The use of heat and chemical penetration enhancers to increase the follicular delivery of erythromycin to the skin

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Abstract

The effect of heat on the follicular absorption of drugs into the skin has not previously been investigated. In comparison to drug delivery across the continuous stratum corneum (SC), follicular absorption is known to be relatively rapid and therefore the use of short durations of heat may be particularly useful for enhancing drug delivery to the hair follicles, as well as being practical for patients to use. In this study erythromycin has been used as a model drug and the combined use of heat and chemical penetration enhancers was found to be able to synergistically increase the penetration of erythromycin into human skin via the follicular route. Moreover durations of heat application as short as 10 minutes in combination with particular enhancer systems were found to be sufficient to significantly increase erythromycin delivery to the skin. Overall the data indicate that the use of heat with chemical penetration enhancers offers a potentially valuable strategy for delivering drugs via the follicular route.

Introduction

Delivering drugs into the hair follicles and their associated pilosebaceous units is highly desirable, including for example in the treatment of conditions such as acne and alopecia. The pilosebaceous units are filled with sebum, which is secreted by the sebaceous glands into the hair duct and then excreted to the surface of the skin [1]. In addition to protecting the body from microbial infection and acting as a natural emollient, sebum is believed to have the ability to interact with topically applied substances on the skin surface and in the upper part of the follicular duct, thus potentially influencing transport of drugs across these appendages of the skin [2, 3]. Focusing drug delivery to the hair follicles for the treatment of conditions such as acne should not only reduce the need for systemic administration of drugs with their associated systemic side effects but may also help to reduce side effects such as skin irritation. The effect of heat on improving drug delivery in general across the skin is well established [4, 5], however its ability to enhance drug delivery to the hair follicles is as yet untested. Previous literature has reported that the viscosity of human sebum is reduced by approximately two-fold, as the temperature of the tissue is increased from 26.5 to 38 °C [6], suggesting that as a penetration enhancement strategy, heat may have particular potential to increase follicular drug delivery by rendering the sebum more permeable to the transport of drugs. Moreover given the relatively rapid delivery of drugs across the hair follicles in comparison to the continuous SC [7], short durations of heat application may enhance drug delivery to the hair follicles to a greater extent than the continuous SC, preferentially directing drug delivery to these locations in the skin. A number of chemical penetration enhancers have been shown to interact with sebum raising the possibility of using these two penetration enhancement strategies, heat and chemical penetration enhancers together, to significantly increase follicular drug delivery [8]. To test this hypothesis erythromycin, a topical antibiotic used in acne treatment was selected for use in this study as a model drug along with a range of chemical penetration enhancers to investigate

whether skin absorption and in particular follicular drug transport could be improved using a combined approach with heat.

Materials and Methods

Materials

Erythromycin (Ph. Eur. grade) was obtained from Sigma-Aldrich (Gillingham, UK). Methanol (99.9%), acetonitrile (99.9%), absolute EtOH (99.5%), phosphoric acid (≥ 85%), formic acid (98-100%), (all HPLC grade), sodium hydroxide pellets (≥ 97%), hydrochloric acid (HCl) ACS reagent, acetic acid (≥ 99.7%), sodium dihydrogen phosphate (99%), phosphate buffer solution (PBS) tablets, hexane (≥ 97%) and IPM (96%) and ammonium formate (99%) were supplied by Fisher Scientific (Loughborough, UK). ^{14}C-erythromycin (0.1 mCi/mL, ethanolic solution, 99% purity) was sourced from American Radiolabeled Chemicals (St. Louis, USA). IPM (96%) and scintillation cocktail, ScintiSafe 3, were supplied by Fisher Scientific (Loughborough, UK). Transcutol (TC, 99.9%) and propylene glycol dipelargonate (DPPG, 96%) were supplied by Gattefossé. Diisopropyl adipate (DPA, 99%) was obtained from Croda. The 2-octyl cyanoacrylate glue was sourced from Adhezion Biomedical (Wyomissing, USA). Soluene® 350 tissue solubiliser was obtained from PerkinElmer (Boston, USA).

High Performance Liquid Chromatography (HPLC)

Quantitative analysis of erythromycin for saturated solubility experiments and infinite dose Franz cell studies was performed using HPLC with an Agilent 1260 infinity system and a Phenomenex, Gemini™ C-18, 150 mm x 4.6 mm, 5 µm particle size. An isocratic mobile phase of 60: 40 phosphate buffer (20 mM PBS, adjusted to pH 7.5, using sodium hydroxide solution 1M): acetonitrile was used. The UV detection wavelength, flow rate and injection volume were 205 nm 1.2 ml/min and 100 µL, respectively. The retention time of erythromycin under these

conditions was approximately 13 min. The HPLC methods were validated for linearity, precision and accuracy according to the ICH guidelines [9]. The calibration curve produced was linear over the concentration range 1–100 µg/ml, with a coefficient of determination (r^2) of 0.99978. The limits of detection (LOD) and quantification (LOQ) were 2.1 and 6.4 µg/mL respectively. Intra- and inter-day precision (% RSD) for three standards representative of high, medium and low drug concentrations ranged from 0.5 to 0.9% and 0.4 to 1.4% respectively. Accuracy of the same three concentrations ranged between 98.7 and 100.1%.

Liquid Scintillation Counting

Quantitative analysis of erythromycin for cyanoacrylate biopsy and finite dose Franz cell studies was performed using radioactivity. Samples containing the radiolabelled drug (^{14}C-erythromycin) were analysed in a liquid scintillation counter (Beckman LS 6500), after addition of 4 mL of scintillation cocktail. The limit of quantification (LOQ; 39 disintegration per minute; dpm) of the method was calculated as the mean concentration of the blank (n=7) plus 10 times the standard deviation of the blank (4 mL of scintillation fluid) [9].

Erythromycin saturated solubility and stability determination

The saturated solubility of erythromycin in a range of solvents including chemical penetration enhancers was determined using HPLC. An excess of erythromycin was added to vials containing the relevant solvent and the samples were stirred for 24 h at room temperature (21 ± 2°C) or the in a water bath at the temperature of interest, following the formation of a drug suspension. For aqueous systems the pH of the solutions was checked pre and post-saturation. Prior to the HPLC analysis, each sample was filtered using 0.2 µm PTFE filters (Fisher Scientific, UK) and following this the filtrate was diluted with mobile phase.

In vitro drug penetration into human skin

Human skin preparation

Human scrotal skin was obtained from white European male donors with ethics approval granted by the National Research Ethics Service (NRES) Committee London, Camberwell St Giles (10/H0807/51). Subcutaneous fat was carefully removed from the skin samples using forceps and a scalpel within Class 2 laminar flow cabinet. The skin was stored in a freezer at -20 °C until it was needed for experimental work.

Preparation of test formulations for infinite dose Franz cell studies

A series of saturated solutions of erythromycin were prepared by adding an excess of drug (determined on the basis of the solubility data) to each of the following vehicles: IPM, DPPG, DPA, EtOH, TC, PBS and TC: DPPG (50:50). The suspensions were then allowed to stir for 24 h at the temperature of the membrane it was applied to (32 and 45 °C), prior to dosing the Franz cells.

Preparation of test formulations for cyanoacrylate biopsy and finite dose Franz cell studies

To prepare the formulations spiked with radiolabelled drug, ^{14}C-erythromycin in EtOH (0.1 mCi/mL) was transferred to a glass vial and the solvent evaporated at ambient temperature; the test formulations were then individually added to the deposited crystals. After stirring at room temperature, the homogeneity of each formulation was checked by transferring aliquots corresponding to either infinite (100 µl/cm^2) or finite doses (10 µl/cm^2) applied in the *in vitro* studies, from the top, middle and bottom of the formulation, into individual scintillation vials. The radioactivity of each sample was quantified using a liquid scintillation counter to confirm whether the homogeneity was within ±10% of the target radioactivity.

Erythromycin penetration studies: epidermal, dermal and receiver fluid drug recovery

Sections of full thickness skin were mounted, SC side up, in individually calibrated unjacketed upright Franz type diffusion cells with an average receiver volume of 2.7 mL and an average surface area of 0.6 cm^2. Skin sections from different skin donors were allocated to experiments in a manner that ensured equivalent use of the different donors across the experiments. The receptor compartments were then filled with PBS (pH 7.4) and the cells placed on a stirring plate placed in a waterbath (Grant Instruments, UK). The receptor fluid was continuously stirred using a small magnetic bar, during the whole duration of the experiment. After allowing the skin to equilibrate with the receiver fluid for 0.5 h, 1 mL of an erythromycin suspension was introduced into the donor chamber. Franz cells (n=6 for each formulation) were occluded to prevent solvent evaporation and immersed in waterbaths controlled at 37 and 50 °C so that the skin temperatures obtained were 32 and 45 °C respectively. These temperatures were monitored throughout the duration of the study using a digital thermometer, with a type K probe (Fisher Scientific, UK). At 24 h, the receiver fluid was withdrawn and transferred to a glass vial, prior to HPLC analysis. In addition to running the Franz cell experiments for 24 hours additional experiments were conducted for a shorter duration (1 hour).

At the end of the experiments, the cells were dismantled and the skin was secured on the bench. Any residual formulation was then removed from the surface of the skin by wiping it with alternate wet (with mobile phase) and dry cotton buds a total of 4 times. The skin was wrapped in aluminium foil and placed in deionised water at 60 °C, for one minute in order to heat-separate the epidermis from the dermis [5, 10]. The epidermis and the remaining dermis were transferred to individual glass vials to which 1 mL of the extraction media (40: 60 acetonitrile: PBS, pH 7.5) was added. These samples were left in a sonicator for 15 minutes and then transferred to a roller mixer overnight. The extraction fluid left in contact with epidermis and dermis was assessed using the HPLC method developed for the quantification of erythromycin.

Prior to HPLC analysis, the samples were filtered using 0.22 µm PTFE (polytetrafluoroethylene) syringe filters.

Effect of different times of heat application on drug delivery

Separate Franz cells diffusion studies were carried out for 1 hour using the method described above, to test the effect of different heating exposure times on follicular transport. The cells were initially placed in a waterbath (Grant Instruments, UK) set at 37 °C and allowed to equilibrate for 0.5 h, in the presence of receiver fluid (PBS, pH 7.4). The temperature of the skin was then increased up to 45 °C by placing the Franz cells in a different waterbath that was set at 50 °C, prior to dosing the donor chamber of each cell with 1 mL of saturated solution of erythromycin in DPPG. The Franz cells (n = 6, for each heating treatment) were then occluded and left in the waterbath set at 50 °C for 5, 10 and 20 minutes respectively. At completion of each individual heating treatment, the cells were moved to a second waterbath (Grant Instruments, UK) previously set at temperature of 37 °C. After the allotted time (1 h), the receiver fluid was taken (and analysed by HPLC) and the cells were dismantled to remove the skin; the amount of drug penetrated into epidermis and dermis was then assessed, as described above.

Tape stripping studies with cyanoacrylate biopsy of the hair follicles

Tape stripping experiments were performed using skin mounted in Franz-type diffusion cells as previously described. The donor chamber of each cell was dosed with 100 µl/cm^2 of a saturated solution of erythromycin, previously spiked with radiolabelled drug. This dose is the minimum required for infinite dose testing conditions; as recommended in the Organisation for Economic Co-operation and Development (OECD) guidelines for skin absorption *in vitro* tests [11]. Franz cells (n = 6) were then occluded and left in the waterbath, for a total duration of 1 h. The amount of drug penetrated into and through the skin was measured when the temperature

of the membrane was maintained at 45 °C for 10 minutes (and 50 minutes at 32 °C) compared to when no additional heat was applied (32 °C for 1 hour).

A multi-step recovery process was utilised to analyse the delivery of erythromycin in different skin layers and in the appendages. At the end of the diffusion experiment, the receiver fluid was taken and then transferred to scintillation vials. The Franz cells were dismantled and the skin was secured to the bench. The donor and receiver chambers were each wiped down using alternate wet (with receiver fluid) and dry cotton buds a total of 4 times. The cotton buds used to recover the drug from receiver and donor chamber were then pooled and collected into separate scintillation vials. Next the skin was wiped with dry cotton buds and two tape strips (Scotch® Tape strips, 3M Center, USA) were taken [12] to recover unabsorbed formulation left on the surface (both cotton buds and tape strips were pooled and added to the same scintillation vial). The 'differential tape stripping' approach that was used in the present work to recover the drug from the appendages involved first removing the SC by tape stripping (10 tape strips). Following this, a drop of 2-octyl cyanoacrylate glue (Adhezion Biomedical, USA) was applied on the viable epidermis, allowed to spread and then the bottom of a glass vial (Fisher Scientific, Loughborough, UK) was pressed against the surface of the viable for either 0.5, 2 or 5 minutes; within this time the glue polymerizes and adheres to the SC. The glass vial was gently lifted and peeled from the skin [13] and then directly transferred to a scintillation vial. Comparative tape stripping experiments were also performed using the same procedure as described above with the exception that the cyanoacrylate glue was not used. To each of the scintillation vials containing samples for analysis, 4 mL of scintillation fluid was added. The viable epidermis and dermis from each of the skin samples were separated mechanically using tweezers and then digested in presence of 4 mL of Soluene® 350, at 50°C for 48 h. Aliquots of these samples were transferred into individual scintillation vials prior to analysis. With the

cyanoacrylate biopsy procedures, the total recovery of the erythromycin was always within the limits, 100 ± 10%, as recommended by the OECD guidelines [11].

Finite dose experiments

For *in vitro* finite dose experiments, Franz cells should be dosed with a small amount of formulation that mimics as closely as possible the *in vivo* application of topical products. In the case of topical solutions, this has been recommended to be 10 µL/cm^2 [11]. The volume of each test formulation, previously spiked with the radiolabelled drug, was applied on the full thickness skin sections placed between the donor and receiver chamber of each Franz cell (n = 6). The cells were left in the waterbath un-occluded for 1 h; the absorption of the erythromycin from each formulation was tested with and without the application of external heat (skin temperature maintained at 45 °C for 10 minutes).

At completion of the experiments at 1h, the multi-step recovery procedure described in the previous section with the exception that the cyanoacrylate biopsy was not performed, was used to assess the recovery of the drug from the SC, epidermis, dermis and receiver fluid.

Data treatment and statistical analysis

Statistical analysis of all the data was performed using Graphpad Prism software (version 7.0 for Windows, La Jolla, USA). The *in vitro* skin permeation and penetration data was initially analysed for normality using the Shapiro-Wilk test to determine if the data was either parametric or non-parametric, where the determined p value was either $p > 0.05$ or $p \leq 0.05$, respectively. The initial screening of the effect of temperature and the full range of penetration enhancers was performed using a two way ANOVA. Subsequent statistical comparisons were then performed using a one way ANOVA for parametric data or Kruskal-Wallis test for non-parametric data. Post hoc comparisons were made with either Fishers LSD test or Dunn's for parametric and non-parametric data respectively. Similarily pair wise comparisons were made

either a t test or Mann Whitney U test as appropriate. Statistically significant differences were assumed at the 95% confidence level, i.e., when $p < 0.05$.

Enhancement ratios were determined as:

$$ER = \frac{Q(E)}{Q(C)}$$

Equation 1

where Q (E) and Q (C) are the amount of the drug permeated into and across the skin when using enhancement strategies (i.e. heat, chemical penetration enhancers and heat with chemical penetration enhancers) and control (no heat or chemical penetration enhancers) respectively.

Results

Saturated solubility of erythromycin

The solubility values of erythromycin in various chemical penetration enhancers, in addition to PBS pH 7.4 are reported in Table 1. Erythromycin showed high solubility in EtOH and Transcutol, intermediate solubility in DPPG, DPA and IPM and relatively lower solubility in PBS.

Erythromycin in aqueous solution is most stable at pH around 7.0-7.5 [14]. As such PBS, in which the drug exhibits a solubility of 7.85 mg/mL at room temperature (Table 6-1) was used as a control formulation (no heat or chemical penetration enhancers) and as receiver fluid in the in vitro studies. Given its pKa of 8.9, erythromycin is expected to be almost fully ionised (96%) in the control formulation. The solubility of erythromycin in the receiver fluid, (PBS, pH 7.4) was also tested at 37 and 50 °C (Table 1) to provide confirmation that erythromycin concentrations in the receiver compartment always remained less than 10% of the saturated solubility of the drug, maintaining sink conditions during the *in vitro* permeation studies.

In vitro erythromycin percutaneous absorption: effect of heat and chemical penetration enhancers

In order to initially screen the effect of heat and chemical penetration enhancers on the skin absorption of erythromycin, the amount of erythromycin that accumulated in the epidermis and dermis along with that which had diffused to the receptor compartment from saturated solutions of the drug in EtOH, TC, IPM, DPPG, DPA and PBS was determined after 24 h, at both 32 and 45 °C. The resulting data is shown in Figure 1. As limited permeation of the drug into the receiver fluid was obtained from several of the test formulations, the drug was allowed to accumulate in the receiver fluid to levels above the LOQ and only a single time point sample was taken at the end of the experiment and therefore no permeation profiles are provided here. In general, considerably more erythromycin was found in the dermal layer in comparison to the epidermis or in the receiver fluid for many of the systems at 24h. The exception to this was for certain vehicles (DPPG, DPA, IPM and EtOH) at 45 °C for which delivery to the receiver fluid was greater than that to the dermis. These differences may relate to the capabilities of the enhancers to promote drug transport out of the hair follicles into the dermal tissue and receiver fluid.

When considering the total amount of drug delivered in Figure 1, that is the quantity of erythromycin recovered from the epidermis, dermis and receiver fluid together, using a two way ANOVA, the effect of both heat and chemical penetration enhancers were found to significantly increase ($p \leq 0.05$) erythromycin delivery. The analysis did not find an interaction between heat and chemical penetration enhancers indicating that the effectiveness of either strategy was not dependent on the other. However when combined together the total magnitude of drug delivery enhancement (~40-70-fold) provided by using both heat and chemical penetration enhancers was found to be greater than the use of heat (~3-12-fold) and chemical penetration enhancers (~6-17-fold) separately, suggesting that the combined penetration enhancing strategies work synergistically together. Amongst the chemical penetration enhancers tested, EtOH at 45 °C and TC at 45 °C provided the highest overall delivery of erythromycin, whereas at 32 °C, TC and IPM provided the highest delivery.

Absorption of erythromycin into skin tissue following different durations of application: 1 h vs 24 h

Given that erythromycin is a relatively large molecular weight molecule (RMM 733Da), it was hypothesised that it would only have low levels of transport across the continuous SC and would be more likely transported via appendages such as the hair follicles, which extend across the full thickness of the skin. Previous studies have suggested that the transport through such units should be rapid [7, 15, 16], consistent with a lower resistance to diffusion provided by the shunt routes in comparison to the continuous SC. The penetration of drugs such as, erythromycin through the hair follicles would therefore be expected to be relatively high at early time points. To confirm this hypothesis, the delivery of the drug from three different vehicles was tested at 1 h, in addition to 24 h, to help provide insight into erythromycin transport into skin. For this work, TC and DPPG, alone and in combination (50:50), were selected as vehicles for the drug. Of the hydrophobic chemical penetration enhancers tested

(DPPG, IPM, DPA), DPPG was found to work best with heat as demonstrated by the enhancements in erythromycin delivery at the higher temperature, which were 6, 4 and 3-fold for DPPG, DPA and IPM respectively. DPPG should therefore be best to aid identification of the impact heat can have on drug delivery into and across the skin. Both of the hydrophilic enhancers tested, TC and ethanol were effective at delivering erythromycin into the skin, however TC was selected as it was found to provide higher drug concentrations in the dermis.

Figure 2 shows the recovery of erythromycin from the skin strata and receiver fluid after both 1 and 24 h following the application of erythromycin in the co-solvent system TC: DPPG (50:50) and the corresponding drug containing neat solvents TC and DPPG, at 32 °C. The penetration of the drug into the epidermis, dermis and receiver fluid under the same conditions but at a skin temperature of 45 °C is shown in Figure 3. What is initially apparent from Figures 2 and 3 is that even after 1 hour the amount of drug in the dermis was considerable with statistical analysis indicating that this was equivalent to that obtained from the dermis at 24 hours ($p > 0.05$) for all systems at 32 °C and TC and DPPG separately at 45 °C. A small increase was observed with TC:DPPG at 45°C. In contrast increased amounts of erythromycin were present in both the epidermis and receiver fluid for many of the systems at 24 hours in comparison to 1 hour. This suggests that erythromycin was able to penetrate rapidly in to the dermis, with subsequent slower permeation of the drug of the drug in to the epidermis (possibly via the continuous SC) and into the receiver fluid and occurring between 1 and 24 h. This seems to support the hypothesis that erythromycin is mainly delivered though the “low resistance” routes of permeation, the skin appendages, which originate in the dermis.

As was previously observed with experiments run over 24 hours, increasing the temperature of the skin increased significantly ($p \leq 0.05$) the overall delivery of erythromycin from each of the chemical penetration enhancers tested (4-6 fold) over 1 hour, compared to normal physiological skin temperature. The use of the mixed solvent system TC:DPPG appeared to

show improved delivery into the skin strata in comparison to the neat solvents TG and DPPG, however this was not statistically significant except at 32 °C over 24 hours suggesting any benefit obtainable from using the mixed solvent system is likely to be relatively small.

Effect of different durations of heat exposure on erythromycin transport

Application of heat to the skin for long periods, even for example 1 h is likely to limit its practical usefulness as an enhancement strategy. However given that the data showed that the application of heat for only 1 h was effective at promoting erythromycin delivery into the skin it was decided to perform further investigations to understand whether it would be feasible to further reduce the duration of heat application, without compromising the effectiveness of using heat as an enhancement strategy. For this work erythromycin in DPPG was selected, as this solvent produced highest enhancement in drug delivery with heat (6-fold) compared to that calculated from TC (4-fold) and the co-solvent system TC: DPPG (4-fold) and as discussed previously should therefore offer the best opportunity to understand the effect of shorter durations of heat can have on skin transport.

The effect of different heating durations, 5, and 10 minutes on erythromycin delivery from a suspension of the drug in DPPG is illustrated in Figure 4. Statistical comparison indicated that to observe the enhancement effect of heat ($p \leq 0.05$) on drug transport, the systems had to be maintained at 45 °C for a period of only 10 min (ER: 5-fold), compared to being held continuously at 32 °C. No further significant increases in the amount of erythromycin absorbed into dermis were observed when the skin was heated for the whole 1 hour time course of the experiment (Figure 3).

Localisation of erythromycin in the hair follicles: cyanoacrylate biopsy

To confirm whether the drug was being delivered via the skin's appendages a cyanoacrylate glue biopsy as a means to specifically quantify appendageal/follicular drug penetration, was

performed. This procedure involves the application of cyanoacrylate glue to the skin; the glue is initially a free flowing liquid that can penetrate into the skin appendages and subsequently polymerises in the presence of air to form a solid mass. This solid mass can be removed from the skin to provide an indication of the amount of drug present in the appendages.

The 'differential stripping' technique, where the cyanoacrylate biopsy procedure is performed after removing the SC was chosen to remove the possibility of drug content in the SC interfering with the assay. A saturated solution of erythromycin in DPPG was used and again the Franz cells were held at 45 °C for 10 minutes prior to being transferred to a water bath at 32 °C for the remaining 50 minutes. The experiments were performed in a manner similar to that reported by Teichmann et al, and in addition a range of application times of the glue were tested to examine whether this affected the data obtained [17].

The results are depicted in Figure 5 and show that following the removal of the SC and then the cyanoacrylate glue, the underlying viable epidermis did not contain any drug. Increasing the contact time between the cyanoacrylate glue and the skin from two to five minutes had no significant effect on the recovery of the active from the follicles, whereas when it was used for only 0.5 minutes a lower recovery was obtained. The amount of drug that was obtained from the cyanoacrylate biopsy was the same as that extracted from the viable epidermis when the conventional tape stripping procedure was used without the cyanoacrylate glue. There were no significant differences in the amount of erythromycin sampled from the dermis using the different tape stripping approaches. These data suggest that all of the erythromycin within the viable epidermis was within the appendages providing strong evidence that this was the main route of the drug into the skin.

Finite dose experiments

In the view of these promising data, which indicated that the use of a short duration of heat along with chemical penetration enhancers was effective at improving drug delivery into the skin via the follicular route, further experiments were designed to provide an understanding of the performance of a heated system under finite dose conditions. As the differential stripping process, although useful in helping identify the route of erythromycin transport into the skin, was not thought to fully quantify drug in the appendages, it was therefore decided to use the simpler conventional tape stripping method for these experiments. DPPG was again used as a formulation vehicle for erythromycin and additionally a mixed vehicle containing DPPG and ethanol was also investigated. Previously in the infinite dose experiments the hydrophilic chemical penetration enhancer, TC was used in combination with DPPG. However although this combined system showed good delivery of erythromycin into the skin it did not appear to offer significant advantages over DPPG alone. Ethanol was also seen to be a good penetration enhancer in the infinite dose studies, moreover it was thought that ethanol volatility could be beneficial in the finite dose investigations as with ethanol evaporation the thermodynamic activity of erythromycin in the formulation would be expected to increase, and could thereby further increase delivery of erythromycin into the skin.

Erythromycin (at both 2% w/w and as saturated suspension) in DPPG and as a 2% solution in DPPG: ethanol (34:66) was applied to the skin with and without the application of a controlled level of heat (45 °C, for 10 min) over a 1 hour period. The results are shown in Figure 6 as absolute amounts of drug and to aid comparison between the formulations as a percentage of the applied dose in Table 2. If the total delivery is examined (SC + viable epidermis + dermis) a significant improvement in erythromycin delivery was observed with heat for each of the three formulations. Comparison between the three formulations indicated that there were no significant differences in overall delivery between them at the respective temperatures. However the use of heat provided significantly more delivery to the dermis from both the

saturated and 2% solutions of erythromycin in DPPG (3.5 fold and 5.4 fold respectively), whereas the 2% DPPG:EtOH formulation did not significantly improve delivery of erythromycin to the dermis with heat but did provide 4.3 fold enhancement of delivery to the epidermis. The saturated solution of erythromycin in DPPG also provided significantly increased delivery to the SC at the elevated temperature (2.7 fold), whereas there were no significant differences between erythromycin delivery to the SC with heat were observed for the other two systems.

The same data included in Figure 6 is shown as the percentage of the applied dose in Table 2. As the doses applied to the Franz cell from the saturated system in DPPG at 32 °C and 45 °C were different (the formulations were saturated the respective temperatures) it aids comparison between delivery at these two temperatures. The saturated solubilities of erythromycin at 32 °C and 45 °C were 44 and 73 mg/mL respectively, and therefore a considerably higher dose was applied when the additional heat was used. However the data in Table 2 show that a greater proportion of the applied dose was delivered from the heated saturated erythromycin in DPPG system, indicating that the increased delivery obtained with heat is not simply a result of an increased dose having been applied. Table 2 also clearly shows that the 2% w/v loaded erythromycin formulations (both DPPG alone and DPPG:EtOH) delivered a greater proportion of the applied dose than the saturated system despite the thermodynamic activity of the drug in the formulation being lower at the higher temperature.

Discussion

The focus of this study was to examine whether heat in combination with chemical penetration enhancers could improve delivery of erythromycin to the skin and in particular via the follicular pathway. A skin temperature of 45°C was chosen to mimic conditions provided by a heated

formulation as this is believed to be a relatively high but physiologically tolerable temperature. For example, the marketed system CHADD® (controlled heat aided drug delivery) is known to produce a maximum temperature on the skin of approximately 45 °C [18]. Technologies that can generate short durations of heat and can be contained within flexible packages are commercially available and other suitable phase change materials have been proposed for heating applications in topical drug delivery [19]. It should be possible to design drug delivery systems using these materials that are practical for patients to be able to use, an important consideration for topical treatments which often have poor patient adherence [20].

Follicular absorption is expected to be important not only for the management of conditions associated with these skin appendages but also for the absorption of relatively high molecular weight drugs including erythromycin [21]. That the differential tape stripping technique used here found no drug in the viable epidermis tissue following the cyanoacrylate biopsy provided a clear indication that the transport of erythromycin to the dermis had occurred via the appendageal route, rather than through traversing the continuous SC and viable epidermis. However as a similar amount of erythromycin was recovered from the dermis regardless of whether the glue was used or not, suggested that the biopsy did not sample the drug from the full length of the appendages which extend into the dermis. This is in agreement with previous work, which found that the cyanoacrylate glue enabled drug sampling from the appendageal infundibulum only, which constitutes the outermost 20% of the total hair follicle length [17]. The rapid delivery of erythromycin into the dermis and its enhancement with durations of heat as short as 10 minutes of heat again further supported the conclusion that erythromycin absorption largely occurred via the skin's appendages given the relatively rapid absorption of this route [7, 15]. The subsequent, slower, delivery of erythromycin through to the receiver compartment, being consistent with slower transport out of the hair follicles and via other permeation pathways.

Heat has been shown to be effective at promoting the skin transport of a range of drug molecules with differing physiochemical properties previously [4]. It is thought to improve drug diffusivity, as described through the Stokes- Einstein equation but may also have effects on drug partitioning and alter the skin's properties for example rendering the intercellular lipids of the continuous SC more permeable [22]. In contrast literature on the ability of heat to work with different formulation vehicles is less well described nor has this method of enhancement previously been investigated for its effect specifically on follicular drug delivery. In this work heat was observed to improve delivery from both hydrophilic and hydrophobic vehicles. The beneficial effect of the chemical penetration enhancers alone on follicular erythromycin delivery likely relates to their ability to interact with sebum, in turn facilitating the solubilisation of the drug within the "sebum-enhancer" matrix [3], increasing follicular drug uptake. The existence of synergy between heat and chemical penetration enhancement is likely to be produced, if as well as directly assisting delivery of the drug into the SC, heat also improved delivery of the chemical penetration enhancer and the extent of enhancement observed is likely to be dependent on the properties of the particular chemical penetration enhancer and how it interacts with sebum [23, 24]. Combinations of hydrophilic and hydrophobic chemical penetration enhancers may provide synergistic improvements in drug delivery across skin, however the mixed solvent comprised of both DPPG and transcutol used here did not provide large improvements in delivery over the individual solvents with heat [25]. To exploit any potential for synergy between combinations of chemical penetration enhancers and heat would require further work and most likely careful consideration of the properties of the drug and chemical penetration enhancers selected as such effects are not automatically obtained [26, 27].

Finite dosing mimics the *in vivo* situation more closely and provides increased confidence in the therapeutic success of prototype formulations for topical drug delivery. For the vehicle

consisting of DPPG alone, similar enhancement ratios with heat were observed as with infinite dosing supporting the use of this approach for initially screening the vehicles. The similar total drug delivery obtained from the 2 % w/v loaded and saturated erythromycin systems in DPPG alone was surprising as the applied dose was considerably smaller for the 2% w/v loaded system. As the saturated systems were saturated at the temperature to which they were applied to the skin, the initial thermodynamic activity of the drug in the saturated vehicles are equivalent at the different temperatures, whereas that of the fixed dose 2% w/v loaded vehicle was lower, particularly for the heated system [28]. As both the heated systems delivered increased amounts of erythromycin and a greater percentage of the applied dose than the unheated systems, this indicated that the increased erythromycin skin absorption in the presence of heat increased cannot be explained through an increased dose being applied at the higher temperature. It also indicated that the reduction in drug thermodynamic activity for the 2% w/v loaded vehicle that occurs at elevated temperature did not negate the beneficial impact of heat in promoting drug uptake into skin. The 2 % w/v loaded DPPG:EtOH vehicle was designed to make use of ethanol's volatility so that any reduction in erythromycin thermodynamic activity in a fixed dose formulation with increased temperature could be compensated for, as the drug's thermodynamic activity would be raised as the solvent evaporated [29, 30]. Although this formulation did work with heat, it did not increase total erythromycin delivery above that of the 2% w/v DPPG formulation. Moreover differences were observed in the distribution of erythromycin in the skin, with the heated DPPG:EtOH formulation providing significant improvements in delivery to the epidermis in particular, but not to the dermis. Consequently this formulation approach utilizing a volatile solvent appears to be less suitable for working with heat to provide optimal erythromycin delivery. It is possible that evaporation of ethanol from the formulation may have induced crystallisation of the drug within the skin, reducing its delivery to the deeper dermal tissues [31]. The increased drug

delivery observed with heat is likely to be related to other effects such as more favourable chemical penetration enhancer uptake into skin [32, 33]. It has previously been shown when chemical penetration enhancers are used, improved drug delivery may be obtained from lower levels of drug saturation if this is associated with improved delivery of the chemical penetration enhancer [23]. Similar behaviour may occur with heat, allowing equivalent delivery to be provided by subsaturated (2% w/w) and saturated erythromycin formulation vehicles.

The relatively large molecular weight of erythromycin, the model drug used here may have disposed its delivery towards targeting of the follicular route of absorption as it is unlikely to be well absorbed through the continuous SC. However the human scrotal skin used in this study is generally considered to be considerably more permeable than that from other body sites [34], despite the density of hair follicles and other appendages being comparatively lower than that of other body areas [35]. This suggests that the data reported may underestimate the impact of heat and chemical penetration enhancers on follicular absorption at body sites with a higher density of hair follicles such as the face or scalp which are commonly associated with conditions such as acne vulgaris and alopecia. For other molecules that exhibit better absorption via the continuous SC, short durations of heat are likely to still be of benefit in preferentially directing drug delivery to the hair follicles through enhancing delivery through this rapid transport pathway to a greater extent than through the SC.

Most of the previous studies that have investigated the effects of heat on drug transport across skin or model membranes have used water baths as a model system to simulate heat producing formulations as was done here [4, 5]. Further work investigating the use of heat generating formulations materials to improve topical drug delivery will be beneficial not just to more closely mimic the *in vivo* situation but also to beneficially utilize the ‘Soret effect’, where molecules exhibit a drift mobility along a thermal gradient in addition to Brownian diffusion [36, 37]. Applying heat to the skin in vivo is thought to increase cutaneous blood flow [38]

which could increase drug clearance from the site of action, decreasing topical effectiveness. However the success of a heated topical patch containing lidocaine and tetracaine suggest that this is not necessarily an overriding consideration [39, 40]. As such it is likely that the increases in drug absorption into the skin that can be produced by heat and chemical penetration enhancers are likely to be capable of improving clinical outcomes of therapeutic treatment. In conclusion the results from this work have demonstrated for the first time the positive impact heat can have on follicular drug absorption into the skin. The application of a moderate level of heat in combination with penetration enhancers was able to synergistically increase absorption of the selected model drug, erythromycin. Moreover durations of exposure to heat as short as 10 minutes were sufficient to improve follicular delivery supporting the suitability and highlighting the potential of this approach to develop practical, improved topical treatments for the management of skin conditions.

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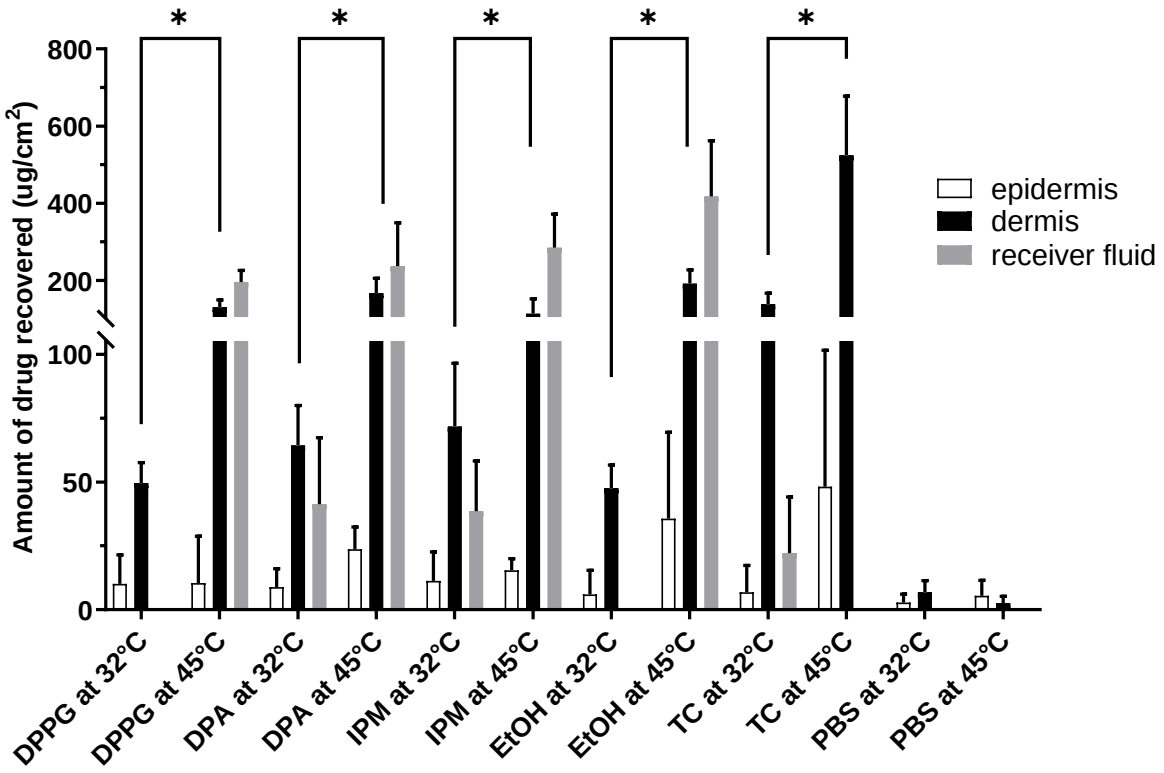

Figure 1. Mean amount of erythromycin recovered from epidermis, dermis and receiver fluid following the application of a saturated solution of erythromycin in DPPG, DPA, IPM, EtOH, TC and PBS, at 32 and 45 ºC, after 24 h (mean ± SE, n=6). * indicates a significantly lower total absorption (epidermis, dermis and receiver fluid combined) for experiments conducted at 45 °C compare to those conducted at 45 °C.

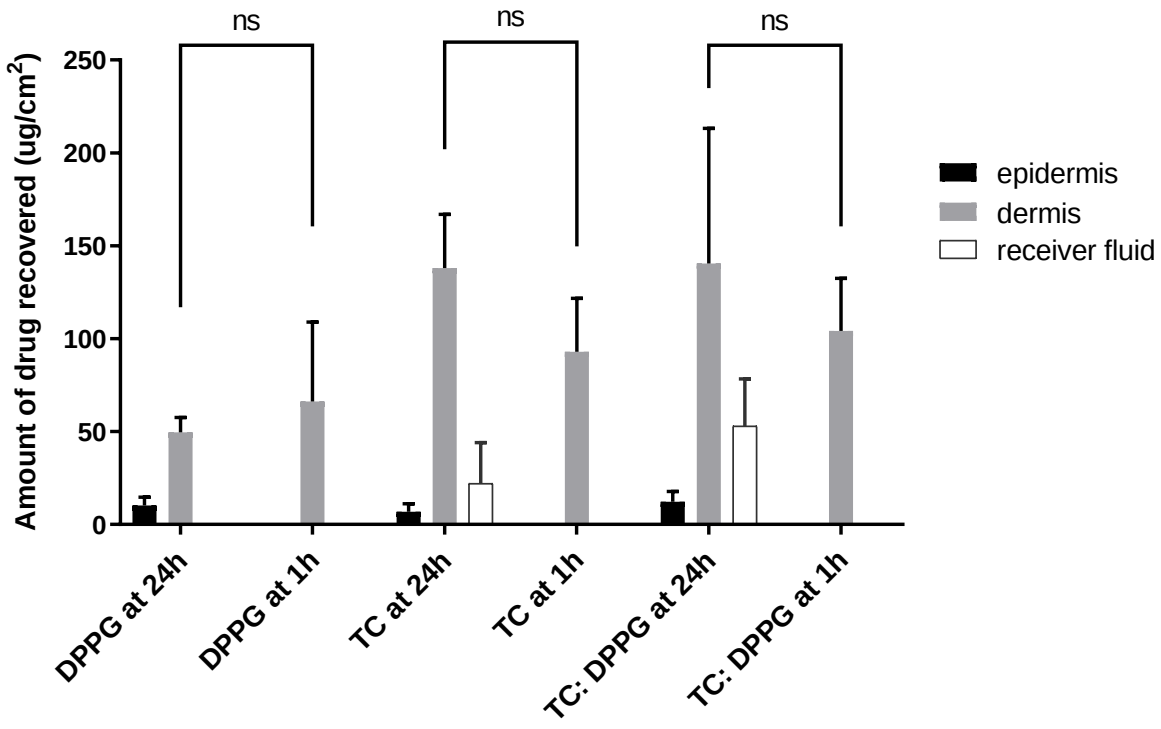

Figure 2. Mean cumulative amount of erythromycin recovered from epidermis, dermis and receiver following the application of saturated solution of the drug in TC: DPPG (50:50), DPPG and TC, at 32 ºC, after 1 and 24 h (mean ± SE, n=6). ns indicates no significant difference in dermal absorption at 1 hour compared to that from the same vehicle at 24 hours.

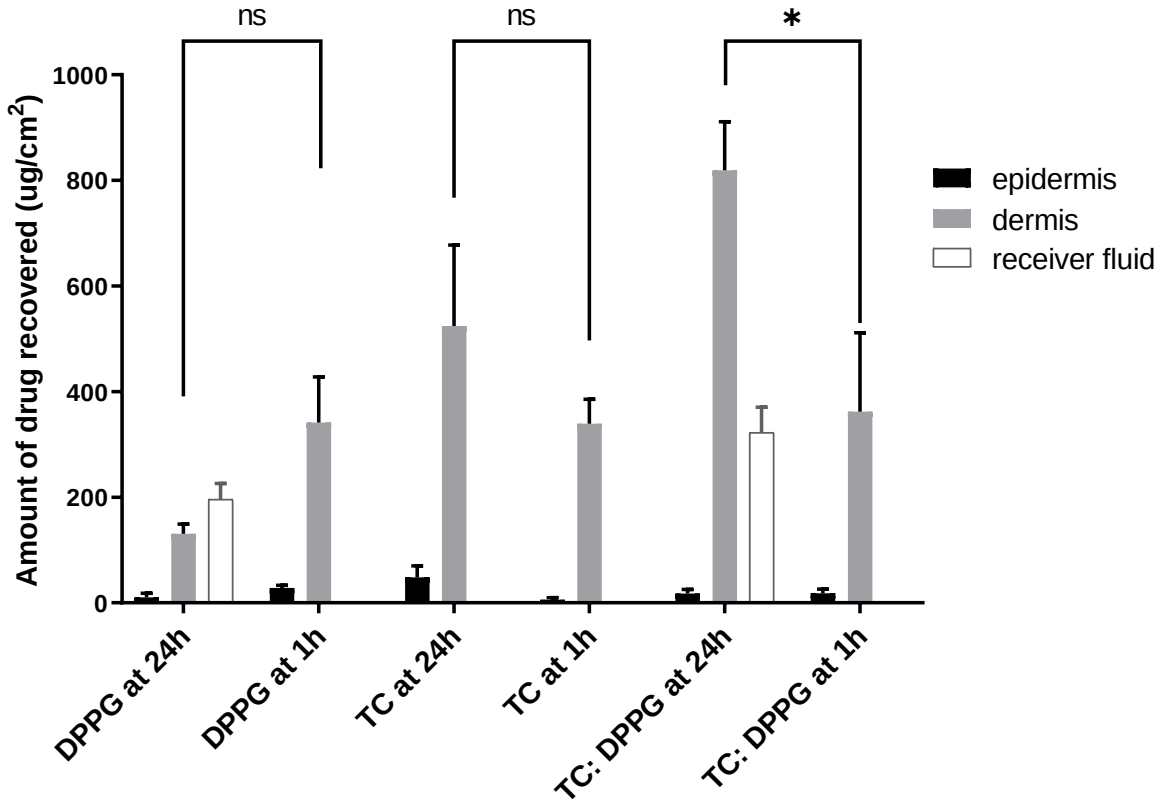

Figure 3. Mean cumulative amount of erythromycin recovered from epidermis, dermis and receiver following the application of saturated solution of the drug in TC: DPPG (50:50), DPPG and TC, at 45 ºC, after 1 and 24 h (mean ± SE, n=6). * indicates a significantly higher dermal absorption at 1 hour compared to that from the same vehicle at 24 hours.

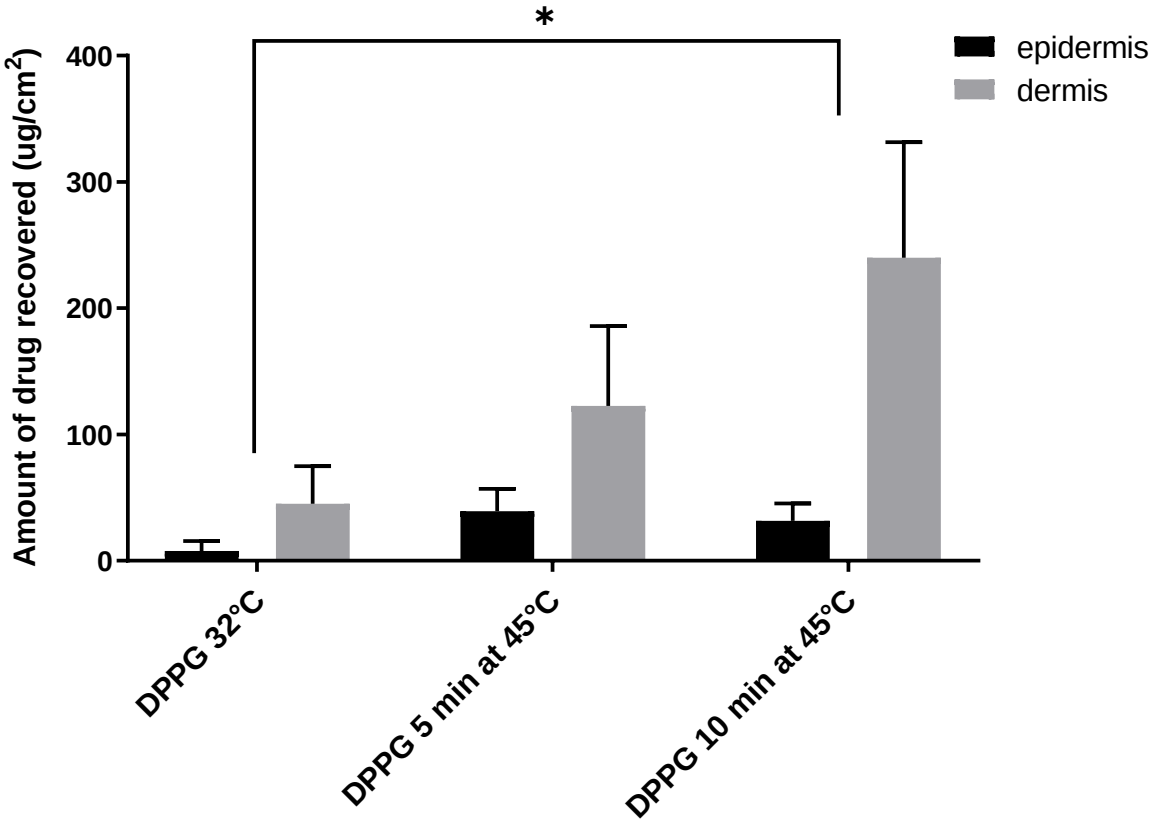

Figure 4. Effect of the duration of heat exposure on the delivery of erythromycin to the epidermis and dermis, from saturated solutions of the drug in DPPG after 1 hour (mean ± SE, n=6). * indicates a significantly higher total absorption (epidermis and dermis combined) at 1 hour compared to the erythromycin in DPPG formulation at 32°C.

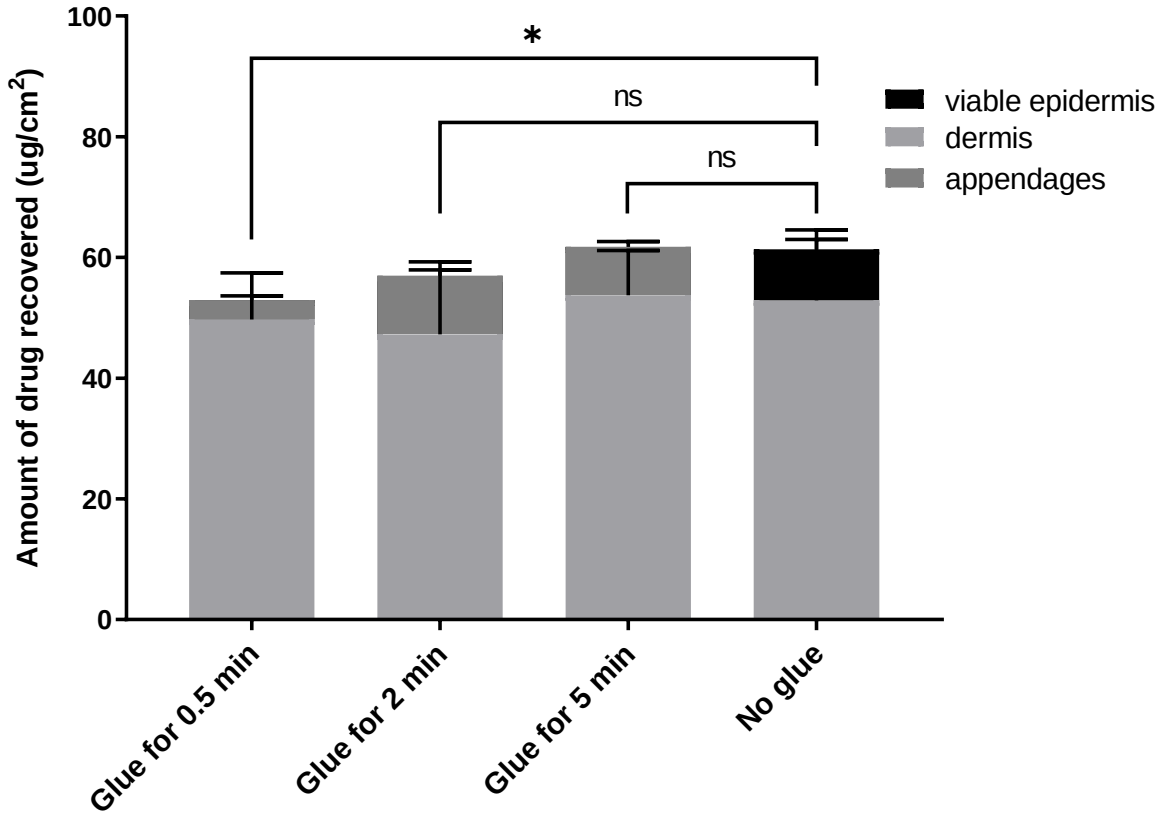

Figure 5. Comparison (a) of the amount of erythromycin recovered from the viable epidermis, dermis and appendages when using both conventional (labelled as "no glue") and differential tape stripping procedures (glue, applied for 0.5, 2 and 5 min), 1 h after the application of a saturated solution of the drug in DPPG, at 45°C (for 10 min (mean with range, n=3). The glue was applied for different durations, after removing the SC. * indicates a significantly lower recovery than was obtained when the cyanoacrylate biopsy was not used.

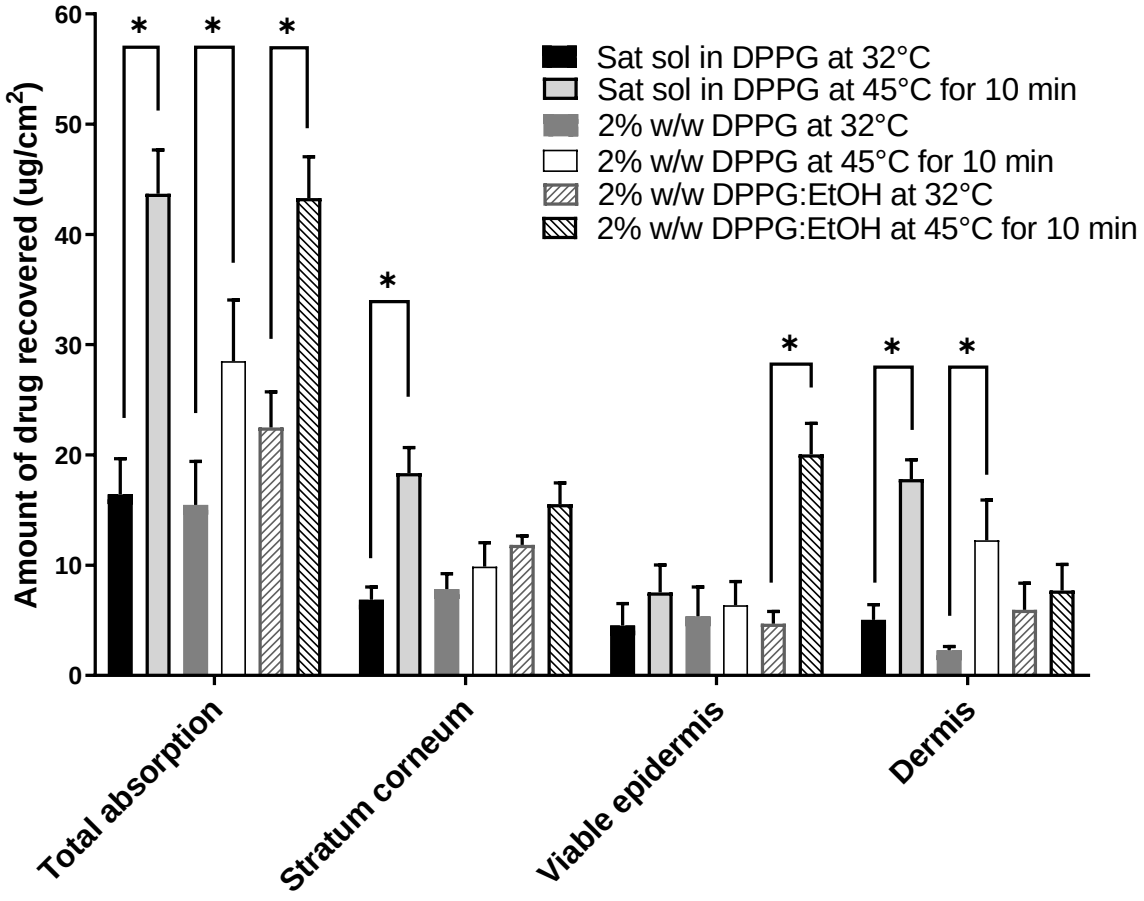

Figure 6. Total mean amount of erythromycin recovered from the skin, and that from the separate skin layers the SC, epidermis and dermis 1 h after the application of a finite dose of a 2% w/v solution and saturated suspension of the drug in DPPG and a 2% solution in DPPG: EtOH (34:66), with and without application of heat (45 °C for 10 min) (mean + SE, n=4-6). * indicates a significant difference between the experiment conducted at 32 °C for one hour and that where the Franz cell was exposed to 45 °C for 10 minutes, before being transferred to the 32 °C conditions.

Table 1. Erythromycin saturated solubility values at room temperature unless otherwise stated. Values are reported as the mean ± range (n=3).

Solvent	Drug Solubility (mg/mL)
EtOH	560.7 (± 7.6)
Transcutol	477.2 (± 5.4)
IPM	23.6 (± 1.4)
DPPG	43.5 (± 1.8)
DPA	62.4 (± 2.2)
PBS	7.85 (± 0.13)
PBS (37°C)	8.24 (± 0.21)
PBS (50°C)	9.72 (± 0.19)

Table 2. Percentage of applied erythromycin recovered from skin layers 1 h post application of all solvent systems. Values are reported as the mean ± SEM (n=4-6).

Formulation vehicle and conditions	Total drug absorption (%)	Stratum corneum drug content (%)	Viable epidermis drug content (%)	Dermis drug content (%)
2% w/w DPPG at 32°C	15.36 (± 3.94)	7.81 (± 1.41)	5.36 (± 2.67)	2.28 (± 0.36)
2% w/w DPPG at 45 °C for 10 min	28.51 (± 5.55)*	9.87 (± 2.17)	6.38 (± 2.13)	12.26 (± 3.65)*
Sat sol in DPPG at 32°C	3.54 (± 0.70)	1.49 (± 0.22)	0.97 (± 0.42)	1.09 (± 0.28)
Sat sol in DPPG at 45 °C for 10 min	10.00 (± 0.91)*	4.20 (± 0.54)*	1.73 (± 0.57)	4.07 (± 0.39)*
2% w/w DPPG: EtOH at 32°C	18.75 (± 2.70)	9.87 (± 0.68)	3.92 (± 0.92)	4.96 (± 2.02)
2% w/w DPPG: EtOH at 45 °C for 10 min	36.08 (± 3.12)*	12.94 (± 1.61)	16.71 (± 2.34)*	6.43 (± 1.96)