Determination of skin penetration profiles by confocal Raman microspectroscopy: Evaluation of interindividual variability and interlab comparability

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Abstract
Confocal Raman microspectroscopy is being increasingly used to investigate the skin penetration of actives and excipients. The aim of the present study was to compare results obtained with skin from different donors, acquired with two Raman systems: the RiverD gen2 Skin Composition Analyzer and the WiTec alpha 500 instrument. To elucidate the interindividual variability between the skin donors and to find out whether the two systems would give similar results in skin penetration analysis, we compared the penetration of the model drug procaine from hydrogel formulations with or without the penetration enhancer POE-23-lauryl ether. Penetration was investigated with both systems on skin from three different skin donors, each. Penetration plots were prepared by plotting the signal intensity of a typical peak of procaine against the skin depth. We then calculated the enhancement ratio from the areas under the curves of the penetration plots of procaine that had penetrated from the formulation with or without the enhancer. They ranged between 2.0 and 3.1 for the WiTec system and between 2.1 and 3.1 for the RiverD system. Thus, we found that both systems provide similar estimations of the skin penetration of procaine.

KEYWORDS
enhancer, penetration, procaine, skin

1 INTRODUCTION

Confocal Raman microspectroscopy has been increasingly used in the past years to investigate the skin penetration of pharmaceutical or cosmetic actives and excipients.1–6 It makes use of the characteristic Raman spectrum of the penetrating substance to monitor its relative concentration across the skin depth and thus does not require any labels, which might interfere with skin penetration. Furthermore, confocal Raman microspectroscopy is a non-destructive technique that can be performed in a non-invasive way, thus offering the
possibility of ex vivo as well as in vivo investigations. Due to its growing use, an increasing number of instruments, instrument set-ups and procedures have been applied. A comprehensive overview is given in a previous publication.\textsuperscript{[3]} Unfortunately, a standardisation of the technique is still missing. In a first attempt to assess the impact of different instrument set-ups, we compared a range of objectives and pinholes and investigated their impact on skin penetration profiles. We could thereby show that the objective and pinhole used impacts the results of skin penetration analysis. The eligibility of the microscope set-up was investigated by comparing results from depth profiling to results from lateral scanning of skin cross sections. Thus, we found two combinations of objectives and pinholes that lead to valid estimations of the penetration profile \textsuperscript{[3, 4]} Other researchers compared different wavelengths of incident light.\textsuperscript{[7]} They found that spectra of the skin are comparable between the wavelengths of 532 and 785 nm. In contrast, irradiation at 633 nm results in differences due to resonant effects.\textsuperscript{[7]}

In the present study, we aimed to compare results obtained with two of the most frequently used Raman systems: the RiverD gen2 Skin Composition Analyzer and the WiTec alpha 500 instrument. The RiverD system is a Raman microspectrometer that was specially designed to analyse skin in vivo. Its software allows for the automated analysis of skin related parameters such as skin hydration, natural moisturising factor and stratum corneum thickness.\textsuperscript{[8, 9]} Besides in vivo application, it can also be used to analyse skin ex vivo. The WiTec alpha 500 is a confocal Raman microscope built as a multi-purpose system. It offers the possibility to acquire Raman spectra in three dimensions. The software allows to calculate the relative concentrations of substances in the measured area and to visualise the built-up of the sample in 2D by chemical imaging. It facilitates the analysis of a wide range of samples. In pharmaceutical research, it is for example used to analyse the drug distribution in tablets or to investigate the inner structure of semisolids.\textsuperscript{[10–12]} It can also be utilised to analyse biological samples and to monitor skin penetration.\textsuperscript{[2–4, 13]}

The aim of the current study was to find out whether the two systems would give similar results in skin penetration analysis. To this end, we compared the penetration of the model drug procaine from hydrogel formulations with or without the penetration enhancer POE-23-lauryl ether. In prior investigations we already gathered strong knowledge about the penetration of procaine from these formulations and its detection by Raman spectroscopy.\textsuperscript{[3, 4]} Therefore, it was used also in the current investigation. Further, skin from three different porcine donors was used to account for inter-individual variability.

2 | MATERIALS AND METHODS

2.1 | Materials

The materials used were procaine HCl (Ceasar&Loretz GmbH, D-Hilden), hydroxypropyl methylcellulose (HPMC, Shin Etsu Chemical Co. Ltd, J-Tokyo), polyoxyethylene polyoxypropylene copolymer (Poloxamer 407, BASF SE, D-Ludwigshafen), and polyoxyethylene-23-lauryl ether (POE-23-lauryl ether, Croda GmbH, D-Nettetal-Kaldenkirchen). Disodium hydrogen phosphate, potassium dihydrogen phosphate, and sodium chloride were Ph. Eur. grade. Pig ears were supplied by the Department of Experimental Medicine at the University of Tuebingen and from a local butcher (EU-Schlachthof Gantner, Hollabrunn, Austria). The Department of Pharmaceutical Technology at the University of Tuebingen is registered for the use of animal products at the District Office of Tuebingen (registration number: DE 08 416 1052 21).

2.2 | Preparation of procaine HCl-containing formulations

HPMC poloxamer gels were prepared according to the method published by Shin et al.\textsuperscript{[14]} The method is also described in our previous publications.\textsuperscript{[2–4]} The formulation without penetration enhancer contained 7 g of procaine HCl, 0.2 g of HPMC, and 2 g of poloxamer 407 in 10-g water. The formulation with enhancer additionally contained 0.5-g POE-23-lauryl ether. The water content was thus reduced to 9.5 g.

2.3 | Incubation of pig ear skin in Franz diffusion cells

Excised pig skin from the postauricular area of the ears was used. The skin had been stored frozen; hair was trimmed before use without injuring the skin and was cut into appropriate size. Then, the skin was mounted onto Franz diffusion cells filled with isotonic phosphate buffer pH 7.4 (phosphate-buffered saline, Ph. Eur. 8.0). An amount of 0.25 g/cm\(^2\) of the formulation was applied and the donor compartments were capped with Parafilm\textsuperscript{®} to prevent water evaporation. Incubation lasted for 14 hr and was performed at 32°C under constant stirring. The exact procedures are described in previous publications.\textsuperscript{[15–20]}

After incubation, the skin samples were removed from the Franz diffusion cells and the remaining formulation was wiped off using phosphate-buffered saline and
cotton swabs. For the investigation with the RiverD instrument, the skin was gently placed into contact with the measurement window, applying minimal pressure to ensure a good contact between skin and glass and prevent sample damage. In the case of the WiTec instrument, the skin was mounted into a specially designed device that excluded drying of the sample during the analysis and thus ensured good contact between the skin and the glass (cover slip). For a description of the device, please refer to our previous publications.[3, 4]

2.4 Confocal Raman microscopic analysis of the skin samples

Two Raman instruments were used: an alpha 500 confocal Raman microscope (WiTec GmbH, D-Ulm) equipped with a 532-nm excitation laser, UHTS 300 spectrometer, 1,800 g/mm grating and DV401-BV charge-coupled device (CCD) camera, and a gen2 Skin Composition Analyzer (River Diagnostics, Rotterdam, The Netherlands) with two incorporated lasers, operating at wavelengths of 785 and 671 nm. Data were collected by a CCD detector.

Using the WiTec instrument, a 100 × 1.25 NA oil immersion objective (Carl Zeiss Jena, Germany), 50-μm optical fibre, and 5-mW laser power were used. Spectra were recorded in the fingerprint region between 719 and 1,820.5 cm⁻¹ and in the high-wavenumber region between 2,776 and 3,581.6 cm⁻¹ using the previously described method.[3, 4] Integration time was two times 8 s in the fingerprint as well as in the high-wavenumber region. 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 (2,940 cm⁻¹). The spectrum that gave the highest keratin signal and the following 19 spectra (fingerprint and high-wavenumber region) were used to calculate the penetration profiles of procaine in the skin. Spectra were recorded in three randomly selected spots on three skin samples, resulting in nine measurements.

To calculate the penetration profiles, all spectra were processed by cosmic ray removal and baseline correction with the software Project Plus 4 (WiTec GmbH, D-Ulm) or using SkinTools® software version 2.0 (River Diagnostics, Rotterdam, The Netherlands). Then, the spectra were exported and the peak areas of the procaine peak (1,612 cm⁻¹; ν(NH) δ(C=O) vibration) and that of keratin (2,940 cm⁻¹; CH-stretching mode) were calculated as the area under the curve (AUC) by the trapezoidal method using MATLAB (The MathWorks, Inc.) as previously described.[3, 4] Peak areas were normalised to the keratin signal to account for depth attenuation.[1, 3, 4]

Furthermore, all spectra were normalised to the mean of the keratin signal at the skin surface to account for intra-individual and interindividual variability of the Raman signal intensity. From the thus corrected procaine peak areas, the mean ± standard deviation was plotted against the skin depth to obtain the penetration profile of procaine.[3, 4] The AUC was used as a surrogate parameter to describe the total amount of procaine penetrated into the skin. It was calculated from penetration plots using the trapezoidal method.[3, 4]

2.6 Statistics

Data were obtained from n = 9 measurements. From the penetration plots, the AUC was calculated as a means to describe the penetrated amount of procaine across the investigated stratum corneum depth. To compare the penetration from the formulations with and without penetration enhancer, an enhancement ratio was calculated using the means of the AUC from the penetration plots obtained from measurements on skin samples that had been incubated with formulations with and without enhancer. Furthermore, the single AUC values of the two times nine measurements were compared using Kruskal–Wallis test. Enhancement ratios obtained from measurements with the WiTec system and the RiverD system were also compared using Kruskal–Wallis test. p values < .05 were regarded as significant difference.
3 | RESULTS AND DISCUSSION

We compared the skin penetration of the model drug procaine from two different hydrogel formulations analysed by two commonly used Raman instruments (RiverD gen2 Skin Composition Analyzer and the WiTec alpha 500). The enhancement ratios of the procaine penetration from the formulations with and without the penetration enhancer POE-23-lauryl ether were used to compare results obtained with the two different Raman systems. As the penetration varies to some extent with the skin donor used, skin from three different donor pigs was used. In doing so, we also evaluated the inter-individual variability in the model drug penetration by confocal Raman microscopy (CRM). Due to the different integration times and detectors and wavelengths used, the absolute values of the Raman signal (shown as CCD cts) were expected to differ between the two instruments. Accordingly, the absolute values were considered unsuitable for a comparison of the findings. Therefore, the AUC values were calculated from the penetration plots and enhancement ratios were derived therefrom to compare the results obtained with the two systems.

First, we compared the spectra of skin incubated with the procaine HCl-containing formulations with and without the penetration enhancer. Figure 1 gives typical spectra of skin incubated with procaine in the formulations with and without penetration enhancer assessed with the two Raman systems.

The typical peak of procaine (1,612 cm\(^{-1}\); \(\nu(\text{NH})\) \(\delta(C=\text{C})\) vibration), which was used to monitor skin penetration, is more pronounced in skin samples that had been incubated with the formulation containing enhancer. In this case, some additional peaks appear at 1,180 and 1,280 cm\(^{-1}\) as well as two signals that mix with the signals of the skin at 860 and 1,690 cm\(^{-1}\). They can only be detected in skin samples that contain higher amounts of procaine, as is the case for samples that have been incubated with the formulation with penetration enhancer. Spectra obtained with the two Raman systems are essentially similar with respect to peak locations and relative intensities.

**FIGURE 1** Typical spectra of skin incubated with procaine in the formulations with and without penetration enhancer assessed with the WiTec system (left) and the RiverD system (right)

**FIGURE 2** Penetration profiles of procaine HCl in porcine skin acquired with the WiTec system (left) and RiverD system (right). Comparison of penetration from formulation with enhancer (red diamonds) and without enhancer (black squares). \(n = 9\); mean ± standard deviation [Colour figure can be viewed at wileyonlinelibrary.com]
Next, penetration plots acquired with the two instruments were evaluated. Figure 2 gives typical examples of penetration profiles obtained by using either the WiTec system or the RiverD system.

The model drug procaine can be detected in the stratum corneum by both systems. As expected, its concentration decreases with increasing skin depth. In both cases, the penetration depth is approximately 8–10 μm. This shows that both systems give a similar approximation of the penetration depth of procaine. Further, it becomes clear that the formulation with enhancer delivers more procaine to the stratum corneum than the formulation without enhancer. This is in line with previous findings.\cite{2}

The AUC of the penetration plots was used to compare the penetrated amount of procaine. Figure 3 shows the AUC values obtained with the two systems and three porcine skin donors.

It can be seen that the AUC values vary between the skin donors and the two instruments. The mean values of procaine penetration (expressed as AUC) from the formulation without enhancer differ slightly between the skin donors; the mean values of procaine penetration from the formulations with penetration enhancer vary more strongly. The variability in skin penetration within skin from one donor is related to slight differences in the skin at different sites and is well known to experienced researchers in the field of skin penetration research. The fact that the variability was found to be higher for skin samples treated with the formulation with penetration enhancer is related to the diverging extent of the effect that the penetration enhancer exerts on the individual skin samples. Together with the variability of the skin sites themselves, an overall higher variability is obtained.

Variability of AUC values was found to be differently pronounced both between and within different skin donors. This is an effect that depends on the skin donor and the state of its skin. It may happen that the skin contains some invisible injuries that may cause this variability. The use of a penetration enhancer further aggravates this variability; thus, the effect becomes more evident in skin samples incubated with the formulation that contains the penetration enhancer.

For each skin donor, AUC values differed significantly between the skin areas that had been incubated with and without penetration enhancer (Kruskal–Wallis test, \( p < .05 \)). This shows that the penetration enhancer did enhance the skin penetration of procaine significantly. To evaluate the dimension of the effect, enhancement ratios were calculated from the mean AUC values for each skin donor. They were 2.0, 2.1, and 3.1 for the WiTec system and 2.1, 2.7, and 3.1 for the RiverD system. They do not differ significantly (Kruskal–Wallis test, \( p > .05 \)). These enhancement ratios are approximately in the same range as found in earlier research using CRM and conventional permeation and penetration experiments.\cite{2} The ratios vary between different donors; this is very typical for skin penetration experiments and has already been described in previous studies.\cite{2, 5} Altogether, this clearly shows that similar results can be obtained using the two systems in the described configuration.

4 | CONCLUSION

In the present study, we compared results on skin penetration of the model drug procaine HCl after spectra acquisition by two frequently used Raman microspectrometers: the WiTec alpha 500 instrument and the RiverD gen2 Skin Composition Analyzer. As the overall spectral intensity depends on multiple factors, we used the enhancement ratio as a means to compare results from both instruments. Our findings show that we obtained essentially the same enhancement ratio values, independent of the instrument used.
This was a quite unexpected outcome, as there is a well-known inherent variability in skin penetration analysis. Furthermore, discrepancies between results obtained in different laboratories have been reported. The most prominent example led to the withdrawal of the 1998 Food and Drug Administration draft guidance that recommended tape stripping to investigate topical dermatological drug products. After two research groups had found different results in tape stripping experiments with the same formulations, the draft guidance was withdrawn and revised.[21–23] A new guidance has recently been drafted, taking into account the lessons learned from 1998. In the field of Raman spectroscopic skin penetration research, where many different approaches have come forth within recent years, it may sometimes be doubted whether a research group may reproduce results from another group, using different instruments. We used instruments of two different suppliers that operate with different objectives, pinholes, and laser wavelengths, located in separate laboratories. In the course of the study, we were able to obtain similar results regarding the skin penetration of procaine. In this context, our investigation shows that it is possible to obtain comparable results with different instruments, even though they differ substantially in their built-up. Therefore, this work will further enhance the trustworthiness of Raman spectroscopic skin penetration analysis. Nevertheless, it may only serve as a first step to prove the comparability of Raman spectroscopic results concerning skin penetration. Comparative studies between other instruments as well as with other model drugs and formulations are highly recommended. Especially in the case of substances that show resonant effects, various factors such as the laser wavelength may still affect the results.

Another aspect that should not be neglected are diverging methods of data evaluation. In the present case, we chose to analyse the obtained data outside the instruments’ software with a custom-made MATLAB script. This was done in order to cancel out any bias due to different data treatment. When looking into the literature, one will find an increasing number of possibilities to analyse skin penetration data from CRM experiments. Whether these different data treatments give similar results and which method is superior remains subject to further research.

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CONFLICT OF INTEREST
The author reports no conflicts of interest.

STATEMENT OF ETHICS
Pig ears were received from the Department of Experimental Medicine of the University Hospital Tuebingen or from a local butcher. 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 Tubingen. 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 Tuebingen (registration number: DE 08 416 1052 21).

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