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Permeability of frog skin to chemicals: effect of penetration enhancers

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ABSTRACT

Rarely do commercial chemical products contain solely the active chemical/ingredient. It is therefore important to consider whether ingredients other than the active may: 1) alter absorption of the active chemical, or 2) be absorbed themselves, resulting in systemic effects. Frogs have highly permeable skin and are routinely exposed to commercial chemical products in the environment or therapeutically. Ethanol and propylene glycol (PG), which have known penetration-enhancing effects, are commonly included in such products. The current study has therefore investigated the *in vitro* absorption kinetics through *Rh. marina* skin of three model chemicals – caffeine, benzoic acid, and ibuprofen – formulated individually as solutions containing: 1%, 10% or 30% v/v ethanol, or 20% v/v PG. Differential scanning calorimetry and histology were used to characterise fresh frog skin, investigate the mechanism of these enhancers in frog skin, and to determine whether these enhancers significantly affected skin structure. Results showed that the extent of absorption enhancement was influenced by chemical, enhancer and skin region, and that enhancement was generally not consistent for individual enhancers or skin regions. The exception was 1% v/v ethanol, which did not significantly alter flux across the skin for any of the chemicals evaluated. Caffeine absorption was not enhanced by any of the investigated penetration enhancers, and was in fact significantly reduced by 30% v/v ethanol and PG. Ethanol caused concentration-dependant changes in skin morphology and should be avoided in concentrations ≥10% v/v. PG, however, caused minimal changes to the skin and consistently improved absorption of benzoic acid and ibuprofen through all skin regions. Owing to the significant changes in skin structure following ≥10% v/v ethanol exposure, it is recommended to avoid its use in frogs. For enhancement of penetration of moderately-to-highly lipophilic chemicals, this study has identified 20% v/v PG should to be the enhancer of choice.

1. Introduction

Penetration enhancers, substances that can partition into the skin and increase the absorption of chemicals, are commonly included in agricultural, industrial and therapeutic formulations. There is substantial evidence that these substances are able to significantly reduce the barrier function of the mammalian epidermis, resulting in greater systemic exposure to topically-administered chemicals. However, despite the common inclusion of penetration enhancers in formulations, their impact on absorption in most non-mammalian species remains unknown.

Frog skin is highly-permeable and structurally different from mammalian skin, owing to the role of frog skin in maintaining physiological homeostasis (fluid, electrolyte and acid/base balance). The stratum corneum (SC), accepted as the primary barrier to percutaneous absorption in mammals, is much thinner in frogs. While in mammals the SC is often 10–20 cell layers thick, in frogs it commonly consists of only one or two cell layers [1]. Thus, it is unsurprising that the limited comparative studies of chemical absorption in frog and mammalian skins have reported much higher absorption in frog skin [2, 3]. As absorption rates are already heightened in frogs compared to in mammals, it is likely that inclusion of a penetration enhancer in a chemical formulation will also have a heightened effect in frog skin.

Frogs are experiencing significant population declines and extinctions worldwide. While much of the decline has been attributed to disease, especially infectious chytridiomycosis, habitat change and contamination have also been identified as causative factors [4]. The dynamic interaction between frogs' skin and the immediate environment is the reason for their heightened sensitivity to environmental contaminants, and this heightened sensitivity has, in turn, led to frogs being considered indicators of the relative health of an ecosystem. They therefore represent an ideal non-mammalian candidate in which to investigate the impact of penetration enhancers on percutaneous absorption, as they are

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likely to encounter these substances in formulations for treatment of disease and in also in the wild.

A variety of ingredients with potential penetration-enhancing effects are regularly included in commercially-available products, particularly as co-solvents (to assist in the dissolution of the active chemical). In therapeutic drug products, the most common co-solvent used is ethanol, although propylene glycol (PG), owing to its relatively lower toxicity *in vivo*, is also widely included, particularly in topical and cosmetic formulations [5]. Further, as commercially-available drug products are often inappropriate for administration directly to exotic species including frogs, clinicians are often required to compound their own formulations. Owing to their availability and favourable solubility profiles, ethanol and PG also represent the most-commonly used solvents used when compounding drug products for topical application. Ethanol also finds extensive use in agricultural and industrial formulations. In particular, the identification of ethanol as a "green" solvent has increased its use in manufacturing, inclusion in fuels, and as a solvent in agrichemicals [6]. PG is most commonly included in antifreeze for aircraft, and so can easily contaminate surrounding environments when in use [7]. While neither PG nor ethanol persist in the environment, acute impacts on wildlife when products containing these ingredients are introduced into the environment cannot be ignored.

Penetration enhancers may influence absorption by allowing higher concentrations of active chemical to be dissolved in the formulation itself, and also by altering the barrier properties of the skin – usually by inducing changes in the lipoid structure/packing of the epidermal layer. Almost all penetration enhancers act via more than one mechanism, and sometimes the primary mechanism of enhancement changes, depending on the concentration of enhancer used. Ethanol is postulated to improve absorption by: increasing chemical solubility in formulation, diffusing itself into the SC thereby improving solubility of the active chemical in the SC, and having a multitude of effects on intradermal lipids, causing lipid fluidisation, restructuring of the lipids, and at high concentrations, lipid extraction from the SC [8]. The mechanism of PG in improving percutaneous absorption is similarly debated; its effects have been attributed to: diffusion into the SC, improving solubility of the active chemical in the SC, interaction with the polar headgroups of the lipid bilayers of the skin altering the lipid packing, and alteration of protein composition in the skin [9, 10]. In order to elucidate the underlying mechanism of penetration enhancers, studies often consider the results of in vitro absorption studies alongside investigations of changes in the skin structure following exposure to the penetration enhancers. Various techniques have been used to study the effect of penetration enhancers on skin structure, including light, electron and confocal microscopy, Raman and Fourier transform infrared spectroscopy, differential scanning calorimetry (DSC) and X-ray diffractometry [11].

As penetration enhancers have different effects depending on the characteristics of the skin to which they are applied and the formulation in which they are included, it is difficult to consistently predict the impact an enhancer will have on skin structure and function. As frog skin is structurally different from mammalian skin, and penetration-enhancers likely to be included in therapeutic formulations used in frogs, and also present in frog habitats, studies are needed to investigate their effect on chemical absorption through frog skin. Further, given the vital role of frog skin in maintaining physiological homeostasis, it is also important to ensure that the integrity of the skin is not significantly altered following application of penetration enhancers.

The current study investigated the effect of the addition of ethanol or PG to an aqueous formulation containing one of three model chemicals on the absorption kinetics through frog skin. In order to further understand the underlying mechanism of penetration enhancement in frog skin, DSC and histology were used to characterise frog skin, prior to and after exposure to these penetration enhancers.

2. Material and methods

2.1. Chemicals and solutions

Model chemicals used were reagent grade caffeine, ACS reagent grade benzoic acid (both Sigma-Aldrich) and ≥98% ibuprofen (Sigma). Amphibian Ringer's solution (ARS), used in both donor and receptor solutions, was prepared according to Wright and Whitaker [12] containing: 113 mM sodium chloride, 2 mM potassium chloride, 1.35 mM calcium chloride, and 2.4 mM sodium bicarbonate. For all ibuprofen experiments, ARS was spiked with 2-hydroxypropyl-beta-cyclodextrin (HPβCD; Aldrich Chemistry) 2.75 mg/ml to ensure adequate solubilization. Donor solutions (Table 1) comprised a saturated ("infinite dose") solution of each of the model chemicals in either: (a) 1% v/v ethanol, (b) 10% v/v ethanol (ibuprofen only), (c) 30% v/v ethanol, or (d) 20% v/v PG, all prepared in ARS ± HPβCD. As the study included prolonged exposure, the penetration enhancers chosen, and concentrations used, were selected due to their inclusion in commercially-available drug products, at amounts reported to be safe in frogs. 10% v/v ethanol was included as a mid-range concentration in the ibuprofen studies, after preliminary studies showed almost no influence of 1% v/v ethanol and extremely high absorption from 30% v/v ethanol. To ensure solubilisation of the chemicals in the receptor solution, all receptor solutions comprised ARS spiked with 2-hydroxypropyl-beta-cyclodextrin (HPβCD; Aldrich Chemistry): 2.75 mg/ml HPβCD was used for the caffeine and ibuprofen experiments, and 5.75 mg/ml HPβCD for benzoic acid experiments. Euthanasia of animals was carried out by bathing in a solution of 0.2% w/v ethyl 3-aminobenzoate methanesulfonate solution (MS-222; Aldrich Chemistry), buffered to pH 7.3 with sodium bicarbonate.

Methanol and acetonitrile used were high-performance liquid chromatography (HPLC) grade (Fisher Chemicals, Trinidad and Thermo

Table 1
LogP of each model chemical, and the composition, saturation solubility data and sampling times for each donor solution used in the absorption kinetics/diffusion cell experiments *values from [17].

Model drug	LogP	Donor solution composition (% v/v)			Saturated solubility (g/L)	Sampling times (hr)
		ARS	Ethanol	PG		
Benzoic acid	1.87	100%	–	–	3.972*	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6
Benzoic acid		99%	1%	–	5.240	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5
Benzoic acid		70%	30%	–	12.101	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5
Benzoic acid		80%	–	20%	2.619	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5
Caffeine	-0.07	100%	–	–	20.298*	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6
Caffeine		99%	1%	–	19.238	0, 0.5, 1, 1.5, 2, 2.5, 3, 4
Caffeine		70%	30%	–	20.046	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5
Caffeine		80%	–	20%	17.947	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5
Ibuprofen	3.97	100%	–	–	0.490*	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6
Ibuprofen		99%	1%	–	0.243	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6
Ibuprofen		90%	10%	–	0.810	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5
Ibuprofen		70%	30%	–	1.338	0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6
Ibuprofen		80%	–	20%	0.262	0, 0.5, 1, 1.5, 2, 2.5, 3, 4

Fisher Scientific, Australia), formic acid was analytical grade (Thermo Fisher Scientific, Australia) and water used for HPLC was ultrapure (Milli-Q Integral, Millipore Australia). All solutions were freshly prepared.

2.1.1. Determining saturation solubility of donor solutions

The saturation solubility of each chemical in each penetration enhancer solution was determined by placing an excess of chemical into 5–100 mL of penetration enhancer solution. The resultant mixture was then sonicated at room temperature for 24 hr before centrifuging at 12,000 RCF for 15 minutes. Supernatant was removed, diluted appropriately with ARS + HPβCD, and analysed using previously-validated HPLC methods [13].

2.2. Study animals

Adult male *Rhinella marina* (cane toads), wild-caught in the Townsville region (Australia) were used in this study. The cane toad has a wide distribution, being a native species in Central and South America, and also having been introduced several nations in the Asia-Pacific region [14]. Extensive biological and ecological research exists for this species, including baseline kinetics of skin absorption. Cane toads are also generally not adversely affected by chytridiomycosis infection (only metamorphs and subadults succumb to the disease [15]), and so present a suitable model for design and trial of therapeutic treatments for this infection. This species, therefore, represents an appropriate model species when investigating the impact of penetration enhancers on the skin of frogs. Thirty-four toads, ranging from 54.5 – 128.65 g body weight (mean = 89.74 g) were randomly allocated to one of the penetration enhancer/chemical treatments. Twenty-five toads were used for the in vitro penetration enhancer experiments, and the remaining toads provided skin samples both for histological analysis (light microscopy) and DSC experiments. Toads were handled carefully in order to minimize potential damage to the skin. Euthanasia was carried out within 24 hr of collection. Full-thickness skin samples were excised immediately after euthanasia. For all studies, each toad provided five skin samples from a combination of the following skin regions: dorsal bilaterally from the central dorsal truncal midline, ventral pelvic bilaterally along the pelvic truncal midline, ventral thoracic bilaterally from the central ventral truncal midline. All studies were completed in accordance with Animal Ethics approval A2222 from James Cook University, Australia.

2.3. Diffusion cell experiments

The effect of penetration enhancers on the absorption of model chemicals in static Franz diffusion cells (Permegear, USA) was investigated. Each diffusion cell consisted of a 1 ml donor chamber with a 9 mm orifice, and a 5 ml receptor chamber. The donor chamber was filled with a saturated solution of one of the model chemicals (benzoic acid, caffeine, or ibuprofen) in ARS (± HPβCD) + penetration enhancer, as outlined in section 2.1. Receptor solution (ARS + HPβCD) was magnetically stirred and allowed to equilibrate in the diffusion cell for 30 minutes prior to skin mounting. Prior to mounting, each skin sample was rinsed in ARS and microscopically inspected for signs of damage. Samples were mounted on diffusion cells with the external skin surface facing the donor chamber. Samples with signs of damage were not used in the study.

After mounting skin samples on the diffusion cell, 1 ml of donor solution was applied to the donor chamber, and the chamber was occluded by application of laboratory film (Parafilm M™, Pecheney Plastics Packaging, Chicago) to the external donor chamber orifice. 1 ml samples were then collected from the receptor chamber until steady-state was achieved for at least four sampling points, as indicated in preliminary studies (data not included). Samples were collected from the center of the diffusion cell, by inserting a 200mm long stainless-steel needle via the sidearm and withdrawing the sample from directly above the stir bar into a glass syringe. Samples were collected at t = 0, 0.5, 1, 1.5, 2, 2.5, 3 and 4 hr for caffeine in 1% v/v ethanol. All other caffeine experiments followed the same schedule with an additional sample taken at t = 5 hr. All benzoic acid samples were taken at t = 0, 0.5, 1, 1.5, 2, 3, 4 and 5 hr. Ibuprofen samples were withdrawn at t = 0, 0.5, 1, 1.5, 2, 3, and 4 hr when dissolved in 20% v/v PG, with an additional sample withdrawn at t = 5 hr for the 10% v/v ethanol-containing samples, and two additional samples (t = 5 and t = 6 hr) for ibuprofen in 1% v/v or 30% v/v ethanol (Table 1). Immediately following sample collection, fresh receptor solution was added to the receptor chamber using a clean needle and glass syringe, the chamber inverted to ensure no air bubbles were present, and the chamber then returned to its holder. This allowed replenishment of lost receptor chamber volume from sample collection, while also ensuring that fresh receptor solution was mixed into the remaining donor chamber solution. Collected samples were analyzed for the chemical content using previously-described HPLC methods [13].

2.3.1. High-performance liquid chromatography (HPLC)

The HPLC system comprised a Shimadzu Nexera-i LC-2040C 3D equipped with a photodiode-array detector. Post-run analysis was performed using Labsolutions 5.82 (Shimadzu). All HPLC methods have been validated and previously described [13]. As the penetration enhancers used in this study could potentially interfere with the analysis, specificity was investigated. Blank samples containing 100% penetration enhancer, samples of individual penetration enhancers spiked with known quantities of each of the chemicals, and samples run in unspiked ARS were injected onto the column to ensure no interference with the chemical peak occurred. All methods remained specific for the determination of the respective chemicals and no interference was detected, thus specificity was assured. HPLC methods were therefore used as previously reported. Calibrations were performed daily ($r^2 > 0.999$), and all samples measured in triplicate.

2.3.2. Data analysis and statistics

Data were examined and analyzed using R [16]. Previously-collected data involving percutaneous absorption of model chemicals from ARS [17] were included in data analyses to determine any differences in absorption due to the penetration enhancers.

Cumulative absorption versus time plots were produced for each sample, and any curves with significant deviation suggestive of skin damage were excluded. Consequently, results from four samples were excluded from further analysis.

Flux (mcg/cm^2/hr; J_{ss}) was determined for each sample from the steady-state slope of the cumulative absorption versus time plot. Steady state was identified as the slope taken from at least four consecutive sampling points, after initial equilibration had occurred. In the case of 30% ethanol, where a distinct lag phase was noted, we ensured that the first sampling timepoint for calculation of the linear portion of the curve was no less than 2.3 x lag time (as per [18]). Permeability coefficient (K_p) was calculated by dividing flux by the concentration of chemical in the donor solution ($K_p = J_{ss}/C_v$). Concentration of drug in the donor solution (C_v) was the saturation solubility of each chemical in the penetration enhancer as determined in stage 1 of this study (sections 2.1.1 and 3.1; Table 1). In addition to data collected in this study, K_p was calculated from previously-collected data involving percutaneous absorption of the model chemicals from ARS [17].

The effect of the penetration enhancer and skin region on chemical flux through the skin was determined for each individual chemical by fitting a series of linear mixed-effects models using the nlme package [19]. Each model used penetration enhancer as the fixed factor, included the individual animal as a random effect, and allowed for heteroskedasticity in the data.

Enhancement ratio (ER) was calculated for each drug/penetration enhancer formulation, as the ratio of K_p of each drug in penetration

enhancer divided by its K_p in ARS (i.e., ER = K_{pPE}/K_{pARS}). This allowed comparison of relative effect of each penetration enhancer to each other and ARS, for a specific skin region and model chemical. ERs reported are the mean ratios from at least four replicates.

2.4. Differential scanning calorimetry (DSC)

The thermal behavior of dorsal and ventral frog skin was investigated using differential scanning calorimetry (DSC; Mettler Toledo DSC822^e with STARe version 14.00). Full-thickness skin samples were used in the study, either freshly-excised or skin that had been exposed to ARS or penetration enhancer solutions for 6 hr. Skin was prepared and mounted on diffusion cells as described in sections 2.2 and 2.4. Donor solutions were as outlined in section 2.1, however model chemicals were not included in any solutions. The donor solutions thus comprised of: (a) ARS, (b) 1% v/v ethanol, (c) 10% v/v ethanol, (d) 30% v/v ethanol, or (e) 20% v/v PG, all prepared in ARS. The receptor solution for all studies was ARS to maintain viability of the inner skin surface. Following the 6-hr exposure time, skin samples were removed from the diffusion cells, rinsed with ARS, and blot-dried. Exposed skin was then excised from surrounding (non-exposed) tissue, cut into pieces weighing approximately 20 mg, and sealed in 40 mcl aluminium crucibles. Baseline samples of freshly-excised frog skin were used in the investigation of the ramp rate on DSC output. Heating rates of 1 °C, 5 °C and 10 °C/min were investigated, with optimal balance between resolution, sensitivity, and reproducibility seen with the 5 °C/min heating rate. Thus, all samples were analysed over the temperature range 30–150 °C, with a heating rate of 5 °C/min under nitrogen flow. Transition data represent the average of at least three skin samples.

2.5. Histology

Ventral and dorsal skin samples, either fresh or following exposure to ARS or penetration enhancers as part of the DSC experiments, were preserved in 4% phosphate-buffered formaldehyde. Preserved skin was then dehydrated and processed for histology using standard methods to produce 5 mcm sections on slides stained with haematoxylin and eosin.

3. Results

3.1. Saturation solubility studies

Table 1 presents the solubility data for each chemical in each of the penetration enhancer solutions. Increasing concentrations of ethanol increased the solubility of both benzoic acid and ibuprofen, with solubility increasing 3-fold for benzoic acid in 30% v/v ethanol, and 2-fold for ibuprofen. 20% v/v PG decreased the solubility of all chemicals investigated (Table 1).

3.2. Diffusion cell studies - absorption kinetics

3.2.1. Cumulative absorption versus time for each model chemical

The cumulative absorption of model chemicals from the different penetration enhancers changed markedly depending on the skin region and model chemical applied. Of note, 30% v/v ethanol demonstrated a distinct lag phase in absorption profile through all skin regions and for all chemicals, with negligible absorption occurring in the first 30 minutes of the experiments. The lag phase was most obvious in the benzoic acid experiments. Following this lag phase, however, absorption rapidly increased. This lag phase was absent for other enhancers. Discussion of results will therefore focus on absorption following resolution of the lag phase.

3.2.1.1. Caffeine. The addition of 1% v/v ethanol consistently showed greater absorption compared to ARS alone for all skin regions, whereas the relative effect of PG and 30% v/v ethanol differed depending on the skin region (Fig. 1). In dorsal skin, the inclusion of 30% v/v ethanol slightly reduced absorption compared to ARS alone, whereas PG reduced absorption by approximately half.

Ventrally, absorption of caffeine was essentially unchanged for PG compared to ARS, through both the thoracic and pelvic skin regions. In 30% v/v ethanol, caffeine absorption was similar to both ARS and PG through thoracic skin, however the absorption profile changed in the later sampling times in pelvic skin. Specifically, pelvic absorption of caffeine was similar between PG, ARS and 30% v/v ethanol over the first 3 hr, however absorption rate of caffeine from 30% v/v ethanol was lower from t = 3 hr, with the final total cumulative amount absorbed ~30% lower than that from PG.

3.2.1.2. Benzoic acid. The addition of any of the investigated penetration enhancers resulted in greater absorption than in ARS alone, in all skin regions. In contrast to caffeine, 30% v/v ethanol consistently resulted in the most rapid uptake of benzoic acid through all skin regions. There was also very little difference in absorption profiles between skin regions, with 30% v/v ethanol consistently having the highest absorption, followed by PG, and then 1% v/v ethanol (Fig. 2). Notably, in dorsal skin the improvement in absorption compared to ARS alone were similar for PG and 1% v/v ethanol, whereas in ventral skin the difference between these enhancers was more prominent, with PG resulting in markedly higher absorption.

3.2.1.3. Ibuprofen. The cumulative absorption versus time curves for

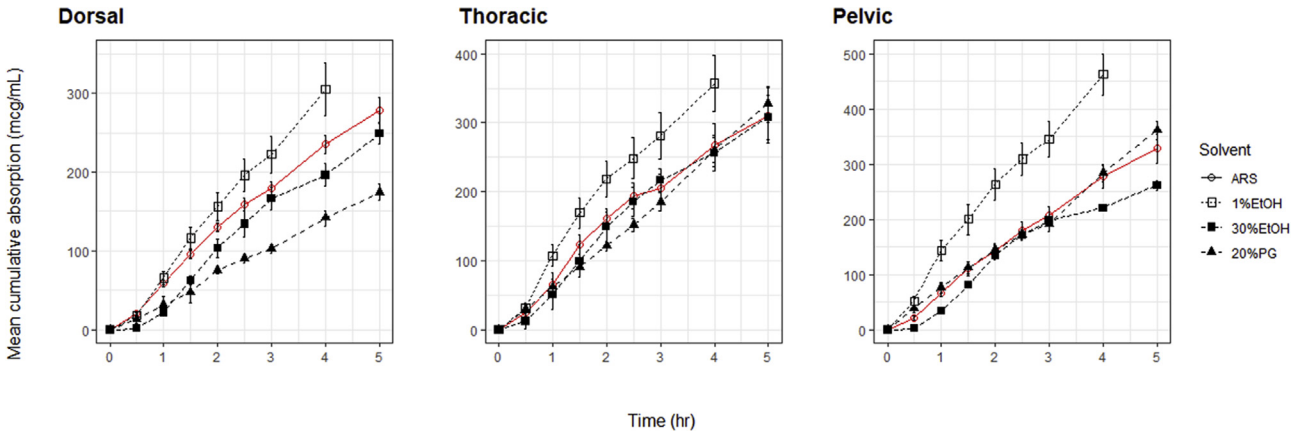

Fig. 1. Cumulative absorption versus time curves for absorption of caffeine for the various penetration enhancers through dorsal, ventral thoracic and ventral pelvic *Rh. marina* skin. Error bars show standard error. ARS data from [17].

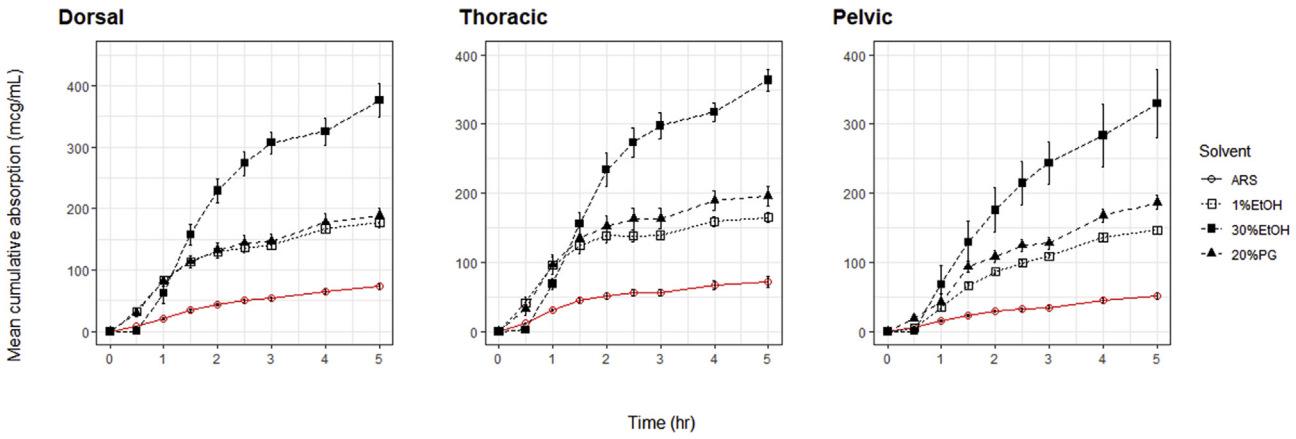

Fig. 2. Cumulative absorption versus time curves for absorption of benzoic acid for the various penetration enhancers through dorsal, ventral thoracic and ventral pelvic *Rh. marina* skin. Error bars show standard error. ARS data from [17].

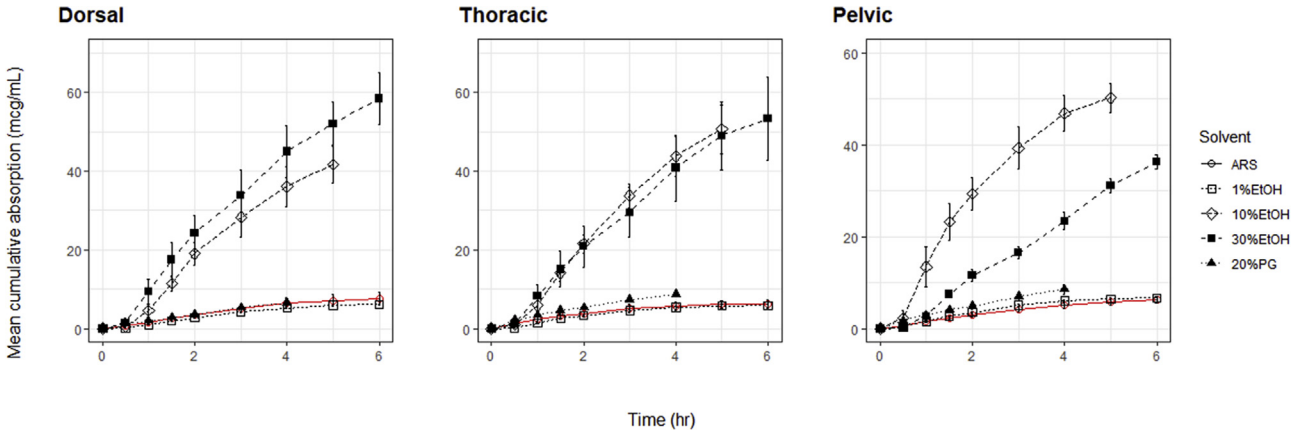

Fig. 3. Cumulative absorption versus time curves for absorption of ibuprofen for the various penetration enhancers through dorsal, ventral thoracic and ventral pelvic *Rh. marina* skin. Error bars show standard error. ARS data from [17].

ibuprofen through dorsal skin showed a much higher absorption of ibuprofen from 10% and 30% v/v ethanol compared to 1% v/v ethanol, PG and ARS (Fig. 3), with highest absorption from 30% v/v ethanol. Absorption of ibuprofen from PG, 1% v/v ethanol, and ARS in dorsal skin was similar.

The absorption trends in the ventral skin (both thoracic and pelvic) were different from dorsal absorption. While absorption remained low from both PG and 1% v/v ethanol, PG does appear to improve absorption marginally compared to 1% v/v ethanol and ARS, especially through thoracic skin.

Although thoracic absorption was highest for 10% and 30% v/v ethanol, no discernible difference was noted between these enhancers. Of interest is the observation that although the absorption characteristics of ibuprofen from the lower ethanol concentrations and PG remain relatively constant for both ventral skin regions, absorption changes significantly between these regions for the 30% v/v ethanol, with pelvic absorption being much lower than thoracic absorption.

3.2.2. *Influence of penetration enhancers on the flux of model chemicals*

1% v/v ethanol did not significantly influence flux for any of the chemicals, and the ability of the other enhancers to significantly affect flux was inconsistent between chemicals and skin regions.

Of interest, 30% v/v ethanol and PG produced significant reductions in flux of caffeine (Table 2), with PG significantly reducing flux through the dorsal skin ($t = -4.387$, $df = 8$, $p = 0.0023$), and 30% v/v ethanol reducing flux through both dorsal ($t = -2.909$, $df = 8$, $p = 0.0196$) and ventral pelvic skin ($t = -5.223$, $df = 8$, $p = 0.0008$). Thoracic application of 30% v/v ethanol reduced caffeine flux substantially, however this failed to reach significance ($t = -2.097$, $df = 9$, $p = 0.0655$).

For benzoic acid and ibuprofen (Tables 3 and 4), 30% v/v ethanol increased flux significantly for both chemicals through all skin regions. PG had variable effects, significantly improving flux of benzoic acid through the dorsal and thoracic skin regions and marginally improving flux through the pelvic skin ($t = 2.063$, $df = 10$, $p = 0.0661$). Its ability to improve flux of ibuprofen was only significant following thoracic

Table 2
Flux and permeability coefficients for caffeine from a saturated solution of different penetration enhancers through dorsal, ventral thoracic and ventral pelvic *Rh. marina* skin. J_{ss} and K_p reported as mean ± standard error. N = 4. *indicates solvent flux values that are significantly different ($p < 0.05$) to ARS flux values reported in [17].

Solvent	Dorsal		Thoracic		Pelvic	
	J_{ss} (mcg/cm^2/hr)	K_p (cm/hr) × 10^{-3}	J_{ss} (mcg/cm^2/hr)	K_p (cm/hr) × 10^{-3}	J_{ss} (mcg/cm^2/hr)	K_p (cm/hr) × 10^{-3}
1% ethanol	72.747 ± 7.632	3.781 ± 0.793	68.939 ± 7.670	3.584 ± 0.797	102.391 ± 7.108	5.322 ± 0.739
30% ethanol	47.871 ± 2.388*	2.547 ± 0.254	52.286 ± 5.294	2.786 ± 0.561	40.627 ± 4.325*	2.162 ± 0.460
20% PG	33.650 ± 2.643*	1.903 ± 0.299	69.622 ± 6.353	3.937 ± 0.718	73.625 ± 6.097	4.163 ± 0.689

Table 3
Flux and permeability coefficients for benzoic acid from a saturated solution of different penetration enhancers through dorsal, ventral thoracic and ventral pelvic *Rh. marina* skin. J_{ss} and K_p reported as mean ± standard error. N = 4. *indicates solvent flux values that are significantly different ($p < 0.05$) to ARS flux values reported in [17].

Solvent	Dorsal		Thoracic		Pelvic	
	J_{ss} (mcg/cm^2/hr)	K_p (cm/hr) × 10^{-3}	J_{ss} (mcg/cm^2/hr)	K_p (cm/hr) × 10^{-3}	J_{ss} (mcg/cm^2/hr)	K_p (cm/hr) × 10^{-3}
1% ethanol	17.759 ± 2.155	4.416 ± 0.536	11.910 ± 2.420	2.961 ± 0.602	19.800 ± 2.463	4.924 ± 0.612
30% ethanol	37.841 ± 4.669*	3.127 ± 0.386	33.981 ± 5.051*	2.808 ± 0.417	44.994 ± 8.686*	3.718 ± 0.718
20% PG	21.221 ± 1.447*	9.891 ± 0.675	17.086 ± 0.980*	7.964 ± 0.457	27.187 ± 1.856	12.672 ± 0.865

Table 4
Flux and permeability coefficients for ibuprofen from a saturated solution of different penetration enhancers through dorsal, ventral thoracic and ventral pelvic *Rh. marina* skin. J_{ss} and K_p reported as mean ± standard error. N = 4 except for dorsal PG where N = 3. *indicates solvent flux values that are significantly different ($p < 0.05$) to ARS flux values reported in [17].

Solvent	Dorsal		Thoracic		Pelvic	
	J_{ss} (mcg/cm^2/hr)	K_p (cm/hr) × 10^{-3}	J_{ss} (mcg/cm^2/hr)	K_p (cm/hr) × 10^{-3}	J_{ss} (mcg/cm^2/hr)	K_p (cm/hr) × 10^{-3}
1% ethanol	1.406 ± 0.061	5.791 ± 0.501	1.303 ± 0.142	5.365 ± 1.167	1.504 ± 0.071	6.196 ± 0.582
10% ethanol	8.346 ± 0.760*	10.306 ± 1.876	10.841 ± 1.832*	13.387 ± 4.524	8.314 ± 0.779*	10.266 ± 1.925
30% ethanol	9.887 ± 0.527*	7.389 ± 0.788	9.711 ± 1.506*	7.258 ± 2.251	6.573 ± 0.212*	4.913 ± 0.316
20% PG	1.557 ± 0.076	6.069 ± 0.514	1.787 ± 0.083*	6.964 ± 0.649	1.883 ± 0.252	7.339 ± 1.967

application (t = 2.222, df = 11, p = 0.0482). Finally, and interestingly, while both 10% and 30% v/v ethanol significantly improved flux of ibuprofen, the effect was greater for the lower (10% v/v) ethanol concentration, following both thoracic and pelvic application.

3.2.3. Enhancement ratio – comparing the effects of penetration enhancers

Direct comparison of the magnitude of enhancement for each penetration enhancer can be made by considering the enhancement ratio (ER) for a specified chemical and skin region (Fig. 4).

As expected from the flux and K_p data, none of the enhancers were effective in improving the penetration of caffeine, and in some cases, a significant reduction in caffeine penetration was observed.

The effects of ethanol were varied, and dependent upon the concentration applied. 1% v/v ethanol only improved permeability for ibuprofen, with a 2-fold increase for ventral application and 1.7-fold increase following dorsal application. 30% v/v ethanol effectively increased permeability of ibuprofen through all skin regions, although the effect was lower in ventral pelvic skin compared to dorsal or thoracic application (ER = 1.5 for ventral skin, 2.16 and 2.68 for dorsal and thoracic skin, respectively). 30% v/v ethanol was not an effective enhancer for benzoic acid, in all cases having an ER < 1.2, nor for caffeine (all ER < 0.75). Interestingly, 10% v/v ethanol was the most effective enhancer for ibuprofen, with at least a 3-fold increase in absorption (dorsal), extending to a 5-fold increase following thoracic application.

PG generally improved absorption, except for caffeine, which did not change appreciably following ventral application (ER = 1.04 and 0.90 for thoracic and pelvic application, respectively), and was reduced by almost 50% through dorsal skin (ER = 0.49). Dorsal and ventral absorption was enhanced by PG for both benzoic acid and ibuprofen, with a 2.5- to 3.95-fold increase for benzoic acid and 1.8- to 2.6-fold increase for ibuprofen, depending on the skin region. Ventral applications showed the largest increases, with ER ranging from 2.28 – 3.95.

3.3. Differential scanning calorimetry (DSC)

Three endothermic transitions were observed in fresh dorsal *Rh. marina* skin samples ($T2_{frog}$, $T3_{frog}$, $T4_{frog}$; Fig. 5) while only two transitions were noted in fresh ventral skin ($T2_{frog}$ and $T4_{frog}$; Fig. 6). The transition temperature for $T2_{frog}$ was consistent between skin regions (62.8 ± 0.4 °C), whereas the transition temperature of $T4_{frog}$ was higher in dorsal skin (124.3 °C versus 116.4 °C). $T3_{frog}$ occurring at 79.7 °C in fresh dorsal skin.

Following exposure to ARS or penetration enhancers, changes in all three transitions were noted in both dorsal and ventral skin. In dorsal skin samples (Fig. 5), $T2_{frog}$ transition temperature was unaffected by

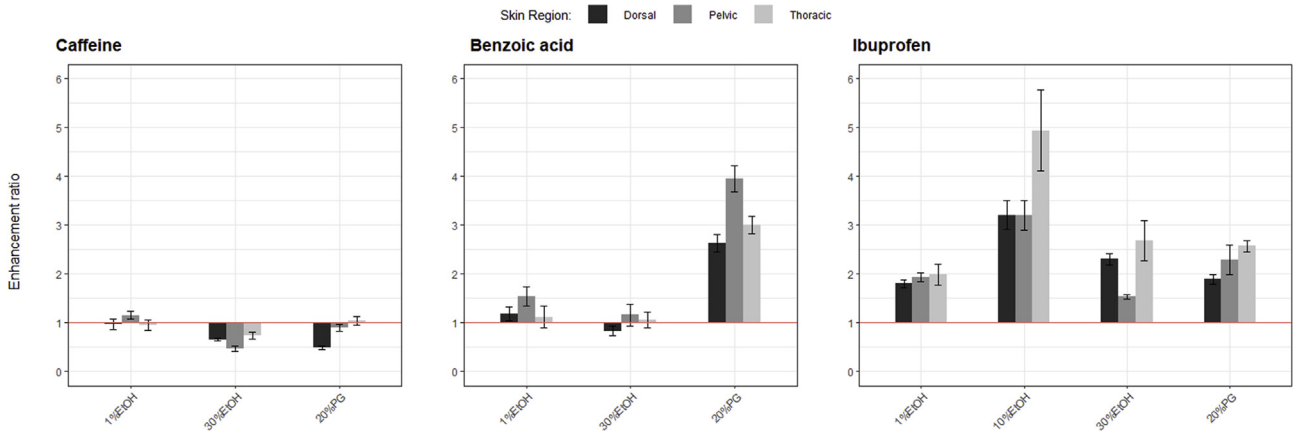

Fig. 4. Effect of ethanol and PG on the penetration of caffeine, benzoic acid, and ibuprofen. Red line indicates ER = 1 (i.e., no change in penetration compared to ARS); error bars are standard error.

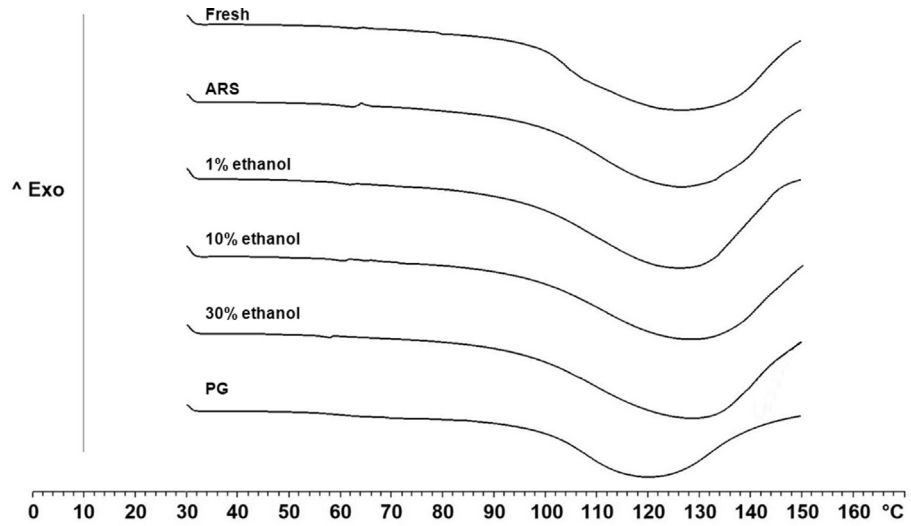

Fig. 5. Representative DSC thermoanalytical curves of dorsal full-thickness Rh. marina skin. From top: fresh skin; skin exposed to: ARS; 1% v/v ethanol; 10% v/v ethanol; 30% v/v ethanol; 20% v/v PG.

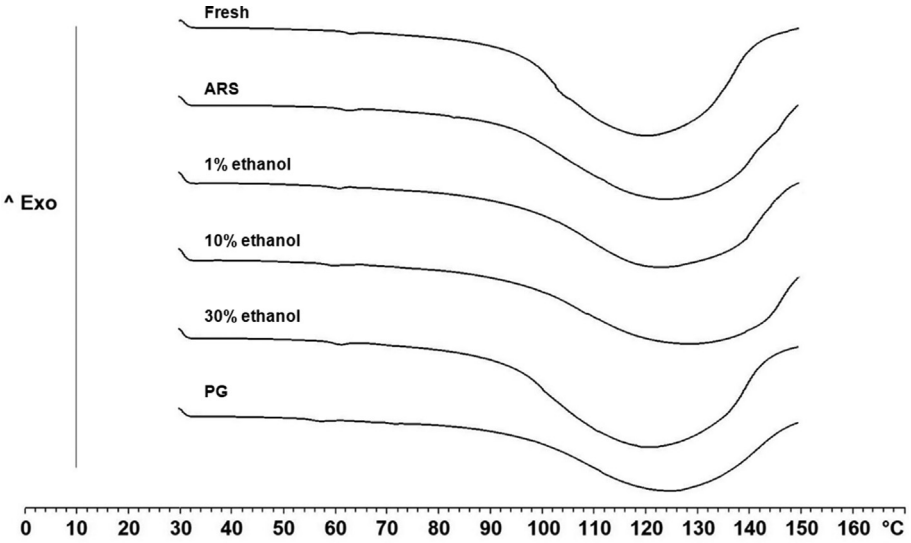

Fig. 6. Representative DSC thermoanalytical curves of ventral pelvic full-thickness Rh. marina skin. From top: fresh skin; skin exposed to: ARS; 1% v/v ethanol; 10% v/v ethanol; 30% v/v ethanol; 20% v/v PG.

ARS, PG or 1% v/v ethanol. As ethanol concentration increased, there was a sequential decrease in transition temperature, with a marked difference in transition temperature, compared to fresh skin, noted for both 10% and 30% v/v ethanol (60.6 °C and 60.0 °C, respectively). Notably, although the transition temperature for ARS-exposed skin did not change, the absence of $T3_{frog}$ was noted, with a corresponding increase in the enthalpy of $T2_{frog}$. A similar phenomenon was observed for the 30% v/v ethanol-exposed skin, where both $T2_{frog}$ and $T3_{frog}$ were present, however the enthalpy of $T2_{frog}$ was increased with a corresponding decrease in the enthalpy of $T3_{frog}$. The opposite trend was observed for PG; although transition temperature of $T2_{frog}$ was essentially unchanged, the enthalpy of the transition decreased and an increase in $T3_{frog}$ enthalpy was noted. $T4_{frog}$ transition temperature was essentially unchanged for most exposed skin; the exceptions were ARS and PG-exposed samples, which showed decreases in transition temperatures (122.6 °C for ARS, and 120.1 °C for PG). Enthalpy of these transitions remained essentially unchanged.

In ventral skin (Fig. 6), the $T2_{frog}$ transition temperature was unaffected by ARS, however the enthalpy of the transition doubled in size compared to fresh skin. For ethanol-exposed skin, decreases in transition temperature were noted for all concentrations, although in contrast to dorsal skin, 10% v/v ethanol had the lowest transition temperature compared to fresh skin (60.3 °C), followed by 30% v/v ethanol and then 1% v/v ethanol. The enthalpy of transition increased by ~50% for all ethanol exposures. Of note, in comparison to dorsal skin where PG did not affect transition temperature of $T2_{frog}$, in ventral skin PG recorded the greatest reduction in transition temperature, compared to fresh skin (57.7 °C). Interestingly, while $T3_{frog}$ was absent in fresh ventral skin, it did appear in all PG-exposed ventral skin, but at a lower temperature than that seen in dorsal skin (72.4 °C). The effects of exposure on $T4_{frog}$ were different from those observed in dorsal skin, with increases in transition temperature and peak broadening noted for all skin exposures. The magnitude of these temperature increases ranged from 119.4 °C for the 30% v/v ethanol concentration to 123.2 °C for ARS-exposed skin. All of the increases in transition temperature were accompanied with a reduction in enthalpy, except for 1% v/v ethanol, which was essentially unchanged.

3.4. Histology

Changes suggestive of disruption to skin integrity and function were noted for all skin samples exposed to all penetration enhancers. The most severe changes were noticeable with ethanol exposures, with changes observable in both epidermal and dermal skin layers. Epidermal changes, including pale nuclei and loss of nuclear and cellular outlines, were most obvious in dorsal skin following exposure to ethanol (Fig. 7), and the severity of these effects increased with increasing ethanol concentrations. PG-exposed skin was noted for swelling/expansion of ventral epidermal keratinocytes resulting in a thickened epidermis (Fig. 8). None of these effects were observed in fresh or ARS-exposed skin samples, indicating they were not associated with the delay between sample collection and preservation, nor with specific ARS-induced changes in the skin.

The most obvious impact of both ethanol and PG on the skin were the effects on the dermis, with marked separation of dermal fibrocytes by oedematous spaces and expansion of melanocytes observed in both dorsal and ventral skin (Fig. 9). The severity of these changes increased with higher concentrations of ethanol, being particularly noticeable in dorsal skin exposed to 30% v/v ethanol. In ventral skin samples, this appeared to reach maximal effect with exposure to 10% v/v ethanol; no further deterioration of the dermis was observed in ventral skin exposed to 30% v/v ethanol. Skin exposed to PG showed similar separation effects, however the severity was similar to that seen following exposure to 1% v/v ethanol.

4. Discussion

4.1. Summary of key findings

While the SC provides the primary barrier to absorption in mammals, this layer is often only 1–2 cell layers thick in frogs, contributing to the heightened permeability of frog skin. As the SC in frogs provides only a modest barrier to absorption, any changes to it, or other skin regions induced by exposure to penetration enhancers is likely to significantly impact absorption through, and regulatory function of, the skin. The DSC and histology findings reported herein found that exposure to penetration enhancers resulted in significant changes to the skin structure, especially in the case of ethanol. 30% v/v ethanol caused loss of cellular outlines in the keratinised cells of the epidermis, leading to coalescence of this layer, and severe changes to the dermis. Even 10% v/v ethanol appeared to induce significant changes in the dermis, and so ethanol in concentrations $\geq$10% v/v should be avoided, as these stark anatomical changes may negatively affect the animal's ability to maintain physiological homeostasis. A minimal effect on the skin was seen following dorsal application of 1% v/v ethanol, however, this concentration of ethanol was ineffective in enhancing penetration for all chemicals, so its value in a therapeutic formulation would primarily be as a co-solvent, enabling reduction in dosing volumes and not enhancement of absorption kinetics.

The best solvent for absorption of a hydrophilic chemical was ARS alone, as none of the enhancers improved penetration of caffeine. 30% v/v ethanol and PG effectively reduced caffeine absorption (30% v/v ethanol following both dorsal and ventral application, PG only following dorsal application). Ergo sequitur, from an environmental toxicology perspective, hydrophilic chemicals used as overhead sprays would be best formulated with PG to reduce absorption of these chemicals in frog skin. Despite the reduction in absorption of hydrophilic chemicals from both dorsal and ventral skin regions due to 30% v/v ethanol, this cannot be recommended, as the histology results suggest that prolonged skin contact with such formulations adversely affect the skin structure in frogs, increasing the absorption of most other chemicals in the environment, and may also negatively affect the frog's ability to maintain physiological homeostasis.

PG was the most consistently-effective enhancer for moderately and highly lipophilic chemicals (all skin regions), while causing the least observable skin changes. As the majority of therapeutic chemicals are of moderate to high lipophilicity, PG represents the safest and most effective option for penetration enhancement for a wide range of therapeutic chemicals in frogs.

4.2. In vitro studies – absorption kinetics

As these experiments included saturated solutions of each chemical, flux results that differ significantly from the use of ARS alone suggest modification of the skin barrier by the penetration enhancers [20]. It is unsurprising that higher concentrations of ethanol improved flux for benzoic acid and ibuprofen in the current study, considering the obvious changes in frog skin structure observed (Fig. 9). Of note is the finding that 10% v/v ethanol was more effective in improving ibuprofen flux than 30% v/v ethanol for ibuprofen. Similar results were observed in human epidermis in a study by Watkinson et al. [21], who reported that flux of ibuprofen from binary ethanol/water solutions increased rapidly up to a 50/50 v/v mixture, thereafter remaining relatively steady up to 75/25 v/v, before decreasing significantly in 100% ethanol. The authors

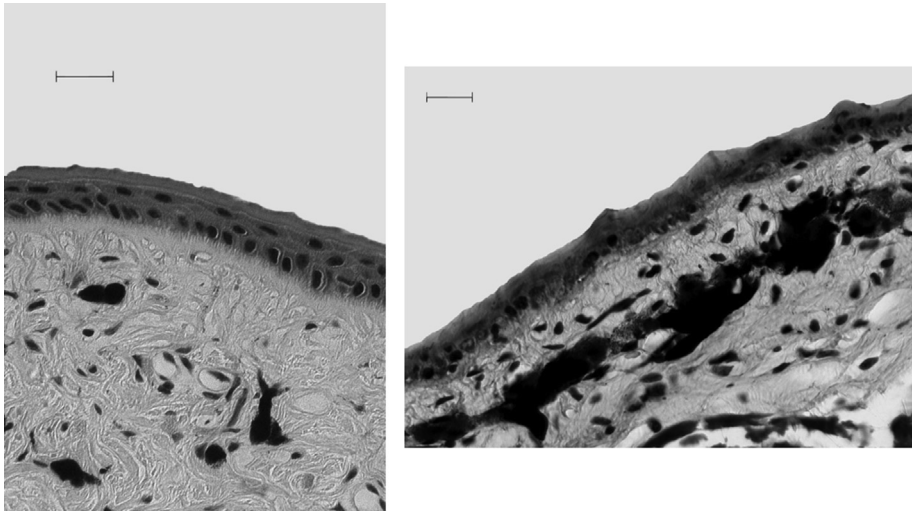

Fig. 7. Histological sections of dorsal skin in *Rh. marina* showing effect of ethanol exposure on epidermal structure. Left: fresh *Rh. marina* skin. Right: skin exposed to 30% v/v ethanol for 6 hr. Note loss of cellular outlines in epidermis. Scale bar = 20 mcm.

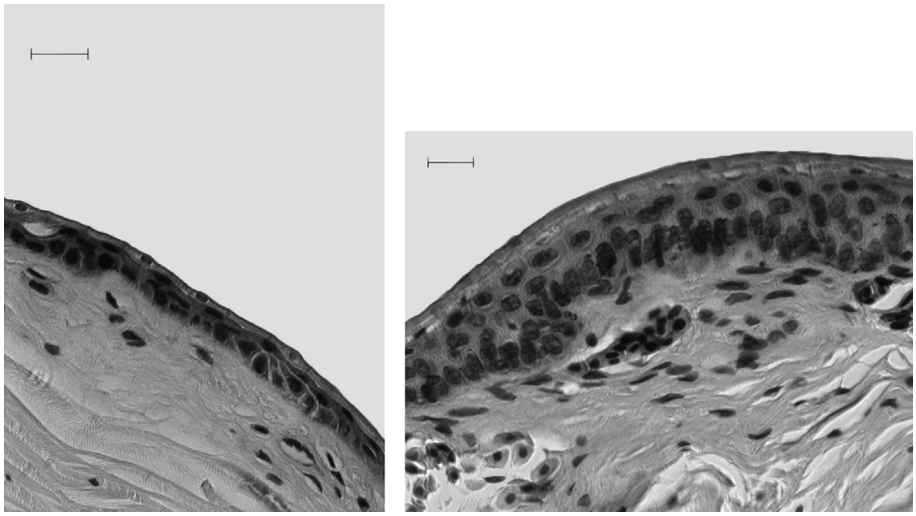

Fig. 8. Histological sections of ventral skin in *Rh. marina* showing effect of PG exposure on epidermal structure. Left: fresh *Rh. marina* skin. Right: skin exposed to 20% v/v PG for 6 hr. Note swelling of keratinocytes compared to control. Scale bar = 20 mcm.

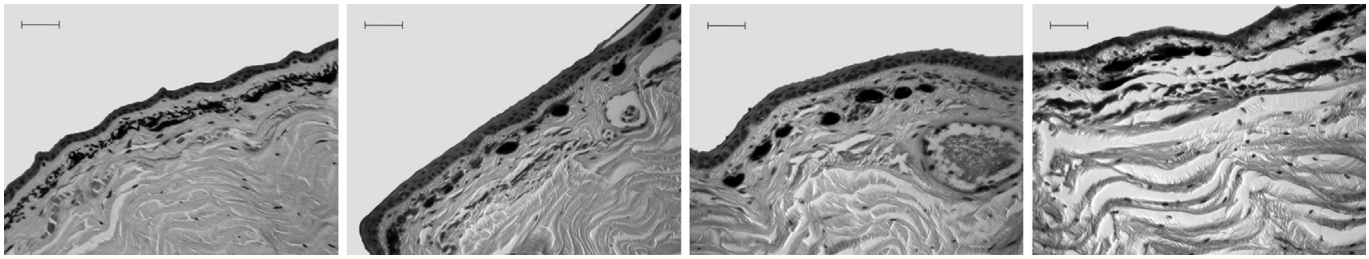

Fig. 9. Effect of ethanol exposure on dorsal skin from *Rh. marina*. Ethanol concentrations increasing from left. Left-to-right: fresh skin, 1% v/v ethanol, 10% v/v ethanol, 30% v/v ethanol. Note separation of dermal fibrocytes by oedematous spaces. Scale bar = 50 mcm.

postulated that this effect could be due to dehydration of the SC at higher ethanol concentrations. However, as can be seen from the histological sections of frog skin (Fig. 7), it is likely the reduction in flux demonstrated with the higher ethanol concentration is due to the homogenization of the SC, creating a solid, relatively lipophilic barrier to absorption. It is likely, given the lipophilic nature of ibuprofen, that it may partition from the dosing solution into the SC/epidermal layer, however the epidermal restructuring caused by the ethanol may cause ibuprofen to be less likely to partition further into the more hydrophilic dermis. Further studies, including fixed-dose applications with full mass balance to determine skin ibuprofen content may provide clarification of this hypothesis. Regardless, the use of ethanol in formulations for application to frog skin cannot be recommended at these higher concentrations, as such changes to the skin structure is likely to have serious implications for the animal's ability to maintain homeostasis.

In mammalian skin studies, a lag phase for absorption is regularly observed, as an applied chemical must diffuse from the donor, across the thick SC and the remaining skin barrier before it reaches the receptor. However, as frog skin is much thinner than mammalian skin, lag times have not previously been reported when measuring transdermal absorption from simple aqueous solutions [13, 17, 22]. A lag phase was, however, noted in the current study when chemicals were formulated in 30% ethanol. In mammals, coformulation with ethanol has different effects on the lag phase depending on the ethanol concentration, due to the concentration-dependant effects of ethanol on the skin. Typically, ethanol at lower concentrations causes fluidisation of the skin membrane, and this is associated with decreased lag time and improved absorption. Conversely, at higher concentrations ethanol is known to extract skin lipids; this is typically observed as a significantly increased lag time followed by rapid flux [8, 23]. In mammals, the first mechanism (membrane fluidisation, decreased lag time and increased permeability) is dominant for ethanol concentrations up to ~60% [21, 24], with lag times increasing higher ethanol concentrations. In reptiles, no lag phase was reported in shed snake skin when an aminophylline gel was formulated with the penetration enhancers lauric acid, sodium taurooglycocholate (a surfactant), or PG, however a lag time was reported when formulated in 60% ethanol [25]. It is therefore not surprising that a lag time was observed with the higher ethanol concentration in the current study, but not with the lower concentrations. We predict that the underlying mechanism is the same in both animal classes, simply that the thinner nature of frog skin means that lower ethanol concentrations are needed for these effects to be observed. Indeed, significant changes to the keratinized cells of the epidermis were noted in skin samples exposed to 30% ethanol (Fig. 7).

PG was the most consistently-effective enhancer for benzoic acid and ibuprofen, although the magnitude of improvement changed depending on the skin region of application. It was the most effective penetration enhancer for benzoic acid, and while not able to enhance penetration as much as ethanol for ibuprofen solutions, induced less severe changes in the skin, and so provides a safer alternative for penetration-enhancement. These results contribute to the contradictory reports in the literature as to the effectiveness of PG as a penetration enhancer on its own [26]. In mammals, often the effect of PG on absorption kinetics is negligible when formulated alone, with enhancement only occurring when used in combination with another known enhancer, such as fatty acids. It is possible that the effectiveness of PG demonstrated in the current study is due to the thinner barrier provided by frog epidermis, and that modulating effects of PG are insufficient to significantly alter the barrier properties of the thicker SC in mammals. The findings in the current study reinforce the caution required by clinicians when using

formulations (containing penetration-enhancing ingredients) designed for use in mammals in frogs, as although these formulations may be reported to not influence absorption in mammals, different effects may be observed in frogs [27].

Finally, penetration enhancers investigated in the current study had different effects on absorption, depending on both the chemical being applied to the skin and the skin region. As skin region influenced the absorption kinetics from the different solvents, the application site needs to be considered together with the formulation composition when developing therapeutics for use in frogs. This finding is of interest, as a previous study by this research group in *Rh. marina* found that the flux of benzoic acid and ibuprofen did not significantly change between skin regions [17] when formulated in ARS alone.

4.3. DSC and histology

This study is the first to characterise full-thickness frog skin, including the effect of penetration enhancers, using DSC. Previous studies have reported findings in a variety of mammalian skins (for review, see Babita et al. [28]), and snake skin has also been reported [3]. Most studies in mammals identify three or four main transitions common to all investigated skin types ($T1_{mammal}$-$T4_{mammal}$), occurring at ~40 °C ($T1_{mammal}$; sometimes absent in studies), 70–75 °C ($T2_{mammal}$), 80–85 °C ($T3_{mammal}$) and 105 °C ($T4_{mammal}$) [28]. T2-T4 were evident in fresh dorsal frog skin, and T2 and T4 in ventral frog skin, however T1 was not observed in either skin region. This may be due to the starting temperature in the current experiment being necessarily high owing to the ambient laboratory conditions, preventing observation of the transition. In addition, $T2_{frog}$ and $T3_{frog}$ were found to be 10–15 °C lower than reported in mammalian SC, so it is possible that this first transition may also have occurred at a reduced temperature, below the temperature range investigated in the current study.

$T2_{mammal}$ and $T3_{mammal}$ have been attributed to intracellular lipids changes, and lipid-protein complex changes, respectively, whereas $T4_{mammal}$ is associated with protein denaturation. It is interesting that the lipid-associated transitions in frog skin occur at lower temperatures, and the protein denaturation at higher temperatures, than seen in mammalian skin. Differences in lipid-associated transitions between species are likely due to differences in constituent skin lipids and their arrangement in the epidermis. In particular, lower phospholipid content of the skin has been reported to correlate with reduced transition temperatures, and ceramides with increased transition temperatures [28]. While the exact lipid composition of *Rh. marina* skin has not been reported, the lipid composition of epidermis in a group of frogs from *Cyclorana* spp. reported 0.86% phospholipid content and a complete absence of ceramides [29]. Comparatively, human SC is reported to contain 3–5% phospholipids and 27% ceramides, depending on the skin region investigated [30]. Additionally, more permeable skins typically exhibit looser packing of the epidermal lipids and as frog skin is highly permeable, it is likely this also contributed to the lower transition temperatures observed for $T2_{frog}$ and $T3_{frog}$ in the current study.

An interesting finding in the current study was the observation that for dorsal skin samples, exposure to ARS or 30% v/v ethanol resulted in a reduction in enthalpy of $T3_{frog}$ with a corresponding increase in the enthalpy of $T2_{frog}$. This has been reported previously in porcine skin [31], whereby heating of the skin resulted in a loss of T3 and an increase in the enthalpy of T2 equivalent in size to that of T3. Of note in the current study was the opposite finding in PG-exposed dorsal skin – where $T3_{frog}$ increased in enthalpy, with a corresponding decrease in enthalpy of $T2_{frog}$. Studies utilizing different methodologies to investigate changes in frog skin following PG-exposure will assist in explaining this phenomenon.

$T4_{mammal}$, in contrast, is associated with the hydration status of skin; it is absent in dehydrated skin and skin with a total water content of <15%, and the temperature of this transition declines continuously with increasing hydration of the skin [28]. Further, it has been suggested that higher transition temperatures of $T4_{mammal}$ with broad peaks indicates dehydration of the membrane. As the pelvic ventral patch in frogs is physiologically designed to optimize water uptake, it is perhaps unsurprising that the transition temperature was lower for fresh ventral skin samples than for the dorsal samples. Similarly, this may also explain the reduction in $T4_{frog}$ temperature following exposure of dorsal skin to ARS (which would be expected to increase hydration of the skin), and the increases in $T4_{frog}$ with accompanying broadening of the peak, for all ventral skin exposures, as presumably fresh ventral skin would already be optimally-hydrated.

For penetration enhancer-exposed skin samples, the thermal analysis curves for dorsal skin exposed to ARS or 1% v/v ethanol were essentially the same, corresponding with the in vitro absorption studies and histology, which suggested that 1% v/v ethanol only marginally affects skin structure and absorption kinetics. Of note, considering the in vitro absorption results for PG in the current study, is the DSC and histology results for PG-exposed skin samples, which showed a reduction in transition temperature for $T2_{frog}$ in both dorsal and ventral skin. These findings concur with those of Brinkmann and Müller-Goymann [9], who found a similar reduction in transition temperature for T2 when investigating the impact of PG pretreatment on human skin. Following x-ray diffraction studies on the pre-exposed skin, the authors concluded that PG integrates into the hydrophilic regions of the SC lipid bilayer, causing expansion to this region and thus disturbing the lipid organization. This conclusion also agrees with the histology findings in the current study, which showed distinct swelling of the epidermal layers of the frog skin following PG exposure.

An ideal chemical penetration enhancer should enhance penetration, without permanently disrupting the skin structure. However, the histology results for ethanol demonstrated significant changes in both the epidermal and dermal skin layers. At higher concentrations, ethanol is reported to extract lipids from the SC in mammals [8]; in the current study higher ethanol concentrations altered the cellular outlines of the epidermal keratinocytes, causing coalescence of the keratinocytes. These effects have numerous safety implications: lipid extraction can create pores in the SC, allowing unimpeded passage of chemicals into the dermis and supratherapeutic (and potentially toxic) drug levels, whereas disruption of the keratinocyte outlines and coalescence of the keratinocytes may result in the formation of a relatively impervious barrier, which may either impede drug absorption (resulting in sub-therapeutic effects) or could provide a depot for sustained drug release. Such damage to the skin is irreversible and would remain until new epidermal turnover is complete. Additionally, such skin damage in a frog would be likely to substantially impact on the ability of the frog to maintain physiological homeostasis, leading to impaired fluid and electrolyte balance. While skin changes, including expansion of the epidermal keratinocytes, were noted in PG-exposed skin, these changes are likely to be more readily reversible than those identified in ethanol-exposed skin.

4.4. General discussion

While the focus of this study has been on the impact of penetration enhancers on model chemical absorption, the ability of the enhancers themselves to be absorbed must be considered. Ethanol and PG rapidly penetrate mammalian skin; indeed, the initial rapid increase in absorption noted with benzoic acid and ibuprofen when formulated in ethanol in the current study is likely due to initial solvent drag. These results emphasise the need for the clinician and the environmental toxicologist to consider the impact formulation of chemical(s) with penetration enhancers may have on systemic absorption of the chemical, while also considering that the enhancers themselves are also likely to be absorbed, having local or systemic effects including potential toxicity. Krause et al. [32] reported on toxicity resulting in death of a group of red-eyed tree frogs (*Agalychnis calidryas*) following topical application of ivermectin diluted in PG. Necropsy results showed supra-therapeutic levels of both the ivermectin and PG, and the authors concluded that toxicity was likely

caused by incomplete mixing of the solution. Ivermectin is a large molecule with a logP (estimated) of 5.83 [33]. In the current study, ibuprofen, the most lipophilic chemical investigated, showed a 1.8–2.6-fold increase in penetration following formulation in 20% v/v PG. It is therefore likely that the penetration-enhancing effects of the PG contributed to the toxicity reported by Krause et al. [32]. Similarly, toxicology researchers have highlighted that the inclusion of surfactants in the herbicide glyphosate contributes to the toxicity of these formulations to amphibians [34]. Howe et al. [35] investigated individually the toxicity of neat glyphosate, the surfactant used in some glyphosate formulations, and five commercial glyphosate formulations in four frog species. Acute toxicity was highest with the surfactant alone, and lowest with the neat glyphosate. However, this and other studies have been primarily concerned with the toxicity caused by the surfactant itself, and have not considered that the surfactant is also likely increasing the absorption of the glyphosate in the formulation, contributing to the toxicological profile. While the current study did not investigate surfactant action on percutaneous absorption in frogs, surfactants are known penetration-enhancers and should be expected to influence absorption of both the chemical and the enhancer itself. Thus, both the contribution of penetration enhancers to altered absorption and the individual toxicity of the enhancers themselves must be considered when formulating chemicals which may be administered to frogs (whether intentionally, for therapeutic purposes, or inadvertently following exposure in their habitat). Studies that investigate other penetration enhancers that are commonly included in agricultural and industrial chemical formulations would therefore provide valuable data regarding risk assessment and management when using these products in frog habitats.

5. Conclusions

The penetration enhancers investigated in this study are the most common agents used in commercial and compounded therapeutic liquid formulations administered to frogs, and are also often included in agrichemicals and other industrial products and so are likely to be present in frog habitats. The results herein provide information on the absorption-enhancing effects of these agents when included in formulations, and can be used to guide dose/application site adjustment when used in frogs. In particular, the use of ethanol in concentrations over 10% v/v cannot be recommended, despite demonstrated penetration-enhancing effects, owing to the severe skin changes caused by ethanol at these concentrations. 1% v/v ethanol may find use in formulations as a co-solvent, with minimal impact on both absorption kinetics and skin morphology. PG can enhance percutaneous absorption of moderately and highly-lipophilic chemicals, with minimal impact on the skin, and so should be considered when penetration enhancement of these chemicals through frog skin is required. Finally, hydrophilic chemicals may have significantly reduced absorption when included in formulations containing PG or higher concentrations of ethanol, particularly when administered to the ventral skin surface, and so these enhancers should be avoided unless reduced absorption is desired, for example in retarding absorption of hydrophilic environmental contaminants.

Declarations

Author contribution statement

Victoria K Llewelyn: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Lee Berger: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Beverley D Glass: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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