A preliminary investigation into the use of amino acids as potential ion pairs for diclofenac transdermal delivery

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Abstract:
Ion pairing is a potential strategy used to increase the partition and permeation of ionisable drug molecules. This work outlines the process of identifying, selecting and testing potential counter ions for diclofenac (DF). Three screening criteria were considered in the initial selection process. The first, toxicity, was used to eliminate counter ion candidates that could not be used in topical formulations. The second related to the balancing of charges. As DF is a free acid in its unionised state, counter ions should be of a basic character. Finally, molecular size, as represented by molecular mass (Da), was used. Because of the impact on ion pair formation, the counter ion was required to have a lower molecular weight than diclofenac. Basic amino acids L-Arginine, L-Histidine, L-Lysine and their salts were chosen. The selection process concluded with Partition Coefficient (PC) studies. These were used to identify any counter ions able to interact electrostatically with the ionised DF, enabling the 'neutral' ion pair to partition from an aqueous into an organic layer. Permeation studies using porcine skin were performed to test the efficacy of any selected counter ion. These preliminary studies suggest that amino acids may be used as counter ions to increase the partition and permeation of ionisable drugs.
Keywords: Ion pairs; diclofenac; diclofenac sodium; amino acids; partition coefficient studies; porcine skin; permeation; mass balance.

1. Introduction

The topical application of drugs provides many advantages, as evidenced in the case of non-steroidal anti-inflammatory drugs (NSAIDs). A study considering hospitalisation due to adverse drug reactions (ADR) found, in the case of prescribed drugs, that NSAIDs accounted for 30% of hospital admissions. Reactions included gastrointestinal bleeding, peptic ulceration, haemorraghic cerebrovascular accident and renal damage (Pirmohamed et al., 2004). A similar study investigated ADRs requiring hospital admissions due to over-the-counter (OTC) medication or self-medication using previously prescribed drugs. It was determined that 30% of the adverse reactions were gastrointestinal complaints caused by NSAIDs (Schmiedl et al., 2014). It is unsurprising therefore that in cases of osteoarthritis, the National Institute for Health and Care Excellence (NICE) recommends offering a topical NSAID (and/ or paracetamol) ahead of any oral NSAIDs, COX-2 inhibitors or opioids (The National Institute for Health and Care Excellence (NICE), 2021).

However, as the uppermost layer of the skin, the stratum corneum (SC), provides a very effective barrier to any exogenous substances, numerous strategies have been explored to increase the permeation of active ingredients. These strategies can be divided into active or passive methods. Active methods, which include iontophoresis, phonophoresis and the use of microneedles, are categorised as physical techniques used to overcome the SC (Mitragotri, 2013). Passive methods are described as those that focus on the formulation itself, i.e. the active ingredient in combination with several excipients (Hadgraft, 1999; Lane et al., 2011). These include increasing the thermodynamic activity of drugs in the formulation (Hadgraft, 1999), the use of skin penetration enhancers (Lane, 2013) and also the use of ion pairs (Cristofoli et al., 2021).

As mentioned previously (Cristofoli et al., 2021), ion pairing has been explored to both increase and regulate the passage of ionised drugs that are applied for topical or transdermal use. This preliminary study outlines the initial steps taken to identify and test potential counter ions for the NSAID diclofenac (DF), with the purpose of increasing the permeation of this active pharmaceutical ingredient (API).

The selection of prospective counter ions for DF involved several important considerations, the first being toxicity. An understanding of the toxicity of counter ions is critical, as they may co-partition with the API into the skin (Cristina et al., 2012). Thus, a prospective counter ion that would not be acceptable from a safety perspective should be disregarded. The second factor relates to the balancing of potential ionic charges. Because the DF molecule is a free acid in its unionised state (Figure 1), the counter ion should be a basic molecule. With a pK_a range reported from 3.8 – 4.2 (Avdeef et al., 1998; Chiarini et al., 1984; Fini et al., 1995; Llinàs et al., 2007; Moffat et al., 2010; Sallmann, 1986), DF in solution is negatively charged at physiological pH. Counter ions should therefore be positively charged. The final consideration is related to the size of the molecule. Bjerrum, who introduced the concept of ion pairs, identified size as a factor that influenced the association or dissociation of ion pairs (Bjerrum, 1926). This was later confirmed by Fini *et al* in their work which examined the formation of ion pairs of diclofenac salts in aqueous solutions (Fini et al., 1999).

The basic amino acids L-Arginine, L-Histidine, L-Lysine and their salts (Figure 2), were selected as the potential counter ions. With reference to toxicity, the amino acids and their salts are generally recognised as safe (GRAS) when used as food additives by the FDA (Office of the Federal Register National Archives and Records Administration, 2011). They have also been used as cosmetic ingredients according to information given to the FDA via the Voluntary Cosmetic Registration Program (VCRP). Human Repeat Insult Patch Test Studies have concluded that products containing these ingredients resulted in no dermal irritation or sensitisation. The Cosmetic Ingredient Review panel after studying this information, considered them to be safe in present practices of use and concentrations in cosmetics (Burnett et al., 2013).

In relation to charge, Table 1 shows that the isoelectric points, (pH (I) or p(I)), i.e. the points at which these amino acids have no net electrical charge, exceed the physiological pH. For L-histidine (L-His) the pI value of 7.6 was not much greater, but it increased with L-Lysine (L-Lys) having a pI value of 9.5 and L-Arginine (L-Arg) with a pI of 10.8. Regarding size, Table 2 indicates that the molecular weight (Da) of DF (296.15 Da) exceeds that of L-His (155.16 Da), L-Lys (146.19 Da) and L-Arg (174.20 Da). For the purposes of clarity, Table 3 confirms that the molecular weight (Da) of diclofenac sodium (DNa) (318.10 Da) exceeds that of L-histidine hydrochloride monohydrate (L-HSS) (209.63 Da), L-Lysine monohydrochloride (L-LSS) (182.65 Da) and L-Arginine monohydrochloride (L-ASS) (210.66 Da). These values however, are more aligned to those of the free acid and free bases, due to dissociation of the ionic salts in solution.

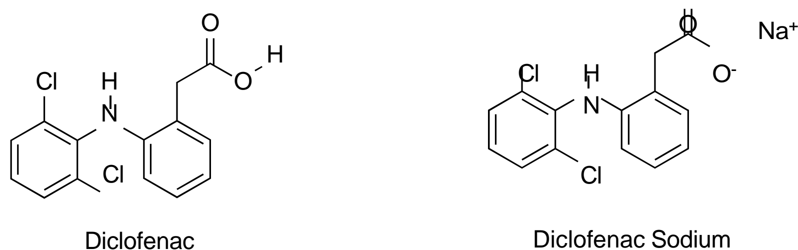

Figure 1. Structures of diclofenac free acid and diclofenac sodium

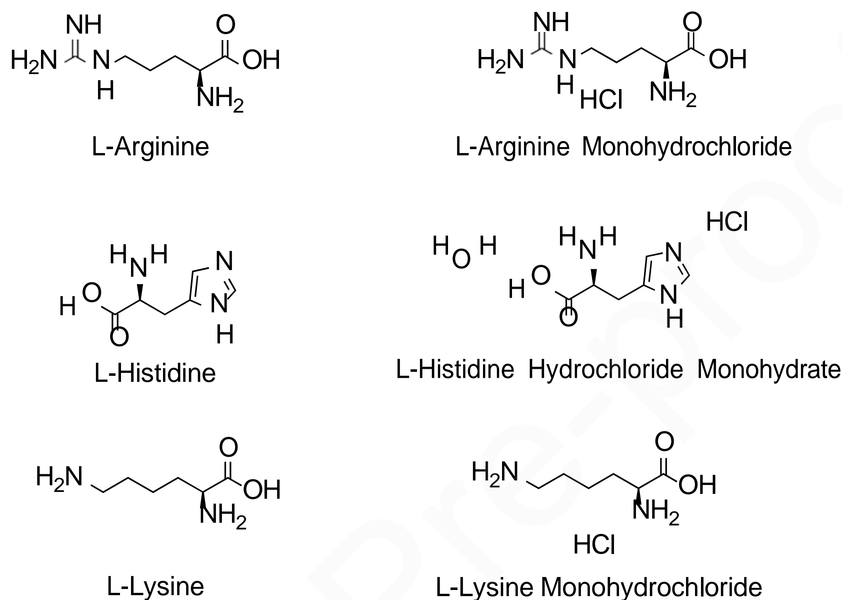

Figure 2. Structures of L-Arginine, L-Arginine Monohydrochloride, L-Histidine, L-Histidine Hydrochloride Monohydrate, L-Lysine and L-Lysine Monohydrochloride

Table 1. pK_a and pI values for DF, L-Arg, L-His and L-Lys

Compound	pK_a	pI
Diclofenac free acid	4.2	N/A
L-Arginine	2.17, 9.04, 12.48	10.8
L-Histidine	1.82, 6.00, 9.17	7.6
L-Lysine	2.18, 8.95, 10.53	9.5

Table 2. Molecular mass of DF, L-Arg, L-His and L-Lys

Compound	Molecular mass (Da)
Diclofenac free acid	296.15
L-Arginine	174.20
L-Histidine	155.16
L-Lysine	146.19

Table 3. Molecular mass of DNa, L-ASS, L-HSS and L-LSS

Compound	Molecular mass (Da)
Diclofenac Sodium	318.10
L-Arginine Monohydrochloride	210.66

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L-Histidine Hydrochloride Monohydrate	209.63
L-Lysine Monohydrochloride	182.65

The selection process concluded by testing whether the counter ion candidates could interact electrostatically with the DF anion, creating an ion pair. If successful, the apparently neutral species should be able to partition from an aqueous into an organic layer (Inagi et al., 1981). Simple partition coefficient (PC) studies, were used for this purpose. When an ion pair is formed, individual ions mask their charges through electrostatic interactions. While these experiments do not account for interactions of the molecules with biological membranes, different solvent systems and other extrinsic factors; they are useful as a screening mechanism to identify prospective ion pairs for permeation studies (Auner et al., 2003; Green et al., 1988; Megwa et al., 2000; Sarveiya et al., 2005; Trotta et al., 2003; Valenta et al., 2000).

The aims of this work were (i) to determine the PC for DF (a) as the free acid, and in combination with the amino acid free bases and (b) as the salt form (DNa), as shown in Figure 1, and in combination with the amino acid salts. The combination achieving the highest PC values would then be used to (ii) investigate the effect on porcine skin permeation of DF using (a) a total amount of DF similar to that contained in a finite dose application of a 1% commercial DF-containing product (± 100 µg) and (b) a total amount of DF closer to that contained in an infinite dose application of a 1% commercial DF-containing product (± 350 µg).

2. Materials and Methods

2.1 Materials

DNa 98% and the amino acids, L-Arg 98+% and L-Lys 98% were produced by Acros Organics (VWR, Leceistershire, UK). DF was synthesised and characterised in house. L-His high purity grade I was ordered from VWR (Leceistershire, UK). The amino acid salts L-ASS 98+% and L-LSS 99+% were obtained from Alfa Aesar and L-HSS 98% was from Acros Organics, all supplied by VWR (Leceistershire, UK)). High vacuum grease was obtained from Dow Corning (Seneffe, Belgium). Oxoid™ Phosphate buffered saline (PBS) tablets were purchased from Thermo Fisher Scientific (Lancashire, UK). HPLC grade acetonitrile (ACN), hydrochloric acid (HCl) (37%) and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Lancashire, UK). 1-Octanol 99% was obtained from Alfa Aesar (VWR, Leceistershire, UK). Ethanol 99.8% and 0.45 µm Millex® HV syringe filter units were supplied by Sigma Aldrich (Dorset, UK). Filter paper 150 mm diameter was obtained from Fisher Scientific (Lancashire, UK).

2.2 Methods

2.2.1 HPLC Analysis

The analysis of diclofenac was performed using an Agilent 1100 HPLC. This was equipped with an Agilent G1379A degasser, a G1311A quaternary pump, a G1313A autosampler and a G1316A thermostat (Agilent Technologies, Santa Clara, CA, USA). The column used was a Shiseido CAPCELL PAK C18 MGIII, with a length of 250 mm, an internal diameter of 4.6 mm, a 5 µm particle size and 100 Å pore size (Osaka Soda, Osaka, Japan). A universal HPLC guard column (Phenomenex, Macclesfield UK) containing a SecurityGuard™ cartridge (Phenomenex, Macclesfield UK) was attached to the column. The mobile phase comprised acetonitrile (ACN): 0.1% trifluoroacetic acid in water (70:30). The flow rate was 1 mL/min and the column temperature was set to 25 °C. A detection wavelength of 277 nm was chosen for the acquisition of chromatograms. The injection volume was 10 µL, with the DF peak eluting at 6 min and each sample having a total run time of 10 min. Calibration curves for the detection of diclofenac, ranging from 0.05 - 400 µg/mL were prepared using DNa. This method was validated in accordance with ICH (2005) guidelines (International Conference on Harmonisation Expert Working Group, 2005) for linearity, accuracy, precision, robustness, limit of detection (LOD) and limit of quantification (LOQ). LOD was 0.12 µg/mL and LOQ was 0.37 µg/mL.

2.2.2 Synthesis of DF from DNa

A solution of DNa was prepared using distilled water. Various concentrations of HCl were made up, including 0.01 M, 0.5 M and 0.0001 M. HCl was slowly added dropwise to a stirring aqueous solution of DNa. At regular intervals, the pH of the solution was determined using a VWR SymPhony pH Meter (VWR, Leicestershire, UK). Once a ~ pH 4 was achieved, the solution was filtered, and the precipitate recovered. The precipitate was then washed three times using low pH (0.0001 M) HCl solution that had been maintained at a temperature of ± 8 °C. This was followed by drying using a desiccator for 24 h. Confirmation that the product, DF, had been synthesised from DNa, was obtained by use of ^{1}H-NMR. The appearance of a broad peak at approximately 12 – 13 ppm, as shown in Figure S2, indicated the protonation of the carboxylic acid species, confirming free acid formation. The ^{1}H-NMR spectrum of DNa, with no peak in the same area is shown in Figure S1.

2.2.3 Confirmation of the non-interference of amino acids with HPLC method for quantification of diclofenac

Absorption spectra (200 – 800 nm) were obtained for DNa, as well as the free base and salt forms of all potential amino acid counter ions, using a Jenway 7315 UV spectrophotometer (Cole-Parmer, Staffordshire, UK). Individual samples of the free bases and their salts using molar equivalent concentrations of 100 ug/mL of DF were run using the HPLC methodology described above. This was repeated after increasing the concentration of the counter ions by 50 times the previous amount. Samples containing known amounts of diclofenac with amino acids in 1:1 and 1:50 molar ratios were analysed using the HPLC method. The solvent mixture used to prepare all samples was ACN:H_2O (70:30). Solutions were filtered using a 0.45 µm Millex® HV syringe filter unit.

2.2.4 Measurement of partition coefficient (PC) between octanol and PBS (pH 7.3 ± 0.2)

The PCs for DF alone and with free base versions of the potential counter ions (L-Arg, L-His and L-Lys) were investigated using the counter ions at the molar ratios 1:0; 1:0.5; 1:1; 1:5; 1:10 and 1:50. As the free acid is only slightly soluble at pH 7.4 (Durairaj et al., 2009), DF was dissolved in ethanol. Octanol saturated PBS was added to the ethanol solution resulting in an ethanol: PBS (20:80 v/v) aqueous phase having a DF concentration of 100 µg/mL. This was divided between 6 vials, containing different concentrations of counter ions resulting in the final molar ratios of DF: amino acid 1:0; 1:0.5; 1:1; 1:5; 1:10 and 1:50. Equal amounts of the aqueous phase and PBS saturated octanol were added to screw topped glass tubes (n=3). These were sealed with Parafilm™ and shaken overnight on a Stuart Orbital Incubator S150 (Cole-Parmer, Staffordshire, UK) at 25 °C at 225 RPM. Samples were then centrifuged at 3000 RPM for 20 min at 25 °C. The PC for each combination of free acid: free base was calculated using Equation 1:

$$P_c = (C_i - C_f) / C_f \quad \text{Equation 1}$$

Where C_i represents the initial concentration of DF in the aqueous phase before partitioning and C_f represents the concentration in the aqueous phase after partitioning. $C_i - C_f$ corresponds to the amount that has partitioned into the octanol phase.

The same experiment was repeated using DNa with the salt versions of the basic amino acids. In this set of experiments no ethanol was used, and the aqueous phase was octanol saturated PBS.

2.2.5 Preparation of PBS formulations for *in vitro* permeation studies (IVPS)

For the initial permeation study (IVPS-100) a stock solution of 107.41 µg/mL of DNa in PBS (pH 7.3 ± 0.2), equivalent to 100 µg/mL of DF in PBS, was prepared. L-HSS (0.0014 g) was added to a vial containing 20 mL of the stock solution resulting in a 1:1 ratio of DF:L-HSS. L-HSS (0.0707 g) was added to a second vial containing 20 mL of the stock solution resulting in a 1:50 ratio of DF:L-HSS. These were stirred overnight.
For the second permeation study (IVPS-350) a stock solution of 376 µg/mL of DNa in PBS (pH 7.3 ± 0.2), equivalent to 350 µg/mL of DF in PBS, was prepared. L-HSS (0.0024 g) was added to a vial containing 10 mL of the stock solution resulting in a 1:1 ratio of DF:L-HSS. L-HSS (0.1238 g) was added to a second vial containing 10 mL of the stock solution resulting in a 1:50 ratio of DF:L-HSS. These were stirred overnight.
The samples representing DNa alone (DNa:L-HSS (1:0)), DNa and L-HSS at a 1:1 molar ratio (DNa:L-HSS (1:1)) and DNa and L-HSS at a 1:50 molar ratio (DNa:L-HSS (1:50)) were tested to determine the amount of DF in solution at the time of application.
For IVPS-350, samples were also filtered to determine the amount in solution at time of application.

2.2.6 *In vitro* porcine skin permeation studies (IVPS)

Infinite dose IVPS were conducted using vertical glass Franz diffusion cells (Soham Scientific, Cambridgeshire, UK). The diffusion area for each Franz cell was approximately 1 cm^2, as calculated using digital calipers (Fisher Scientific). The number of replicate experiments was n = 4.

Full thickness porcine ear skin, obtained from a local abattoir, was selected as the closest model for human skin (Dick and Scott, 1992; Jacobi et al., 2007; Starr et al., 2022). Upon receipt, the skin was separated from the underlying tissue, wrapped in aluminium foil and stored in the freezer at -20 °C. Immediately prior to permeation, the skin was cut to size, excess hair was trimmed, rinsed in PBS (pH 7.3 ± 0.2) and dried using filter paper. The membrane was sandwiched between the donor and receptor compartments of the pre-greased Franz cells, which were then secured with a clamp. The receptor compartment of the clamped unit was filled with approximately 2 mL of PBS (pH 7.3 ± 0.2) and stirred using a Teflon™ coated magnetic micro stirrer bar. This was placed into a Grant Sub Aqua 26 water bath (Grant Instruments, Cambridgeshire, UK) preheated to ~ 37 °C, to achieve a skin surface temperature of 32 ± 1 °C. Once the temperature of the skin was confirmed, using a Digitron TM22 digital thermometer (British Rototherm Company Ltd, Port Talbot, UK), an unfiltered 1 mL dose of the formulations was applied to the donor compartment. The donor compartment was covered using Parafilm™. Samples of 200 µL were taken hourly for 6 h for IVPS-100 and for 7 h for the IVPS-350. A final sample was taken at 24 h for both studies. Equal volumes of PBS (pH 7.3 ± 0.2), maintained at the same temperature in the water bath, replaced the sample volumes taken. All samples were analysed using the previously described HPLC method.

2.2.7 Mass Balance Studies

At the end of each permeation experiment a mass balance study was undertaken. Any remaining solution in the donor compartment was removed and weighed. The membrane surface was washed with methanol (1 mL) three times and dried with a cotton bud. Franz cells were disassembled, and remaining DF was extracted from the membrane using methanol (1 mL). Wash and membrane samples were placed in an orbital shaker (Orbital Mini Shaker, VWR Leceistershire, UK) overnight at room temperature. The mass balance samples were centrifuged at 13 200 rpm for 15 min at room temperature. Aliquots (200 µL) of the supernatant were taken, diluted where necessary and analysed using HPLC.

2.2.8 Data Analysis

Each set of experiments comprised a minimum of three replicates. The mean and standard deviation (SD) were calculated using Microsoft Excel ® version 16.55 (Microsoft Corporation, Washington, US). SPSS Statistics® Version
28.0 (IBM, New York, US) was used to perform any additional statistical analysis. The normality of distribution of the data sets was determined using the Shapiro-Wilks test. For parametric data, a one-way analysis of variance (ANOVA) combined with Tukey's post hoc test was used to determine the statistical significance between groups. For non-parametric data, the Kruskal-Wallis one-way ANOVA (k-samples) with multiple pairwise-comparisons was performed. A

of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Confirmation of the amino acids non-interference with HPLC method for quantification of diclofenac

Absorption spectra for DF indicated two peaks, the highest at 222 nm and the second highest, chosen for analysis, at 277 nm. The λ-max values for the amino acid free bases and salts were: L-Arg 228 nm, L-ASS 225 nm, L-His 222 nm, L- HSS 223 nm, L-Lys 223 nm and L-SS 221 nm. Absorption at 277 nm was negligible and this was confirmed using the HPLC method described above. No absorption at 277 nm occurred when individual samples of the free base and salt versions of the amino acids were run at molar equivalent concentrations of 100 ug/mL of DF or at 50 times that amount. When samples combining DF and amino acid free bases in 1:1 and 1:50 molar ratios were run, amounts of DF remained constant. The same result occurred when DNa was combined with the amino acid salts.

3.2 Measurement of PC between octanol and PBS (pH 7.3 ± 0.2)

The results of the PC studies are shown in Tables 4 and 5. The PC of DF alone (14.21 ± 0.36 - 15.24 ± 0.33) decreased with the addition of increasing amounts of L-Arg, L-His and L-Lys. The decrease in the PC for DF was minimal as increasing amounts of L-His were added. When a DF:L-His 1:50 molar ratio was achieved, the PC was 13.32 ± 0.21. This was not the case when L-Arg (6.88 ± 0.24) and L-Lys (7.61 ± 0.20) were added at 50 times the molar concentration of DF. The reduction in the PC for DF when L-Arg and L-Lys counter ions were added at 50 times the molar ratio of DF, was 50% or greater.

Salt combinations appeared to increase, rather than decrease, the PC of diclofenac. Combinations of DNa with 50 times molar ratios of L-ASS or L-LSS resulted in modest increases in the PC of DNa:L-HSS (1:0), of approximately 10%. The combination of DNa with L-HSS provided a much greater impact relative to the PC of DNa alone, increasing it by more than 8-fold when a 50 times molar ratio of the counter ion was used. The DNa:L-HSS combination was therefore chosen for the preliminary IVPS.

Table 4. PC studies for DF and amino acid free bases in different molar ratios (n=3; mean ± SD).

nter ion	DF:L-Arg	DF:L-His	DF:L-Lys
	PC ± SD	PC ± SD	PC ± SD
	14.36 ± 0.27	14.21 ± 0.36	15.24 ± 0.33
	12.85 ± 0.07	14.60 ± 0.07	14.96 ± 0.07
	12.97 ± 0.26	14.42 ± 0.18	14.44 ± 0.15
	8.58 ± 0.09	14.14 ± 0.23	10.33 ± 0.10

7.36 ± 0.06	14.41 ± 0.14	8.71 ± 0.15
6.88 ± 0.24	13.32 ± 0.21	7.61 ± 0.20

Table 5. PC studies for DNa and amino acid salts in different molar ratios (n=3; mean ± SD).

nter ion	DNa:L-ASS	DNa:L-HSS	DNa:L-LSS
	PC ± SD	PC ± SD	PC ± SD
	15.33 ± 0.27	14.79 ± 0.31	17.97 ± 0.24
	15.34 ± 0.41	16.51 ± 0.14	17.98 ± 0.20
	15.38 ± 0.26	17.27 ± 0.18	17.28 ± 0.20
	15.78 ± 0.13	28.72 ± 0.40	18.09 ± 0.09
	15.85 ± 0.33	45.24 ± 2.76	18.18 ± 0.18
	17.00 ± 0.45	121.43 ± 14.56	19.92 ± 0.26

3.2 *In Vitro* Porcine Skin (IVPS) Permeation Studies

3.2.1 IVPS-100

The concentration of DF in solution for the various formulations at the time of application was 99.63 ± 0.12 µg/ mL (DNa: L-HSS (1:0)), 99.34 ± 0.38 µg/mL (DNa:L-HSS (1:1)), and 62.40 ± 0.41 µg/mL (DNa:L-HSS (1:50)). Formulations
were applied unfiltered. No permeation was detected during the first six hours of sampling. As shown in Table 6, at 24 h the permeation of DF from DNa:L-HSS (1:0) was comparable to that of DNa:L-HSS (1:1), namely 0.50 ± 0.20 µg/cm^2 and 0.54 ± 0.14 µg/cm^2, respectively ($p > 0.05$). The permeation from DNa:L-HSS (1:50) for the same time point was significantly higher at 5.39 ± 1.00 µg/cm^2 ($p < 0.05$). This amounted to approximately a 10-fold increase when assessed relative to the other formulations. Likewise, when expressing cumulative permeation as a percentage (%) of the amount of DF originally detected in solution at application, there was very little difference between DNa:L-HSS (1:0) and DNa:L- HSS (1:1). These values were 0.51% and 0.54% respectively ($p > 0.05$). The value for DNa:L-HSS (1:50) was significantly higher at 8.7% ($p < 0.05$).

Figure 3 represents the amount (µg) of DF recovered from mass balance calculations for the various formulations. The figure distinguishes between DF retrieved through washing, extracted from the membrane, retrieved from the residual formulation in the donor compartment and the cumulative amount of DF that had permeated at 24 h.
The amounts detected in the membrane for DNa:L-HSS (1:0) and DNa:L-HSS (1:1) were 2.44 ± 0.59 µg and 2.10 ± 0.23 µg, respectively ($p > 0.05$). The amount detected in the membrane for DNa:L-HSS (1:50) was significantly higher at 8.61
± 0.66 µg ($p < 0.05$).

Combining the amount of DF that permeated with the quantity extracted from the membrane, indicates the potential of the formulation to both partition into and permeate through the membrane. As shown in Table 6, when represented as a percentage of the amount of DF in solution at the time of application, this amounts to 2.96% for DNa:L-HSS (1:0), 2.66% for DNa: L-HSS (1:1) and 22.5% for DNa:L-HSS (1:50).

DF recovered through washing and the formulation remaining in the donor compartment, amounted to 4.95 ± 1.39 µg and 92.10 ± 1.85 µg respectively for DNa:L-HSS (1:0). For DNa:L-HSS (1:1), washing accounted for 5.05 ± 3.44 µg and

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the remaining formulation amounted to 93.51 ± 1.32 µg DF. In the case of DNa:L-HSS (1:50) washing recovered 11.01 ±
1.22 µg while the remaining formulation comprised 60.86 ± 4.93 µg DF.

Total recovery of DF for DNa:L-HSS (1:0) was 100 ± 4.05 µg, for DNa:L-HSS (1:1) was 101 ± 5.17 µg and for DNa:L-HSS (1:50) was 85.90 ± 12.59 µg. While the recovery of DF for the first two formulations accounted for 100% and 102% of the amount in solution at the time of application, the result was much higher for the third formulation. Here, the unfiltered formulation delivered an amount of DF (85.90 ± 12.59 µg) to the donor compartments that exceeded the amount in solution at the time of application (62.40 ± 0.41 µg). It was, however, less than the amount originally added to the formulation (100 µg). This total recovery result confirms that despite a reduced amount of DF having been applied (whether in solution or not), the use of the counter ion in a 1:50 molar ratio significantly increased the partition of DF into the membrane as well as the permeation of the drug, relative to formulations that comprised higher initial concentrations of DF, namely DNa:L-HSS (1:0) and DNa:L-HSS (1:1) .

Table 6. (i) Amount of DF in solution at time of application (µg/mL) (ii) Amount of DF (µg/cm²) that permeated at 24 h after unfiltered solvents were applied and (iii) Amount of DF (µg) in membrane at 24 h (iv) Total amount of DF (µg) that permeated and in membrane at 24 h ((n=4; mean ± SD) .

Ratio DNa:L-HSS	Amount of DF in solution at time of application (µg/mL) ± SD	Permeation (µg/cm²) ± SD at 24 h	Amount in membrane (µg) ± SD at 24 h	Total amount permeated and in membrane (µg) ± SD at 24 h
1:0	99.63 ± 0.12	0.50 ± 0.20	2.44 ± 0.59	2.95 ± 0.75
1:1	99.34 ± 0.38	0.54 ± 0.14	2.10 ± 0.23	2.64 ± 0.25
1:50	62.40 ± 0.41	5.39 ± 1.00	8.61 ± 0.66	14.04 ± 1.67

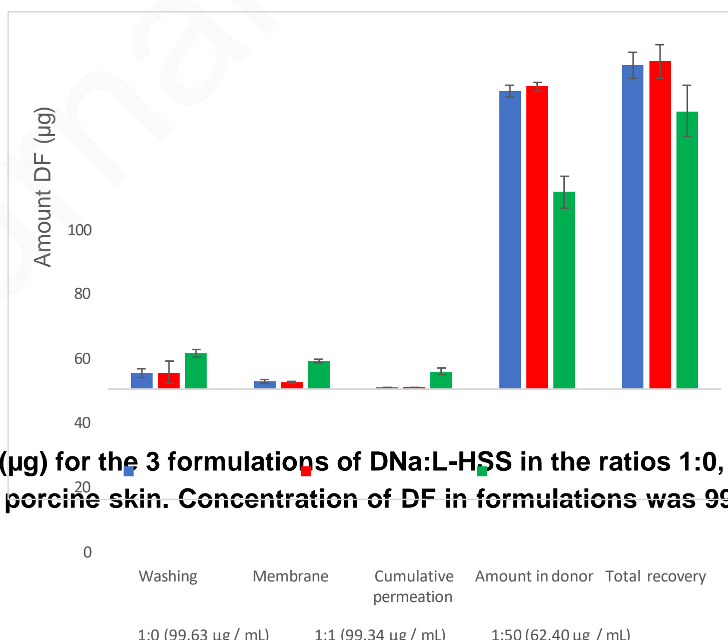

Figure 3. Recovery of DF (µg) for the 3 formulations of DNa:L-HSS in the ratios 1:0, 1:1 and 1:50 following mass balance studies in porcine skin. Concentration of DF in formulations was 99.63 ± 0.12 µg/mL for DNa:L-HSS (1:0),

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99.34 ± 0.38 µg/mL for DNa:L-HSS (1:1) and 62.40 ± 0.41 µg/mL for DNa:L-HSS (1:50) at time of application. Unfiltered formulations (1 mL) were applied. (n=4; mean ± SD).

3.2.1 IVPS-350

As shown in Table 7, the concentration of DF in solution for the various formulations at the time of application was 347.52 ± 0.18 µg/mL (DNa:L-HSS (1:0)), 318.94 ± 0.64 µg/mL (DNa:L-HSS (1:1)), and 9.52 ± 0.22 µg/mL (DNa:L-HSS
(1:50)). The amount in solution for DNa:L-HSS (1:50) was far lower than when the equivalent of only 100 µg of DF was added. Formulations were applied unfiltered. Traces of DF were detected in receptor solutions for samples containing counter ions at 7 h. These were, however, below the limit of quantification. At 24 h, permeation from DNa:L-HSS (1:0) was 1.65 ± 0.57 µg / cm^2. This differed significantly from permeation values for both DNa:L-HSS (1:1) and DNa:L-HSS (1:50), which were 6.93 ± 2.97 µg/cm^2 and 6.91 ± 3.61 µg/cm^2, respectively ($p < 0.05$).

At 24 h, DNa:L-HSS (1:0) permeation was equivalent to 0.47% of the amount of DF in solution at application, comparable to IVPS-100 which showed 0.51% permeation. For the DNa:L-HSS (1:1) formulation, permeation appeared to increase to 2.17% compared with 0.54% for IVPS-100. In the case of the DNa:L-HSS (1:50) formulation, the cumulative amount of DF that permeated (6.91 µg) as a percentage of the amount that was in solution at the time of application (9.52 µg) was 72.55%. Despite the low amount in solution, the quantity of active that permeated was four times the amount that permeated for DNa without the presence of the counter ion.

Figure 4 shows the results of mass balance calculations for the various formulations. Amounts (µg) of DF recovered from washing, residual formulation in the donor compartment, cumulative permeation results (as discussed above), as well as amounts of DF detected in the membrane are presented.

The amounts of DF detected in the membrane for DNa:L-HSS (1:0), DNa:L-HSS (1:1) and DNa:L-HSS (1:50) were not significantly different at 10.01 ± 0.72 µg, 12.74 ± 3.32 µg and 13.46 ± 2.83 µg ($p > 0.05$).

The combination of the amount of DF that permeated together with the amount extracted from the membrane indicates the potential of the formulation to both partition into and to permeate through the membrane. As shown in Table 7, the total for DNa:L-HSS (1:0) amounts to 11.66 ± 0.38 µg. This is significantly lower ($p < 0.05$) than the comparable amounts of 19.67 ± 5.19 µg for DNa:L-HSS (1:1) and 20.37 ± 2.49 µg for DNa:L-HSS (1:50) ($p > 0.05$).

Washing and the amount of DF formulation remaining in the donor compartment for DNa:L-HSS (1:0) accounted for 10.75 ± 4.12 µg and 328.27 ± 9.66 µg of DF respectively. For DNa:L-HSS (1:1), washing accounted for 7.47 ± 1.09 µg and the remaining formulation contained 320.61 ± 8.05 µg of DF. In the case of DNa:L-HSS (1:50), washing recovered
36.87 ± 12.02 µg and the remaining formulation included 17.34 ± 0.67 µg DF.

Total recovery of DF for DNa:L-HSS (1:0) was 350.68 ± 6.59 µg, equivalent to the total amount applied, and slightly higher than the amount originally detected in solution (347.52 ± 0.18 µg). For DNa:L-HSS (1:1) the total amount recovered, 347.75 ± 2.82 µg, exceeded the amount in solution at the time of application (318.94 ± 0.64 µg). This suggested that the unfiltered DNa:L-HSS (1:1) formulation that was applied to the donor compartments of the Franz cells, contained a quantity of DF that was not in solution. The total recovery of DF for the final formulation, DNa:L-HSS (1:50),

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was 74.58 ± 11.33 µg, notably less than the 85.90 ± 12.59 µg recovered from the IVPS-100. Despite the low quantity retrieved, it greatly exceeded the amount detected in solution at the time of application, namely 9.52 ± 0.22 µg. Notwithstanding the exceptionally low amount of DF added to the donor compartments for DNa:L-HSS (1:50), both the amount of DF in the membrane and amount permeated are comparable to those DNa:L-HSS (1:1) where much higher quantities were applied. Furthermore, the use of the counter ion at both a 1:1 and 1:50 molar ratio significantly increased the combined partition of DF into the membrane and permeation of the drug, relative to the formulation that contained no L-HSS ($p < 0.05$).

Table 7. (i) Amount of DF in solution at time of application (µg/mL) (ii) Amount of DF (µg/cm^2) that permeated at 24 h after unfiltered solvents were applied and (iii) Amount of DF (µg) in membrane at 24 h (iv) Total amount of DF (µg) that permeated and in membrane at 24 h (n=4; mean ± SD) .

Ratio DNa:L-HSS	Amount of DF in solution at time of application (µg/mL) ± SD	Permeation (µg/cm^2) ± SD at 24 h	Amount in membrane (µg) ± SD at 24 h	Total amount permeated and in membrane (µg) ± SD at 24 h
1:0	347.52 ± 0.18	1.65 ± 0.57	10.01 ± 0.72	11.66 ± 0.38
1:1	318.94 ± 0.64	6.93 ± 2.97	12.74 ± 3.32	19.67 ± 5.19
1:50	9.52 ± 0.22	6.91 ± 3.61	13.46 ± 2.83	20.37 ± 2.49

Figure 4. Recovery of DF (µg) for the 3 formulations of DF:L-HSS in the ratios 1:0, 1:1 and 1:50 following mass balance studies in porcine skin. Concentration of DF in formulations was 347.52 ± 0.18 µg/mL for DNa:L-HSS (1:0),
318.94 ± 0.64 µg/mL for DNa:L-HSS (1:1) and 9.52 ± 0.22 µg/mL for DNa:L-HSS (1:50) at time of application. Unfiltered formulations (1 mL) were applied. (n=4; mean ± SD).

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4. Conclusion

This study outlined the process of identifying, selecting and testing potential counter ions for DF. Screening criteria based on molecules of required charge, size and toxicity were used to identify potential counter ion candidates. This process was subsequently followed by PC studies which concluded the selection procedure. The results suggested that a combination of DNa and L-HSS were able to interact electrostatically within an aqueous solution, resulting in the masking of charges and enabling the partition of the apparently neutral species into an organic phase. This ion pair combination was tested using IVPS.

Despite a reduction in the solubility of DF as the amount of the counter ion increased, the IVPS clearly showed the potential indicated by the PC experiments to increase the partition of DF. Reduced amounts of DF both in solution and applied, as confirmed by mass balance studies, prevented an effective comparison of the impact of the counter ion. Notwithstanding, the addition of the counter ion increased both the ability of DF to partition into the membrane and to permeate through it. For IVPS-100, the amount of DF that permeated with the DNa:L-HSS (1:50) formulation was ~11 times greater than the amount that permeated without the counter ion. The amount of DF in the membrane was 3.5 times greater in the DNa:L-HSS (1:50) samples, than for DNa:L-HSS (1:0).

In relation to IVPS-350, the very low amount of DF applied to the DNa:L-HSS (1:50) samples resulted in less extreme differences in the results. The amount that permeated with the DNa:L-HSS (1:50) formulation was four times greater than the amount that permeated without the counter ion. The amount in the membrane was also 1.3-fold greater. This should, however, be considered in context. For the DNa:L-HSS (1:50) samples, only 9.52 ± 0.22 μg was in solution at the time of application and the total amount of DF applied was 74.58 ± 11.33 μg.

While a 1:1 DF:counter ion ratio may not be optimal, the effect of increasing solubility was outlined in IVPS-350, where DF:L-HSS (1:1) increased the combined amount permeated and in the membrane significantly (19.67 ± 5.19 μg), when compared with DF:L-HSS (1:0) (11.66 ± 0.38) ($p < 0.05$). As large quantities of L-HSS hindered the solubility of DNa, future work should consider different ratios of DNa:L-HSS to determine the efficacy of lower quantities of L-HSS. This could be done by performing additional PC studies using the DF:L-HSS molar ratios of 1:20; 1:30 and 1:40, to supplement the existing ones, namely 1:0; 1:0.5; 1:1; 1:5; 1:10 and 1:50. The impact of each increase on the gradient of a curve can be used to determine at what point increasing the amount of the counter ion reduces the rate at which the PC increases. Furthermore, solvent systems that facilitate the solubility of both DF and L-HSS require investigation and are on-going in our laboratory. This is being done with a view to creating simple solvent systems as potential precusors to topical formulations.

Because of the well-documented adverse effects of oral NSAIDs, topically-applied NSAID preparations are increasingly being used as alternatives. These investigations suggest a simple approach to identifying and selecting sustainable counter ions that may be used to increase the partition and permeation of a topically-applied NSAID. The use of ion pairs may therefore increase both the efficacy of formulations and/or result in a reduction in the amount of the active pharmaceutical ingredient being required. Moreover, the use of amino acids as potential counter ions not only satisfy toxicity requirements, but comply with the need to use renewable resources that are economical and have a low environmental impact.

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Graphical abstract

Potential of ion pairs in topical applications:

The ion pair comprises two charged ions, where the charges are masked. This representation does not refer to any specific type of ion pair.

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CRediT author statement

Mignon Cristofoli: Conceptualisation, Methodology, Validation, Investigation, Formal Analysis, Visualisation, Writing Original Draft **Jonathan Hadgraft:** Conceptualisation, Writing - Writing - Reviewing and Editing **Majella Lane:** Conceptualisation, Resources, Supervision, Writing - Reviewing and Editing **Bruno Sil Dos Santos:** Conceptualisation, Resources, Supervision, Reviewing and Editing

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Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Co-authors Dr Majella E. Lane and Professor Jonathan Hadgraft both serve in an editorial capacity for the journal.

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