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Mechanistic insight into heat enhanced permeation of diclofenac and piroxicam in combination with chemical penetration enhancers across skin

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ABSTRACT

The topical application of heat offers considerable potential for enhancing the delivery of non-steroidal anti-inflammatory drugs across the skin barrier. A better understanding of the mechanisms underpinning the improved skin permeation and how heat can be best used to work with complementary enhancement strategies would help to realise this potential. In this study the effect of heat on the permeation of diclofenac and piroxicam across different membranes, including human skin was investigated along with use of complementary enhancement strategies including selection of formulation pH, drug salt form and inclusion of chemical penetration enhancers. Heat alone improved drug delivery across human skin for both drugs, with larger increases for piroxicam. This increase was produced by improvements in drug release, molecular diffusivity and partitioning into the stratum corneum. In combination with chemical penetration enhancers, heat synergistically increased the skin permeation of diclofenac and piroxicam up to 13 and 40-fold respectively, with the increase in permeation being ascribed primarily to improvements in drug and enhancer partitioning into the stratum corneum. An Arrhenius plot of diclofenac permeation across skin was linear indicating that the orthorhombic to hexagonal stratum corneum lipid packing transition did not have a significant effect on skin permeation in response to heat.

1. Introduction

The use of externally applied heat offers considerable potential for enhancing the delivery of drugs across skin. In particular, it offers an exciting opportunity for improving the delivery of non-steroidal anti-inflammatory drugs (NSAIDs) as the application of heat alone may also be beneficial for treating conditions that topical NSAIDS are commonly used for, such as musculoskeletal pain (Clijsen et al., 2022).

Heat has been shown to be effective at enhancing the skin permeation of a wide range of drugs; however, the mechanisms through which it exerts action have not been fully elucidated (McAuley and Caserta, 2015). Considerable attention has been placed on the effect of heat changing the SC lipid structure from orthorhombic to hexagonal packing at approximately 40 °C (Boncheva et al., 2008). As the lipid chains in the hexagonal arrangement are less densely packed, drug transport across the membrane is expected to increase in comparison to when the lipids are in an orthorhombic state (Pilgram et al., 1999). Some studies however have not observed an alteration of SC barrier properties associated with temperature increases above this transition (Ogiso et al., 1998; Groen et al., 2011). Other mechanisms through which heat is likely to increase drug permeation across the SC are through increasing drug molecular mobility (in accordance with the Stokes–Einstein equation) in both the formulation and the skin, along with improved drug partitioning into the stratum corneum (Akomeah et al., 2004).

A fundamental understanding of heat enhanced permeation of drugs across the skin and how it can work with other formulation strategies will aid the development of improved drug delivery systems that could provide benefits over the current available approaches. A number of different NSAIDs are available for topical use and formulation parameters such as pH and the presence of chemical penetration enhancers (CPEs) are known to affect absorption across skin (Hadgraft et al., 2000; Ma et al., 2010; Herkenne et al., 2008). Systematic reviews of clinical trials of topical NSAIDs have demonstrated the efficacy of several of these products and their relative lack of side effects (Derry et al., 2017). However, they have also highlighted the critical function of their formulation on efficacy and the variability that exists between products.

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Moreover, despite the wide range of formulation approaches that have been investigated to improve the delivery of NSAIDs such as diclofenac across the skin, the amount that penetrates is typically low (Goh and Lane, 2014). Piroxicam and diclofenac are two clinically effective NSAIDS that have similar molecular weights but different lipophilicities, with log P values of 1.2 and 4.3 respectively (Wenkers and Lippold, 1999; Pénzes et al., 2005). In this work, the effect of heat and formulation parameters on the permeation of piroxicam and diclofenac, across human skin has been examined. Moreover, mechanistic studies were conducted with diclofenac to provide a greater understanding of how heat affects the dermal delivery process and to elucidate how heat can be utilised alongside other formulation approaches to improve drug permeation across skin.

2. Materials and methods

2.1. Materials

Diclofenac diethylammonium salt (DDEA) (BP grade), diclofenac free acid (BP grade) and piroxicam (BP grade) were a gift from MedPharm Ltd (Guildford, UK). Methanol (99.9 %), acetonitrile (99.9 %), absolute ethanol (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 ammonium formate (99 %) were supplied by Fisher Scientific (Loughborough, UK). Transcutol (TC) (99.9 %) was provided by Gattefossé (France). Regenerated cellulose dialysis tubing with molecular weight cut off of 12–14 KDa was purchased from Medicell International (UK).

2.2. High-performance liquid chromatography (HPLC)

Quantitative analysis of diclofenac and piroxicam 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. For diclofenac, an isocratic mobile phase of 35: 65 ammonium formate (20 mM) in water (adjusted to pH 2.5, using formic acid): ammonium formate (20 mM) in methanol was used. The UV detection wavelength, flow rate and injection volume were 280 nm 1.0 mL/min and 20 μL, respectively. Similarly, the analysis of piroxicam carried out isocratically (35: 65 PBS (20 mM, adjusted to pH 2.5, using phosphoric acid: acetonitrile) setting UV detector wavelength, flow rate and injection volume at 355 nm, 1.0 mL/min and 20 μL, respectively. Under these conditions diclofenac and piroxicam showed retention times of approximately 14 and 11 min respectively. The HPLC methods were validated for linearity, precision and accuracy according to the current ICH guidelines (Q2A, 1995; Q2B, 1997). The calibration curves produced for diclofenac and piroxicam were linear over the concentration range 0.1 – 100 μg/ml, with a coefficient of determination (r2) of 0.99998 and 0.99996 respectively. The limits of detection (LOD) and quantification (LOQ) were 0.51 and 1.54 μg/mL respectively for diclofenac and 0.72 and 2.19 μg/mL respectively for piroxicam. Both assays were shown to be 'fit for purpose' in accuracy (100 ± 2%) and precision (CV < 2.0 %) meeting the standards described by the ICH guidelines.

2.3. Skin tissue 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). 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). Subcutaneous fat was carefully removed prior to use. Post-mortem full-thickness dorsal skin was obtained from three month-old Yorkshire Large White cross pigs (Matrix Biologicals, Yorkshire, U.K.). Porcine skin was clipped and defatted. Both pig and human skin samples were stored at −20 °C prior to use.

2.4. Preparation of donor suspensions

All in vitro permeation studies were conducted using saturated solutions of the model drugs at the temperature the experiment was performed in order to provide a constant drug thermodynamic activity in the vehicles at the respective temperatures. Suspensions of diclofenac and piroxicam were prepared by adding an excess of each individual drug to the vehicle. The suspensions were then allowed to stir for 24 h at the temperature of the membrane it was applied to. The temperatures used in this study were in the range of 28 to 45 °C. The pH of the suspensions were confirmed pre- and post-saturation.

2.5. Drug saturated solubility determination

The saturated solubility of both drugs in a range of vehicles was determined using HPLC. An excess of each drug was added to vials containing the relevant solvent and the samples were stirred for 24 h in a water bath at the temperature of interest, following the formation of a drug suspension. 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.

2.6. Drug release and skin permeation studies

Drug release and skin permeation experiments were carried out using individually calibrated unjacketed upright Franz type diffusion cells with an average receiver volume of 3.0 mL and an average surface area of 1.0 cm^2. For drug release experiments, porous regenerated cellulose membrane (RCM), which was pre-soaked at 70 °C for 60 min in deionised water, was used as a release membrane. For skin permeation experiments human skin from both the abdomen and the scrotum and pig skin were used. With scrotal skin, where more than one skin donor was required, skin was allocated from several donors across the experiments so that introduction of bias was avoided. Human abdominal skin was from a single donor. Each membrane, whether RCM or skin was cut and placed between the donor and the receptor compartments of each cell. The receptor compartments were filled with 80: 20 PBS: EtOH (pH 7.4) and the cell placed on a stirring plate immersed in a waterbath (Grant Instruments, UK). This receiver fluid was selected to provide sink conditions and suitable drug stability for the duration of the experiments. A small magnetic bar was inserted into each receptor compartment, to enable continuous stirring during the duration of the experiment. Franz cells were occluded and immersed in a waterbath (Grant Instruments, UK) which was set up such that pre-determined membrane experimental temperatures (28, 32, 35, 38, 41, 43 and 45 °C) were reached and maintained for the course of the experiments. For drug release experiments this was for 4 h whereas skin permeation experiments were run for either 34 or 48 h. These temperatures were monitored throughout the duration of the study using a digital thermometer, with a type K probe (Fisher Scientific, UK). After allowing the membrane to equilibrate with the receiver fluid for 0.5 h, 1 mL of the saturated drug containing solution of interest was introduced into the donor chamber. At pre-determined time intervals, 200 μL of receiver fluid was withdrawn and immediately replaced by an equivalent volume of receptor solution, which was equilibrated at the required temperature. The samples were then analysed by HPLC.

2.7. ATR-FTIR spectroscopy

ATR-FTIR spectroscopy was performed using a PerkinElmer Frontier FT-IR spectrometer equipped with VeeMAX III ATR accessory (Pike Technologies, UK). Small sections of skin were cut to an average surface

area of 1cm^2 and were cleaned using a cotton swab, that had been soaked in hexane in order to remove any potential sebum present on the surface of the skin (Nordstrom et al., 1986). Spectra of the skin samples were collected by placing them, SC side-down, on a zinc selenide crystal, the temperature of which was increased from 28 to 60 °C at a rate of 15 °C per hour. Spectra were collected between 4500 and 650 cm^{-1} with a resolution of 2 cm^{-1} and each spectrum was an average of 16 accumulations taken every 2 °C. The second derivative of within the CH_2 scissoring region (1480 cm^{-1} and 1460 cm^{-1}) was calculated using Spectrum software (PerkinElmer, UK). The bandwidths of the peak within the CH_2 scissoring region were then calculated, taking the values at 50 % of the peak height ($BW_{50\ \%}$), from the second derivative spectra that had been normalised to identical maxima and minima, within the CH_2 scissoring region (Boncheva et al., 2008).

2.8. Factorial experimental design

Minitab software (version 15.0; Minitab Inc., State College, PA, USA) was used to set two individual 2-level full factorial designs with the aim of identifying the effects of the independent variables skin temperature, the presence of EtOH and TC, vehicle pH and the use of active in salt form (diclofenac diethylamine), on the cumulative amount (Q) of piroxicam (factorial design 1) and diclofenac (factorial design 2) permeated over 34 h. The chosen levels for the experimental input variables are reported in Table 1.

The low and high levels of skin temperature of 32 and 45 °C were set as normal skin temperature and a high but physiologically tolerable skin temperature respectively, similarly the high and low formulation vehicle pH values were selected to be physiologically appropriate and allow investigation of NSAID ionisation state on permeation. The high level of the chemical penetration enhancers was set at 50 % as this enabled the effects of individual enhancers and their combination to be assessed. According to the rule K^n, where n is the number of factors being investigated at K different levels (high and low, Table 1), the factorial design for piroxicam generated 16 runs. Each run shows the conditions to be employed in the in vitro diffusion studies, such as skin temperature, pH and composition of piroxicam donor solutions. The response (Q_{34h}) was then measured and assigned to the corresponding experimental run generated from the factorial design.

Since in the case of diclofenac, five parameters were selected, the second factorial design generated 32 runs. Six different human scrotum skin donors were used during the diclofenac diffusion studies and four different donors were used for the piroxicam diffusion studies. Skin samples from the different donors were assigned randomly to the experimental runs. The variability between each donor was measured, by performing the centre point of the experimental design, using skin tissue from each of the donors. The given centre point was automatically calculated by the programme as the median; between the lowest and highest value for each factor. In this factorial design the centre point experiment was a saturated solution of either diclofenac or piroxicam in a formulation vehicle consisting of 25 % EtOH, 25 % TC and 50% pH 5.5 buffer and was run at a membrane temperature of 38.5 °C. Individual experimental runs, set variables in accordance with the factorial design, e.g. when all parameters were at the low level, the formulation consisted of a saturated solution of diclofenac or piroxicam in pH 3.5 buffer with 0 % EtOH, 0 % TC and was run at a membrane temperature of 32 °C. In contrast when all parameters were at the high level, the formulation consisted of a saturated solution of diclofenac or piroxicam in 50 % EtOH, 50 % TC adjusted to pH 7.5 with a low volume of acid/base and was run at a membrane temperature of 45 °C.

Table 1
Experimental input variables.

Parameters	Low level	High level
Skin temperature (°C)	32	45
pH of the vehicle	3.5	7.5
EtOH (%)	0	50
TC (%)	0	50
Use of diclofenac diethylamine (DDEA)*	0	100

* Only in the case of diclofenac).

2.9. Data modelling and statistics

To model in vitro experimental permeation data so that a mechanistic evaluation could be made on how the application of heat enhances percutaneous absorption, Fick's first law was used. Eq. (1).

$$J = \frac{D \times K \times C_V}{h} \tag{1}$$

According to this equation the flux of a drug across the membrane under steady-state conditions (J) is directly proportional to the partition (K) and diffusion (D) coefficient of the compound. Since the J and the lag time, T_L can be readily determined from the diffusion profile "cumulative mass permeated per unit area (Q) vs. time (t)", as the gradient and *x*-intercept of the linear portion of the graph respectively, K and D can in theory be obtained. However, as the diffusional pathlength across the SC is not known, these instead must be calculated as pathlength normalised values (D/h^2 and Kh) as shown in Eqs. (2) and (3). The Franz cell experiments using skin tissue were run for either 34 or 48 h to ensure steady-state drug flux was reached during the duration of the experiment, which is achieved typically at 2.7 times T_L, avoiding inaccuracies in the determination of Kh and D/h^2 (Crank, 1975).

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

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

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

The use of C_V in calculating Kh was expected to affect calculation of the Kh values because of altered drug solubility in the vehicles at the higher temperature. The use of C_V in Fick's law is common, however it is the thermodynamic activity of the drug in the vehicle/formulation that is more appropriately fully accounts for the diffusional gradient across the skin, (Higuchi, 1960). It was therefore decided to use the thermodynamic activity (α) of the drugs 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) (Farah et al., 2019).

Prior to the analysis of the factorial design, the normality (Shapiro-Wilk test) and homogeneity (Levene's test) of variance of skin permeation data was checked using Minitab software. As the data was found to violate the assumption of normality, a logarithmic (Log10) data transformation was carried out in order to transform the data from a non-normal distribution into a normal distribution (Lewis et al., 1998). Factorial ANOVA (analysis of variance) was performed to identify the most significant factor affecting the response, Log10 of Q_{34h} (of either diclofenac or piroxicam). The chosen level of significance was $p \leq 0.05$. Following the factorial designs analysis, further Franz cells experiments were performed. In this case, the flux (J), pathlength normalised values of partition (Kh) and diffusion (D/h^2) coefficients of diclofenac and piroxicam were compared using the *t*-test or Mann-Whitney (when data were found to be not normally distributed). The enhancement ratios (ER) were calculated by dividing each skin parameter (i.e. Kh, D/h^2 and J) of diclofenac and piroxicam determined following the application of all the saturated solutions tested here, at 32 and 45°C, with respect to the control (PBS, pH 7.5 at 32°C) as shown in Eq. (5):

$$ER = \frac{P(E)}{P(C)} \quad (5)$$

where P (E) and P (C) are the specific skin parameter of the drug (i.e. Kh, D/h^2 and J) measured when using enhancement strategies (i.e. heat, CPEs and heat-CPEs) and control (no heat and CPEs) respectively.

3. Results

3.1. Factorial experimental design: screening factors

The influence of temperature, use of the drug in a salt form, pH of the formulation and CPEs, on skin permeation of piroxicam and diclofenac were investigated through a factorial experimental design. Each parameter was studied at a low and high level, with a centre point using parameters midway between each of the factors also being run and the effect of any given parameter was determined as the average variation in the response associated with a change from the low to the high level of that factor.

Permeation profiles for typical experiments in the factorial design are shown in Figs. 1 and 2 for diclofenac and piroxicam respectively. The experiments were run for 34 h to confirm that the permeation followed expected infinite dose profiles and that steady state flux had been achieved. Fig. 1 shows that the permeation of diclofenac is dependent on the temperature of the skin, with the cumulative amount of drug permeating increasing from 32 to 45 °C from a EtOH: buffer (50:50), pH 3.5 vehicle. Similarly Fig. 2 shows that piroxicam permeation is greatly enhanced at the higher temperature from a saturated solution of EtOH: TC (50:50), pH 7.5 at 45 °C (high level, Table 1) compared to 32 °C and to the centre point of the factorial design (saturated solution of piroxicam in EtOH: TC: PBS (25:25:50)), 38.5 °C and pH 5.5).

Figs. 3 and 4 show the results of the factorial designs and illustrate the impact of each of the investigated parameters on diclofenac and piroxicam percutaneous absorption respectively (at high and low level, with respect to the centre point), after a 34 hour experimental period.

The statistical analysis of these results showed that temperature, pH, EtOH and TC (both alone and in combination) were significant ($p \leq 0.05$) factors affecting the permeation of diclofenac across the skin. The transport of piroxicam was influenced significantly ($p \leq 0.05$) by temperature, pH and the combination of EtOH and TC (50:50 mixture). Temperature had the greatest effect on the permeation of both drugs across the skin. Both diclofenac and piroxicam permeation displayed a significant ($p \leq 0.05$) dependence on the pH of the donor suspension, with higher transport at the higher pH.

The chemical penetration enhancers EtOH and TC, individually and in combination, significantly increased ($p \leq 0.05$) the transport of diclofenac; whereas piroxicam permeation was only significantly enhanced when the CPEs were used together. The use of DDEA did not significantly increase diclofenac permeation over the free acid, however since the salt forms (i.e., sodium salt and/or diethylammonium salt) are more commonly used in topical formulations, the diethylamine salt form was selected for the remainder of this study.

3.2. Mechanistic investigation of the effects of heat on diclofenac permeation

To investigate the mechanisms by which heat exerts its effects on drug permeation across skin, further experiments were conducted looking at permeation across RCM, pig skin, human scrotal skin and human abdominal skin. These experiments were performed with DDEA in PBS pH 7.5 and were designed to also confirm the relevance of using human scrotal skin as a model membrane to understand the effects of heat on skin permeation.

3.2.1. Effect of heat on diclofenac release from a formulation vehicle

The effect of heat on the release of diclofenac from the vehicle was assessed using RCM. The release of diclofenac from PBS gave linear profiles at the two experimental temperatures, 32 and 45 °C (Fig. 5). The gradient of the plot, which is proportional to the release rate of the drug from the vehicle, was calculated by linear regression ($R^2 \geq 0.99$) and showed that there was a significant ($p \leq 0.05$) increase in diclofenac release of approximately 2-fold, in response to the 13 °C rise in temperature (Fig. 5).

3.2.2. Effect of heat on the delivery of diclofenac across different skin types

The application of heat (45 °C) was found to enhance significantly ($p \leq 0.05$) the cumulative amount of diclofenac transported through both human (abdominal and scrotal) and pig skin, as shown in Fig. 6. DDEA permeation was higher across human scrotal skin than pig skin, which was higher than that across human abdominal skin. This is to be expected though care should be taken with interpretation given the larger number of donors contributing to the scrotal skin data. This skin experimental data was modelled to calculate the permeation parameters of diclofenac at 32 and 45 °C. D/h^2 and Kh The average values of J, D/h^2 and Kh of diclofenac determined at 45 and 32 °C, across human scrotal, human abdominal and pig skin are listed in Table 2.

Comparison of the data (Table 2) indicates that the effect of heat on human scrotal skin caused the flux of diclofenac to increase significantly ($p \leq 0.05$) by 2.7-fold whereas the increases in flux observed when using pig and abdominal skin were slightly larger (3.7 and 5.6-fold

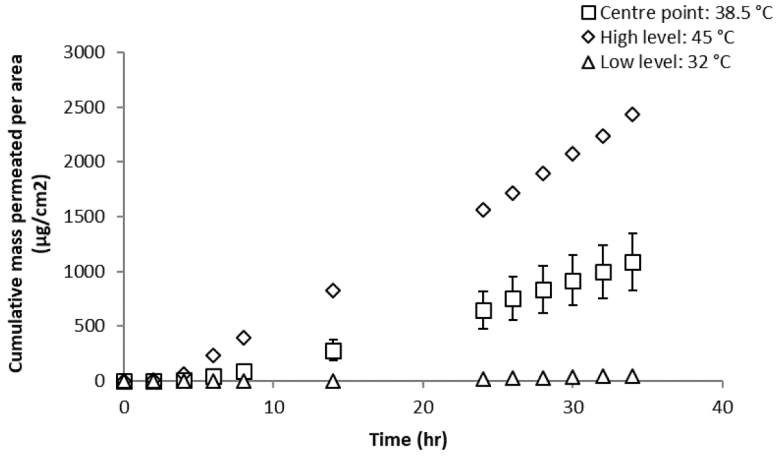

Fig. 1. Mean cumulative amount of diclofenac diethylamine permeated across full thickness scrotal human skin from a saturated solution of EtOH: buffer (50:50), pH 3.5 at 45 °C (high level, $n = 1$) and 32 °C (low level, $n = 1$), with respect to the centre point (saturated solution of diclofenac diethylamine in EtOH: TC: PBS (25:25:50)), 38.5 °C and pH 5.5, mean ± SE, $n = 6$).

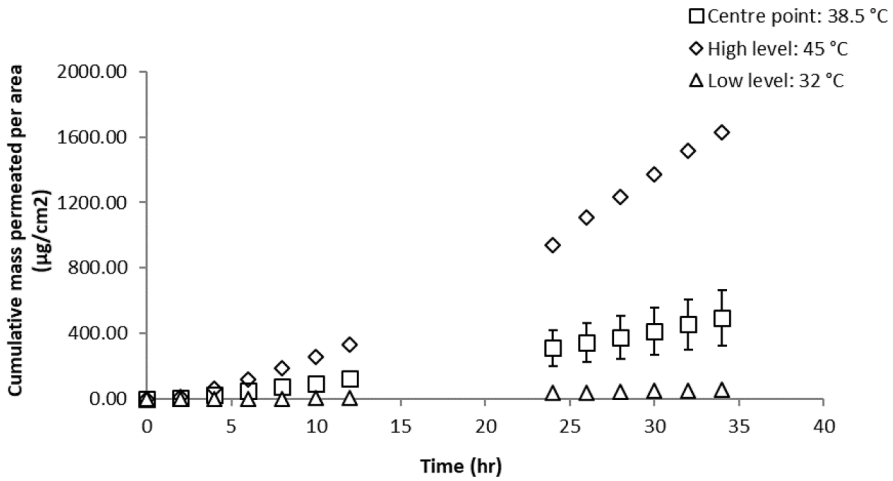

Fig. 2. Mean cumulative amount of piroxicam permeated across full thickness human scrotal skin from a saturated solution of EtOH: TC (50:50), pH 7.5 at 45 °C (high level, $n = 1$) and 32 °C (low level, $n = 1$), with respect to the centre point (saturated solution of piroxicam in EtOH: TC: PBS (25:25:50)), 38.5 °C and pH 5.5, mean ± SE, $n = 6$).

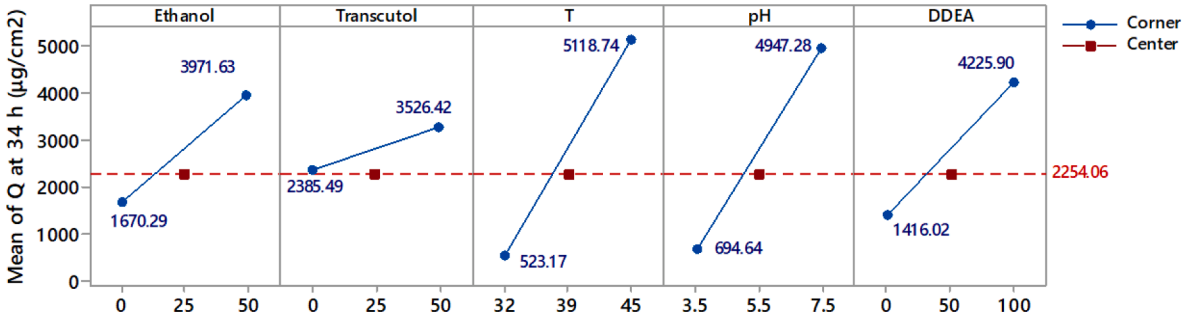

Fig. 3. Main effects plot showing the average change in response (Q at 34 h of diclofenac) when moving from the low to the high level of each factor investigated.

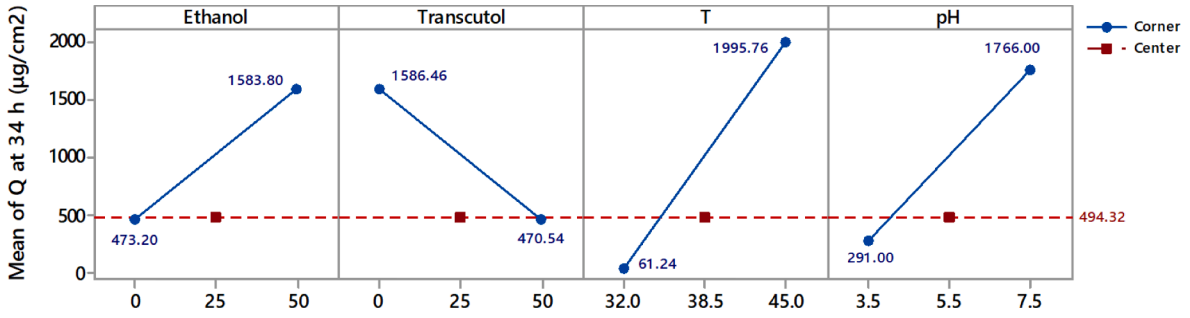

Fig. 4. Main effects plot showing the average change in response (Q at 34 h of piroxicam) when moving from the low to the high level of each factor investigated.

respectively). The pathlength normalised diffusion coefficient (D/h^2) values increased 1.7- 2.0-fold observed at the higher temperature, whereas normalised partition coefficients (Kh) of diclofenac, increased 1.9 – 3.4 fold when the temperature was increased from 32 to 45 °C.

3.2.3. *Arrhenius plots of diclofenac skin permeation*

A series of diclofenac in PBS buffer pH 7.5 permeation experiments were conducted using human scrotal skin between 28 and 45 °C and Arrhenius plots of the permeation parameters J and D/h^2 were constructed and are shown in Fig. 7. The data for both plots show a linear trend suggesting that there is no mechanistic change in the permeation behaviour of the drug over the temperature range examined. This might be expected if the change in SC lipid structural organisation from orthorhombic to hexagonal packing either side of the 37–42 °C range, had a significant effect on the permeation process. Similar observations can be made for the parameter J (that considers both the partitioning and diffusion parameters of diclofenac). Second derivative IR spectra of the CH_2 scissoring region of the SC of scrotal skin (Fig. 8) showed that with the increased temperature the two characteristic peaks of the orthorhombic lattice below the transition temperature, merged into a single, narrower peak, indicative of the presence of the hexagonal organisation of the SC lipids, at around 1468 cm^{-1} (Boncheva et al., 2008).

3.3. *Heat and chemical penetration enhancement of diclofenac and piroxicam skin permeation*

The factor screening study showed that temperature, CPEs and pH had a significant impact ($p \leq 0.05$) on the permeation of diclofenac and piroxicam across the skin. These factors were used to design a prototype

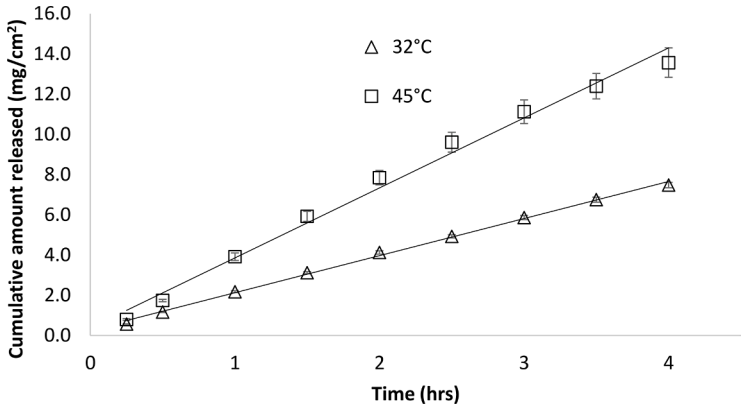

Fig. 5. Mean cumulative amount of diclofenac diethylamine released from a saturated donor solution in PBS (pH 7.5) across RCM, at 32 and 45 °C (mean ± SE, $n = 6$).

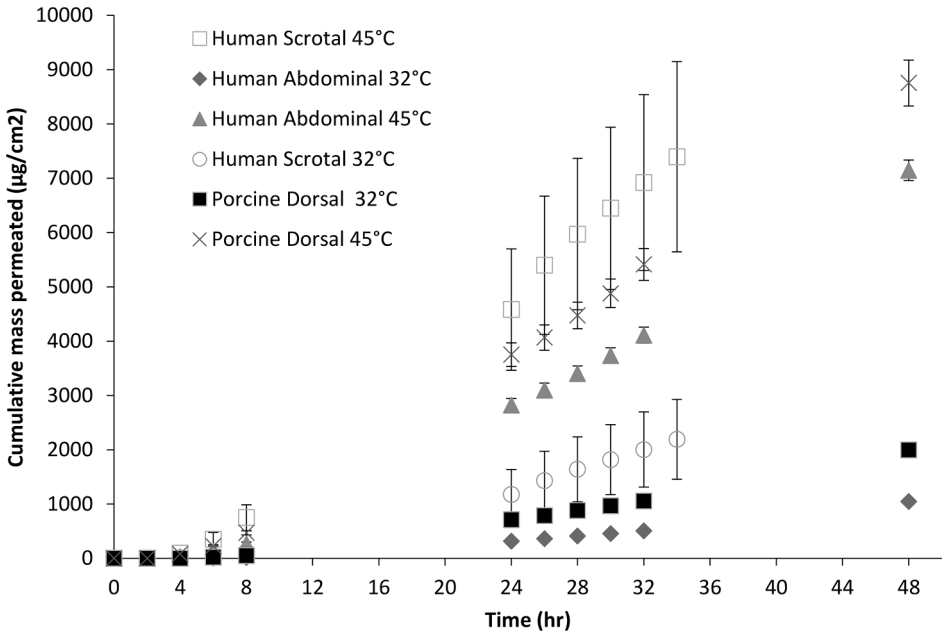

Fig. 6. Mean cumulative amount of diclofenac diethylamine permeated across pig and abdominal human skin over 48 hr and scrotal human skin over 34 hr from a saturated donor solution in PBS (pH 7.5), at 32 and 45 °C (mean ± SE, $n = 6$).

Table 2
Skin permeation parameters determined for diclofenac diethylamine transport across different skin membranes using Fick's first law, at 32 °C and 45 °C.

Membrane	Membrane temperature (°C)	J(µg/cm^2/hr)	ER	D/h^2(x10^{-2}hr^1)	ER	Kh (10^3 cm)	ER
Human abdominal skin	32	33.3 ± 1.27	5.6	1.05 ± 0.01		3.16 ± 0.34	
	45	187.0 ± 3.90		1.79 ± 0.13	1.7	10.7 ± 1.6	3.4
Human scrotal skin	32	99.5 ± 27.2	2.7	2.53 ± 0.25		1.71 ± 1.05	
	45	272.9 ± 61.4		1.41 ± 0.14	1.8	0.84 ± 0.80	2.0
Pig dorsal skin	32	56.6 ± 2.3	3.7	1.24 ± 0.09		4.65 ± 0.83	
	45	210.5 ± 8.7		2.40 ± 0.15	2.0	8.87 ± 1.07	1.9

formulation with the aim of further increasing percutaneous absorption compared to when only heat was applied. A formulation vehicle consisting of EtOH: TC (50: 50) that was adjusted to pH 7.5 was selected to be used in combination with heat. The permeation profiles of diclofenac and piroxicam from these vehicles at 32 and 45 °C and from a control formulation of a suspension of the drug in PBS at pH 7.5 are shown in Figs. 9 and 10.

A significant ($p \leq 0.05$) synergistic increase of approximately 13 and 40-fold in the cumulative amount permeated at 34 h for diclofenac and piroxicam respectively was recorded with a skin temperature of 45 °C when using CPEs (EtOH: TC, at pH 7.5), compared to when enhancers and heat were not applied (saturated solution of the drug in PBS, pH 7.5 at 32 °C, Table 3). In agreement with the results of the factorial design experiments, temperature had a more pronounced influence on the transport of piroxicam than diclofenac (from saturated solution in PBS, at pH 7.5); whereas the proportional effect of the CPEs (32 °C) was found to be lower for both permeants (Table 3).

These data were modelled using Fick's first law so that the skin permeation parameters flux (J), pathlength normalised values of partition (Kh) and diffusion (D/h^2) coefficients, of both drugs, in the presence

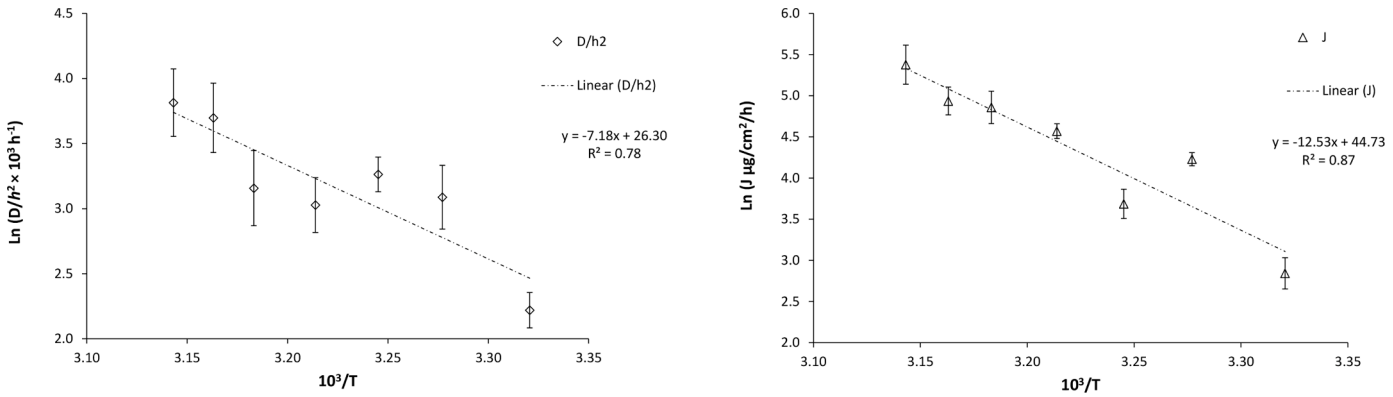

Fig. 7. Arrhenius plots of the normalised diffusion coefficient (D/h^2, on the left) and flux (J, on the right) of diclofenac from a saturated donor solution in PBS (pH 7.5) across human scrotal skin at membrane temperatures of 28, 32, 35, 38 41, 43 and 45 °C. Each point represents mean ± SE ($4 \leq n \leq 6$).

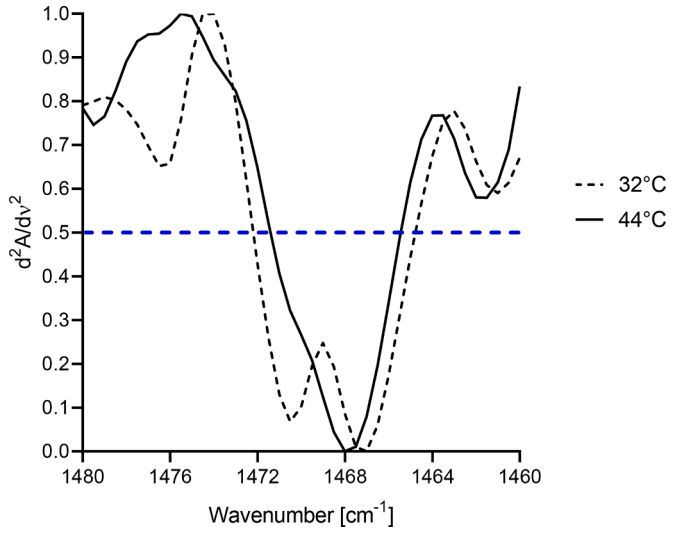

Fig. 8. Second-derivative spectra of the CH_2 scissoring region (1480 cm^{-1} to 1460 cm^{-1}) of the stratum corneum of human scrotal skin collected at 32 °C and 44 °C.

of heat and CPEs could be determined. The average values of these parameters are reported in Table 3.

The data show that at 32 °C there was a significant ($p \leq 0.05$) increase in K*h* of both drugs into the SC (approximately 2–3 fold), from the vehicle including CPEs such as EtOH and TC, compared to the control (PBS, 32 °C). The application of external heat (45 °C) on the control formulation (PBS, no CPEs) showed a significant increase ($p \leq 0.05$) in the D/h^2 of diclofenac (1.8 fold), but not for piroxicam. However, a pronounced significant ($p \leq 0.05$) increase in the partitioning of piroxicam was observed, with K*h* increasing by 14.5-fold, with the increase on diclofenac partitioning being significant but at 2.7-fold, much smaller. When heat was used along with CPEs (at pH 7.5), the enhancement produced in the K*h* of diclofenac and piroxicam was found to be higher than the additive effect of each enhancement strategy alone. The ability of heat-CPE_S to increase synergistically the partitioning of both drugs into the skin resulted in increases of diclofenac and piroxicam flux of approximately 13 and 40 -fold respectively, as evident from the analysis of the ER values listed in Table 3.

The solubility values of diclofenac and piroxicam in the vehicles and temperatures used are reported in Table 4. Piroxicam had a lower solubility than diclofenac in both vehicles tested at both temperatures. The use of the penetration enhancers increased drug solubility considerably in the vehicles. The increase in solubility of diclofenac and piroxicam in the vehicles at the higher temperature was much smaller, ranging

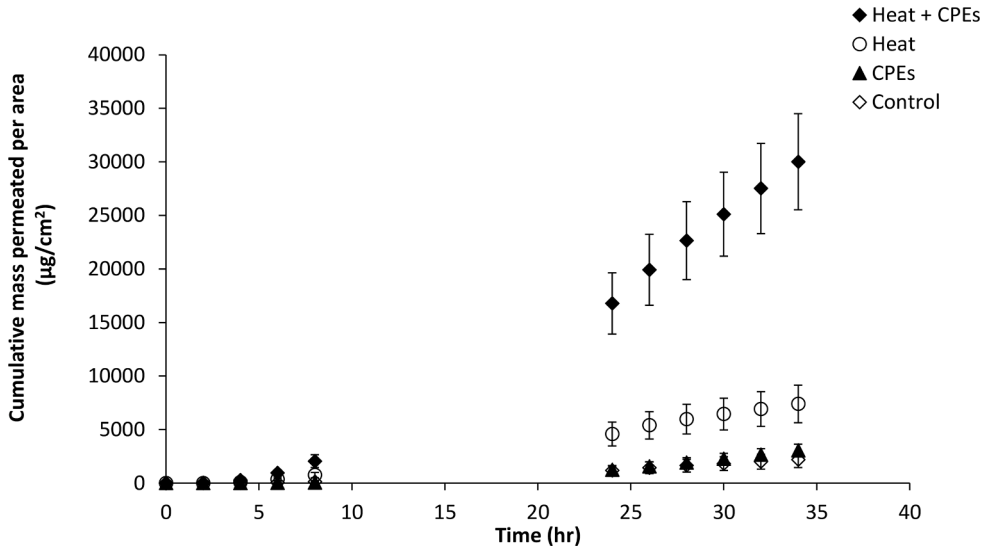

Fig. 9. Mean cumulative amount of diclofenac diethylamine permeated across full thickness human skin (4 donors) from a saturated solution of EtOH: TC (50:50) at 45 °C, pH 7.5 (heat + CPEs) compared to that from PBS, pH 7.5 at 45 °C (heat), EtOH: TC (50:50) at 32 °C, pH 7.5 (CPEs) and PBS, pH 7.5 at 32 °C (control) (mean ± SE, $n = 6$).

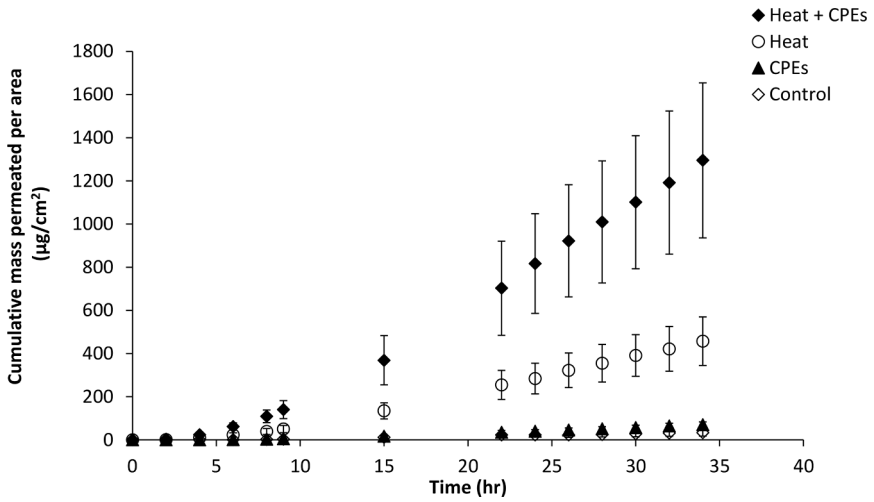

Fig. 10. Mean cumulative amount of piroxicam free acid permeation across full thickness human skin (4 donors) from a saturated solution of EtOH: TC (50:50) at 45 °C, pH 7.5 (heat + CPEs) compared to that from PBS, pH 7.5 at 45 °C (heat), EtOH: TC (50:50) at 32 °C, pH 7.5 (CPEs) and PBS, pH 7.5 at 32 °C (control) (mean ± SE, $n = 6$).

Table 3
Mean values of J, Kh and D/h2 of diclofenac and piroxicam measured when using CPEs (50 % EtOH and 50 % TC adjusted to pH 7.5) and PBS, pH 7.5 at skin temperature of 32 and 45 °C (mean ± SE, $n = 6$).

Drug	Application vehicle	J (µg/cm^2/h)	ER*	*Kh* (10^3 cm)	ER*	D/*h*^{2} (10^{-2} h^1)	ER*
Diclofenac	EtOH: TC, pH 7.5 at 45 °C	1305.4 ± 158.7*	13.1	10.5 ± 8.2*	12.6	1.53 ± 0.07	1.1
	PBS at 45 °C	272.9 ± 61.4*	2.7	1.71 ± 1.05*	2.0	2.53 ± 0.25*	1.8
	EtOH: TC, pH 7.5 at 32 °C	182.3 ± 21.8*	1.8	1.86 ± 1.25*	2.2	0.96 ± 0.08	0.7
	PBS at 32 °C	99.5 ± 27.2	1.0	0.84 ± 0.80	1.0	1.41 ± 0.14	1.0
Piroxicam	EtOH: TC, pH 7.5 at 45 °C	47.0 ± 12.6*	39.7	0.29 ± 0.27*	42.2	2.55 ± 0.11	0.9
	PBS at 45 °C	17.1 ± 4.1*	14.5	0.11± 0.16*	16.2	2.29 ± 0.34	0.8
	EtOH: TC, pH 7.5 at 32 °C	3.1 ± 0.6*	2.6	0.03 ± 0.02*	3.6	1.50 ± 0.08	0.5
	PBS at 32 °C	1.2 ± 0.6	1.0	0.01 ± 0.01	1.0	2.88 ± 0.16	1.0

Table 4
Diclofenac (as diethylamine salt – DDEA) and piroxicam saturated solubility values. Values are reported as the mean ± range ($n = 3$).

Application vehicle	Diclofenac (mg/mL) (µg/cm^2/h)	Piroxicam (mg/mL) (10^{-2} h^{-1})
EtOH: TC, pH 7.5 at 45 °C	458.6 ± 5.7	175.9 ± 2.4
PBS, pH 7.5 at 45 °C	8.1 ± 0.4	1.6 ± 0.4
EtOH: TC, pH 7.5 at 32 °C	379.5 ± 4.8	129.5 ± 3.7
PBS, pH 7.5 at 32 °C	4.9 ± 0.3	1.2 ± 0.2

between 1.2 – 1.6-fold.

4. Discussion

The factorial design indicated several factors to be relevant in increasing the permeation of diclofenac and piroxicam across the skin, including vehicle pH, inclusion of the chemical penetration enhancers EtOH and TC and the use of heat. The pH values selected for this study of 3.5 and 7.5 are suitable for topical formulation and considerably influence the ionisation state of both diclofenac and piroxicam with both drugs dissociating to a greater extent into the ionised species at the higher pH. With pKa values of 4.1 and 5.5 respectively diclofenac dissociation increases from 25 % at pH 3.5 to > 99 % at pH 7.5, whereas piroxicam dissociation changes from 0 to 99 % across the same pH range. The higher pH of 7.5, where both drugs were ionised increased skin permeation of both NSAIDs. Although this contrasts with pH-partitioning theory, from which unionised drugs would be expected to show increased transport across the lipophilic SC, similar observations have previously been reported with NSAIDs (Shore et al., 1957; INAGI et al., 1981; Hadgraft et al., 2000). These findings of higher permeation of the ionised form have been hypothesised to be a result of the improved solubility of the ionised species, compensating for its lower permeability across the stratum corneum. EtOH and TC were selected as commonly used chemical penetration enhancers and increased significantly ($p \leq 0.05$) the transport of diclofenac and piroxicam across the skin (Williams, 2003). EtOH and TC are thought to be able to act by modifying the solubility parameter of the SC (Pershing et al., 1990; Harrison et al., 1996) so that the uptake of the drug within the membrane is enhanced. This agrees with the data in this study which found that the enhancers improved Kh. EtOH, may also act through extracting extracellular SC lipids and through interacting with the polar heads of the SC lipid bilayer facilitating the diffusion of drugs across the SC lipid chains (Bommannan et al., 1991; Ghanem et al., 1992). These effects may impact drug partitioning behaviour, but no increase in D/h^2 was observed here for either piroxicam or diclofenac in response to EtOH and TC.

Raising the skin surface temperature to 45 °C, was found to be an effective strategy to increase the permeation of both diclofenac and piroxicam across the skin. This temperature is thought to be physiologically tolerable by the skin, and has been widely used in studies investigating heat effects on skin permeation, though some have suggested that 42 °C, may be a preferable upper limit for commercial application (Caserta et al., 2019; Hao et al., 2016). Moreover, increased temperature was found to be able to work synergistically with chemical penetration enhancement to produce large increases in drug delivery. To gain an improved understanding of the role of heat as a penetration enhancement strategy, the effects of temperature on three potential rate-controlling steps of the transdermal process (formulation release, partitioning and diffusivity of the drug through SC) were investigated

using diclofenac as a model drug. Heat can promote diffusion through the skin in response to increased molecular mobility of the drug in accordance with the Stokes-Einstein equation, which would be expected to improve drug release from the formulation and diffusion through the stratum corneum. RCM was used to provide assessment of the influence of heat on molecular diffusion and drug release, removing any interference of partitioning effects or structural alterations of the membrane that can occur with skin (INAGI et al., 1981). At the higher temperature, drug release was increased approximately 2-fold suggesting that this was a contributory factor but did not fully account for the larger effect of heat on increasing drug permeation. The SC lipids are known to undergo a phase transition between 37 and 42 °C, moving from orthorhombic to the less tightly packed hexagonal packing at the higher temperature (Boncheva et al., 2008). This phase change has been hypothesised to contribute to the increased permeation across skin in response to heat. The SC lipids of human scrotal skin were confirmed to undergo the orthorhombic to hexagonal phase transition. The work presented here has used human scrotal skin as a model skin tissue membrane. Scrotal skin is histologically similar to skin from other body regions and although it is typically more permeable to drugs, it still presents a barrier to drug penetration (Caserta et al., 2019; Smith Jr et al., 1961). The tissue has been used previously for permeation studies and that the impact of heat on diclofenac permeation across scrotal, human abdominal and pig skin suggests that human scrotal skin may be used as an alternative skin source to evaluate heat enhanced drug permeation across the skin (Beebeejaun et al., 2022; Beebeejaun et al., 2023a, b). The Arrhenius plots constructed from diclofenac permeation showed a linear response to temperature. The slope of an Arrhenius plot is proportional to the activation energy (E*a*) required for the permeation across skin to occur (Blank et al., 1967). Thus, a change in the slope would be expected if alterations in the SC properties in response to increased temperature affected the permeation of compounds across the membrane. Neither the Arrhenius plot constructed using flux (J), which includes contribution from both the diffusion and partition of diclofenac or the plot constructed using D/h^2, which relates to diffusion alone, exhibited a change in the gradient of the plot. This suggests that the increased permeation of diclofenac in response to heat is not significantly affected by heat-induced changes in the SC lipids. The increases in diclofenac diffusion through both human and pig skin and across RCM were of comparable magnitude. This further suggests that the shift in the human SC lipids arrangement produced by heat had a negligible impact on the diffusion coefficient of diclofenac and that the enhancement effect of temperature on D/h^2 was mainly the result of changes in the mobility of the drug molecules at 45 °C, as pig skin lipids are known to be predominantly in the hexagonal form (Boncheva et al., 2008). Other authors have also reported that heat induced structural changes in the lipid packing of the SC did not affect drug transport across the skin, for example the Arrhenius plot of the permeability coefficient of terodiline yielded a straight line between 25 and 50 °C (Ogiso et al., 1998).

The use of heat in combination with CPEs (at pH of 7.5) produced a synergistic enhancement in permeability of each of the model drugs tested (ca. 13-fold and 40-fold for diclofenac and piroxicam respectively). This was much greater than the increase in drug solubility in the vehicles at the higher temperature. Analysis of the transport data suggested that this synergistic effect was mostly a consequence of the ability of the system heat-CPEs to increase the partitioning of diclofenac and piroxicam to a greater extent than the additive effect of each enhancement strategy alone. This has been seen previously with isotretinoin and finasteride delivery to skin (Farah et al., 2020; Farah et al., 2019). As mentioned previously, the CPEs tested in this work, have been previously shown to increase the solubility of several compounds into human SC (Harrison et al., 1996; Watkinson et al., 2009). This effect is likely to be a feature of both the significant uptake of the CPEs into the membrane, along with a good solubility of the drugs in these chemicals (McAuley et al., 2010). The increases in drug partitioning with the application of heat are often thought to rely on the changes induced in the free volume within the SC lipids chains (Kirjavainen et al., 1999), subsequently favouring the sorption of the drug into the skin. In general heat (when used individually as and enhancement strategy) is thought to increase drug permeation approximately 2–3 fold, however for piroxicam (log P 1.6) which is more hydrophilic than diclofenac (log P 4.2), the effect was considerably larger (McAuley and Caserta, 2015; Hao et al., 2016). This suggests that heat, as well as working as a general enhancement strategy for most drugs, may be able to improve skin permeation of some molecules with less favourable molecular characteristics for skin permeation to a greater extent. As well as promoting the transfer of the drug into the skin, the heat-induced changes in the free volume are likely to increase the uptake of the EtOH and TC into the SC, which can increase permeation across the membrane (Twist and Zatz, 1990). Others who have investigated the effects of heat on commercially available products, have found the effect of heat on penetration enhancement to be larger for a product containing the recognised penetration enhancer DMSO (Thomas et al., 2020). It is likely that the synergistic increase in K*h* observed here arose mostly from the ability of heat to increase the uptake of EtOH and TC into the SC, further favouring the transfer of the drug into the stratum corneum. Heat can have other effects on drug permeation across skin, for example it has been shown to affect follicular drug delivery, which may contribute to the increased absorption seen here (Caserta et al., 2019). In addition, the application of heat is also thought to increase cutaneous blood flow (Minson et al., 2001) which could increase drug clearance from the skin. Understanding the impact of this on the use of topical NSAIDs would require further in vivo or clinical studies.

5. Conclusions

The use of heat was found to significantly increase the skin permeation of both diclofenac and piroxicam to the skin. Heat increased drug release from the vehicle, increased the diffusion coefficient of the drugs in the skin and improved their partitioning into the stratum corneum. In addition to heat, an elevated vehicle pH and the inclusion of the CPEs, EtOH and TC, were also found to be beneficial in increasing the transport of these drugs through the skin. Heat was effective in conjunction with these formulation approaches and was able to work synergistically with CPEs in greatly increasing drug permeation across the skin. This synergistic effect was likely to be a consequence of the ability of heat to improve the partitioning of the CPEs into the skin that in turn facilitated the further partitioning of the drugs into the skin.

CRediT authorship contribution statement

F. Caserta: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **M.B. Brown:** Writing – review & editing, Supervision, Conceptualization. **W.J. McAuley:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Conceptualization.

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Data availability

Data will be made available on request.

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