The Complementary Role of ToF-SIMS in the Assessment of Imiquimod Permeated into the Skin from a Microemulsion Dosage Form

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Abstract:

The assessment of drug permeation into/across the skin is traditionally accomplished using Franz diffusion cells with subsequent analysis by conventional chromatographic methods such as HPLC and more recently using advanced imaging techniques. In this context, time of flight-secondary ion mass spectrometry (ToF-SIMS) offers distinctive advantages in mapping drugs within skin with high sensitivity and chemical specificity without the need for fluorescent tags or radiolabels. The work in this paper uses the combination of conventional and advanced methods to evaluate imiquimod permeation into the skin. This approach provides complementary and detailed information regarding the permeated mass, the permeation depth and the spatial distribution and localisation of drugs within skin.

Imiquimod is an immune modulator drug approved by the FDA for the treatment of superficial basal cell carcinoma (BCC) but not the nodular lesions. As other studies have reported that Aldara™ cream (imiquimod 5% w/w) has some limitations in the treatment of nodular BCC lesions due to the cream's inability to deliver imiquimod into the deeper more invasive nodular lesions, an enhancement of imiquimod permeation is thought to be useful to overcome these limitations. Therefore, an attempt to improve delivery of imiquimod into the deeper skin layers using microemulsions was investigated.

Imiquimod microemulsions were formulated and characterised in our previous work are now tested for skin permeation enhancement. However, the assessment of imiquimod permeation from the formulated microemulsions using HPLC and ToF-SIMS demonstrated a limited ability of the microemulsions to improve delivery of imiquimod over Aldara™ cream. This was attributed to the poor release of imiquimod from the microemulsion formulas due to the high affinity of imiquimod for the oil phase and the encapsulation of the oil droplets by the S/Co-S mixture. This is thought to be, the first time that ToF-SIMS has been used to assess permeation of imiquimod from a microemulsion dosage form.

Key words: Imiquimod, HPLC, Microemulsions, Skin permeation, ToF-SIMS.

الدور التكميلي لجهاز ToF-SIMS في تقييم نفاذية دواء الايميكويمود الى الجلد من المستحلب الدقيق

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الخلاصة:
أن تقييم نفاذية الدواء الى داخل أو عبر الجلد يتم بصورة تقليدية بأستخدام خلايا فرانز ومن ثم التحليل بجهاز ال HPLC وحديثا تم أستخدام تقنيات التصوير المتقدمة وفي هذا السياق يوفر جهاز الToF-SIMS فوائد مميزة في تتبع الادوية داخل الجلد بحساسية عالية ومحددة للدواء نفسه بدون الحاجة الى مواد مضيئة أو مشعة. أن العمل في هذا البحث يدمج أستخدام الطرق التقليدية والمتقدمة لتقييم نفاذية دواء الimiquimod داخل الجلد وهذا النهج يوفر معلومات تكميلية ومفصلة بشأن الكمية النافذة من الدواء والعمق والتوزيع المكاني داخل الجلد. الimiquimod هو دواء مغير للمناعة وافقت عليه أدارة الادوية والاغذية الامريكية لعلاج سرطان الخلايا القاعدية السطحي للجلد ولكن لم تتم الموافقة لعلاج سرطان الجلد العقدي وكما أفادت دراسات أخرى فأن (imiquimod 5% w/w Aldara cream) ليس له القابلية على النفاذ العميق داخل الجلد لعلاج السرطان العقدي الاكثر عمقا لذلك تمت المحاولة لزيادة النفاذية الى طبقات الجلد العميقة بأستخدام المستحلب الدقيق(Microemulsions).المستحلب الدقيق للimiquimod تم تحضيره وتمييزه في بحث سابق والان تم فحص نفاذيته داخل الجلد. أن تقييم نفاذية ال imiquimod من المستحلب الدقيق بأستخدام ال HPLC وال ToF-SIMS أظهرت القابلية المحدودة للمستحلب الدقيق على تحسين نفاذية ال imiquimod للجلد مقارنة مع ال Aldara cream ويعزى ذلك الى قلة تحرر ال imiquimod من المستحلب الدقيق بسبب الارتباط العالي بين الimiquimod والطور الدهني وتغليف دقائق الطور الدهني بال S/Co-S mixture.

الكلمات المفتاحية: ايميكويمود،HPLC،المستحلب الدقيق،نفاذية الجلد،Tof-SIMS.

Introduction

Due to the difficulties associated with *in vivo* experiments, most of the topical and transdermal drug permeation studies use *in vitro* experiments as an alternative method. *In vitro* permeation experiments can provide a platform for the initial evaluation of the permeation of topical and transdermal formulations in addition to their application in risk assessment experiments for many drugs and chemicals. This method has the advantages of a quick and simple application with the ability to perform multiple repeats without the need to enrol large numbers of subjects. *In vitro* permeation experiments are usually performed using static diffusion cells (Franz cell) with subsequent analysis by HPLC [1]. HPLC represents the conventional chromatographic method that is widely used for the determination of drug concentration permeated into or across the skin [2-5]. Although, HPLC offers a relatively quick, quantitative and versatile method for analysis, it suffers from issues in the detection and quantification of a permeant if it presents at low concentrations (low sensitivity) [1]. Additionally, in liquid chromatography the sample should be in a solution and therefore an efficient extraction step with solvents is required prior to injection into the HPLC system. This increases the complexity of the method and the time needed for the analysis. Furthermore, as the HPLC does not have any imaging capability, it is unable to identify the exact depth of permeation or provide information about the spatial distribution of a permeant within skin for the topically applied formulations.

Advanced imaging techniques with relatively high spatial resolution have been successfully used either to analyse native skin or to map the permeation and visualise the spatial distribution of a permeant within skin such as confocal laser scanning microscopy (CLSM) [6-8], Raman spectroscopy [9-11] and time of flight-secondary ion mass spectrometry (ToF-SIMS) which offers numerous advantages that can be exploited for the analysis of biological samples. However, to date there are only a small number of studies that utilise ToF-SIMS to explore drug permeation into the skin [12, 13].

ToF-SIMS is a highly sensitive surface analysis technique that is used to characterise the surface chemistry of a sample including illustrating the spatial distribution. It involves the use of a primary ion beam to bombard the surface of a sample under ultra-high vacuum conditions to sputter secondary ions which are detected and separated according to their mass. When a high energy beam of ions in the order of 25 keV bombards a surface, the particle energy is transferred to the atoms of the solid by billiard-ball-type collisional

process called 'collision cascade'. In this cascade of collisions that occurs between the atoms in the solid, some collisions return to the surface and result in the emission of secondary ions (Fig. 1). These secondary ions consist of either whole ionised molecules or fragment ions. [14].

In addition to the mass spectra obtained from ToF-SIMS analysis, the ToF-SIMS has the capability to produce ToF-SIMS ion images that illustrate the spatial distribution of different ions present in a sample by rastering (scanning) the primary ion beam onto the sample surface. ToF-SIMS instrument has the ability to map each detected ion as a function of its mass and a spatial resolution of 400 nm to 1 µm can be achieved with ToF-SIMS depending on the primary ion mode [15]. ToF-SIMS ion images represent chemical maps which not only show the distribution and localisation of an exogenous compound within a tissue but also they highlight the co-localisation of the native components of that tissue and therefore permit the depth of permeation to be estimated [12].

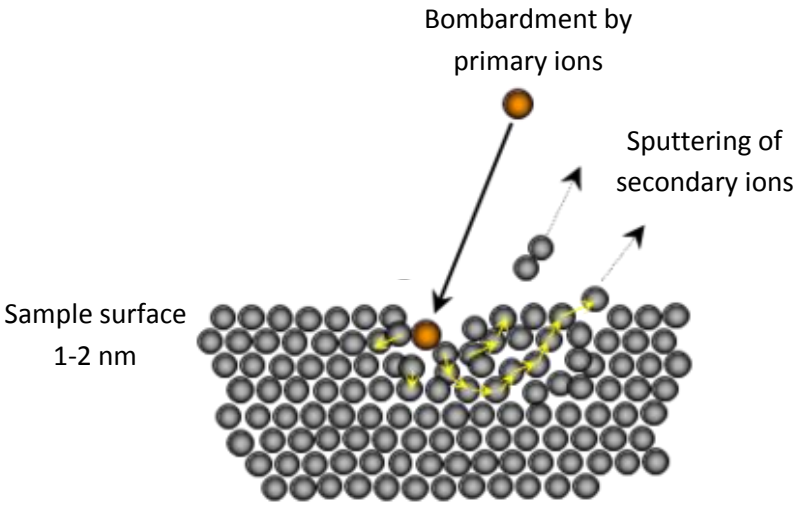

Figure 1: A schematic showing the collision cascade (yellow arrows) that occurs by bombardment of a sample surface by a primary ion beam which leads to sputtering of secondary ions during SIMS analysis. Adapted from the National Institute of Standard and Technology (NIST)

ToF-SIMS provides a high mass resolution and chemical specificity for materials analysis which is important to discriminate between the complex structures in biological tissues. In addition, it produces high spatial resolution images of 1 µm for the complex multi-layered structures with sensitivity at ng/mL levels [16-18]. Furthermore, the preparation of samples for ToF-SIMS analysis is simple and does not require any extraction process often used in chromatographic methods or the addition of fluorescent tags or radio-labels [12, 15, 19] except the removal of the excess moisture from the samples prior the analysis [20]. The successful application of ToF-SIMS to analyse biological materials such as proteins [21, 22] and lipids [23-28] from either healthy [29-31] or diseased tissues [32-34] have been demonstrated in several studies. There is presently a limited number of studies that investigate the application of ToF-SIMS to map drug permeation into the skin [12, 13].

Imiquimod is an immune response modifier drug with antiviral and antitumor activity. Its action is indirect through stimulation of the immune system to kill viruses and tumour cells. It was initially approved by the FDA in 1997 for genital and perianal warts treatment and marketed as Aldara™ cream (5% w/w imiquimod) by 3M Pharmaceuticals, USA. In 2004, the FDA approved the use of imiquimod for the treatment of actinic keratosis and

superficial basal cell carcinoma (BCC) [35]. Imiquimod was approved by the FDA for the treatment of small, primary, superficial BCC but not nodular BCC. The lesions in nodular BCC show deeper invasion within the dermis with the inability of imiquimod to reach the tumour's full depth [35].

Microemulsions are transparent, thermodynamically stable, isotropic mixtures of an oil and aqueous phase with a surfactant and co-surfactant [36-39]. Microemulsions have received considerable attention as vehicles for drug delivery because of their high solubilisation capacity, thermodynamic stability, ease of formation and optical transparency [36, 37]. They have an ability to improve the solubilisation of both lipophilic and hydrophilic drugs, which results in a high concentration gradient (thermodynamic activity) towards the skin. In addition, the microemulsion components such as the oil phase, surfactants and co-surfactants may serve as penetration enhancers to increase drug permeation [40]. Several studies have been shown the ability of microemulsions to increase transdermal drug delivery of both hydrophilic and lipophilic drugs in comparison to conventional vehicles [41-50].

The aim of this research is to show the valuable and complementary role of ToF-SIMS analysis to HPLC in the determination of imiquimod permeated into the skin when applied from a microemulsion in an attempt to improve its delivery into the deeper layers of the skin.

Materials

Imiquimod was purchased from Bioscience Life Sciences, UK. Aldara™ 5% cream, MEDA Company, Sweden was purchased from Manor pharmacy, UK. HCl, perchloric acid, triethylamine (HPLC grade) were purchased from Sigma-Aldrich, UK, Germany and USA. Acetonitrile (HPLC grade). Sodium 1-octanesulfonate was obtained from VWR International Ltd. England and Belgium. Teepol solution (Multipurpose detergent) was ordered from Scientific Laboratory Supplies, UK. D-Squame standard sampling discs (adhesive discs) were ordered from CUDERM corporation, USA. Deionised water was obtained from an ELGA reservoir, PURELAB® Ultra, ELGA, UK. All reagents were of analytical grade, unless otherwise stated.

Methods

Formulation of microemulsions

Nine microemulsion formulas were formulated and characterised according to our previous work [51]. These microemulsions containing different oil (oleic acid) percentages 1%, 3% and 5% w/w and different S/Co-S mixture (Tween 20/PG) percentages were selected from the microemulsion region in the pseudo-ternary phase diagram of Tween 20/PG at ratio of 3:1 as shown in Table 1. Imiquimod was loaded into the microemulsion formulas that showed appropriate stability. Formulas F3, F6 and F9 were loaded with 0.1%, 0.3% and 0.5% w/w of imiquimod respectively.

Table 1: Formulas of the microemulsions selected from the microemulsion region in the pseudo-ternary phase diagram of Tween 20/PG at ratio of 3:1.

Formula No.	Oil% w/w	S/Co-S% w/w	Water% w/w
1	1	10	89
2	1	12	87
3	1	14	85
4	3	28	69
5	3	30	67
6	3	32	65
7	5	43	52
8	5	45	50
9	5	47	48

Microemulsions permeation study

All *in vitro* permeation experiments were carried out using the following regulatory protocols as a guide:

OECD Guideline for the Testing of Chemicals, Skin Absorption: *in vitro* Method, No. 428 (2004), [52].

OECD Guidance Document for the Conduct of Skin Absorption Studies, No. 28 (2004), [53].

These guidelines are accepted as a reliable standard for conducting *in vitro* permeation experiments.

The three microemulsion formulas F3, F6 and F9 loaded with 0.1%, 0.3% and 0.5% w/w of imiquimod respectively were used in the permeation study.

Skin preparation

Skin samples were prepared from the dorsal side of pig ears of six-month-old obtained from a local abattoir prior any steam cleaning process. The skin was washed with distilled water and dried using tissue. Hair was carefully cut by scissors to avoid any damage to the *stratum corneum* and the subcutaneous fatty layer was removed using a scalpel. After that, the skin samples were wrapped in aluminium foil and stored at -20 °C. Skin samples were used within six weeks of being frozen.

Assembly of diffusion cells

Franz diffusion cells with an exposed surface area of 0.64 cm^2 were used for the permeation study. Skin samples were mounted on Franz cells with the *stratum corneum* facing upwards. The receptor chamber was filled with 10 mL of 0.1N HCl used as receptor fluid to keep sink conditions because of the high solubility of imiquimod (basic compound) in this acidic medium 9.5 mg/mL (tested experimentally). Franz cells were then placed in a stirring water bath (Cleaver Scientific Ltd., UK) at 37 °C for 30 minutes to equilibrate before applying the formulations. The experiment run time was 24 hours and the receptor fluid were stirred continuously by a small Teflon-coated magnetic stirrer bar at 600 rpm. The microemulsion formulas F3, F6 and F9 were tested in comparison to Aldara™ cream in the same run using the same piece of skin (n = 6). Microemulsions were dosed on the weight basis of 1000 mg. In addition, F9 microemulsion formula was tested with an additional 6 Franz cells for ToF-SIMS analysis.

Measurement of mass balance

When the Franz cell experiments were completed (after 24 hours), the excess formulation was removed from the surface of the skin by careful application of a combination of very soft dry and moistened sponges with 3% v/v Teepol® detergent solution. The sponges were combined and analysed for imiquimod by HPLC as a total skin wash. In addition, any formulation on the donor chamber inner surface was also removed by the sponges and analysed for imiquimod by HPLC as a donor chamber wash.

Tape stripping

After removing the excess formulation from the skin surface, the skin was dismantled from the Franz cell assembly and left to air dry at ambient temperature for approximately 2 hours. Following this, a tape stripping technique was employed using adhesive tapes (D-Squame, Standard Sampling Discs, USA) with a diameter of 22 mm. The adhesive tapes were applied and removed successively from the same treated skin area for up to 20 strips with the aid of a roller to press the adhesive tape 10 times onto the skin surface to stretch it to avoid the effects of furrows and wrinkles on the tape stripping procedure [54]. Adhesive tapes were removed from the skin surface by tweezers in one swift motion [54] which were then placed in Eppendorf vials and stored at -20 °C until required for analysis. Tape strips obtained from 6 Franz cells were analysed by HPLC in which the tape strips from each cell were pooled together for analysis, the remaining skin after tape stripping (i.e. stripped skin without *stratum corneum* layer) was also reserved for HPLC analysis. In addition, tape strips from 3 Franz cells were analysed individually by ToF-SIMS in which the tape strips were attached to glass slides with a double-sided adhesive tape where the removed *stratum corneum* facing upwards. Following this, the slides were placed in plastic petri dishes and liquid nitrogen was carefully poured into the petri dish to freeze tape strips on the slides, then the frozen slides were kept in a freeze dryer for 1 hour prior to ToF-SIMS analysis.

OCT embedding and skin cryo-sectioning

Skin samples removed from 3 Franz cells were placed in a plastic block containing optimum cutting temperature (OCT) gel (VWR International Ltd., Belgium) which is an inert mounting medium for cryotomy that solidifies upon rapid cooling. Therefore, the plastic block containing skin immersed in OCT was placed in a beaker of isopentane pre-cooled with liquid nitrogen to solidify. After solidification, the OCT blocks were wrapped in aluminum foil, placed in an airtight plastic bag and stored at -80 °C. Cryo-sectioning of skin samples were carried out by placing the OCT block in a cryostat chamber (Thermo Cryotome™, UK) at a temperature of -20 °C. The block was allowed to equilibrate within the cryostat chamber for 30 minutes and then sectioned using a steel blade into vertical cross sections of 20 µm thickness. Following this, the cryo-sections were mounted onto clean polysine microscope adhesion slides (ThermoFisher Scientific) and freeze dried for 1 hour prior to ToF-SIMS analysis.

HPLC analysis

HPLC analysis was carried out using an Agilent 1100 series instrument (Agilent Technologies, Germany) equipped with degasser, quaternary pump, column thermostat, autosampler and UV detector. System control and data acquisition were performed using Chemostation software. The details of the HPLC chromatographic conditions are as follows: column C_{18} (150 × 4.6 mm) ACE3/ACE-HPLC Hichrom Limited, UK. Mobile phase of buffer:acetonitrile (70:30 v/v), the buffer is of 0.005 M sodium 1-octanesulfonate in water containing 0.1% triethylamine adjusted with dilute perchloric acid to pH of 2.2, flow rate of 0.8 mL/minute, UV detection at λ max. 226 nm, injection

volume of 10 µL and column temperature at 25 °C [55].

Determination of the percentages of imiquimod recovered by HPLC

The amount of imiquimod from the different Franz cell elements (skin wash, donor chamber wash, pooled tape strips and remaining skin after tape stripping) was extracted by the addition of 20, 10, 5 and 3 mL of the extraction solvent MeOH mixture (MeOH 90%: Water 9%:0.1N HCl 1%) respectively. They were then vortexed for 2 minutes and left overnight. Following this, they were sonicated for 30 minutes, filtered through a 0.45 µm syringe filter and injected into the HPLC system. Receptor fluid samples were filtered through a 0.22 µm centrifuge tube filter and injected directly into the HPLC system without any dilution.

ToF-SIMS analysis

ToF-SIMS analysis was performed using a ToF-SIMS IV instrument (IONTOF, GmbH) with a Bi_3^+ cluster source. A primary ion energy of 25 keV was used, the primary ion dose was preserved below 1×10^{12} per cm^2 to ensure static conditions. The resolution was 100 pixels/mm and charge compensation were applied in the form of a low energy (~ 20 eV) electron flood gun. Data was acquired and analysed using SurfaceLab6 (IONTOF GmbH) instrument software. All peak intensities were normalised to the total ion count of the spectra.

ToF-SIMS was used to analyse individual tape strips and cryo-sectioned skin samples obtained from Franz cell testing. The tape strips and cryo-sectioned skin samples were placed in a freeze dryer for 1 hour prior to ToF-SIMS analysis to remove any moisture present in the samples. An ion representing biological material and therefore indicative of skin (skin marker) was identified as CH_4N^+ and was used to threshold the data sets. After that, the data was reconstructed to remove the data from the adhesive tape material found between the fissures in the stripped skin (removing the substrate data) and therefore the data was only analysed from the skin material.

Statistical analysis of the data

The statistical tests used to compare ion intensities were one-way ANOVA with Tukey's multiple comparisons test or t-test. All data is presented as the mean ± SD of ($n = 4$ or 6) with P values of ≤ 0.05 being regarded as significant using GraphPad Prism 7 software (USA). Prior to statistical analysis all data was tested for normality using Shapiro-wilk normality test.

Results and discussion

Microemulsions permeation study

Determination of the percentage of imiquimod recovered from microemulsions permeation study by HPLC

Imiquimod loaded microemulsion formulas F3, F6 and F9 which showed appropriate stability were used to assess the permeation of imiquimod from microemulsions into porcine skin in comparison with Aldara™ cream. The mean recovery percentage of imiquimod from the different Franz cell elements (donor solution, donor chamber wash, skin wash, tape strips, remaining skin and receptor fluid) of the permeation study of the microemulsions and Aldara™ cream are reported in Table 2. It is apparent from the data presented in Table 2, that the recovery percentage of imiquimod from microemulsion formulas is highest in the donor solution (the dosed microemulsion solution into the donor chamber recovered following completion of Franz cell run) as compared to other elements. In addition, very minor percentages are recovered from the tape strips, remaining skin and receptor fluid which are even less than the recovery percentages obtained from Aldara™ cream. This indicates the very limited permeation of imiquimod from these microemulsion formulas and suggests the inability of these microemulsion formulas to deliver more imiquimod into the skin compared to

Aldara™ cream although they are formulated at a high thermodynamic activity of imiquimod and contain multiple ingredients regarded as penetration enhancers such as oleic acid, Tween 20 and propylene glycol.

An assessment of the mean recovery percentage of imiquimod in the remaining skin element from the different microemulsion formulas shows that the recovery percentage from the microemulsion formulas F3, F6 and F9 is significantly less than that of Aldara™ cream as illustrated in Figure 2. Moreover, the comparison among the different microemulsion formulas demonstrates that the recovery percentage of imiquimod from F9 formula (0.33% ± 0.08) is higher than F3 (0.15% ± 0.08) and F6 (0.18 ± 0.07) formulas. F6 formula, in turn yields a slightly higher recovery percentage than F3 formula. This can be attributed to the increase in oleic acid and Tween 20/PG mixture content from F3 to F9 formulas. However, these differences in the recovery percentage among the different microemulsion formulas are found to be non-significant as shown in Figure 2. It is apparent from the HPLC results, that microemulsion formulas F3, F6 and F9 do not have the capability to improve the delivery of imiquimod into the skin over Aldara™ cream.

Table 2: Mean recovery percentage of imiquimod from the different Franz cell elements of the permeation study of microemulsions in comparison with Aldara™ cream when analysed by HPLC. Data is presented as the mean % ± SD (n = 6). The donor solution represents the dosed microemulsion solution into the donor chamber recovered following completion of Franz cell run.

Analysed Element	F3 microemulsion Formula	F6 microemulsion Formula	F9 microemulsion Formula	Aldara™ Cream
Donor solution	84.10 ± 3.42	88.17 ± 6.40	81.73 ± 5.40	
Donor wash	1.22 ± 0.53	2.76 ± 0.65	3.82 ± 0.90	6.41 ± 2.01
Skin wash	6.19 ± 0.58	4.27 ± 0.53	9.82 ± 0.30	87.48 ± 8.64
Tape strips	0.10 ± 0.07	0.08 ± 0.05	0.11 ± 0.09	0.95 ± 0.56
Remaining skin	**0.15 ± 0.08**	**0.18 ± 0.07**	**0.33 ± 0.08**	**0.99 ± 0.18**
Receptor fluid	0.14 ± 0.05	0.17 ± 0.04	0.28 ± 0.03	0.47 ± 0.29

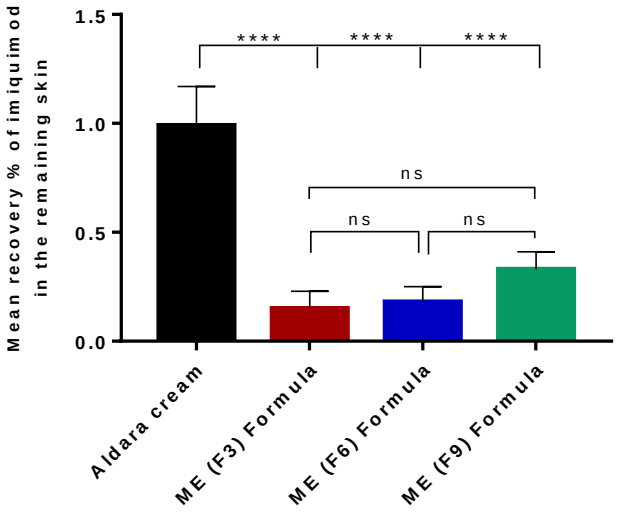

Figure 2: Mean recovery percentage of imiquimod in the remaining skin obtained from the permeation study of microemulsion formulas F3, F6 and F9 in comparison with Aldara™ cream when analysed by HPLC. Data is presented as the mean ± SD (n = 6).

ToF-SIMS analysis of microemulsion tape strips

The ToF-SIMS spectra that highlight the $[M+H]^+$ of imiquimod $C_{14}H_{17}N_4^+$ (at m/z = 241) in tape strips 2 and 10 obtained from the permeation study of F9 microemulsion formula are shown in Figure 3. The $[M+H]^+$ of imiquimod is detected with a moderate intensity at TS 2 but with a very low ion intensity at TS 10. In addition, an examination of the ToF-SIMS ion image of the $[M+H]^+$ of imiquimod in TS 10 (Fig. 4c) which represents a reflection of the imiquimod spectra shows the presence of imiquimod at a very low intensity. These results indicate the very limited permeation of imiquimod into the *stratum corneum* from the F9 microemulsion formula which are in close agreement with the HPLC results.

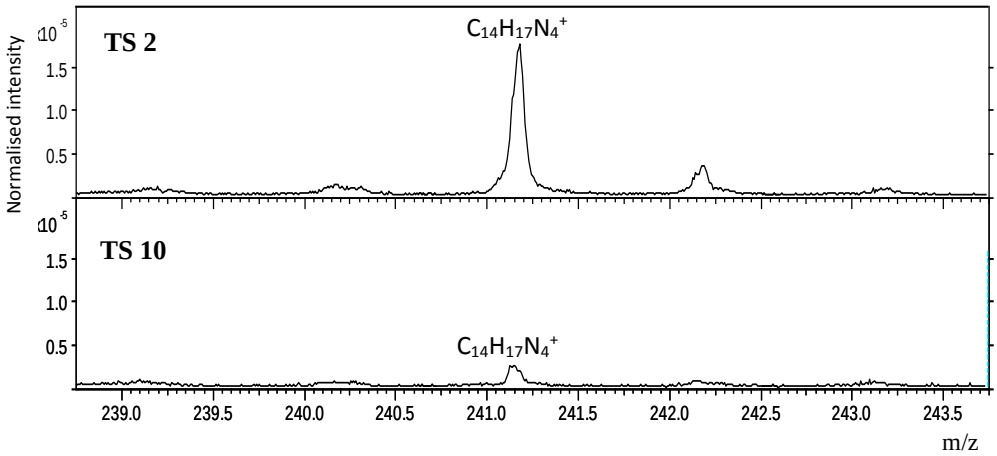

Figure 3: Stacked ToF-SIMS spectra of the $[M+H]^+$ of imiquimod in tape strips 2 and 10 of F9 microemulsion formula, showing the peak of the $[M+H]^+$ of imiquimod ($C_{14}H_{17}N_4^+$) at m/z = 241.

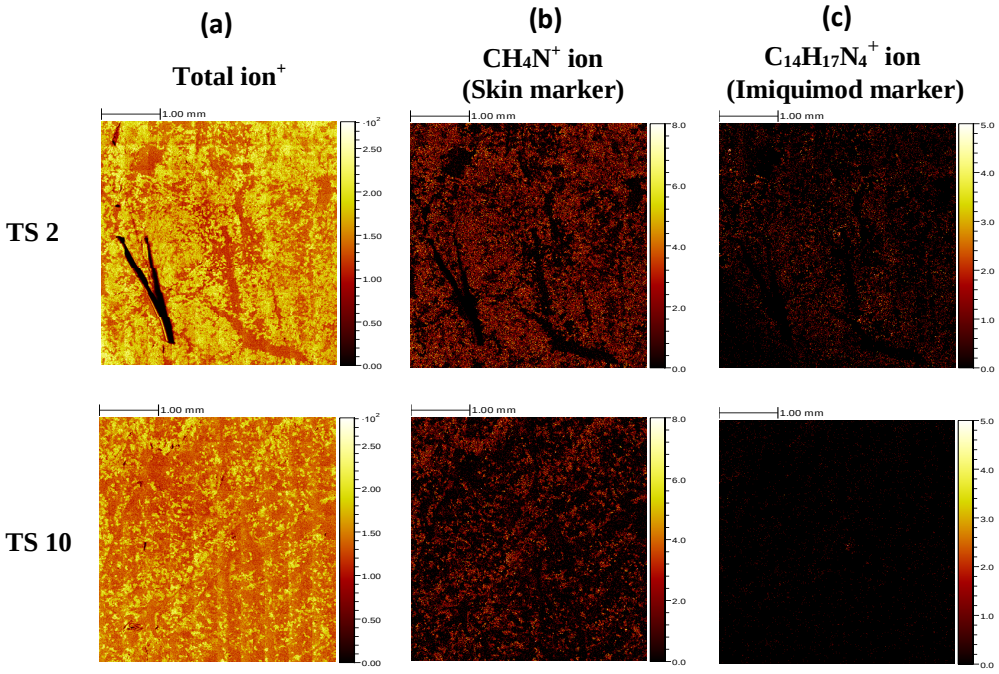

Figure 4: ToF-SIMS ion images of tape strips 2 and 10 of F9 microemulsion formula, showing: (a) the total ion$^+$, (b) the skin marker (CH_4N^+) and (c) the imiquimod marker ($C_{14}H_{17}N_4^+$).

ToF-SIMS analysis of microemulsion cryo-sectioned samples

The ToF-SIMS spectrum of the cryo-sectioned skin samples of F9 microemulsion formula is shown in Figure 5b, in which the normalised ion intensity of the $[M+H]^+$ of imiquimod is detected with a very low intensity close to the ion intensity of the skin control sample (Fig. 5a). This very low ion intensity is also observed in ToF-SIMS ion image of the $[M+H]^+$ of imiquimod (Fig. 6c) which illustrates the absence of imiquimod from the cross-sectioned samples.

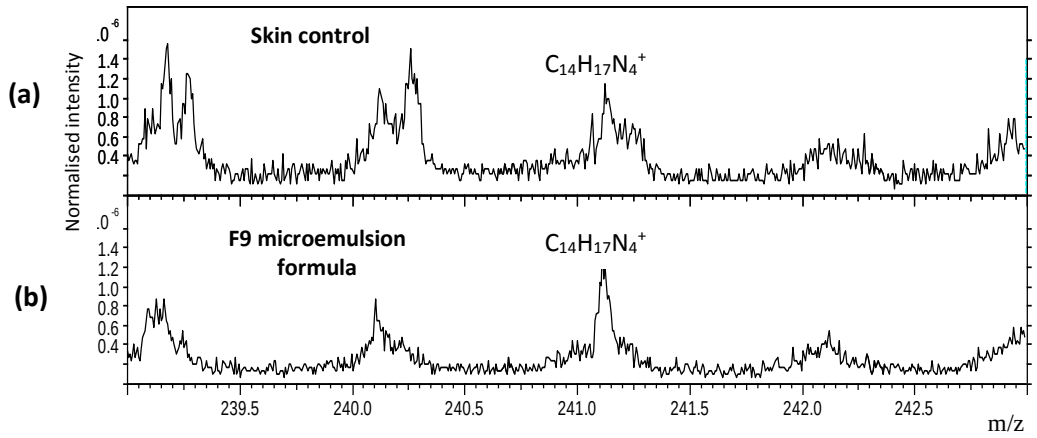

Figure 5: stacked ToF-SIMS spectra of the cryo-sectioned samples for (a) skin control and (b) F9 microemulsion formula, showing the peak of the $[M+H]^+$ of imiquimod ($C_{14}H_{17}N_4^+$) at m/z = 241.

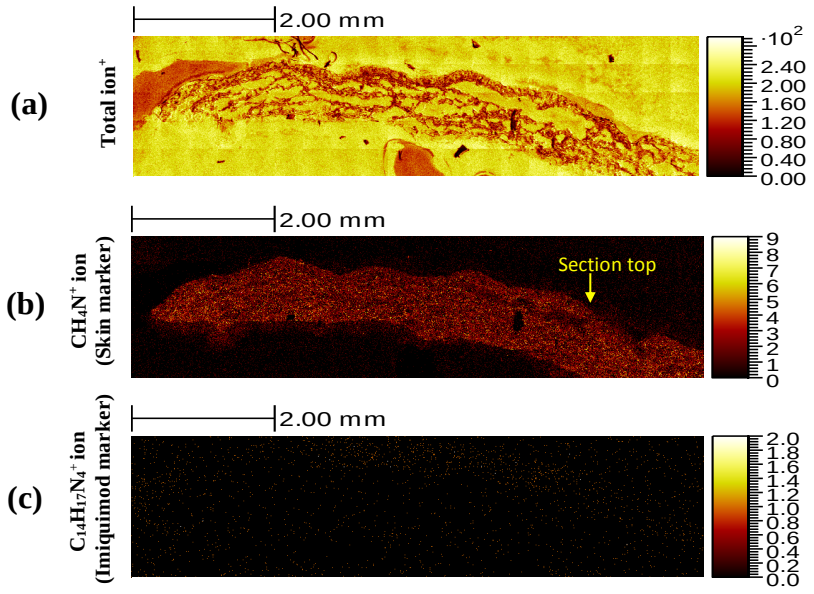

Figure 6: ToF-SIMS ion images of the F9 microemulsion formula cryo-sectioned samples showing (a) the total ion$^+$, (b) the skin marker (CH_4N^+) and (c) the imiquimod marker ($C_{14}H_{17}N_4^+$).

The HPLC and ToF-SIMS results confirm the very limited permeation of imiquimod from microemulsions and this suggest that there is an issue with the release of imiquimod from the microemulsion formula.

The drug release from the formulation should be at an optimum rate and encourage partitioning into the skin. This can be achieved by using a solvent in which the drug is only moderately soluble (the vehicle should allow some solubility of the drug but should not retain the drug) [56]. In the case of imiquimod's microemulsions, imiquimod is a lipophilic drug with a partition coefficient of 2.7 [57] which showed the highest solubility of 170 mg/mL in oleic acid. Therefore, it is proposed that the high solubility of imiquimod in oleic acid significantly decreases the release of imiquimod from the microemulsion formulations (i.e. oleic acid retains imiquimod and reduces its partition out to the skin). It was reported that the high affinity of a drug to its vehicle can inhibit skin permeation due to the slow release and transfer of the drug from the vehicle to the skin [58].

Although, the high solubility of imiquimod in oleic acid can reduce the release and skin permeation of imiquimod from the microemulsion formulas, it is proposed that this is not the only factor which influences the poor release of imiquimod from the microemulsions. it is thought that the microstructure of the microemulsion and more specifically the presence of S/Co-S mixture surrounding or encapsulating oleic acid droplets may additionally impart a negative influence on the release of imiquimod from the microemulsions (i.e. make it more difficult for imiquimod to be released from the microemulsions). A drug may be held within microemulsion droplets leading to a reduction in its permeation into the skin [49]. Chen *et al*. studied the skin permeation of triptolide microemulsions using oleic acid as an oil phase, they found that the encapsulation of oleic acid by the S/Co-S mixture (Tween 80/PG) reduced the penetration of oleic acid into the *stratum corneum* [59]. In another study conducted by Paolino *et al*. they observed that there was no significant enhancing effect obtained by the addition of oleic acid to ketoprofen lecithin microemulsions due to the strong

binding of oleic acid to the microemulsion structure [60].

It is possible that the high affinity of imiquimod for oleic acid and the encapsulation of oleic acid droplets by S/Co-S mixture results in retaining imiquimod within oleic acid droplets with a reduced ability to partition out that leads to the poor release and transfer of imiquimod from microemulsions to the skin. An optimisation of the microemulsions formulation is required as future work, perhaps focusing on areas such as changing the type of oil used to one in which imiquimod is just only moderately soluble to facilitate its release to the skin.

Conclusions

The assessment of drug permeation into/across the skin is traditionally performed using Franz diffusion cells with subsequent analysis of the different Franz cell elements by HPLC. Although, HPLC represents a quick, quantitative and versatile analysis method, it suffers from issues of low sensitivity and the requirement of an efficient sample extraction step with solvents prior to injection into the HPLC system. This increases the complexity of the method and the time needed for the analysis. Additionally, as the HPLC does not have any imaging capability, it is unable to identify the exact depth of permeation or provide information about the spatial distribution of a permeant within skin for the topically applied formulations. ToF-SIMS offers numerous advantages of high sensitivity and chemical specificity that can be exploited for the analysis of biological samples without the need for fluorescent tags or radiolabels. This work shows the valuable complementary role of ToF-SIMS technique in the analysis and imaging of imiquimod permeation into the skin. ToF-SIMS analysis has confirmed the HPLC results in that imiquimod has a limited permeation into the skin from the prepared microemulsions.

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