Heat enhanced follicular delivery of isotretinoin to the skin

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ABSTRACT

Purpose: The aim of this work was to evaluate the use of short durations of externally applied heat with chemical penetration enhancers to improve delivery of isotretinoin to the skin and in particular via the follicular route.

Methods: A range of chemical penetration enhancers were screened for their ability to improve isotretinoin delivery into human skin with heat using infinite dose, Franz cell experiments conducted in a water bath at a higher temperature to simulate heated conditions. Following this a prototype external heating system was developed that provided short durations of heat and its ability to improve delivery of finite doses into the skin and hair follicles was assessed.

Results: The magnitude of the effect of heat on drug delivery was influenced by the choice of vehicle with changes in isotretinoin flux across skin ranging from not statistically significant to 25 fold increases with heat in the infinite dose studies. The prototype heating system provided significant increases in the total delivery of isotretinoin into the skin from an optimised vehicle. Drug distribution in the skin revealed significant increases in isotretinoin delivery to the hair follicles, and deeper skin layers, but not to the stratum corneum, providing strong evidence that the enhancement in delivery occurred mainly via the hair follicles.

Conclusion: These data indicate that the use of short durations of heat combined with chemical penetration enhancers offers a valuable strategy for improving the delivery of drugs such as isotretinoin to the skin via the hair follicles.

Keywords: Acne, chemical penetration enhancers, follicular drug delivery, heat, isotretinoin, skin permeation, topical drug delivery.

1 Introduction

Topical delivery of drugs for the treatment of skin diseases associated with the hair follicles and sebaceous glands is desirable to reduce the side effects associated with systemic treatment. The skin however presents a major barrier to topical drug delivery and may prevent a treatment from being effective or limit its efficacy when administered topically. An example is isotretinoin which is used topically to treat mild to moderate acne but it and other drugs are administered systemically for more severe acne. Improved topical delivery of isotretinoin could reduce the need for systemic treatments and therefore development of their associated systemic side effects. When applied topically drugs such as isotretinoin are also associated with localised side effects such as skin irritation and peeling, though these still occur to a lesser extent than with systemic delivery [1]. If their delivery could be focused through the follicular route to the pilosebaceous units which are the site of the disease, this should not only increase therapeutic effectiveness but may also be able to reduce the development of localized side effects that may otherwise be increased with improved topical drug delivery.

A penetration enhancement strategy that may have the potential to achieve this and yet has not been extensively studied is the use of heat [2-4]. Heat is believed to enhance percutaneous drug absorption by influencing several parameters that may affect drug transport across skin. Heat can increase the release of the permeant from the formulation/vehicle [5, 6], partitioning and diffusion through skin [4, 7] and increase cutaneous blood flow [8]. These mechanisms are not limited to drug delivery via the hair follicles and the majority of studies investigating the effect of heat on drug permeation facilitated research have focused on permeation across the continuous SC. However, heat is thought to have specific benefits for improving drug delivery via the follicular route, for example through lowering the viscosity of sebum which is contained within the pilosebaceous units [9], making it more permeable to the transport of drugs. In addition, given that drugs are known to be transported relatively rapidly across the hair follicles in comparison to the continuous SC [10], short durations of heat are likely to increase drug

transport through them to a greater extent than the continuous SC. This could potentially focus drug delivery to these locations in the skin. Indeed recently, increased follicular delivery of erythromycin, a drug with a relatively large molecular weight and thus would not be expected to permeate well across the SC, showed significantly enhanced delivery with the use of short durations of heat in combination with chemical penetration enhancers [11].

Much of the work investigating the impact of heat on drug delivery to the skin has used water baths to simulate the heated conditions. However, externally applied heat where the skin surface is heated to a greater extent than the deeper skin layers is more relevant to the clinical application scenario, moreover it may offer additional advantages for the delivery of drugs, as the thermal gradient may provide an additional thermophoretic driving force which may be beneficial for drug transport [12].

In this work the potential of heat to improve the delivery of isotretinoin into human skin has been investigated. Isotretinoin is widely used in the treatment of acne and its physiochemical properties do not predispose it specifically to delivery via the follicular route. Therefore, it could aid identification of the potential of heat to improve follicular delivery in comparison to that across the continuous SC for other clinically relevant molecules. Moreover, following initial use of water bath to generate heat to screen chemical penetration enhancers, a prototype external heating system was developed and used to understand the effects of heat on isotretinoin skin permeation in a clinically relevant manner.

2 Materials and Methods

2.1 Materials

Isotretinoin (99.9 %) was purchased from Sequoia Research Products (Pangbourne, UK). Acetonitrile HPLC grade (99.9 %), ethanol HPLC grade (EtOH, 99.9 %), formic acid (98.0-100.0 %), methanol HPLC grade (99.9 %), propylene glycol (PG, > 99.0 %), phosphate buffer saline (PBS) tablets (10 mM, pH 7.4), Dura Seal™ (Diversified Biotech, USA), Hamilton

GASTIGHT® syringes (Hamilton®, Switzerland) and Parafilm M® laboratory film (Bemis® Flexible packaging, USA) were acquired from Fisher Scientific (Loughborough, UK). Isopropyl myristate (IPM, > 96 %) and sodium thiosulfate pentahydrate (ST, 99.5 %) were bought from Acros Organics (New Jersey, USA). Diisopropyl adipate (DPA, > 99.8 %) was supplied by Croda (Barcelona, Spain). Propylene glycol monolaurate type II (PGML, > 99.8 %) and Transcutol® P (TP, > 99.8%) were provided by Gattefosse (France).

2.2 Quantitative analysis of isotretinoin

Isotretinoin was assayed using HPLC (Shimadzu Corp., Kyoto, Japan). The system consisted of a pump (LC-20 AD) with a degasser (DGU-20A_{5R}), auto-sampler (SIL-20A HT), column oven (CTO-20 AC), UV-VIS detector (SPD-20A) and communication module (CBM-20A). Data acquisition was performed on LabSolutions software® version 5.54 SP2. Chromatographic separation was performed using a Phenomenex® HyperClone™ ODS C18 (5 µm, 150 x 4.6 mm, Phenomenex Ltd., Cheshire, UK) at room temperature. The detection wavelength was 355 nm. The mobile phase consisted of 76 % acetonitrile (containing 0.1 % formic acid): 24 % deionized water (containing 0.1 % formic acid) at a flow rate of 1.0 mL/min. The run time was 15 min. The injection volume was 10 µL and the retention time for isotretinoin was 10 min. The HPLC methods were validated for linearity, precision and accuracy according to current ICH guidelines The LOD and LOQ were 0.24 µg/mL and 0.73 µg/mL respectively.

2.3 Determination of isotretinoin solubility and stability in donor vehicles

Solubility studies were conducted by preparing saturated suspensions of isotretinoin in each vehicle to be tested in amber glass vials at both 32 °C and 45 °C (these were the skin surface temperatures employed during the *in vitro* permeation studies). A PTFE coated magnetic stirrer bar was introduced into each vial, after which the vials were tightly sealed and covered with Parafilm. The suspensions were stirred for 24 hours at the stated temperatures, after which samples were removed and filtered using 0.2 µm PTFE syringe filters (dot-red® analytical,

UK). Where appropriate the filtrate was diluted with methanol. The stability studies were performed at 32 °C and 45 °C. The drug content of each sample was analysed at the day of preparation (starting point). This value was quoted as 100 %. Afterwards samples were taken after 24 hours, where a defined amount of formulation was dissolved to 10 mL in methanol and vortexed for approximately 1 min. Samples where then filtered using 0.2 µm PTFE syringe filters. In all the experiments three repetitions (n=3) of each experiment were performed and isotretinoin was protected from light by using amber glassware where possible or covering glassware with aluminum foil.

2.4 Preparation of donor formulations/suspensions

To prepare formulations for the infinite dose *in vitro* permeation experiments, excess drug was added to the required vehicle and stirred for 24 hours in a water bath (Grant Instruments, UK) at the appropriate temperatures (32 °C or 45 °C) to produce saturated solutions which were used directly. For the finite dose *in vitro* permeation studies, isotretinoin at a concentration of 0.05% w/v or as saturated solutions prepared at 32 °C and 45 °C (which were filtered before use) in a 1:1 binary system of EtOH: PGML were prepared in amber glass vials.

2.5 Skin preparation

Full thickness human skin from a single female donor was obtained with informed consent following abdominoplasty. Ethical approval was provided by the University of Surrey Ethics Committee (EC/2012/29/MedPharm). The skin was prepared carefully by removing the subcutaneous fat using a forceps and scalpel and then stored in a freezer at -20 °C until it was needed for use.

2.6 Infinite dose *in vitro* skin permeation studies

Skin samples were placed on the individually calibrated unjacketed upright Franz diffusion cells (diameter 1.0 cm^2, volume 3.0 mL) (Soham Scientific, UK), with the SC facing the donor compartment and the dermis facing the receptor. Both chambers were then wrapped together using Parafilm® (at 37 °C) or Dura Seal™ (at 50 °C) before being clamped together. The

receptor chamber was filled with EtOH: PBS solution (1:1) and stirred with a magnetic bar to ensure adequate mixing (600 rpm). The water bath temperature was maintained at either 37 °C or 50 °C to keep the skin surface at approximately 32 ± 1 °C and 45 ± 1 °C respectively. Prior to dosing, the Franz cells were equilibrated at the appropriate temperature and then the membrane surface temperature was measured from the donor compartment using a Fisher Scientific Traceable Digital Thermometer with a type-K probe. Air bubbles were removed through the sampling arm by carefully tilting or inverting the diffusion cell and checks for leaks were made at the same time. A saturated suspension (0.5 mL) of isotretinoin was then introduced into the donor chamber. Following this the receiver fluid (200 μL) was removed from receptor compartment via the sampling arm at regular intervals and analysed via HPLC. An equal volume of pre-warmed receiver fluid was immediately added to replace the sampled volume. Six repetitions (n=6) of each experiment were performed. In this experiment, the Franz diffusion cells were all occluded and covered with aluminum foil to protect isotretinoin from light.

2.7 Finite dose *in vitro* skin permeation studies

Isotretinoin at a concentration of 0.05% w/v or as saturated solutions prepared at 32 °C (which were filtered before use) in a formulation vehicle consisting of EtOH and PGML (1:1) were prepared and used to dose Franz cells mounted with human abdominal skin with finite doses of 10 μL cm^{-2}. As before the Franz cells had been pre-equilibrated in water baths maintained at either 37 °C or 50 °C to keep the skin surface at approximately 32 ± 1 °C and 45 ± 1 °C respectively. All other parameters, and procedures were consistent with those described for the infinite dosing experiments.

2.8 Determination of drug content in skin

After the completion of the infinite dose diffusion experiments, the skin was removed from the Franz cell and carefully dried by patting with tissue paper. The residual formulation was removed with three separate cleaning phases. The first cleaning phase was conducted by

carefully rolling a dry cotton bud over the skin upwards three times, then downwards three times, then clockwise and anticlockwise along the edges once. For the second cleaning phase, the first cleaning phase was repeated using a wet cotton bud soaked in methanol. For the third cleaning phase, the first cleaning step was repeated using a dry cotton bud. All three buds were then discarded. To remove any remaining surface formulation two tape strips (Scotch® Tape strips, 3 M Center, USA) were taken and discarded. To remove the SC, a further ten tape strips were taken and placed into an amber glass vial. Following SC removal, the skin was placed in a benchtop oven (Binder GmbH, Germany) at 60 °C for 2 min which allowed the epidermis to be separated from the dermis more easily. Epidermal and dermal samples were transferred into individual amber glass vials. Then methanol (2.0 mL) was added to each vial, which was then sonicated for 20 min before being transferred to a Stuart roller mixer SRT9 (Cole-Parmer, UK) overnight (16 hr -18 hr). All samples were then filtered using 0.2 µm PTFE syringe filter (dot-red® analytical, UK) before being analyzed by HPLC.

For the finite dose experiments the same process was followed, with the exception that tissue paper was not used to remove excess formulation from the skin surface and the cotton buds used to remove the residual formulation were not discarded. The extraction procedure was repeated for each stage until the drug content in the extracted samples was no longer detectable or below the LOQ and considered fit for purpose. For all of the finite dose experiments, the level of isotretinoin recovery was over 80 % in accordance with OECD guidelines for drugs with stability issues [13].

2.9 Measurement of heat release profiles generated from a prototype heating system containing sodium thiosulfate pentahydrate (ST)

Sodium thiosulfate pentahydrate (ST) 20.0 g was weighed into glass vials (28 mL volume) and placed into a water bath at 60 °C. Once fully melted aliquots of ST (2.5 mL or 5.0 mL) were placed into glass vials (7 mL volume) and allowed to cool to room temperature. Franz cells were then assembled with digital thermometers with type-K probes (Fisher Scientific, UK)

placed strategically on the skin surface (SC) and through the sampling port just below the membrane to measure the changes in temperature with the application of the ST solution (Figure 1). The Franz cells were then placed into a water bath at 37 °C ± 1 °C to equilibrate, after which the membrane temperature was measured to ensure the skin surface (SC) temperature was 32 °C (i.e., external surface temperature of human skin). Then aliquots (2.5 mL or 5.0 mL) of the cooled ST liquid were poured into the donor chamber, where direct contact between the skin and the heating system was prevented using aluminum foil. A wired probe connected to a thermocouple (Hanna Instruments, UK) was then inserted into the center of the ST solution. A seed crystal was added to the ST liquid to initiate crystallization, and the temperature was recorded over time, in the ST liquid, on the membrane surface (i.e. SC temperature) and in the receiver fluid. The maximum temperature ($T_{\max}$), time to maximum temperature ($t_{\max}$) and duration (DUR) the SC temperature was above 32 °C were recorded for each volume of ST solution investigated. Each measurement was repeated in triplicate.

2.10 The effect of externally applied heat on the uptake of isotretinoin into the hair follicles, skin tissue and receiver fluid

The effect of controlled local heating on drug uptake into the hair follicles and skin tissue was determined by applying 10 µL of a filtered saturated solution of isotretinoin (1.38 % w/v) in EtOH: PGML (1:1) prepared at 32 °C. After applying the formulation to the donor chamber, 2.5 mL or 5 mL of ST solution was introduced into the donor chamber to produce a membrane surface temperature of ~ 43 °C - 44 °C for approximately 20 and 30 min respectively. As illustrated in Figure 1 aluminum foil was used to separate the heating system from the formulation. Control experiments were conducted in a water bath at 37 °C and 50 °C to produce membrane surface temperatures of 32 °C and 45 °C respectively. Aluminum foil was placed in the donor chamber of each Franz diffusion cell to mimic experiments using the ST chemical heating system. The receiver fluid was sampled only at the end of each experiment (24 hr and

1 hr duration experiments). Six repetitions (n=6) were performed for each treatment group. The skin samples were then removed from the Franz diffusion cells and the residual formulation was removed from the aluminum foil, donor chamber and skin surface using the cleaning procedure described for the finite dosing experiments. To remove any remaining skin surface formulation two tape strips were taken. Then a differential tape stripping was performed [11]. Briefly, 10 tape strips were taken to remove the SC and transferred into an amber glass vial. After the SC removal, a drop of cyanoacrylate glue was applied onto the tape stripped skin and was covered with a glass slide under slight pressure. After 5 min, the cyanoacrylate superglue polymerized, and the glass slide was removed with one quick movement taking the follicular casts with the slide. The cast was transferred into amber glass vial using forceps. The remaining skin was heat separated as described for the finite dosing experiments. Isotretinoin was then extracted from the samples as described above and quantified by HPLC.

Data analysis and statistics

2.11 Analysis of permeation parameters

To provide a mechanistic insight into the role of heat and CPEs in enhancing percutaneous absorption, the experimental permeation data was modelled using Fick's first law Eq. (1).

$$J = \frac{D \times K \times C_V}{h} \quad (1)$$

Where J is steady state flux, D the diffusion coefficient of the solute, h the diffusional path length, K the partition coefficient and C_V is the drug concentration in the vehicle. According to Eq. (1), J is directly proportional to the partition (K) and diffusion (D) coefficient of the solute under steady state conditions. Since J and the lag time (T_L) can be readily determined from the diffusion profile (plot of the cumulative amount permeated per unit area (Q) vs time (t)), as the gradient and x-intercept of the linear portion of the graph (18 hr – 24 hr) respectively, D and K can theoretically be obtained. However, since the diffusional pathlength across the SC (h) is

unknown, these cannot be calculated directly, but can be calculated as pathlength normalised values (D/h^2 and Kh) as illustrated in Eq. (2) and Eq. (3) [14].

$$\frac{D}{h^2} = \frac{1}{6 \times T_L} \tag{2}$$

$$K \times h = 6 \times \left(\frac{J}{C_V} \right) \times T_L \tag{3}$$

$$K \times h = 6 \times \left(\frac{J}{\alpha} \right) \times T_L \tag{4}$$

The use of C_V in calculating Kh was expected to lead to under estimation of the Kh values because of increased isotretinoin solubility in the vehicles at the higher temperature. The use of C_V in Fick's law is common as it is convenient to measure, however it is commonly recognised that it is the thermodynamic activity of the drug in the vehicle that fully accounts for the diffusional gradient across the skin, particularly when comparing between different formulations or conditions [11, 15]. It was therefore decided to use the thermodynamic activity (α) of isotretinoin in the vehicles, which as the formulations were saturated solutions has a value of unity, to model the experimental data obtained at both temperatures (32 °C and 45 °C). Thus, C_V in Eq. (3) was replaced with the thermodynamic activity (α) to give Eq. (4).

Statistical analysis

Statistical analysis of all the data was performed using GraphPad Prism version 8.00 for Windows, (GraphPad Software, La Jolla California, USA). 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, Tukey's HSD test or Dunn's for parametric and non-parametric data as appropriate. Similarly pair wise comparisons were made with either a *t*-test or Mann Whitney *U* test as appropriate. Statistically significant differences were assumed at the 95% confidence level, i.e., when $p < 0.05$.

The enhancement ratios (E_R) were determined using Eq. (5) below:

$$E_R = \frac{Q(E)}{Q(C)} \quad (5)$$

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 and CPEs) and control (no additional heat and CPEs) respectively. The permeation parameters obtained at different temperature (J, D/h^2, Kh and T_L) were then compared to the control (32 °C) using the student t-test or Mann-Whitney U test (when data were found to be not normally distributed). Statistically significant differences were accepted at 95% confidence level, i.e., when $p < 0.05$.

3 Results

3.1 Isotretinoin solubility and stability in donor vehicles

The saturated solubility of isotretinoin (at 32 °C and 45 °C) was determined in a range of solvents commonly used in topical/transdermal drug delivery formulations and is shown in Table 1. Isotretinoin solubility was highest in TP, with good solubility observed in a range of solvents including EtOH, PGML and DPA. Isotretinoin solubility was increased at the higher temperature of 45 °C in all cases; the magnitude of this increase ranged between 2.13 fold to 4.63 fold. The stability of isotretinoin in the same solvents was also investigated (at 32 °C and 45 °C) over 24 hours. Overall, isotretinoin was found to have sufficient stability with > 97 % recovery recorded for isotretinoin in all the solvents studied (data not shown).

3.2 Infinite dose *in vitro* skin permeation and distribution studies

3.2.1 Infinite dose *in vitro* skin distribution studies

Infinite dose Franz cell studies using human abdominal skin were conducted using saturated solutions of isotretinoin in each of the solvents above. The total amount of isotretinoin recovered from the skin tissue sum of drug recovered from SC, epidermis and dermis and receiver fluid, as well as in each of these compartments after 24 hours at both 32 °C and 45 °C are shown in Figure 2.

A two-way ANOVA revealed the effects of both vehicle and heat on isotretinoin delivery were statistically significant ($p < 0.05$). At the higher temperature (45 °C), the total amount of isotretinoin delivered was significantly increased from all vehicles except PG. The magnitude of these increases ranged between 2.9 and 1.8 fold with PGML and TP producing the largest and smallest enhancements with heat and total delivery at the higher temperature. At 32 °C, DPA provided the highest total isotretinoin delivery which was significantly, 1.83 fold greater than that from IPM ($p < 0.05$). Total isotretinoin delivery from the remaining vehicles were statistically the same. For each solvent at 32 °C, isotretinoin recovery from the receiver fluid was low in comparison to that of the skin tissue, however PGML provided a greater level of drug delivery to the dermis whereas for all the other vehicles tested higher amounts of drug were extracted from the epidermis. Again in the 45 °C experiment, to some extent the vehicle employed affected drug distribution across the skin. For example, EtOH significantly increased isotretinoin delivery into the receiver fluid in comparison to the other vehicles investigated.

3.2.2 Infinite dose *in vitro* skin permeation studies

The isotretinoin permeation profiles into the receiver fluid over the 24 hours from the distribution experiments above were plotted to help elucidate the mechanisms through which heat improved isotretinoin uptake into the skin. The plots at 32 °C and 45 °C which show apparently typical infinite dose profiles with an initial lag time followed by a linear permeation profile are shown in Figures 3 (A) and 3 (B) respectively.

At the higher temperature a significantly increased amount of isotretinoin was delivered into the receiver fluid from all the vehicles investigated except for PG. In addition, at 32 °C delivery of isotretinoin into the receiver fluid was highest from PG, whereas at 45 °C, it provided the lowest permeation of the solvents tested. In contrast when EtOH was the vehicle the increase in the amount permeated at 24 hours was 31 fold greater at the higher temperature indicating that the different solvents have differing abilities to work with heat to improve the permeation of isotretinoin across the skin.

The inset graphs of in Figures 3 (A) and 3 (B) show an expanded image of the delivery between 0 to 8 hours, allowing differences in delivery at the two temperatures to be discerned more easily at early time points. Analysis of this data revealed that significant differences in delivery with heat were apparent from 1 hour for all the vehicles except for TP. Significantly increased isotretinoin permeation from TP with heat was apparent from 2 hours onwards.

To gain a mechanistic understanding of how heat influenced the transport of isotretinoin across skin when delivered from the various vehicles investigated, Fick's first law was used to model the linear portion of the permeation data between 18 and 24 hours. The pathlength normalised values of partition (Kh) and diffusion coefficients (D/h^2) were determined and are shown in (Table 2). For this analysis, the thermodynamic activity of the drug in each of the solvents was used (α=1) rather than the concentration of the drug in the vehicle. Ideally to use Fick's first law, steady state flux would be assessed from a point on the plot 2.7 times past the lag time [16]. For some vehicles, this was not possible, however an estimated steady state flux value or pseudo steady state was taken from the linear portion of the plots (18-24 hours) as they can still provide mechanistic insight into how heat increase drug permeation across the skin [17].

The flux between 18 and 24 hours ($J_{18\text{-}24}$), was significantly greater for all solvent systems except PG at the higher temperature. This increase in flux could primarily be attributed to improvements in Kh which was significantly increased for all vehicles except PG, with the enhancement being up to 25 fold with EtOH. With regards to D/h^2 the increase in temperature to 45 °C produced what appeared to be small increases in the D/h^2 values (1.04 to 1.65-fold), however these apparent increases were only statistically significant ($p < 0.05$) for IPM.

3.3 Finite dose *in vitro* skin permeation and distribution studies

The findings of the infinite dose permeation and distribution studies showed that heat and EtOH enhanced isotretinoin delivery into the receiver fluid the most and that heat with PGML produced the highest drug tissue concentrations. A combination of both EtOH: PGML in 1:1 ratio was selected as a vehicle in an attempt to provide optimal isotretinoin delivery under finite

dose conditions with heat. Figure 4(A) illustrates the percentage of the applied dose of isotretinoin (0.05 % w/v) recovered from the skin tissue (SC + epidermis + dermis) and receiver fluid at 32 °C and 45 °C using an EtOH: PGML (1:1) binary system.

With the application of heat the total amount of isotretinoin recovered was enhanced significantly by 2.03 fold to 65.40 % of the applied dose. Also, at the higher temperature there appeared to be a change in drug distribution within the skin after 24 hours. In particular significantly larger amounts of isotretinoin were delivered into the dermis (3.67-fold), epidermis (2.33-fold) and receiver fluid (1.85-fold), whilst the quantity measured within the SC was significantly reduced 1.76 fold.

The permeation profiles for isotretinoin at both temperatures are shown in Figure 4B. At 32 °C, isotretinoin was only detected in the receiver fluid from 18 hours onwards, after which the amount permeated continuously increased up to 24 hours. The transport of isotretinoin into the receiver fluid after 24 hours was significantly increased 1.85 fold at the higher temperature but notably time taken for isotretinoin to be detected in the receiver fluid was considerably reduced to only 1 hour after the experiment started.

3.4 Heat profiles generated from the sodium thiosulfate pentahydrate (ST) prototype heating system

Both the infinite and finite dosing experiments indicated that the use of heat was extremely promising for improving the delivery of isotretinoin to the skin. However, the model system used thus far for increasing the skin temperature which used a water bath is not truly representative of what would occur in a clinical application scenario. To address this a more clinically relevant model heating system was developed that would allow the application of an externally applied heat for a practically feasible duration of time. This heating system utilised heat from the recrystallization of sodium thiosulfate pentahydrate. The temperature profiles generated from 2.5 mL (ST 20) and 5.0 mL (ST 30) of ST solutions and their effects on membrane surface and receiver fluid temperatures were recorded over a 30 min period after

crystallization was initiated with a nucleating agent and are shown in Figures 5(A) and 5 (B). The plots show that the heating systems were able to rapidly increase the temperature of the skin and create a thermal gradient where the surface of the membrane is at a higher temperature than the underlying receiver fluid. The maximum temperature ($T_{\max}$), time to maximum temperature ($t_{\max}$) and duration (DUR) above 32 °C (i.e., external surface temperature of human skin) were recorded for each volume of ST solution and are shown in Table 3. For ST 20, a $T_{\max}$ of 47.73 ± 0.10 °C was generated with a $t_{\max}$ of 2.33 ± 0.33 min within the heating system Figure 5(A). On the membrane surface and in the receiver fluid, the application of ST 20 produced a $T_{\max}$ of 42.77 ± 0.50 °C and 41.20 ± 0.15 °C respectively. With the application of ST 20 the temperature remained above 32 °C (DUR) for 15.33 ± 1.45 min within the heating system and for 19.33 ± 0.88 min on the membrane surface. For ST 30, a $T_{\max}$ of 48.43 ± 0.95 °C was produced within the heating system with a $t_{\max}$ of 3.33 ± 0.88 min Figure 5(B). Additionally, with the application of ST 30 a $T_{\max}$ = 44.07 ± 0.27 °C and 42.40 ± 0.12 °C was produced on the membrane surface and in the receiver fluid respectively. The temperature remained above 32 °C (DUR) for 25.67 ± 1.20 min within the heating system and 27.67 ± 0.33 min on the membrane surface with ST 30.

3.5 The effect of local heating on the uptake of isotretinoin into the hair follicles, skin tissue and receiver fluid

The effect of using ST solutions to generate short bursts of localized heat (~ 43 - 44 °C for ca. 20 and 30 min), water bath heating (45 °C) and no 'additional' heat (32 °C) on the delivery of isotretinoin into the hair follicles, skin tissue (SC, epidermis and dermis) and receiver fluid was then investigated. The previous finite dose experiment found that 0.05% w/v isotretinoin in a mixed EtOH: PGML 1:1 binary system was effective in working with heat. To improve delivery from this vehicle further, the thermodynamic activity of isotretinoin was optimised using a filtered saturated solution prepared at 32 °C which had an isotretinoin concentration of 1.38% w/v. The finite dosing results with the 0.05% w/v isotretinoin system indicated that with the use of heat isotretinoin could permeate rapidly across the skin and could be detected in the

receiver fluid after as short a time period as 1 hour. This was hypothesised to be linked to heat promoting delivery through the follicular pathway, as this route is known to provide rapid absorption across skin [18, 19]. To assess the impact heat was having specifically on the follicular pathway the cyanoacrylate biopsy was used and additional experiments were performed for only one hour. This allowed isotretinoin distribution in the skin to be assesssed at an early time points to gain a better understanding of the permeation process under the influence of external heat.

The distribution of isotretinoin in hair follicles, skin tissue (SC, epidermis and dermis) and receiver fluid after 24 hours following application of the 1.38% w/v isotretinoin formulation under different heating conditions as a percentage of the applied dose is shown in Figure 6(A). In this experiment all of the different heating conditions employed significantly increased the total amount of isotretinoin delivered compared to the control 32 °C experiment and there was a trend of improved drug delivery with increased duration of heating. Both of the localised external heating systems provided a significant increase in overall isotretinoin delivery (the sum of delivery to the SC, epidermis, hair follicles, dermis and receiver fluid) compared to the control; in the case of ST 30 this was 1.3 fold. This indicates that even short durations of externally applied heat are effective increasing total drug delivery over 24 hours.

The application of heat was found to alter the distribution of isotretinoin across the different skin layers, hair follicles and receiver fluid. The amount of drug recovered specifically from the hair follicles was significantly enhanced 1.3, 1.4 and 1.4 fold with ST 20, complete system heating and ST 30 respectively compared to the control (32 °C). Likewise, the dermis and receiver fluid concentrations were significantly enhanced with heating whereas in contrast, additional heat did not significantly increase the amount of isotretinoin recovered from either the epidermis or SC.

The influence of ca. 30 minutes of localized heat (ST 30) on drug uptake into the hair follicles, skin tissue and receiver fluid were studied over a shorter period (1 hr) to provide a better

understanding of the potential role of hair follicles in drug transport with the application of external heat. The ST 30 heating system was selected because it increased the delivery of isotretinoin to a greater extent than ST 20 over 24 hours, and so it was believed that the difference between the heated and control (32 °C, no additional heat) systems would be easier to distinguish.

In this 1 hour experiment, the application of localised heat was effective, as the total amount of isotretinoin delivered was enhanced 2.2 fold to 58.5 ± 3.6 μg/cm^2 (42.4 % of the applied dose). As was previously seen in the 24 hour experiment, the increase in isotretinoin delivery to the deeper layers was greater than that of the SC with delivery into the epidermis, hair follicles, dermis and receiver fluid being enhanced significantly, 2.3 fold, 2.1 fold, 3.2 fold and 4.2 fold respectively. There was no significant change in the SC isotretinoin content. Comparing between the 1 hour and 24 hour experiments with the application of localized heat for approximately 30 minutes (ST 30), significantly more isotretinoin was recovered from the hair follicles in the 1 hour study (1.4-fold) in comparison to the equivalent 24 hour study. In contrast the amount of isotretinoin in the SC was not significantly increased.

4 Discussion

The aim of this study was to identify whether heat could improve the delivery of isotretinoin into human skin and if successful whether such an approach could be effective over a sufficiently short period of time to suggest that it could be clinically and therefore commercially relevant. Initially infinite dose Franz cell studies with isotretinoin in a range of chemical penetration enhancers were performed at 32 °C and 45 °C to identify which of these would work best with heat and to start to understand the mechanism through which two penetration enhancement approaches can work together increase drug delivery across skin. The higher temperature that was used, 45 °C has been widely used as a relatively high temperature that helps to elucidate the effects of heat without damaging the stratum corneum, the rate limiting barrier to drug permeation across the skin [4, 7, 11, 12].

The chemical penetration enhancers varied in their ability to work with heat, at the higher temperature PGML provided the greatest total amount of drug delivery to the skin although ethanol provided higher concentrations in the receiver fluid. In agreement with previous work which found PG to be a good enhancer for isotretinoin, PG provided the highest isotretinoin permeation from the enhancers tested here at 32 °C [20]. However, the delivery of isotretinoin from PG was not significantly increased at the higher temperature, highlighting the importance of selecting solvent systems that work well with heat to obtain penetration enhancement [11]. The ability of different chemical penetration enhancers to work well with heat is relatively uninvestigated and the delivery provided by some of the enhancer systems here is considerably larger than heat alone has been typically reported to achieve [21]. This is likely to relate to the different mechanisms through which these enhancers exert their effects, and how these mechanisms can work in conjunction with heat to increase drug permeation across the skin. For example although both ethanol and propylene glycol are thought to improve drug solubility in the stratum corneum, ethanol is also believed to have a capability to interact with and extract SC lipids [22]. Hydrophobic esters such as IPM and PGML which were found here to work well with heat as penetration enhancers here are thought to be taken up in to the SC lipids and perturb their packing [23]. These different mechanisms would not be expected to respond similarly in the presence of heat.

In an attempt to understand how heat improved delivery across the skin the infinite dose permeation profiles were modelled using Fick's first law to obtain pathlength normalised diffusion and partition coefficients. For the infinite dose experiment saturated suspensions were employed to ensure the thermodynamic activity of the penetrant in the donor chamber remained the same at both temperatures investigated (32 °C and 45 °C), as the skin permeation process is known to be dependent on the thermodynamic activity of the drug in the vehicle rather than the its concentration [24]. Previously emphasis has often been placed on the effect of temperature changing the SC lipid structure from the orthorhombic to hexagonal packing,

rendering the lipids more fluid [25]. This effect was expected to be observed through higher diffusion coefficients of permeants across the skin. The increases in steady state isotretinoin flux observed here with heat were predominately associated with improvements in drug partitioning into the skin. Altering lipid structure would also be expected to affect partitioning behaviour, and the variation in the ability of different CPEs to increase permeation may potentially relate to the ability of heat to increase the uptake of the CPE into the skin [26]. In particular the CPEs that performed well here are thought to also interact with the SC lipids and it's possible that these mechanisms are able to work synergistically together. As the vehicles were saturated with isotretinoin at the different temperatures, the isotretinoin concentration was higher in the vehicles at the higher temperature. However, the increases in drug transport across the skin were considerably greater than the increases in isotretinoin solubility at the higher temperature indicating that this cannot explain the increased in drug transport into and across the skin. This analysis relates to drug flux at 'steady state' using Fick's first law of diffusion, however it was evident from the data, that heat did have a significant effect on drug absorption at early time points, yet this did not correlate with significantly shorter lag times from the Fickian analysis. Drug transport across the skin is complex with different diffusion pathways available including the follicular and intercellular pathways. Fickian analysis, although proven to provide useful insight into skin permeation processes, has limitations to explaining fully the transport of drugs across the skin when drug transport can occur via different routes [27]. The pronounced effect of heat on isotretinoin permeation at early time points is likely to be as a result of increased follicular transport across the skin, which is known to be rapid. At steady state, transport across the intercellular pathway of the continuous SC is likely to predominate instead [19].

Initially finite does experiments were performed at 32 °C and 45 °C using a concentration of 0.05% w/v isotretinoin which is similar to that used in commercial formulations, but is considerably below the saturated solubility of the drug in the 1:1 EtOH:PGML vehicle that was

used and so drug permeation would be expected to be lower than that of the infinite dose study. The rapid delivery of isotretinoin into the receiver fluid from the 45 °C experiment suggested that heat facilitated drug transport from the finite doses occurred in particular via the follicular route, producing higher isotretinoin permeation and skin tissue concentrations. Moreover, similar to the findings of Caserta et al (2019) the effect of heat on improving delivery overcame any effect of heat on reducing of drug saturation in the vehicle at the higher temperature which would be expected to reduce drug permeation. These promising data provided the basis for selecting a vehicle to be used with a more clinically relevant heating system that would be applied to the outer surface of the skin, increasing the skin temperature for relatively short and practically feasible durations. Currently there are no regulatory limits governing the temperatures produced by heat generating delivery systems, however both the skin temperature produced and the duration this is held for are known to be important safety considerations [28]. For example thermal injury to the skin occurs rapidly after 3 seconds at a temperature of 60°C. [28]. At lower temperatures such as 44°C thermal injury can still occur, but the skin must be exposed to the elevated temperature for longer than 5 hours can for this to happen. Discomfort in response to heat is also an important consideration, as this may discourage patients from using a heating system. Differences in the literature exist as to the temperature at which skin discomfort may be felt upon application of heat, with some reports indicating this occurs at of approximately 43 °C, and others at temperatures as high as 47.5 °C [12, 28, 29]. The prototype heating system produced maximum temperatures of 43 and 44 °C and maintained the skin temperature above ambient (32 °C) for approximately 20 and 30 minutes respectively. The actual duration the skin was at ‘high’ temperatures was less than this. For example with the skin temperature was at 40 °C or above for 10 and 15 minutes with the two heating systems respectively. Thus the heating system would not be expected to cause thermal injury and could be modified to produce lower skin temperatures if desirable. The significant penetration enhancement seen here with heat providing skin temperatures of 43 and 44 °C is indicative that increased delivery would also be likely if the heating device was modified to produce lower

temperatures. This prototype heating system employed heat from the crystallisation of a phase change material system containing sodium thiosulfate (ST), and has been characterised in more detail for the provision of heat to improve dermal absorption elsewhere [30]. Other methods of providing short durations of heat to drug containing formulations on the skin have been proposed including photothermal enhancement and have shown promise for improving permeation across the skin [31]. Such systems require a light source, which is likely to make them more expensive than chemical heating systems, however they may have specific advantages for particular applications such as being able to readily adjust the amount of heat provided.

The cyanoacrylate biopsy was performed to try to gain a better understanding of whether the drug was being transported via the hair follicles. Isotretinoin delivery to the hair follicles and deeper layers of the skin was increased to a greater extent than the continuous SC when the heating system was used. This provides strong evidence that short durations of heat in combination with particular CPEs has the ability to promote delivery preferentially via the follicular route. Previously when the cyanoacrylate biopsy was used to examine the delivery of erythromycin, into the skin no drug could be detected in the viable epidermis, though it could be detected in the deeper hair follicles and dermis [11]. This was taken to provide evidence that the drug travelled primarily into the skin via the hair follicles. In comparison to erythromycin, isotretinoin is smaller and more hydrophobic and therefore has more favourable properties for transport into the continuous SC. Following the cyanoacrylate biopsy to assay isotretinoin content in the hair follicles, isotretinoin was still found in the viable epidermis, indicating that the drug may have also traversed the continuous SC and/or diffused out of the follicular duct into the viable epidermis. Nonetheless the large increase in the amount of drug in the hair follicles and that the isotretinoin content in the SC was not increased, is indicative that the heat and chemical penetration enhancer system is effective through predominately increasing follicular delivery. The effects of heat and CPEs on increasing drug delivery into the hair

follicles could be due to increased vehicle miscibility with sebum. Some CPEs, such as IPM and TP have been shown to lower the melting points of sebum, and formulation vehicles that interact with sebum have been shown to increase the deposition of salicylic acid in hair follicles [32, 33]. The beneficial effects of heat in combination with the CPEs may relate to improving the interaction of the CPEs with sebum, increasing their uptake into the hair follicles and facilitating the drug transport process through making the sebum less viscous [34]. The insight gained here, that heat and CPEs can improve delivery across the skin via the follicular route, may also help provide mechanistic understanding into others' work. For example how photothermal enhancement in combination Tween 20 was able to provide rapid permeation of ondansetron across the skin [31].

A continuous level of heat provided by having the water bath at a higher temperature to achieve a skin temperature of 45 °C over 24 hours provided a larger amount of delivery than the heating system. This not surprising given the much longer duration at the higher temperature. A difference between using a water bath to generate heat and an external heating system is that the thermal gradient across the skin produced by the heating system has the potential to provide an additional driving force for diffusion through the Soret effect, where molecules show a drift mobility in addition to Brownian motion across a temperature gradient [21]. This effect, which is in theory directly related to the temperature difference cannot be separated from the other effects of heat on these data and therefore its magnitude on isotretinoin diffusion across skin cannot be discerned. Although use of water baths to apply additional heat is not directly equivalent to how heat may be applied in vivo, the data produced here does appear to justify their use as a model experimental setup to aid selection of vehicles that work well with heat.

Comparison between the two finite dose experiments conducted at 45 °C over 24 hours showed that a higher percentage of the applied dose was delivered from the 0.05% w/v formulation in comparison to the saturated (at 32 °C) 1.38% w/v formulation, though the 1.38% w/v formulation still delivered the largest amount of isotretinoin. Understanding such behaviour is

challenging as in addition to the saturation or thermodynamic activity of the drug being different in the two formulations, the thermodynamic activity of the enhancers are different and would also be affected by the different temperatures. This would be expected to alter their delivery and that of the drug into the skin. However similar results have been seen previously, where sub saturated systems deliver a higher percentage delivery of the applied dose [11]. It is possible that at the lower level of isotretinoin saturation, delivery of the chemical penetration enhancers is improved, increasing the percentage of the applied dose delivered [24].

In using human abdominal skin *in vitro* as a model membrane, this work may underestimate delivery via the hair follicles as the follicular reservoir is reduced to about 10 % of the follicular reservoir *in vivo* [35]. Moreover, other body regions which are relevant for acne treatment such as the face will typically have a proportionally greater surface area available for follicular drug absorption [36]. This suggests that the effect of heat and CPEs in promoting follicular drug delivery for treatment of conditions such as acne may in fact be greater that what was observed here. The human skin used in this study came from a single donor. This is preferable in formulation development to reduce inherent variability relating to the skin source allowing the influence of experimental parameters to be more easily discerned. However an understanding of the variability in response in a larger number of donors would be beneficial for understanding the variability of the effect of heat and CPEs in the general population.

5 Conclusion

Overall these data demonstrate that clinically feasible, short durations of applied heat in combination with CPEs can improve the topical delivery of drugs such as isotretinoin into the skin via the follicular route. This approach has potential to reduce the need for systemic treatment and through preferentially delivering isotretinoin via the hair follicles to the target

site, the use of short durations of heat may help to optimise therapeutic effectiveness whilst reducing the incidence of both systemic and localised side effects.

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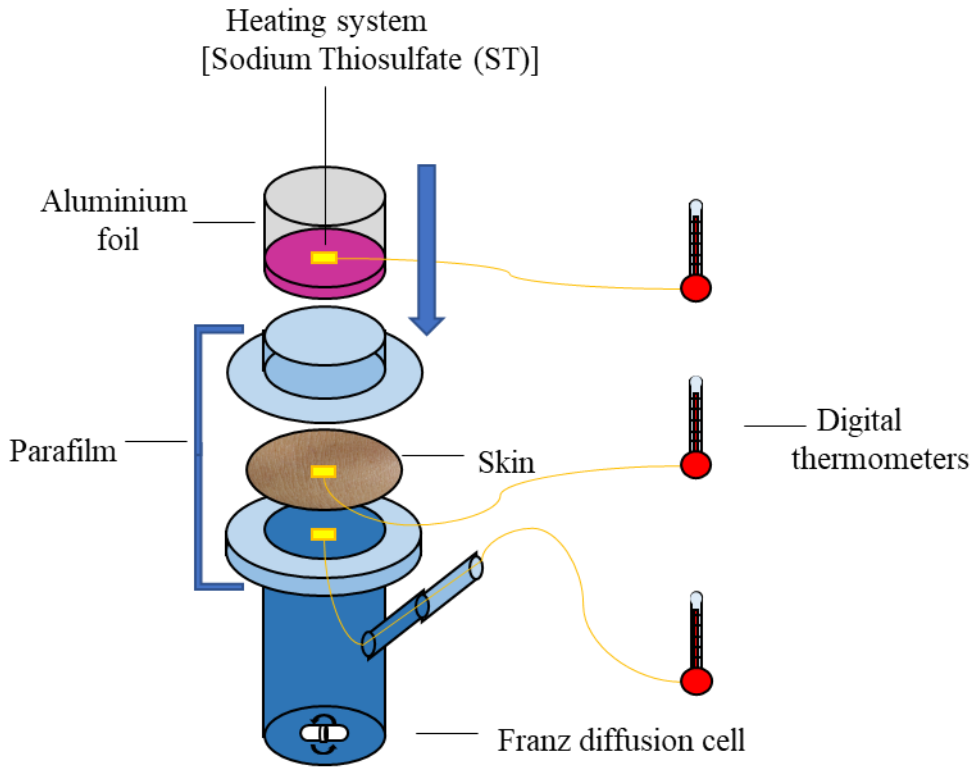

Figure 1. Diagrammatic representation of a Franz diffusion cell assembly used to assess the heating profile generated by the sodium thiosulfate (ST) heating system. The temperature changes were recorded over time in the ST solution, receiver fluid and on the skin surface.

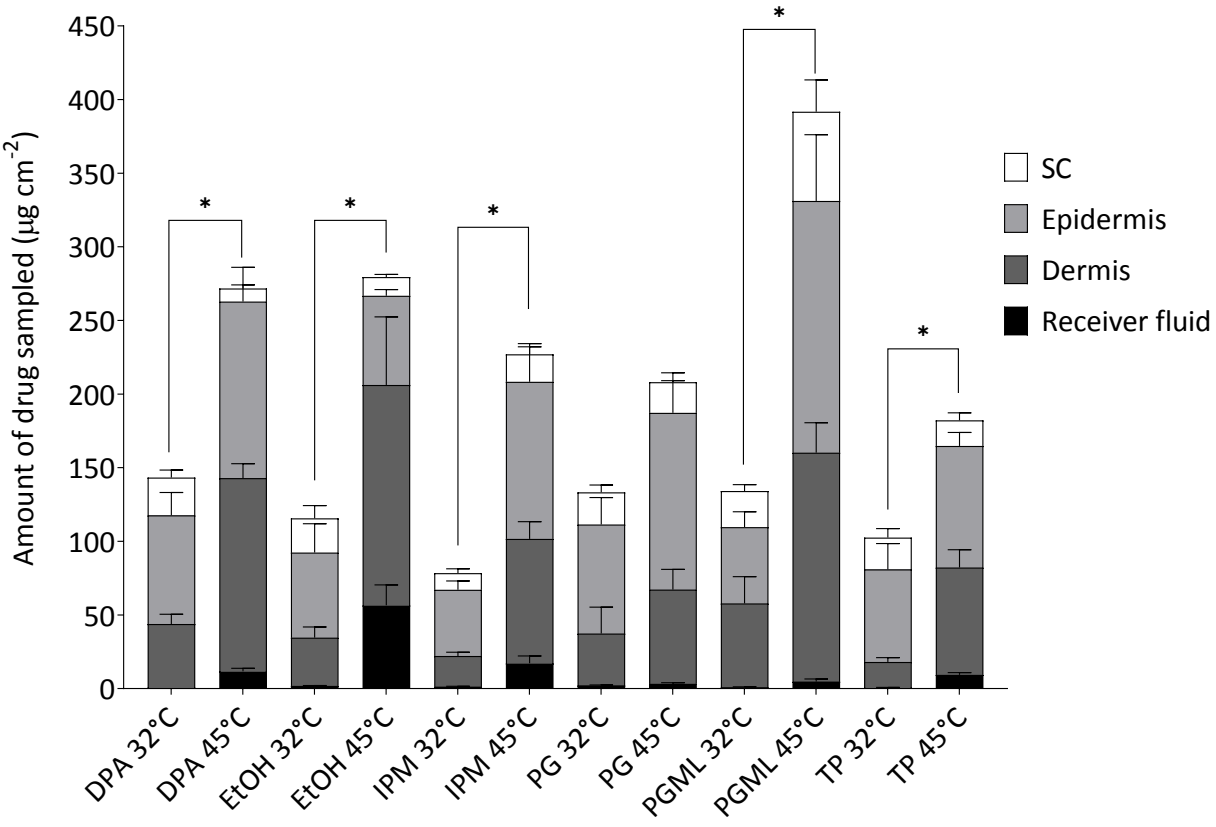

Figure 2. The total amount of isotretinoin recovered from the *stratum corneum* (SC), epidermis, dermis and receiver fluid at the end of the infinite dose permeation study at 32 °C and at 45 °C from diisopropyl adipate (DPA), ethanol (EtOH), isopropyl myristate (IPM), propylene glycol (PG), propylene glycol monolaurate (PGML) and Transcutol (TP) vehicles. * indicates significant differences in total isotretinoin delivery at 45°C. All data bars show the mean + SEM of n=5-6 diffusion cells.

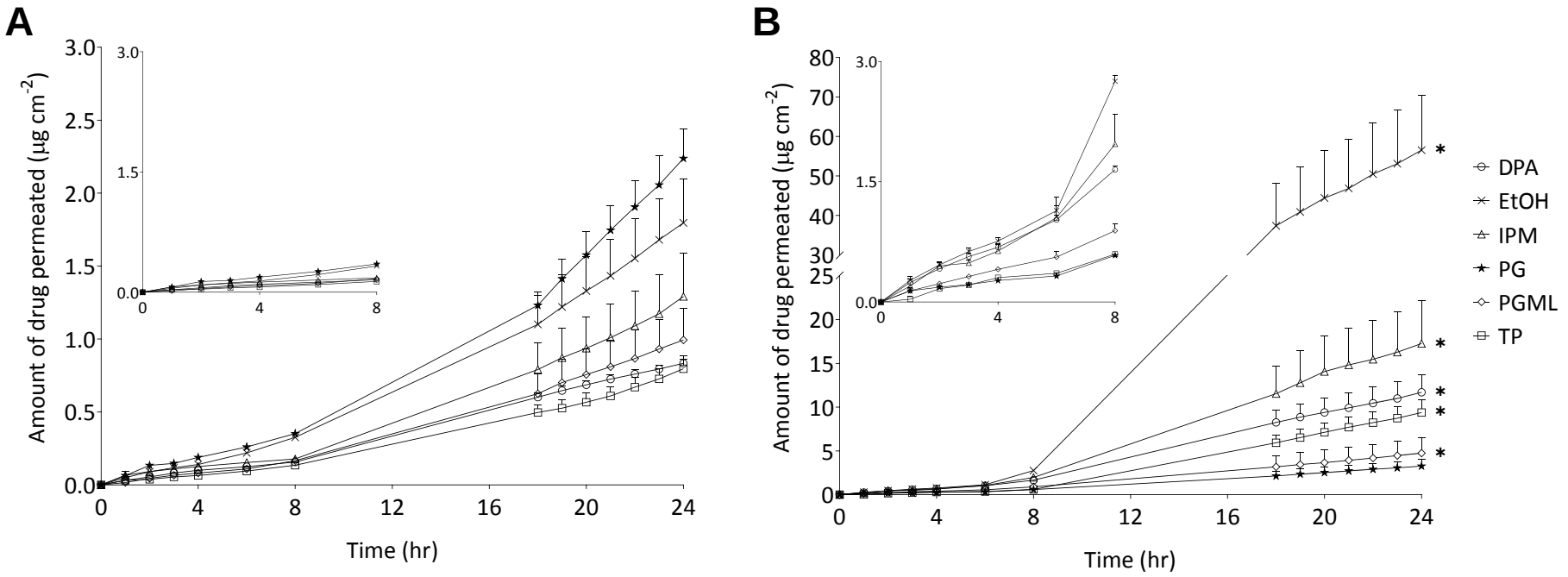

Figure 3. Mean cumulative amount of isotretinoin permeated through human abdominal skin over 24 hrs: (A) at 32 °C; (B) at 45 °C. The insets show an expanded image of the delivery between 0 to 8 hrs**.** Isotretinoin was applied (0.5 mL cm^{-2}) to the skin surface in a previously equilibrated saturated suspension for each vehicle. Vehicles tested were Diisopropyl adipate (DPA), Ethanol (EtOH), Isopropyl myristate (IPM), Propylene glycol (PG), Propylene glycol monolaurate type II (PGML), Transcutol P® (TP). * indicates significant differences in isotretinoin delivery at 45°C. All points are mean + SEM of n=5-6 diffusion cells.

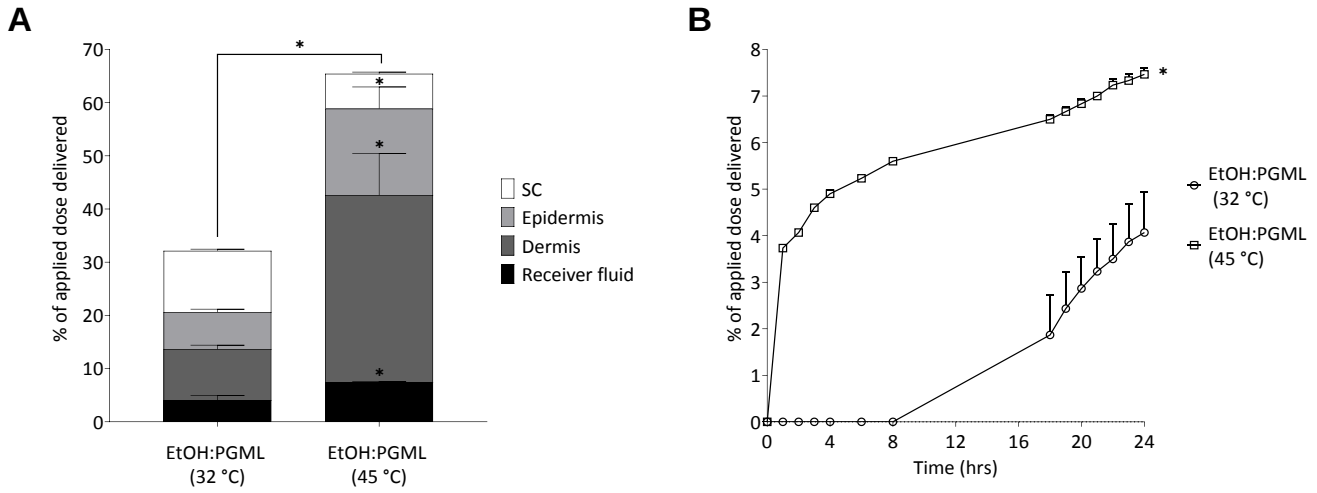

Figure 4. (A) The percentage (%) of the applied dose recovered from the SC, epidermis, dermis and receiver fluid at the end of the 24 hr finite dose permeation study at 32 °C and 45 °C from EtOH: PGML; (B) mean cumulative amount of isotretinoin permeation through human abdominal skin over 24 hr from the EtOH: PGML (1:1) binary system at 32 °C and 45 °C. * denotes significant difference in the amount of drug recovered compared to the control (32 °C, no additional heat). All points are mean + SEM of n=6 diffusion cells.

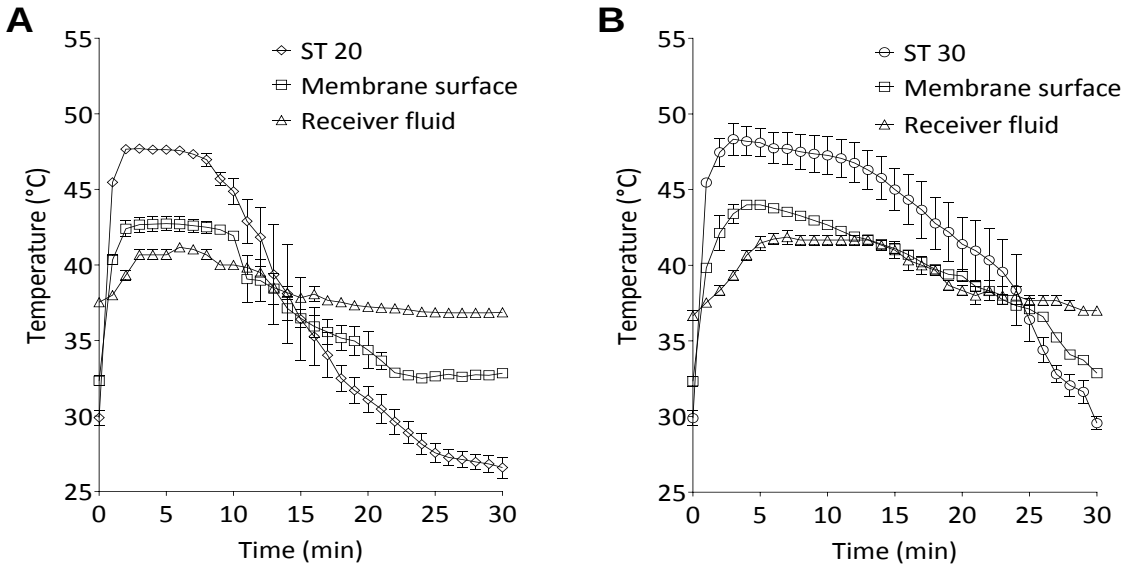

Figure 5. Heat release profiles generated from (A) 2.5 mL of sodium thiosulfate pentahydrate solution (ST 20) (B) 5 mL sodium thiosulfate pentahydrate solution (ST 30) and their effects on membrane surface and receiver fluid temperatures. Results are presented as mean ± SEM of n=3 replicates.

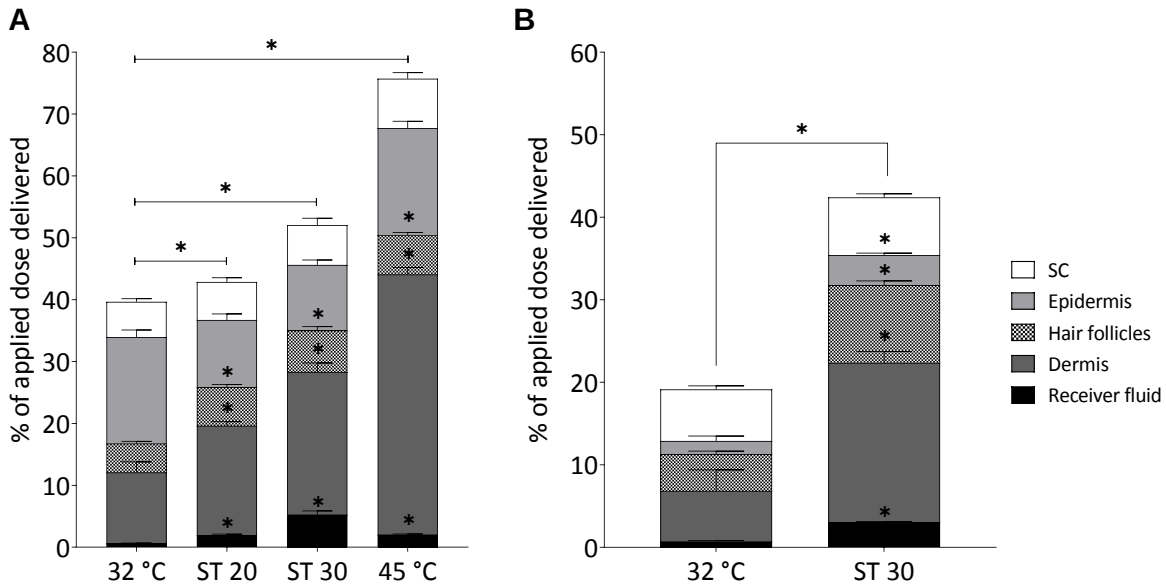

Figure 6. Dose distribution of isotretinoin across full-thickness human abdominal skin and receiver fluid at the end of (A) 24 hr permeation study and (B) 1 hr permeation study. Experiments were conducted at 32 °C with and without ST solution producing heat (~ 45 °C) for Ca. 20 & 30 mins (ST 20 & ST 30 respectively) and at 45 °C (without ST). All bars are mean + SEM of n=6 diffusion cells. * denotes significant difference in the amount of drug recovered compared to the control (32 °C, no additional heat).

Table 1: Saturated solubility of isotretinoin in various vehicles over 24 hr at 32 °C and 45 °C (the temperatures employed in the skin permeation studies). Mean ± SD (n=3).

Vehicle	Saturated solubility of isotretinoin (mg/mL)	
	32 °C	45 °C
DPA	8.42 ± 0.40	19.41 ± 1.15
EtOH	10.66 ± 0.56	33.78 ± 1.03
IPM	4.07 ± 0.80	14.74 ± 1.65
PG	0.71 ± 0.15	3.29 ± 0.63
PGML	10.71 ± 0.65	25.81 ± 1.57
TP	27.63 ± 0.93	58.91 ± 1.21

Table 2: Skin permeation parameters measured for isotretinoin in different dermatological formulations. Cumulative amount permeated (Q_{24}), flux between 18 and 24 hours (J_{18-24}), pathlength normalized partition coefficient (*Kh*), pathlength normalized diffusion coefficient (D/h^2) were measured for both physiological skin temperature (32 °C) and elevated skin temperature (45 °C) using Fick's first law (mean ± SEM, n=6). * denotes significant difference in the parameter at 45 °C for each vehicle compared to the same respective vehicle at 32 °C.

Vehicle	***32 °C***			***45 °C***					
	J_{18-24} (µg/cm^2/hr)	D/h^2(x10^{-2}hr^1)	*Kh* (cm)	J_{18-24} (µg/cm^2/hr)	D/h^2(x10^{-2}hr^1)	*Kh* (cm)	$E_R\, J_{18-24}$	E_R D/h^2	E_R *Kh*
DPA	0.038 ± 0.001	3.35 ± 0.48	1.14 ± 0.08	0.608 ± 0.106*	3.96 ± 0.29	15.46 ± 2.13*	16.00	1.18	13.56
EtOH	0.115 ± 0.020	2.06 ± 0.13	5.59 ± 0.91	3.140 ± 0.557*	2.24 ± 0.10	140.03 ± 32.04*	27.30	1.09	25.05
IPM	0.081 ± 0.018	1.94 ± 0.52	4.17 ± 0.16	0.915 ± 0.267*	3.21 ± 0.31*	28.52 ± 2.96*	11.29	1.65	6.84
PG	0.165 ± 0.022	2.10 ± 0.27	7.94 ± 1.49	0.183 ± 0.039	2.18 ± 0.18	8.47 ± 1.01	1.11	1.04	1.07
PGML	0.060 ± 0.013	2.05 ± 0.18	2.93 ± 0.33	0.258 ± 0.078*	2.47 ± 0.12	10.49 ± 2.24*	4.30	1.20	3.58
TP	0.050 ± 0.008	2.04 ± 0.12	2.45 ± 0.43	0.569 ± 0.100*	2.41 ± 0.16	23.62 ± 3.54*	11.38	1.18	9.64

$E_R\, J_{18-24}$ = J_{18-24} at 45 °C / J_{18-24} at 32 °C

E_R D/h^2 = D/h^2 at 45 °C / D/h^2 at 32 °C

E_R *Kh* = *Kh* at 45 °C / *Kh* at 32 °C

Table 3: The maximum temperature ($\boldsymbol{T_{max}}$), time to maximum temperature ($\boldsymbol{t_{max}}$) and duration (**DUR**) above 32 °C (i.e., external surface temperature of human skin) recorded for 2.5 mL (ST 20) and 5.0 mL (ST 30) of ST solution.

Thermal performance	ST solution 2.5 mL (ST 20)			ST solution 5.0 mL (ST 30)		
	Heating system	Membrane surface	Receiver fluid	Heating system	Membrane surface	Receiver fluid
T_{max} (°C)	47.73 ± 0.09	42.77 ± 0.50	41.20 ± 0.15	48.43 ± 0.95	44.07 ± 0.27	42.40 ± 0.12
t_{max} (min)	2.33 ± 0.33	3.33 ± 0.67	5.33 ± 1.20	3.33 ± 0.88	4.00 ± 0.58	6.00 ± 0.58
DUR (min)	15.33 ± 1.45	19.33 ± 0.88	N/A	25.67 ± 1.20	27.67 ± 0.33	N/A