0.6 NA objective and $50\text{-}\mu\text{m}$ pinhole. **c** 40×0.6 NA objective and $25\text{-}\mu\text{m}$ pinhole. **d** 100×0.9 NA objective and $50\text{-}\mu\text{m}$ pinhole. **e** 100×1.25 NA objective and $50\text{-}\mu\text{m}$ pinhole.

In the case of the 10×0.25 NA objective in combination with a $50\text{-}\mu\text{m}$ pinhole, the procaine signal does not decrease with increasing depth in any of the samples. This clearly shows that no confocality can be attributed to this microscope configuration.

The results obtained with a 40×0.6 NA objective and $50\text{-}\mu\text{m}$ pinhole show that the finite dose samples give the highest procaine signals whereas the signals of the wiped-off samples give values similar to the incubated samples. All profiles show a decrease in the procaine signal with increasing depth. Interestingly, the values of the normalized procaine signal do not reach the values detected in the untreated samples at any penetration depth in the skin. As a result, the penetration depth in the skin estimated with this microscope configuration would be $>20\text{ }\mu\text{m}$.

If a $25\text{-}\mu\text{m}$ pinhole is used with the 40×0.6 NA objective, the amount of procaine detected 'in' the skin decreases in the order finite dose samples $>$ wiped-off samples $>$ incubated samples $>$ washed samples = untreated samples. The procaine signal obtained from the incubated samples reaches the value of untreated and washed samples after approximately $8\text{ }\mu\text{m}$. This suggests that the penetration depth of procaine in the skin is approximately $8\text{ }\mu\text{m}$. Thus, the use of a smaller pinhole clearly improved confocality.

In the case of the use of the 100×0.9 NA objective and $50\text{-}\mu\text{m}$ pinhole, only the incubated samples give a profile which declines with increasing depth. Still, this only holds true for the first $10\text{ }\mu\text{m}$. Thereafter, high fluctuations (i.e. deviations of many points from the expected/estimated curve shape) can be observed. This is related to the high degree of refraction which takes place with this objective, and these results are in accordance with those of other groups [25, 26, 29, 30]. High deviations and fluctuations can be found in the results obtained on finite dose, wiped-off, washed and untreated samples. The results obtained with this microscope configuration can therefore be considered to give very low accuracy.

The results obtained with the 100×1.25 NA objective and $50\text{-}\mu\text{m}$ pinhole show a sharp decline for the profiles of the finite dose samples and the wiped-off samples. Here, the amount of procaine detected 'in' the first $5\text{ }\mu\text{m}$ of the skin is distinctly higher in the case of finite dose and wiped-off samples than in the incubated samples, the washed samples and the untreated samples, i.e. geometric mean values are higher and error bars do not overlap. The procaine signals obtained from the incubated samples reach the values of untreated and washed samples after approximately $8\text{ }\mu\text{m}$. This suggests a penetration depth in the skin of approximately $8\text{ }\mu\text{m}$ as was found with the 40×0.6 NA objective and $25\text{-}\mu\text{m}$ pinhole.

Discussion

Regarding the sample preparation, it can be concluded that the formulation needs to be washed off the skin prior to a CRM analysis. If it is left on the skin or simply wiped off with a dry cotton swab, this will result in an erroneous detection of the active within the skin.

This may be explained by the fact that no matter to which depth the microscope is focused, some amount of the active will always be present in the beam path. Therefore, the active will contribute to the spectrum even if the laser is focused well below the skin surface. This necessarily leads to overestimation of the active pharmaceutical ingredient (API) content in the skin.

With respect to the microscope configuration, it is clear that the 10×0.25 NA objective and the 100×0.9 NA objective should not be used as the $10\times$ objective does not detect the decline of the API concentration with increasing depth and the $100\times$ objective gives very pronounced fluctuations. The results obtained with the 40×0.6 NA objective in combination with the $50\text{-}\mu\text{m}$ pinhole suggested a penetration depth in the skin of $>20\text{ }\mu\text{m}$. This is likely to be an overestimation as previous CRM investigations on cross-sections of skin showed that procaine was only detected in the stratum corneum (approx. $10\text{ }\mu\text{m}$ depth) after incubation with the hydrophilic gel formulation [31]. This finding is likely attributed to the fact that procaine which is present in superficial layers adds to the procaine signal in deeper layers as it is located within the beam path and its signal is not rejected completely from entering the spectrometer by the $50\text{-}\mu\text{m}$ pinhole. In contrast, the 40×0.6 NA objective in combination with the $25\text{-}\mu\text{m}$ pinhole enabled better differentiation between the different sample preparation methods. The 100×1.25 NA objective also gave good results in this aspect. Both microscope configurations suggested a penetration depth in the skin of approximately $8\text{ }\mu\text{m}$, which is in line with the previous study mentioned above [31].

Conclusion

In order to acquire reliable skin penetration profiles, it is pivotal to ensure complete removal of the formulation from the sample surface prior to the analysis as any residual formulation will lead to erroneously high API concentrations 'in' the skin.

The investigation of the impact of different microscope configurations revealed a tremendous effect on the skin penetration profiles. In this study the most accurate

skin penetration profiles could be acquired using the 40 × 0.6 NA objective in combination with a 25-μm pinhole or the 100 × 1.25 NA objective in combination with a 50-μm pinhole.

As the accuracy of depth profiles is influenced by many factors, a thorough analysis of the suitability of the available objectives and pinholes is highly recommended before performing depth profiling of pharmaceutical actives within the skin.

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Statement of Ethics

Pig ears were received from the Department of Experimental Medicine of the University Hospital Tübingen. The live animals were kept at the Department of Experimental Medicine and were sacrificed in the course of their experiments, with the approval of the Ethics Committee of the University Hospital Tübingen. The ears were received directly after the death of the animals. The Department of Pharmaceutical Technology is registered for the use of animal products at the District Office of Tübingen (registration number: DE 08 416 1052 21).

Disclosure Statement

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