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Quantitative investigation of Terbinafine Hydrochloride Absorption into a Living Skin Equivalent Model by MALDI-MSI

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Abstract

The combination of microspotting of analytical and internal standards, matrix sublimation and recently developed software for quantitative mass spectrometry imaging has been used to develop a high resolution method for the determination of Terbinafine hydrochloride in the epidermal region of a full thickness living skin equivalent model. A quantitative assessment of the effect of the addition of the penetration enhancer (dimethyl isosorbide (DMI)) to the delivery vehicle has also been performed and data have been compared to those obtained from LC/MS/MS measurements of homogenates of isolated epidermal tissue. At 10% DMI the levels of signal detected for the drug in the epidermis were 0.20 ± 0.072 mg/g tissue for QMSI and 0.28 ± 0.040 mg/g tissue for LC/MS/MS; at 50% DMI 0.69 ± 0.23 mg/g tissue for QMSI and 0.66 ± 0.057 mg/g tissue for LC/MS/MS. Comparison of means and standard deviations indicates no significant difference between the values obtained by the two methods.

Keywords: MALDI-MSI, quantitation, Terbinafine hydrochloride, microspotting.

Introduction

The skin is used as route of drug administration both for compounds intended as topical treatments and for those intended as systemic treatments where there is a requirement to avoid the first pass effect.[1] Since there are issues in reliably obtaining human skin for pre-clinical studies (and sufficient skin for a representative study given issues including, race, gender, age and genetic polymorphisms) there is a great deal of interest in the use of 3D *in vitro* tissue engineered models of human skin as reproducible and easy to obtain testing platforms.[1] In a recent review by Mathes *et al.* the use of these models in drug development has been described.[2] Skin models commercially available include: human reconstructed epidermis (HRE), 3D differentiated epidermis cultures derived from human keratinocytes, i.e. EpiSkin (Epskin, Lyon, France) and EpiDerm (Mattek, Ashland USA) and full thickness living skin equivalents (LSE) e.g. EpiDermFT (Mattek Ashland USA), T-skin (Episkin, Lyon, France) and Labskin (Innovenn(UK) Ltd York UK).[3]

Mass spectrometry imaging (MSI) is a relatively new and powerful technique able to study intact tissue sections providing ion distribution maps of many non-labelled endogenous and exogenous species simultaneously. This is a distinct advantage in comparison to conventional techniques, such as immunohistochemistry and radiolabelling. The absence of the label makes this technique fast, relatively inexpensive. It has been used extensively in studies of pharmaceutical distribution[4] and its use to study skin absorption was one of the first applications of MSI in pharmaceutical analysis to be reported.[5]

The application of mass spectrometry imaging (MSI) to 3D *in vitro* tissue models of human skin represents a valid approach for investigating either drug absorption or drug biotransformation in skin. Arvey *et al.*[6] were the first to combine MSI with a comercially available 3D tissue model of the epidermis “Straticel” in order to analyse the penetration of the drug imipramine. In later work conducted by Francese *et al.*[7] and Mitchell *et al.*[8] full thickness living skin equivalents (LSEs) were used as models to be analysed by MALDI-MSI. Such models consist of a full differentiated epidermal layer supported by a dermal component generated by seeding fibroblasts into a 3D scaffold. The aim of the work reported by Francese *et al.*[7] was to evaluate the efficiency of curcumin as a MALDI matrix. After the application of this matrix, MALDI-MSI was used to plot the localization of the drug acetretin within Labskin, a commercial living skin equivalent model. This study demonstrated that at 4 hours post treatment the drug had penetrated into the epidermal layer. In a more recent work, Harvey *et al*.[9] used MALDI-MSI to map the same drug acetrin in Labskin models, in which psoriasis was induced by treating the samples with the proinflammatory cytokine interleukin-22. This study showed that at 24 hours post treatment the drug was localised

onto the epidermal layer, whereas after 48 hours the drug had penetrated into the dermal region.[9] Such studies have demonstrated that the widespread adoption of MSI in skin research could contribute to the study of drug/toxicant absorption, drug/toxicant response, infection, would-healing and burns.

In MALDI-MSI the nature of analyte ionization depends strongly on the entity of the analyte as well as the tissue. The same molecule can be subjected to varying ion suppression effects in different tissues or across the same tissue in response to a changeable histological framework as well as to the ionization competition with compounds within the morphological microenviroment.[10] This aspect in addition to the variation of ion signals due to heterogeneity of matrix deposition represent the major issues that need to be addressed in the development MALDI-MSI as a method for quantitative mass spectrometry imaging (QMSI). A recently published review conducted by Rzagalinski *et al.* investigated multiple approches and strategies for quantification of small molecules using MALDI-MSI in order to correct these limitations.[11] Particular attention was focused on the strategy used to create calibration curves based on the use of serial dilution of standard. In order to mimic suppression ion effects within tissue, it has been common to either use mimetic arrays created from tissue homogenates[12] or to spot working standard solutions using a control tissue in two different ways: 1) by spotting a range of standard concentration onto the tissue prior to depositing the matrix or 2) by spotting a range of concentration underneath of tissue prior to positioning the tissue and depositing the matrix. Lagarrigue *et al.* used spotting onto tissue in order to quantify the amount of pesticide chloredecon within mouse liver section.[13] In this study six replicates were performed and a good linearity coefficient was achieved (R^2 from 0.9807 to 0.9981). In contrast Pirman *et al.* spotted a range of calibration standard underneath a control brain tissue in order to quantify levels of cocaine by visualization of the expected major product ion at *m/z* 182 using MALDI-MS/MS imaging.[14]

The effect of tissue composition on signal response in MSI has large implications when skin is the target organ for quantitative experiments. The layers of the skin comprise distinct cell types and hence each skin layer would be expected to give a slightly different response for the same amount of analyte. This implies that mimetic arrays created from skin homogenates would not be a suitable methodology for calibration in this instance. A recent paper by Chumbley *et al.*[15] reported the use of an acoustic micro-spotter[16] for the creation of calibration arrays in a study of the quantification of rifampicin in liver tissues. It was reported that the use of the acoustic spotter enabled picolitre volumes of standard solutions to be reproducibly and accurately spotted onto tissue sections. It was decided to investigate the use of acoustic microspotting of standards as a way of calibrating QMSI experiments where the object was to quantify the amount of a compound in a defined skin layer.

The object of this study is to obtain absolute quantitation of the amount of an antifungal agent, Terbinafine hydrochloride, in the epidermal layer of a 3D living skin equivalent model. Terbinafine is an antifungal belonging to allylamine class and it acts by blocking sqalene epoxidase. The hydrochloride form of Terbinafine has been included in topical formulations for the treatment of dermatophytoses, pityriasis versicolor and cutaneous candidiasis.[17] Since the aim of drug delivery systems for Terbinfine is topical delivery rather than transdermal delivery, in order to assess their efficiency, methodologies for the quantitation of Terbinafine in skin rather than measurements of permeation through skin e.g. Franz-Cell diffusion measurements[18] are required. Of interest in such measurements is how the addition of penetration enhancers e.g. Dimethyl Isosorbide (DMI) to the delivery formulation affects the amount of drug at the target site (*i.e.* the epidermis).[19] The model chosen for this study is Labskin, a full thickness skin model produced by seeding a fibrin scaffold with fibroblasts and keratinocytes. The model has been extensively used for mass spectrometry imaging experiments due to its good physical strength and ease of crysectioning.

In the study reported here the combination of microspotting of analytical and internal standards, matrix sublimation and recently developed software for QMSI[20] has been used to develop a high resolution QMSI method for the determination of Terbinafine hydrochloride in the epidermal region of living skin equivalent model and a quantitative assessment of the effect of the addition of the penetration enhancer DMI to the delivery vehicle. These data have been compared to data obtained from LC/MS/MS measurements of homogenates of isolated epidermal tissue.

Experimental

i. Materials

Alpha cyano-4-hydroxycinnamic acid (α-CHCA), acetonitrile (ACN), phoshorus red, Terbinafine hydrochloride standard (TBF HCl, MW 327.89), isosorbide dimethyl ether (DMI), haematoxylin, eosin, xylene substitute, ethanol (EtOH) and formic acid ≥ 96% (FA) were purchased from Sigma-Aldrich (Gillingham, UK).

Pertex mounting medium was obtained from Leica Microsystems (Milton Keynes, UK).

LC-grade methanol (MeOH) and LC-grade acetonitrile (ACN) were purchased from Fisher Scientific. 18 MΩ water was obtained from an ELGA water purification system (Buckinghamshire, UK).

The internal standard Terbinafine d7 hydrochloride (TBF-d_7 HCl, MW 334.93) was obtained by Clearsynth (Maharashtra, India). Gentian violet 1% was purchased from De La Cruz Laboratories Inc. (Califiornia, USA).

Labskin living skin equivalent (LSE) samples were provided by Innovenn (UK) Ltd (York, England).

ii. Sample treatment

Living skin equivalent (LSE) samples were obtained at day 7 air liquid interface (ALI) and incubated in Labskin maintenance media for 24 hours within 5% CO_2 at 37°C.
For the experiment, three LSE samples were treated with 20 µL of Terbinafine hydrochloride (1% w/w) dissolved in an emulsion made up of water/olive oil (80:20 v/v) with either 10% or 50% DMI and incubated for 24 hours. For the vehicle control group, three LSEs samples were treated with 20 µL of the emulsion water/olive oil (80:20 v/v) alone and incubated for 24 hours.
After incubation, the samples were taken and washed with LC-grade MeOH to remove excess formulation and, then, snap-frozen with liquid nitrogen cooled isopentane (2-5 min) and stored at -80°C.
For cryosectioning, LSEs were transferred into the cryostat (Leica 200 UV, Leica Microsystems, Milton Keynes, UK), mounted onto cork ring using dH_2O at -25°C for 30 min to allow to thermally equilibrate. 12 µm tissue sections were cryosectioned, thaw mounted onto polylysine glass slides and stored at -80°C.
Before matrix application and imaging the samples were freeze-dried under vacuum (0.035 mBar) for 2 hours to avoid delocalization of the analyte and preserve the integrity of the tissues.

iii. Preparation of Standard Curves

For MALDI-MSI experiments, working standards were made to 0.01, 0.1, 1, 10, 100, 500, 1000 and 1500 ng/µL of TBF HCl with 100 ng/µL of the internal standard TBF d7 HCl in MeOH/H_2O (50:50). Calibration standards were applied onto the epidermis area of 12 µm thick sections of blank tissue sections using an acoustic robotic spotter (Portrait 630, Labcyte Inc., Sunnyvale, CA).
9 microspots of internal standard TBF-d7 hydrochloride (100 ng/µL in MeOH/H2O (50:50)) were deposited onto the epidermis of a vehicle control Labskin section treated with water/olive oil (80:20) alone and two Labskin samples treated with Terbinafine hydrochloride 1% w/w in water/olive oil (80:20) with either 10% or 50% DMI.

For application of the standards and internal standard, the number of cycles for each spot was set to 20 for a total volume of 3.4 nL of each deposited solution. 5 extra spots were applied outside the tissue to give a “drying time” between each cycle.

For LC-MSMS, calibration standards were made to 0.001, 0.01, 0.05, 0.1, 0.5, 1, 10 ng/µL of Terbinafine hydrochloride with 0.1 ng/µL of internal standard Terbinafine d7 hydrochloride in acetonitrile + 0.1% formic acid/ultrapure water + 0.1% formic acid (80:20).

iv. Matrix Application

300 mg of matrix α-CHCA was spread evenly at the bottom of the sublimation apparatus (Sigma-Aldrich, Gillingham, UK). Polylysine glass slides containing Labskin tissues were attached to the flat top of the chamber. The flat top of the chamber was then attached to the bottom using the O-ring seal and the vacuum was applied. When a stable vacuum of 2.5×10^{-2} Torr was achieved, the top was filled with cold water (5°C) and the temperature was set up at 180°C. The sublimation process was performed until the optimal amount of α-CHCA (between 0.1-0.2 mg/cm^2) was achieved.

v. Mass Spectrometry Imaging

All tissues were imaged using a Waters MALDI HDMS Synapt™ G2 mass spectrometer (Waters Corporation, Manchester, UK) equipped with a neodynium: yttrium aluminium garnet (Nd:YAG) laser operated at 1 KHz. The calibration of instrument was performed by using phosphorous red. MALDI-MS images were acquired in positive mode, in full scan “sensitivity” mode at a range of *m/z* 100-1500, (resolution 10,000 FWHM) at spatial resolution of 60 µm x 60 µm, and with laser energy set to 250 arbitrary units. The ion mobility function of the instrument was not enabled.

vi. LC/MS/MS

All LC/MS/MS experiments were performed using a Xevo G2-XS QTof (Waters Coorporation, Manchester, UK) with ionization mode ESI+ with analyser in sensitive mode.
The LC conditions were made of an ACQUITY UPLC HSS T3 C18 1.7 µm, 2.1 x 100 mm (p/n 186003539) column. The mobile phase consisted of ultrapure water (solvent A) and acetonitrile (solvent B) containing both 0.1% formic acid. The flow rate and the injection volume were 0.2 mL/min and 2µL, respectively.

The gradient eluition was performed as follows: 0.0-2.0 min (A: 95%, B: 5%), 2.0-12.0 min (A: 5%, B: 95%), 12.0-30.0 min (A: 5%, B: 95%), 30.0-40.0 min (A: 95%, B: 5%), 40.0-44.0 min (A: 95%, B: 5%).
The experimental instrument parameters used were: capillary voltage: 3.0kV, cone voltage: 35.0V, source temperature: 140°C, desolvation temperature: 250°C, desolvation gas:1000L/hr and cone gas: 50L/hr.
Argon was utilized as collision gas and the collision energy was set at 19eV.
A multiple reaction monitoring (MRM) method was used to detect the product ion of Terbinafine (292.3 → 141.1 m/z) and the product ion of Terbinafine d7 (I.S.) (299 → 148 m/z). The retention time was ~ 10.6 min.

vii. Skin extraction

The vehicle control and treated Labskin tissues were placed for 2 min in 1X PBS pre-heated at 60°C; then, the epidermis was separated from the dermis by using forceps, transferred to tubes and weighted.

The tissue homogenisation and drug extraction were performed by a small modification of previously published work carried out by Sachdeva *et al*.[21] The modification made was that after the second extraction, the back extraction was not performed; instead, the organic layer containing the extracted drug was evaporated under nitrogen and, then reconstituted in 1.8 mL of ACN/H_2O (80:20) + 0.1% FA. The solution was filtered through a 0.22 µm filter and 0.2 mL of internal standard TBF d7 hydrochloride (0.1 ng/µL in ACN/H2O (80:20) + 0.1% FA) was added to the solutions prior to analysis.

viii. Histological Staning

LSE sections were stained used Mayer's haematoxylin and eosin solutions. Each slide was rehydrated by submerging in 100%, 95% and 70% EtOH washes for 3 min and they were left for 1 min in deionised water before being stained in filtered Meyer's haematoxylin for 10 min. Tissues were washed in running tap water for 3-5 min and dehydrated using 70% and 95% EtOH solutions and immersed in filtered eosin 100% for 1 min. The last dehydration step was performed using 95% and 100% EtOH solution, each for a period of 3 min. Finally, the slides were submerged in 2 changes of xylene substitute for 5 min each and mounted using Pertex mountant.

Optical images were obtained using a Cytation 5 imaging reader and analysed with Gen5 software (BioTek, Swindon, UK).

ix. Data Analysis

MALDI-MSI data were processed using the HDI 1.4 (Waters Corporation, UK) software tool. Using this software, MSI raw data files were converted in imzML format and imported into MSIQuant software for quantitative investigations.

For LC/MS/MS data, the chromatograms peaks for Terbinafine hydrochloride and Terbinafine d7 hydrochloride were integrated and processed using Mass Lynx (Waters Corporation, UK) software tool.

Statistical analysis was performed using the StatDirectsoftware (StatsDirect, Cheshire, UK). F test and T test were used to evaluate the statistical significance in terms of precision and accuracy, respectively, between the values obtained by MALDI-MSI and LC/MS/MS techniques.

Three replicate measurements (n=3) were used and the level of significance was set to 5%. Outlier point identifications were performed using Prism software. The method selected was Grubbs' test for outliers (α= 0.05).

Results and Discussion

Reproducibility of droplet spot size of the Portrait 630.

Manually spotting of calibrants onto control tissues has constituted one of the major approaches for generating calibration array in previous QMSI experiments.[13,22-24] Although widely practiced, this technique is not without limitations. One of the major drawbacks of manual pipetting is the difficulty of depositing sub-microliter volumes of solutions. This makes it difficult to localize standards to small defined regions of tissue. Furthermore, manually applied spots are susceptible to variations in size and, hence, the amount standards in the spots are difficult to control.

In this study we decided to measure and compare the perimeter and area of the droplet spots generated by the Portrait 630 in order to assess the reproducility and accuracy of this device. In order to perform the experiment, a solution 0.1% of gentian violet in MeOH/H_2O (1:1) was used as a spot size marker and 9 microspots of the solution were deposited onto the epidermal layer of a 12 µm thick blank Labskin section. In each spot the number of cycles was set to 20, with a total deposited volume of 3.4 nL per spot. The experiment was performed twice and, after spotting, the sections were imaged with a Cytation 5 imaging reader equipped with Gen5 software, while the perimeter and area of each spot on recorded images was measured by using ImageJ software (https://imagej.nih.gov/ij/).

The results of these experiments are shown in Supporting Information (Figure S1-A,-C). The presence of the dye in the solution allowed easy visualisation of the spots onto the tissue (Figure S1A). The average perimeter of spots for two Labskin sections was found to be 0.50 ± 0.041 mm and 0.53 ± 0.035 mm, respectively, while the average area was found to be 0.019 ± 0.0027 mm^2 and 0.021 ± 0.0028 mm^2, respectively. The relative standard deviations of the measurements were as follow: 14.35% (area) and 8.21% (perimeter) from section 1; 13.50 % (area) and 6.62 % (perimeter) from section 2 (Figure S1B-C).

These data demonstrate the high reproducibility in the size of the dye spots intra and inter sections when the Potrait spotter was used. The area and perimeter values detected from the spots in two sections of Labskin tissue were not statistically different. The use of Portrait 630 acoustic spotter to generate microspots with constant size and minimal lateral diffusion allowed better control of the concentration of analyte and also avoided the possibility of cross contamination that could occur for direct contact of the pipet with the substrate.

Method used for quantitation

In MALDI-MSI, the optimisation of the matrix disposition method is an essential pre-requisite for reproducible detection of the analyte of interest with the required sensitivity and spatial resolution. In previous work[25] we have reported that matrix sublimation[26] is an excellent methodology for the production of high resolution images of xenobiotic distribution in skin and 3D skin models. Figure 1A-C shows MALDI-MSI images of the distribution of the in source generated Terbinafine fragment ion at *m/z* 141 in three sections of Labskin recorded at 60 μm pixel size following treatment with (A) 20 μl of emulsion water/olive oil (80:20) alone (vehicle control) and 20 μl of Terbinafine 1% (w/w) in water/olive oil (80:20) with (B) 10% or (C) 50% isosorbide dimethyl ether (DMI) for 24 hours. It can be seen that the Terbinafine signal appears to be localized to the epidermis and that there is an increase in its intensity with increasing amount of DMI.

In order to quantify the amount of Terbinafine in the epidermis from such images it is necessary to calibrate the response specifically for signals arising from the epidermis to achieve "matrix matched standards". Previous studies have shown that the epidermis of Labksin consists of a very thin differentiated layer with an average thickness of 32 μm.[9,27] As discussed previously, this makes preparing standards by tissue spotting challenging. Therefore in this work, the use of an acoustic picoliter droplet ejector, used previously as a MALDI matrix deposition device,[16] was used to spot 3.4 nL of the working standard (from 0.01 ng/μL to 1500 ng/μL) in MeOH/H_2O (1:1) onto the

epidermis of a blank section of Labskin to create a calibration array. Internal standard Terbinafine d7 hydrochloride (100 ng/µl) was included into standard solutions prior to spotting and additionally 9 spots of internal standard (100 ng/µl) were applied to the epidermal region of each treated sample for analysis (again using the acoustic picoliter droplet ejector). Figure 2A-D shows the MS image of the distribution of the *m/z* 148 in source generated fragment ion of Terbinafine d7 on (A) untreated sample along with the calibration array, (B) vehicle control skin sample treated with 20 µl of the emulsion water/olive oil (80:20) alone and skin samples treated with Terbinafine 1% (w/w) in water/olive oil (80:20) with either (C) 10% or (D) 50% isosorbide dimethyl ether (DMI) for 24 hours. The distribution of the internal standard can be clearly seen for each spot on each section and hence these data are suitable for the definition of the area of spots created by the acoustic picoliter droplet ejector. The MSIQuant software[20] allows a number of methods for the definition of regions of interest (ROI) and extraction of peak intensities from them for quantitative analyses. Here the methodology used was to exploit signals from endogenous species to define the epidermis and stratum corneum of the tissue section (*m/z* 184 PC to define the tightly packed cells of the epidermis and *m/z* 264 to define the stratum corneum). Then using the software an average intensity for the signals of the Terbinafine and the Terbinafine d7 of a ROI located to solely in the epidermis for each spot could be extracted (Figure 3A-B).

Over the past years, the use of an internal standard has been demonstrated to increase the quantitative capabilities of MSI analysis.[28,29] The internal standard must be a molecule with chemical and physical caracteristics similar to the analyte under study, and, for this reason, most common deuterated analogues are implied. During MSI analysis the internal standard mimics the behavious of the analyte of interest in terms of ionization efficiency and compensates for the ion signal variations of the analyte tissue-dependent. This aspect causes an improvement of relative signal ion reproducibility and image quality due to an increase of pixel to pixel precision.[14,15] Therefore, it was decided to use a deuterated analogue of Terbinafine hydrochloride with seven deuterium ions on naphtalene group in order to distinguish the fragment ion of the internal standard to the fragment ion of the analyte in the mass spectrum, leading an increase of selectivety.

Different approaches for applying a constant concentration of internal standard uniformly to the tissue have been investigated. Most commonly, an automatic spray-coating device is implied to deposit an internal standard either premixed with MALDI matrix[13,24,30] or prior to matrix deposition[31-33] onto the tissue. Instead, we found it beneficial to apply the internal standard onto the tissue by microspotting in order to preserve the localization of the calibration analyte, which, instead it was found to migrate when the solution of Terbinafine d7 hydrochloride was sprayed onto the tissue (data not shown).

The generation of the calibration curve (n=3) was obtained by plotting either the average intensity of *m/z* 141 (Figure 3C) or the average intensity ratio of *m/z* 141/148 (Figure 3D) versus the concentration of Terbinafine expressed in ng/mm^2. In agreement with previous studies, we found that the normalisation of the analyte signal to its deuterated analogue caused a significant improvement in the calibration curve linearity with a correlation coefficient (R^2) from 0.9968 to 0.9992 upon normalisation. The limits of detection (LOD) and quantitation (LOQ) were calculated; from these calibration data: the LOD was found to be 1.30 ng/mm^2 or 0.11 mg/g tissue, whereas, LOQ was found to be 3.93 ng/mm^2 or 0.33 mg/g tissue.

Quantitation of the drug within the tissue.

Using the method described above the concentration of Terbinafine in the epidermis of (A) vehicle control Labskin and Labskin treated with 20 μl of Terbinafine 1% (w/w) in water/olive oil (80:20) with either (B) 10% or (C) 50% isosorbide dimethyl ether (DMI) for 24 hours was determined. In order to perform the experiment, a total of nine microspots with a known concentration of Terbinafine d7 hydrochloride (100 ng/μL) was deposited onto the epidermal layer of the vehicle control and treated Labskin samples. ROIs for each microspot of the TBF d7 fragment ion (*m/z* 148) were drawn in corrispondence of the epidermal layer. Even in this case, the localization of the microspots of the Terbinafine d7 fragment ion onto the epidermis and stratum corneum was visualised by sumperimposing the internal standard fragment ion signal (*m/z* 148) with the phosphocoline ion signal (*m/z* 184) and the ceramide fragment ion signal (*m/z* 264). Using MSIQuant software, the average intensity of the Terbinafine fragment ion on each ROI was extracted and normalised to the average intensity of the Terbinafine d7 fragment ion (*m/z* 141/148). Then, the average intensity ratio (*m/z* 141/148) from each spot was compared to the calibration curve, as shown in Figure 4A-C.

By resolving the calibration equation, the amount of drug from each spot was obtained in ng/mm^2. To calculate the quantitative concentration of Terbinafine hydrochloride in milligramm per gramm of tissue, first, the amount in gramm of tissue in 1 mm^2 was detected. The volume of tissue in 1 mm^2 was calculated multiplying the area (1 mm^2) by the thickness of the section (0.012 mm). Then, the volume (0.012 mm^3) was multiplied by the density of Labskin (1 mg/mm^3) and the amount of tissue (g) in 1 mm^2 was obtained (0.000012 g). By divinding in turn the concentration of Terbinafine from each spot (ng/mm^2) to the gramm of tissue in 1 mm^2, the concentration of the drug was converted in milligramm per gramm of tissue. The values derived from the spots applied onto each Labskin section were averaged and the mean concentration of Terbinafine hydrochloride was calculated.

As shown in Figure 4D, in initial experiments the apparent levels of the drug were found to be 0.15 ± 0.11 mg/g tissue in vehicle control, 0.35 ± 0.047 mg/g tissue within Labskin treated with Terbinafine at 10% DMI, and, 0.84 ± 0.14 mg/g tissue within Labskin treated with Terbinafine at 50% DMI. On investigation it was found that the internal standard solution used contained a small amount of the unlabelled drug. The problem related to deuterium-hydrogen exchange in deuterated compounds was previously described by Chavez *et al.*[34] and can lead to an overestimation of the concentration of unlabelled analyte. To correct for this the amount of Terbinafine detected in the vehicle control was subtracted from the amount of Terbinafine detected in the treated tissues for each QMSI experiment. After this correction, at 10% DMI the concentration of TBF was found to be 0.20 ± 0.072 mg/g of tissue (below the formal limit of quantitation), and at 50% DMI the level was found to be 0.69 ± 0.23 mg/g tissue.

In order to validate the MALDI-MSI data LC/MS/MS experiments were performed using the methodology described by Sachdeva *et al.*[21] LC/MS/MS is a high sensitivity technique, widely used in previous studies for quantification of Terbinafine hydrochloride.[35,36] Although it is common knowledge that LC/MS/MS provides reliable quantitation, analysis using this technique can not be carried out directly on the intact surface skin, but analytes of interest have to be extracted out of the tissue, increasing the complexity of sample preparation, time of analysis and losing spatial information. In addition, another drawback on using LC/MS/MS is represented by the amount of tissue necessary for homogenization (from 0.5 mg to 50 mg) compared to the small amount of tissue that can be analysed using MALDI-MSI (0.010-0.012 mg).

LC/MS/MS experiment was repeated three times per each tissue of Labskin and the limit of detection (LOD) and quantitation (LOQ) were assessed at 0.42 µg/mL and 1.27 µg/mL, respectively. In the vehicle control sample, the levels of Terbinafine were below the limit of detection, whereas, at 10% DMI and 50% DMI the levels were above the LOQ and they were found to be 0.28 ± 0.040 mg/g tissue and 0.66 ± 0.057 mg/g tissue, respectively.

A statistical unpaired T test was perfomed on the data from both tissues treated with Terbinafine with either 10% DMI or 50% DMI. The concentration of the drug resulted statistically increased in the tissue when the percentage of DMI increased in the formulation in both QMSI (two sided P= 0.0256) and LC/MS/MS (two sided P= 0.0007) (Figure 4E-F). Furthermore, in order to compare the values obtained by QMSI and LC/MS/MS, F test and paired T test between the methods were performed. With the F test, the variances between the values of Terbinafine at 10% DMI and 50% DMI were found to be not statistically different between the methods (at 10% DMI; two sided P= 0.478; at 50% DMI, two sided P=0.1116). When the paired T test was performed, also the means between the values of Terbinafine at 10% DMI and 50% DMI were found to be not statistically

different between the methods (at 10% DMI, two sided P=0.0726; at 50% DMI, two sided P=0.8361) (Figure 4G).

These data have demonstrated the development of a QMSI method for the determination of the amount of an active pharmaceutical ingredient in skin. In addition the capability of the penetration enhancer DMI to increasing the drug penetration in the upper epidermis of living skin equivalent has been demonstrated.

Conclusions

In this article a novel approach for quantitative mass spectrometry imaging (QMSI) of Terbinafine hydrochloride in in the epidermal region of a full thickness living skin equivalent model, has been presented. The use of an acoustic spotter turned out to be ideal for applying precise and uniform analytical and internal standards onto a thin and well-defined epidermal layer of the Labksin tissue, leading to mimic cell-type based ionization response of the analyte from the treated tissue sections. The combination of microspotting technique and matrix sublimation allowed preserving the spatial distribution of the analyte and achieving better mass spectral quality and reproducibility. The study presented here also provided an innovative method to assess the performance of the penetration enhancer DMI added to the delivery vehicle. QMSI data demonstrated an increase of concentration of Terbinafine into the upper epidermis of Labskin in response to an increase of percentage of DMI in the delivery vehicle.

QMSI data were satisfactory in showing no statistically significant differences from LC/MS/MS measurements of homogenates of isolated epidermal tissue, leading accuracy and precision between the methods to be the same.

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Supporting Information

Figure S1 (A-C) data from measurement of spot size reproducbility experiments.

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Figure legends

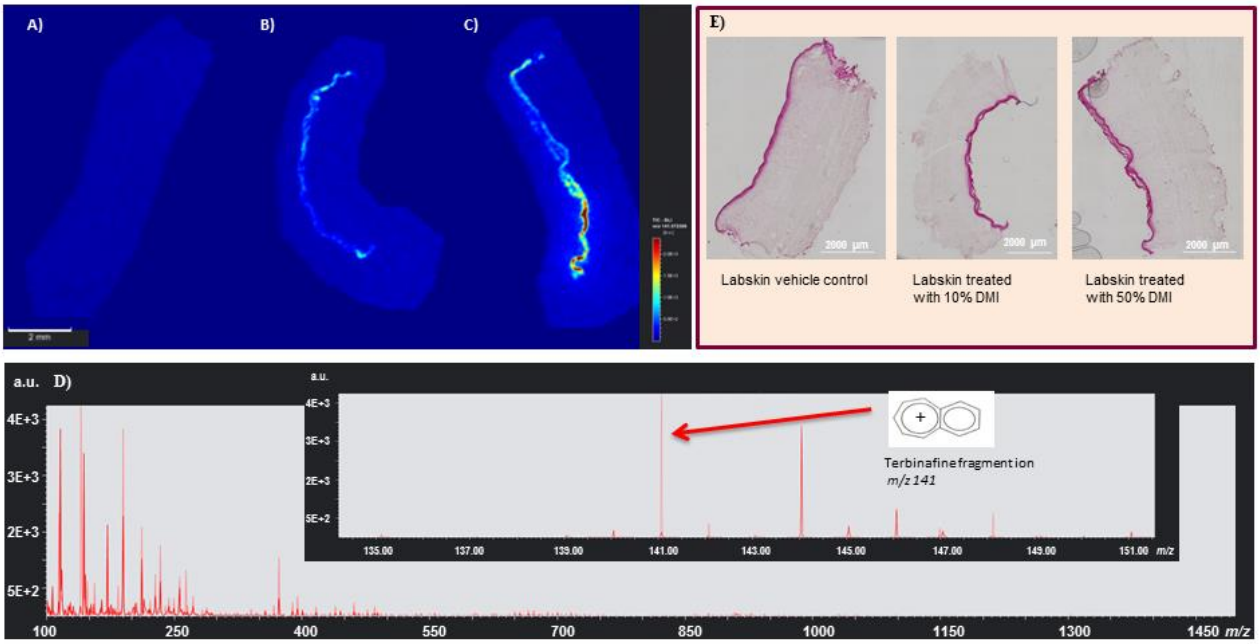
Figure 1. MALDI-MSI at 60 x 60 µm spatial resolution of the Terbinafine hydrochloride fragment ion ($[C_{11}H_9]^+$; *m/z* 141) on (A) vehicle control section and two Labskin sections treated with Terbinafine 1% (w/w) in water/olive oil (80:20) with either (C) 10% or (D) 50% isosorbide dimethyl ether (DMI) for 24 hours. D) Average MALDI-MSI spectra showing the peak of the Terbinafine hydrochloride fragment ion at *m/z* 141. E) Haematoxylin & eosin stained optical image of the sublimated sections after MALDI-MSI (4X magnification).

Figure 2. MALDI-MSI at 60 x 60 µm spatial resolution of the Terbinafine d7 hydrochloride source generated fragment ion ($[C_{11}D_7H_2]^+$; *m/z* 148) microspotted directly on the epidermal layer of A) untreated sample along with the calibration array, (B) vehicle control section and two Labskin sections treated with Terbinafine 1% (w/w) in water/olive oil (80:20) with either(C) 10% or (D) 50% isosorbide dimethyl ether (DMI) for 24 hours.

Figure 3. A) MALDI-MSI of the Terbinafine d7 source generated fragment ion in red (*m/z* 148) superimposed with choline head group in blue (*m/z* 184) and ceramide fragment peak in green (*m/z* 264). B) Haematoxylin & eosin stained optical image of the sublimated section after MALDI-MSI (4X magnification). Calibration curve (n=3) generated using C) the average intensity of *m/z* 141 (no normalisation) and D) the ratio average intensity of *m/z* 141/148. Normalisation to the internal standard *m/z* 148 improved the linearity of the calibration curve.

Figure 4. MALDI-MSI of the Terbinafine d7 fragment ion in red (*m/z* 148) superimposed with choline head group in blue (*m/z* 184) and ceramide fragment peak in green (*m/z* 264) in A) vehicle control section and two Labskin sections treated with Terbinafine 1% (w/w) at (B) 10% or (C) 50% DMI for 24 hours. The intensity of the analyte normalised to the internal standard was extracted from each ROI and compared to the calibration curve. D) Graph showing the initial QMSI levels of Terbinafine from the sections of Labskin. E) Graph showing the final levels of Terbinafine from the sections of Labskin after correction for the degradation of the internal standard. F) Graph showing the levels of Terbinafine obtained from LC/MS/MS measurements of homogenates of isolated epidermal tissue. G) Graph showing comparison between the results obtained from MALDI-MSI and LC/MS/MS, the error bars illustrates the standard deviation of three repeats for each method. No significant differences between the two methods were found.

Figure 1


A)
B)
C)
E)
Labskin vehicle control
Labskin treated with 10% DMI
Labskin treated with 50% DMI
a.u.
D)
4E+3
3E+3
2E+3
5E+2
100
250
400
550
700
850
1000
1150
1300
1450 m/z
Terbinafine fragment ion
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141.00
143.00
145.00
147.00
149.00
151.00 m/z

Figure 2

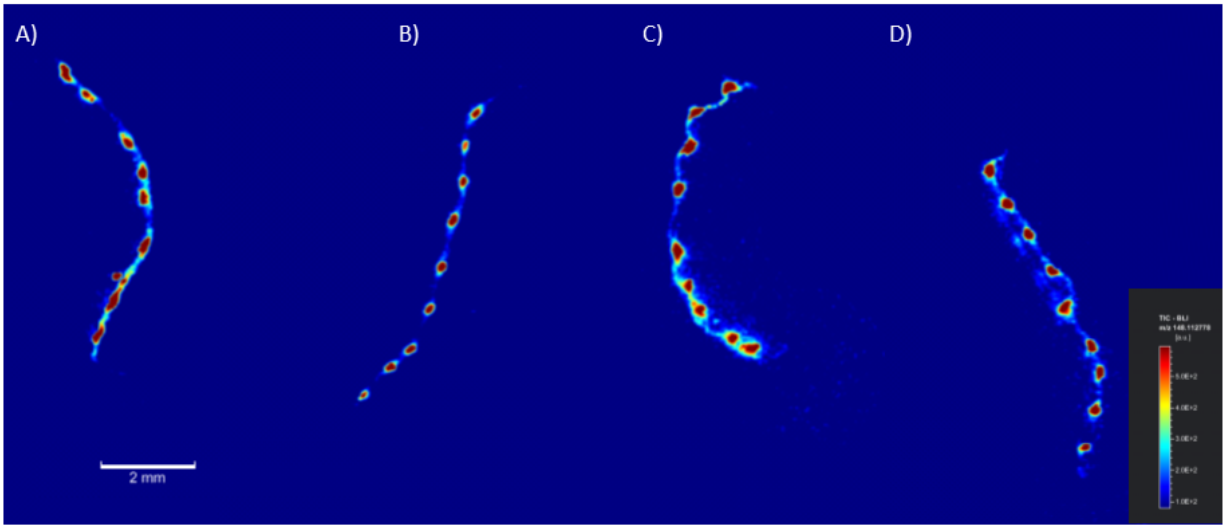


Figure 3

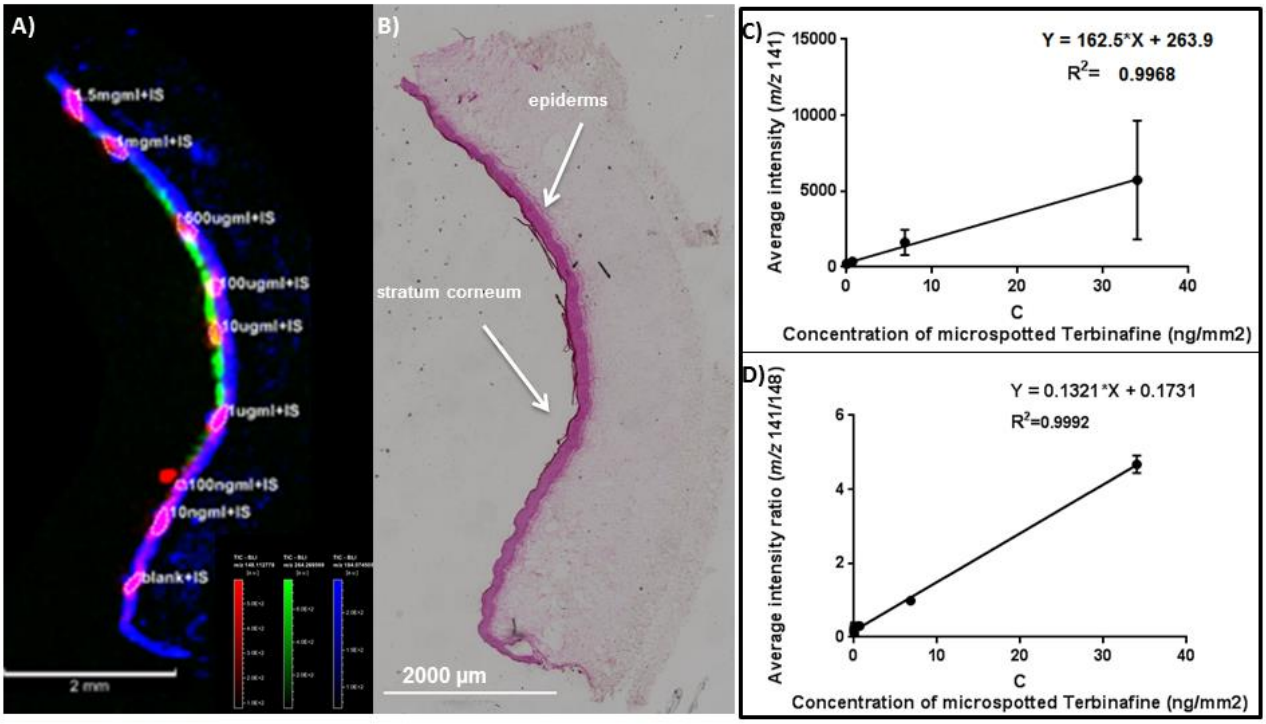
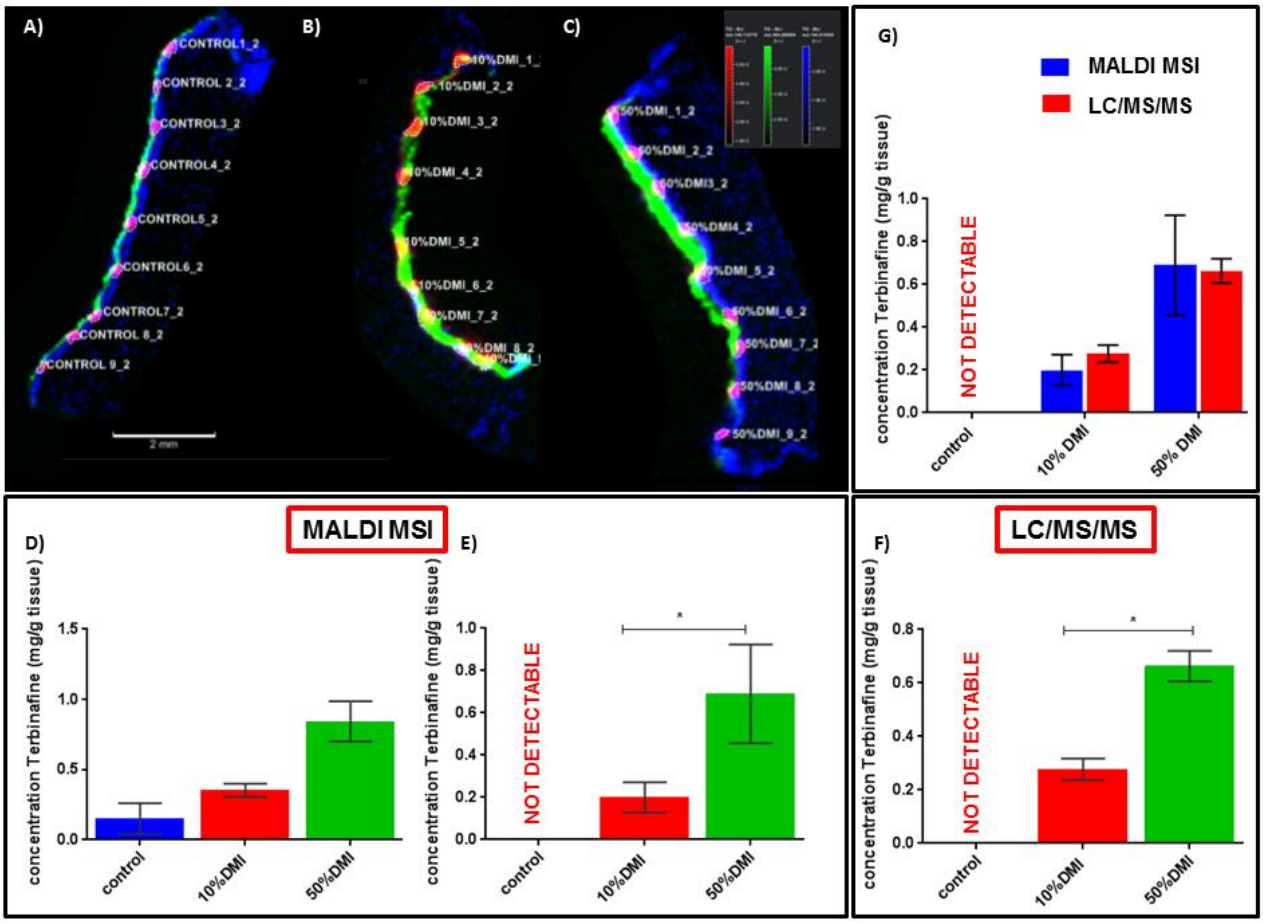

A)
1.5mgml+IS
1mgml+IS
500ugml+IS
100ugml+IS
10ugml+IS
1ugml+IS
100ngml+IS
10ngml+IS
blank+IS
2 mm
B)
epiderms
stratum corneum
2000 µm
C)
Y = 162.5*X + 263.9
R2= 0.9968
Average intensity (m/z 141)
15000
10000
5000
0
0
10
20
30
40
C
Concentration of microspotted Terbinafine (ng/mm2)
D)
Y = 0.1321*X + 0.1731
R2=0.9992
Average intensity ratio (m/z 141/148)
6
4
2
0
0
10
20
30
40
C
Concentration of microspotted Terbinafine (ng/mm2)

Figure 4


A)
B)
C)
CONTROL1_2
CONTROL 2_2
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CONTROL4_2
CONTROL5_2
CONTROL6_2
CONTROL7_2
CONTROL 8_2
CONTROL 9_2
2 mm
10%DMI_1_
10%DMI_2_2
10%DMI_3_2
10%DMI_4_2
10%DMI_5_2
10%DMI_6_2
50%DMI_1_2
50%DMI_2_2
50%DMI3_2
50%DMI4_2
50%DMI_5_2
50%DMI_6_2
50%DMI_7_2
50%DMI_8_2
50%DMI_9_2
G)
MALDI MSI
LC/MS/MS
concentration Terbinafine (mg/g tissue)
NOT DETECTABLE
control
10% DMI
50% DMI
MALDI MSI
D)
E)
LC/MS/MS
F)
*