A useful technique using imaging mass spectrometry for detecting the skin distribution
of topically applied lidocaine

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Abstract

The skin disposition of topically applied lidocaine hydrochloride was determined under steady-state skin permeation by imaging mass spectrometry (MSI). The distribution of lidocaine in pig ear skin was assessed using MALDI-TOF-MS with mass images being examined with BioMap software. Following the detection of intrinsic signals of lidocaine, the skin concentration-distance curve was obtained using brightness analysis ([M+H]+: m/z 235.18). Although the skin concentration profile obtained by MALDI-TOF-MS did not completely correspond to the calculated one obtained from the skin permeation profile, the skin concentration profile could be detected. These results demonstrate that imaging MSI might be a useful method that can be used to determine the skin disposition of topically applied non-radiolabeled or non-fluorescent drugs.

Keywords: imaging mass spectrometry; transdermal delivery; topical application; skin concentration; skin distribution
1. Introduction

The skin disposition of topically applied drugs is known to be strongly associated with local efficacy and toxicity (Holford et al., 1981). Dermatopharmacokinetics (Alberti et al., 2001) (DPK) is now being utilized to evaluate generic topical formulations in Japan (www.nihs.go.jp/drug/be-guide(e)/Topical_BE-E.pdf). The skin concentrations of topically applied drugs have previously been assessed using several methods, including tape stripping (Pershing et al., 1992; N'Dri-Stempfer et al., 2009), heat separation (Surber et al., 1990), and others (Kiistala, 1968; Surber et al., 1993), which generally require drug extraction and tissue homogenization procedures. However, difficulties have been reported in accurately determining the drug distribution or drug concentration-distance profiles in the skin by these methods. The skin permeation profiles of topically applied drugs can be analyzed by Fick’s second law of diffusion under the assumption that the skin consists of one or two homogeneous membranes (Sugibayashi et al., 2010). We recently reported that the skin concentrations of topically applied drugs in a certain logarithmic range of the n-octanol/water coefficient, log$K_{ow}$ ($1.93 < \log K_{ow} < 2.81$), could be calculated with a two-layered diffusion model, with a relationship being identified between the calculated and observed steady-state concentrations of drugs. On the other hand, the skin concentrations of hydrophilic drugs ($\log K_{ow} \leq 0$) could not be accurately determined using the same method. Several studies (Lademann et al., 1999; Toll et al., 2004; Trauer et al., 2009; Marshall et al., 2010) confirmed that hair follicles and sweat ducts were the primary permeation pathways for hydrophilic drugs and macromolecules.
Fluorescence dyes such as calcein, fluorescein, and fluorescein isothiocyanate dextran (FITC-dextran) have been used as model hydrophilic drugs and macromolecules in order to provide insight into their skin disposition following topical application. However, this technique can only be used for fluorescent compounds.

Recently, several studies have used mass spectrometry imaging (MSI) by matrix-assisted laser desorption/ionization (MALDI) to investigate the disposition of a drug after its topical application (Marshall et al., 2010; Enthaler et al., 2011). In addition, D’Alvise et al. have reported that the follicular transport of lidocaine in the deeper skin layers and its metabolism in subcutaneous tissue could be detected by desorption electrospray ionization mass spectrometry imaging (D’Alvise et al., 2014).

The observation of heterogeneous drug distribution in skin, such as localized drug distributions in hair follicles, using a horizontal or vertical skin section with a microscope would provide us with very useful information to reveal the skin permeation route of topically applied drugs. Since the pharmacological and toxicological effects of topically applied chemicals can be determined by their concentration at the viable epidermis/dermis, drug concentration-depth profile in viable epidermis/dermis should be investigated in order to evaluate the safety and effects. Bunch et al. have reported that a quantitative skin profile of ketoconazole was acquired with MALDI quadrupole time-of-flight mass spectrometry (Q-TOFMS) (Bunch et al., 2004). However, no comparison of skin concentration profiles obtained by imaging MALDI-TOF MS and a conventional method has been performed to investigate the usefulness of the novel method.
In the present study, an atmospheric-pressure MALDI quadrupole-ion-trap TOF-MS (AP-MALDI-QIT-TOF-MS) instrument was first used with 10-µm spatial resolution to obtain positional information on the concentration of a drug in skin following its topical application. Lidocaine hydrochloride was used as a model drug to observe the drug concentration-distance profile by MALDI-TOFS because its skin permeation profile could be successfully calculated with Fick’s second law of diffusion from the skin permeation profile.

2. Experimental

2.1. Materials and methods

Lidocaine hydrochloride was obtained as a model drug from Sigma Aldrich (St. Louis, MO, U.S.A.). Other reagents and solvents were of reagent or HPLC grade and used without further purification. Frozen pig ear skin (three-breed cross pigs involving the Landrace, Yorkshire and Duroc breeds) was purchased from the National Federation of Agricultural Cooperative Associations (Tokyo, Japan). The skin samples were stored at -30°C until the skin permeation experiments.

2.2. Preparation of skin membrane

The purchased skin was maintained frozen at -80°C prior to use. The skin was thawed at 32°C and excised from the outer surface of a pig ear after being cleaned with distilled water. Stripped skin was obtained by tape stripping of the stratum corneum
(SC) with adhesive tape (Scotch®; 3M Japan Ltd., Tokyo, Japan) 20 times prior to its excision from the pig ear. Excess fat was carefully trimmed off from the excised skin with a knife.

2.3. Skin permeation experiments for lidocaine

The skin sample was set in a vertical-type diffusion cell (effective diffusion area: 1.77 cm²) in which the skin surface temperature was maintained at 32°C. After 1 h of hydration with phosphate-buffered solution (PB), PB containing 2.5 mg/mL lidocaine (volume: 1.0 mL) was applied to the SC side as a donor solution for 8 h, and PS (volume: 6.0 mL) was added to the dermis side as a receiver solution. The receiver solution was stirred with a stirrer bar on a magnetic stirrer. An aliquot (500 µL) was withdrawn from the receiver chamber and the same volume of PB was added to the chamber to keep the volume constant. The penetrant concentration in the receiver chamber was determined by HPLC. Three skin permeation experiments were conducted to calculate the skin concentration-distance profile.

2.4. HPLC conditions

Ethyl paraben (EB) was used as an internal standard (IS) in the quantitative determination of lidocaine. An aliquot of a sample solution containing lidocaine was mixed with acetonitrile (1:1) and centrifuged (15,000 × rpm, 5 min, 4°C) to obtain supernatant. Then, 20 µL of obtained supernatant was injected into the HPLC system. The HPLC system consists of a pump (LC-20AD), a UV detector (PD-20A), a
system controller (SCL-10AVP), an auto-injector (SIL-20A), a degasser (DGU-20A3), a column oven (CTO-20A), and analysis software (LC solution) (all from Shimadzu, Kyoto, Japan). An Inertsil-ODS-3 5 µm, 4.6×150 mm (GL Sciences Inc., Tokyo, Japan) column was kept at 40°C. The mobile phase was 0.1% phosphoric acid:acetonitrile = 7:3, containing 5.0 mM 1-heptanesulfonate at a flow rate of 1.0 ml/mL. Detection was carried out at UV 230 nm.

2.5. Preparation of skin samples to plot lidocaine calibration curve

The stripped skin was immersed in different concentrations of lidocaine solution (500, 1500, and 2500 µg/mL) for 24 h to obtain skin with homogeneously distributed lidocaine. A calibration curve for the skin concentration of lidocaine was obtained by imaging analysis of the treated skin.

2.6. Skin treatment for MALDI-TOFS observation

After the permeation experiment or after immersion in lidocaine solutions to obtain a calibration curve, the skin surface was washed three times using 1 mL of ultrapure water. The skin was then embedded in 2.0% carboxymethyl cellulose and frozen in isopentane at -80°C. The embedded skin was sliced using a cryostat (CM3050; Leica Microsystems, Wetzler, Germany) to obtain 7-µm-thick vertical sections at -20°C. Then, each skin section was kept at room temperature for 30 min before subjecting it to MALDI-QIT-TOF-MS. These sections were mounted on an indium tin oxide-coated
slide glass (Sigma-Aldrich), dried in a silica gel-containing plastic tube, and then sprayed with 2,5-dihydroxybenzoic acid (DHB; 50 mg/mL in 70% methanol and 30% water containing trifluoroacetic acid at a concentration of 0.1%) using a 0.2-mm-nozzle caliber airbrush (Procon Boy FWA Platinum; Mr. Hobby, Tokyo, Japan) to conduct MALDI-imaging mass spectrometry in the positive-ion mode. MALDI-QIT-TOF-MS equipped with a 355-nm Nd:YAG laser and microscope allowed mass images to be obtained with a high spatial resolution of 10 µm, in the scan range of m/z 200-900, as described previously for a prototype MALDI-imaging mass spectrometry instrument (Marshall et al., 2010) (Mass Microscope; Shimadzu, Kyoto, Japan). Microscopic images were obtained after application of a matrix (300 µL of DHB solution) to the skin sections. Mass spectra were subsequently acquired in the positive-ion mode in the designated areas of a specimen.

2.7. Image analysis of skin sections using BioMap®

The distribution of the specific signal intensity of lidocaine ([M+H⁺]: m/z 235.18) in the skin was assessed using AP-MALDI-TOF-MS with mass images being examined with BioMap® software (Novartis Pharma K.K., Basel, Switzerland). The brightness of the obtained skin section images was analyzed using the image processing software Image J® (National Institutes of Health, Bethesda, MD, U.S.A.). Figure 1a shows microscopic observation results of a skin section. The measurement area in the skin section was decided by the positional information of the obtained image and the area, and was then divided into 5 sections (the size of each section: 260 µm long × 500 µm
wide) from the surface of stratum corneum side \((x = 0)\) to calculate the brightness intensity (Figure 3b). The average brightness value in each section was obtained using Image J software (ver. 1.48). In the case of the full-thickness skin to measure the skin concentration of lidocaine, the outermost section to the stratum corneum side was further divided into two sections; the size of the outermost section was 20 µm long × 500 µm wide and that of the other one was 240 µm long × 500 µm wide.

2.8. Calculation of skin concentration from skin permeation profile

The calculated skin concentration of lidocaine was obtained by curve-fitting the cumulative amount of it that permeated through the full-thickness skin and stripped skin to the theoretical values, where the theoretical values could be expressed by Fick’s second law of diffusion in the stratum corneum and viable epidermis and dermis. Differential equations describing Fick’s second law are as follows.

\[
\frac{dc_{i,j+1}}{dt} = \frac{1}{\Delta t} (C_{i,j+1} - C_{i,j}) \tag{1}
\]

\[
\frac{dc_{i,j}}{dx^2} = \frac{1}{\Delta x^2} (C_{i-1,j} - 2C_{i,j} + C_{i+1,j}) \tag{2}
\]

The mathematical approach for determining skin permeation using a two-layered diffusion model was the same as in our previous method (Sugibayashi et al., 2010). Skin permeation parameters such as permeability coefficient, diffusion coefficient, and partition coefficient were obtained by curve-fitting to the obtained permeation profiles with a two-layered diffusion model.
2.9. Measurement of skin thickness

The thicknesses of the stratum corneum, epidermis, and whole skin in pig ear skin was microscopically determined from microtomed sections after hematoxylin–eosin staining. Five section selected at random from each specimen were used to measure the stratum corneum, and whole skin thicknesses were measured by a light micrograph (IX71; Olympus Corp., Tokyo, Japan) and a calibrated ocular micrometer. The thickness of the epidermis was calculated by subtracting the stratum corneum thickness from the whole skin thickness.

3. Results

3.1. Skin permeation profile of lidocaine

Figure 1 shows the skin permeation profiles of lidocaine through full-thickness skin and stripped skin after its topical application. The cumulative amount of lidocaine that permeated through the stripped skin was much higher than that through the full-thickness skin. Skin permeation parameters obtained from the permeation profiles through the full-thickness and stripped skin are listed in Table 1. Since the skin thickness of stratum corneum and viable epidermis and dermis were 19.0 ± 2.0 µm (mean ± S.E.) and 1406 ± 38 µm (mean ± S.E.), these values were set to calculate the skin permeation parameters. The skin concentration-distance profiles in full-thickness and stripped skin that were calculated with the skin permeation parameters are shown in Figures 6a and b, respectively. The ratio of lidocaine concentration at depth 0 to the
distance from the stratum corneum was much higher than that at other distance points, and the lidocaine concentration in the skin gradually decreased toward the dermis side from the stratum corneum.

Fig. 1

3.2. Fractionation of lidocaine signals by MSI

Figure 2 shows a typical mass spectrum, collected from m/z=200 to 900, of a skin section after the topical application of lidocaine. The mass peak of protonated lidocaine was present (m/z 235.18) in the skin section.

Fig. 2

3.3. MSI observations of pig ear skin sections and image analysis using BioMap®

Figure 3a shows optical images of a skin section after the skin had been immersed in lidocaine solution at a concentration of 1500 µg/mL in order to obtain a calibration curve. The distribution of lidocaine was determined in a designated square area of the skin that is enclosed by black lines in Fig. 3a. Figure 3b shows the distribution of lidocaine, which could be identified by MS analysis and visualized as mass images with BioMap software. The skin distribution of lidocaine is shown as a red spot. The [M+H+] signal was detected in the whole area of the skin.
3.4. Imaging analysis of lidocaine in skin sections using Image J software

To evaluate the skin distribution of lidocaine, the distribution of signals was quantified based on the depth direction using Image J software. Figures 4a and b show optical images of a skin section and the lidocaine distribution in the stripped skin after immersion in solutions with different concentrations of lidocaine (0, 500, 1500, and 2500 µg/mL), respectively. Lidocaine distributions were observed in each skin sample, and the brightness of red spots increased with an increase of the applied concentration of lidocaine. Figure 4c shows the correlation between brightness intensity and skin concentration of lidocaine. A good relationship ($R^2=0.998$) was observed within the range of lidocaine concentrations up to 2500 µg/mL.

Figures 4a, 4b, and 4c

Figures 5 and 6 show lidocaine distribution and its concentration-depth profiles in the stripped skin and the viable epidermis and dermis parts of full-thickness skin. The lidocaine distributions were detected in both stripped skin and full-thickness skin and many bright dots were confirmed in stripped skin compared with those in full-thickness skin. The obtained skin concentrations from MSI analysis were corrected with $K_{ved}$ value of lidocaine and the observed skin concentrations was plotted against the average value of the depth of each skin section. As shown in Fig. 6, calculated lidocaine
concentration in skin was decreased in the stripped skin as well as in the full-thickness skin toward the dermis from the stratum corneum side. The observed lidocaine concentration in stripped skin was lower than calculated one, whereas the observed concentration in full-thickness skin was higher than calculated one. Although the profiles did not correspond to each other, skin concentration-depth profiles obtained from MSI analysis showed almost the same tendencies as the calculated ones.

Fig. 5

4. Discussion

The DPK test in the stratum corneum for topically applied drugs by the tape-stripping method would be helpful for evaluating the bioequivalence of topically applied drugs (Kalia et al., 2000). Although LC/MS, LC/MS/MS and accelerator mass spectrometry are a sensitive technique to detect drug concentration, these are limitations for detecting its disposition in a tissue correctly. It is an evidence that Hill equation could be applied to reveal the relationship between pharmacodynamics or toxicodynamics of drugs and its tissue concentration. Therefore, establishment of drug disposition in skin with an imaging analysis would be very useful for evaluation of safety and effects of topically applied chemicals. In our previous experiment (Kijima et al., 2015), a non-steady state of drug concentration-skin depth profile could be detected by confocal laser scanning microscopy after the application of a fluorescent marker, and DPK parameters could be calculated with Fick’s second law of diffusion. Therefore, in the
present experiment, the skin concentration-depth profile of topically applied non-labeled drug was investigated to confirm the possibility of DPK analysis with MSI analysis.

A calibration curve to detect the lidocaine concentration in the skin was achieved with the combination of MSI observation and imaging analysis using Image J software. In the present experiment, calibration curve was obtained from immersed skin in different concentrations of lidocaine solution. Skin concentration of lidocaine was corrected with the $K_{ved}$ value that obtained from *in vitro* skin permeation profile through stripped skin. Therefore, when another chemical was selected, the skin concentrations to calculate skin concentration curve could be corrected with its partition coefficient value.

Oshizaka et al (2014) reported that skin concentration could be calculated using a skin resistance model. Skin permeation resistance in stratum corneum ($R_{sc}$), which is equal to the reciprocal value of the drug permeation coefficient through the stratum corneum ($1/P_{sc}$), could be calculated by subtraction of the reciprocal value of the skin permeation coefficient through full-thickness skin ($1/P_{tot}$) to that through stripped skin ($1/P_{ved}$) (Scheuplien et al., 1971). A high $R_{sc}$ value provides a high drug concentration gradient across the stratum corneum. $R_{sc}$ of lidocaine showed about 90% of total skin resistance and the remaining 10% was $R_{ved}$ in the present study. Thus, lidocaine concentration would be dramatically decreased across the stratum corneum in Fig. 6b. In our previous study, the lidocaine concentration-distance profile obtained from the skin permeation profile corresponded well to the observed one (Oshizaka et al., 2014). Lidocaine concentration-distance profiles obtained from MSI analysis in the stripped
and full-thickness skin differed from the calculated values. However, these measured values obtained from MSI analysis were relatively close to the calculated ones. Thus, this method would be applicable for the detection of drug concentration in skin. When stripped skin was analyzed with MSI, the observed skin concentrations were lower than calculated values. On the other hand, the observed concentrations in the full-thickness skin was higher than the calculated ones. These results might be related to lidocaine diffusion in the skin from high to low concentrations during matrix coverage process before measurement of lidocaine with MALDI-TOF-MS. Thus, LC concentration in stripped skin might be decreased by the diffusion from the inside to the outside of the skin and the concentration in the full-thickness skin might be increased by the diffusion from the stratum corneum to the viable epidermis and dermis compared with calculated values.

In our previous study (Kijima et al., 2015), drug concentration-depth profiles obtained from imaging analysis by confocal laser-scanning microscopy at non-steady-state and steady-state conditions corresponded fairly well with those obtained from the skin permeation experiments. The measurement by laser-scanning microscopy was conducted without a pretreatment procedure. Thus, improvement of procedure might be necessary to analyze drug concentration correctly with MSI analysis.

Bunch et al. (2004), Sjövall et al. (2014), Judd et al. (2013), and D’Alvise (2014) have investigated the drug distribution in skin using MSI analysis. In addition, many reports have been published on the usefulness of MSI analysis as a potential tool for measurement of the distribution of compounds in tissues (Buck et al., 2015; Hamm et
al., 2012; Grobe et al., 2012). Internal standard was not used to conduct the MSI analysis for detection of lidocaine disposition in the skin in the present study. To compensate for variation of the acquired brightness of red spots images, the average value of brightness was used in the divided skin sections. Although drug-disposition profiles in the full thickness skin was achieved by skin-thickness information obtained from the optical image and the profiles were relatively close to the calculated ones with two-layered model, this approach would be not suitable for detection of drug concentration in specific area such as hair follicles, sweat ducts and interfacial layers between different cells. Furthermore, skin is histologically composed of three layers and the line of these layers are not straight, but markedly wavy. Thus, measurement of drug skin with divided skin section area might be difficult to measure its concentration in each layer of the skin. The use of an internal standard to perform quantitative analysis should be needed with MSI analysis in the further experiment to analyze more detail drug distribution in the skin.

Judd et al (2013) have investigated the detection of drug disposition in the stratum corneum with ToF-SIMS by the combination of tapa-stripping method. Although high-sensitive analysis was successfully conducted with this combination approach, drug concentration in viable epidermis and dermis could not be measured with this approach. Few studies have reported that a comparison of topically applied drug concentrations measured by MSI analysis and conventional techniques such as extraction and tissue homogenization procedures. Thus, the present work showed the usefulness of MSI analysis technique to evaluate drug disposition and concentration in
5. Conclusion

Investigation of the dermatopharmacokinetics (DPK) should be performed to develop topical formulations and drug formulas. In the present study, only the lidocaine distribution in skin was evaluated. To clarify the usefulness of this approach, the skin distribution of compounds with a wide range of physicochemical properties should be investigated. Although further experiments involving DPK analysis with the obtained data are required, this result suggests that the drug concentration profile in viable epidermis and dermis could be obtained, and evaluations of the safety and effect of chemicals as well as evaluation of their disposition in the skin might be conducted by observation using MSI.
References


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Figure 1  Skin permeation of lidocaine through full-thickness skin (●) and stripped skin (○). The mean ± S.E. (n=4).

Figure 2  Typical MALDI mass spectrum, which was collected from m/z= 200 to 900, in skin sections without a) and with b) topical application of lidocaine at a concentration of 2500 µg/ml.

Figure 3  Example of an IMS observation image for lidocaine intensity in a full-thickness pig ear skin section after the permeation experiment. a) Optical image of a vertical skin section. b) MSI image observation of distribution of lidocaine in skin after its topical application. The skin section was decided by the positional information of the obtained image. Abbreviation: SC, stratum corneum.

Figure 4  Calibration curve between applied lidocaine concentration [(i) 0 µg/mL, (ii) 500 µg/mL, (iii) 1500 µg/mL, and (iv) 2500 µg/mL] and its brightness.  a) Optical image of skin (the MSI observed area is enclosed with black lines).  b) MSI observation image in skin.  c) Calibration curve obtained from applied lidocaine concentration and its intensity. Each pixel was 10 µm in width. Each point represents the mean ± S.E. (n=5).

Figure 5  The optical and MSI observations of lidocaine after its topical application to
stripped (a and c) and full-thickness skins (b and d). a) and b) are optical images of a skin section, whereas c) and d) are MSI images. Scale bar shows 100 µm.

Figure 6  Lidocaine concentration-depth profile in a) stripped and b) full-thickness skin and c) enlargement of b) in terms of the scale of the y-axis. The value of abscissa \((x/L)\) calculated from the ratio, which obtained from the positional information. \(L\) is set to be 1300 µm from the surface of the skin.

Symbols: ●, lidocaine concentration calculated from skin permeation parameters; ○, concentration observed by MSI image analysis.
Table 1  Permeation parameters obtained from the skin permeation profile of LC

<table>
<thead>
<tr>
<th>Drug</th>
<th>Skin permeation parameters</th>
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<tbody>
<tr>
<td>LC</td>
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</tr>
<tr>
<td>( P_{\text{tot}} ) (cm/s)</td>
<td>( 7.97 \times 10^{-7} \pm 8.8 \times 10^{-8} )</td>
</tr>
<tr>
<td>( P_{\text{ved}} ) (cm/s)</td>
<td>( 6.37 \times 10^{-6} \pm 4.1 \times 10^{-7} )</td>
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<tr>
<td>( D_{\text{sc}} ) (cm²/h)</td>
<td>( 8.5 \times 10^{-7} )</td>
</tr>
<tr>
<td>( D_{\text{ved}} ) (cm²/h)</td>
<td>( 2.2 \times 10^{-3} )</td>
</tr>
<tr>
<td>( K_{\text{sc}} )</td>
<td>9.5</td>
</tr>
<tr>
<td>( K_{\text{ved}} )</td>
<td>1.7</td>
</tr>
</tbody>
</table>

\( P_{\text{tot}} \): skin permeation coefficient through the full-thickness skin  
\( P_{\text{ved}} \): skin permeation coefficient through the stripped skin  
\( D_{\text{sc}} \): diffusion coefficient of drug in the stratum corneum  
\( D_{\text{ved}} \): diffusion coefficient of drug in the viable epidermis/dermis  
\( K_{\text{sc}} \): diffusion coefficient of drug in the stratum corneum  
\( K_{\text{ved}} \): diffusion coefficient of drug in the stratum corneum  
Thicknesses of the stratum corneum and viable epidermis/dermis were set to be 15 µm and 1485 µm, respectively.
Figure 1

Cumulative amount of lidocaine permeated (µg/cm²) vs. Time (h)
Figure 2

a)

![Graph 1](image1)

b)

![Graph 2](image2)
Figure 3

a) [Image of SC side and Dermis side]

b) [Image of depth markers: 0, 260, 520, 780, 1040, 1300 µm, with SC side and Dermis side indicators]
Figure 4

a) i) ii) iii) iv)  

b) i) ii) iii) iv)  

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Figure 5
SC side  Dermis side  SC side  Dermis side

[Images of tissue samples]
Figure 6

a) Distance ratio from the stripped skin surface

b) Distance ratio from the SC surface