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An in vitro investigation into the effect of glycosaminoglycans on the skin partitioning and deposition of NSAIDs

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Abstract

Recently, Solaraze gel (Bioglan, Herts, UK) a topical hyaluronan (HA)/diclofenac formulation for the treatment of actinic keratosis has received regulatory approval in the US, Canada and Europe for the treatment of actinic keratosis. However, a mechanism of action to explain the topical delivery properties of HA remains to be elucidated. Thus, the aim of this study was to compare the effect of HA with other glycosaminoglycans (chondroitin sulphate (CS), heparin (HP)) and pharmaceutically relevant polysaccharides (sodium carboxymethyl cellulose and pectin) on the dermal partitioning and percutaneous penetration of diclofenac and ibuprofen. The studies demonstrated that HA significantly enhanced the partitioning of both diclofenac and ibuprofen into human skin when compared to an aqueous control, pectin and carboxymethylcellulose ($P < 0.01$). Although the HA vehicle increased the partitioning of both drugs compared to the effects of the other glycosaminoglycans, CS and HP, this difference was not significant ($P > 0.05$). However, the results from the Franz cell diffusion studies showed that HA (1% w/w) significantly enhanced the amount of drug localising within the skin when compared to all of the other polysaccharides ($P < 0.05$). The results suggest that the use of HA as a vehicle excipient offers potential advantages in the dermal delivery and localisation of drugs.

Keywords: Hyaluronan; Glycosaminoglycans; Diclofenac; Ibuprofen; Partition coefficient; Dermal delivery

1. Introduction

The skin can be utilised for drug delivery in two ways, transdermally and dermally. Transdermal delivery involves percutaneous delivery of the drug across the skin into the systemic circulation (i.e. the blood stream). In dermal delivery, the skin itself is the target organ, but often such

Abbreviations: NSAIDs, nonsteroidal anti inflammatory drugs.

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delivery cannot be efficiently achieved systemically because of the avascularity of, or lack of blood supply to this layer. However, the targeted delivery of drugs for the treatment of dermatological conditions is not trivial. An ideal delivery profile for a therapeutic active agent applied topically to treat conditions such as eczema, psoriasis or skin cancer should involve partitioning and diffusion from the skin surface through the stratum corneum, accumulation at the site of drug action and subsequent metabolism to inactive products with little or no systemic absorption of the drug. Such delivery would avoid some of the adverse side-effects associated with some of the drugs used to treat these disease states.

Relatively few investigations have been conducted into the development of delivery systems to optimise drug targeting to the skin, although one such system which is receiving increasing attention is Hyaluronan (HA). HA is a naturally occurring glycosaminoglycan, comprising repeating disaccharide units of *N*-acetyl-D-glucosamine and β-glucoronic acid. It is found in the extracellular matrix of most mammals, including the skin (Laurent and Reed, 1991; Hascall et al., 1998) where its viscoelastic properties stabilise and protect the connective tissue. The unique properties and biocompatability of HA have led to its use in a number of clinical applications including: a vitreous replacement in ophthalmology, the supplementation of joint fluid for osteoarthritis and as a medium in which orthopaedic, ophthalmologic and otologic surgery can be performed (Balazs and Denlinger, 1989; Laurent et al., 1995; Laurent, 1998). More recently, HA has been investigated as a drug delivery agent where its bioadhesive properties have been used to immobilise drug on a mucoadhesive membrane (Vercruysse and Prestwich, 1998; Lim et al., 2000). In addition, the incorporation of HA within a topically applied vehicle containing drug has been shown on the basis of preliminary in vitro studies to be effective in the targeting and localisation of radiolabelled diclofenac within the epidermis of human skin when compared to an aqueous control (Brown et al., 1995; Martin et al., 1999). In addition, these data have been supported in vivo where radiolabelled HA was found not only to penetrate the skin of nude mice but also to aid the transport of diclofenac to the epidermis (Brown et al., 1999). Such targeting has also been reported to occur in human subjects (McKewan and Smith, 1997; Rivers and McLean, 1997) and has helped to facilitate the regulatory approval of Solaraze Gel, a diclofenac/HA topical formulation, in the US, Canada and Europe for the topical treatment of actinic keratoses (AKs), the third most common skin complaint in the US (Fieldman et al., 1998). AKs are malignant intra-epidermal skin lesions, caused by excessive exposure to solar radiation which can, if the basement membrane is penetrated, lead to the development of invasive squamous cell carcinoma (Moy, 2000). However, the mode of action of the HA vehicle still remains unclear. Hence, the aim of the current study was to investigate the effect of HA on both the partitioning and diffusion of diclofenac into human skin. To assess whether any effects were specific to either the drug or vehicle, comparitor experiments were also performed using ibuprofen and a range of polysaccharides including two other lower molecular weight, sulphated glycosaminoglycans, chondroitin sulphate (CS) and heparin (HP), and other pharmaceutically relevant polysaccharide excipients, including sodium carboxymethylcellulose (NaCMC) and pectin.

2. Materials and methods

2.1. Materials

Sodium hyaluronate (Viscosity average Mwt. 600000) and diclofenac, as used in Solaraze Gel, were kindly donated by SkyePharma (London, UK). Ibuprofen, CS (Mwt. 45000) and HP (Mwt. 7500) were obtained from Sigma (Poole, UK). Pectin (Mwt. 50000), sodium carboxymethyl cellulose (high viscosity, Mwt. 700000), disodium hydrogen phosphate, sodium dihydrogen phosphate and all other buffer salts were supplied by BDH (Poole, UK). Collagenase (Type IV) was obtained from ICN Biomedicals (Aurora, USA). All solvents used for chromatography were HPLC grade and purchased from Rathburn (Walkerburn, UK). Deionised water (Option 3 Water

Purifier, Elga, Buckinghamshire, UK) was used throughout the study.

Partition cells were made on site at King's College London and were constructed from polypropylene in two parts. The lower part provided a base to support the skin sample during the partition studies, whilst the upper part, comprising a hollow threaded cylinder, was employed to constrain the skin sample in situ. Franz cells were also made on site from borosilicate glass and comprised a sampling port and a donor (internal diameter 0.85 cm) and receptor compartment (volume 1.80 ml) which were fixed together using a clip.

2.2. Skin preparation

Human skin was obtained from patients undergoing elective abdominoplastic surgery, the donors being female aged between 25 and 40 years. Excised skin was frozen at −20°C within 2 h of surgery and stored until use. Full thickness skin sections (dermal and epidermal layers) were prepared by carefully removing subcutaneous fat and other debris using forceps and scalpel. The skin was then cut into small circular pieces using a template of similar dimensions to the partition or Franz cell. Skin from the same section was used for each drug study, enabling comparison of the formulation effects for the same drug but not between drugs.

2.3. Skin partitioning studies

Donor solutions containing drug (10 μg/ml for diclofenac, 20 μg/ml for ibuprofen) and 1% w/w polysaccharide were prepared in deionised water (control was deionised water alone, DW) and allowed to hydrate for 24 h. The skin was mounted in the partition cells with the epidermal side up, and the unit was then placed into an air tight 50 ml glass jar containing 20 ml of the donor solution such that the layer of skin was completely immersed. The bottle was then transferred to a water bath (32 °C) and shaken for 48 h. Preliminary experiments showed that this time was required for equilibration to be established. Samples were then removed and assayed for drug content by a validated HPLC assay (below). The amount of drug which partitioned into the skin was measured indirectly by the loss of compound from the donor solution. The percentage of skin partitioning was calculated from concentrations of compound in the donor solution before and after equilibrium. Control experiments were performed with no skin in the partition cell to determine any loss due to adsorption to the partition cell or glass. The percentage partitioning into the skin was calculated according to Eq. (1), which takes into account losses due to adsorption.

$$\% \text{ partitioning into skin} = [(C_0 - C_a) - C_f] \times /[C_0 - C_a] \quad (1)$$

where C_0, initial drug concentration in donor solution; C_a, drug concentration lost due to absorption to partition cell or glass; C_f, drug concentration in donor solution at equilibrium.

Results were expressed as mean + SD for $n = 4$ experiments. The student's t-test was used and $P < 0.05$ was taken as an indication of statistical significance.

2.4. Franz cell experiments

The small sections of full thickness skin were mounted, stratum corneum side up, in the Franz cells. The receptor compartment was filled with Hank's balanced salt solution pH 7.4, after which the diffusion cell was placed on a stirring plate in a water bath maintained at 32°C. The receptor fluid was continuously stirred using a small teflon-coated magnetic bar to ensure complete mixing of the receptor fluid. The diffusion cells were allowed to stand for 6 h in order to equilibrate the skin and also to wipe any visceral debris that may have remained on the dermal side of the skin. After equilibration, the receptor fluid was changed by replacing with fresh Hank's balanced salt solution and any air bubbles accumulated inside the receptor compartment or at the skin/receptor interface, were removed by tilting the cell. Subsequently, 50 μg of each formulation containing 1.75% w/w diclofenac or 20% w/w ibuprofen in 1% w/w polysacharides (or deionised water) unless otherwise stated, was applied to the surface of the skin

($n = 4$ for each formulation). Throughout the 48 h of the experiment, the receiver fluid was stirred to ensure homogeneity, and was also maintained at the same level as the skin. After 48 h, the experiment was terminated and the skin removed from the diffusion cell to enable the mass balance study to be performed.

2.5. Mass balance

After completion of the diffusion experiment, a mass balance experiment was performed in order to quantify the amount of drug on the skin, in the skin and in the receiver fluid. At the end of the experiment, the skin was removed and the surface washed eight times with 1 ml of mobile phase, after which any residual formulation was removed by wiping with a cotton bud. The cotton bud was then placed into the combined washings and left overnight at room temperature. The wash/wipe off sample was then syringe filtered (0.45 μm PTFE Whatman filter). The receiver fluid was assayed for drug content by taking a sample and syringe filtering and directly injecting the filtrate onto the HPLC. The skin sample was cut into small pieces using surgical scissors which were then added to solution of collagenase (3650 IU/g of skin) in Hank's balanced salt solution (pH 7.4). The skin and collagenase solution was then placed on an underwater magnetic stirrer in a water bath at 37°C and incubated for 15 h. After incubation 0.7 ml of the sample was thoroughly mixed with 0.3 ml of methanol, centrifuged (13500 rpm for 10 min) and the supernatant assayed as described below. All of the extraction methods were previously validated for both diclofenac and ibuprofen in all of the sample matrices (data not shown) as demonstrated by the fact for all the experiments the mass balance total recoveries was $98.8 \pm 2.8\%$. Statistical analysis was performed using the students t-test with $P \leqslant 0.05$ used to indicate statistical significance.

2.6. HPLC analysis of drug concentration

The HPLC analysis of diclofenac and ibuprofen was performed using a 15 cm × 4.6 mm I.D. Spherisorb RP-C18 (5 μm) column (Hichrom Ltd., Reading, Berkshire, UK), with a 10 mm C18 guard column (S5ODS2-10C5) (Hichrom Ltd.,) using a CM 4000 pump and a CI-4100 integrator connected to a SpectroMonitor 3100 UV detector (all LDC Analytical, FL). Analysis was performed isocratically with a mobile phase comprising 73% phosphate buffer (45 mM pH 7) and 27% acetonitrile:tetrahydrofuran (7:3 v/v). All samples were made up in mobile phase, containing internal standard (20 μg/ml), to concentrations within the predetermined calibration curve. For diclofenac analysis, ibuprofen was used as the internal standard, and vice versa. An injection volume of 100 μl was used, the samples were eluted at a flow rate of 1.10 ml/min and monitored at 273 and 264 nm for diclofenac and ibuprofen, respectively.

3. Results

3.1. Partition studies

The effects of 1% w/w polysaccharide concentrations on the skin partitioning of diclofenac and ibuprofen are given in Figs. 1 and 2, respectively. The results demonstrate that all three glycosaminoglycans: HA, CS and HP significantly increased the partitioning of diclofenac and ibuprofen into the skin compared to the aqueous control. The extent of the increase induced by HA however was greater, although not significantly so, than that for both CS and HP when the latter two were employed at the same w/w concentra-

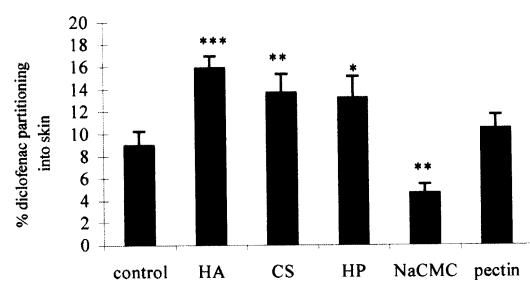

Fig. 1. The effect of 1% w/w polysaccharides on the dermal partitioning of 10 μg/ml diclofenac $n = 4$, mean ± SD (* represents $P \leqslant 0.05$, ** represents $P \leqslant 0.01$, *** represents $P \leqslant 0.001$, level of significant difference compared to aqueous control).

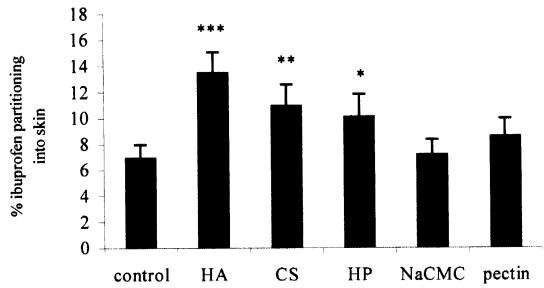

Fig. 2. The effect of 1% w/w polysaccharides on the dermal partitioning of 20 μg/ml ibuprofen, $n = 4$, mean ± SD (* represents $P \leqslant 0.05$, ** represents $P \leqslant 0.01$, *** represents $P \leqslant 0.001$, level of significant difference compared to aqueous control).

tions. In contrast, NaCMC, commonly used as a viscosity enhancer in transdermal formulations, effected a decrease in diclofenac partitioning and had no effect on the partitioning of ibuprofen. Pectin was found to have no significant effect on the partitioning of either diclofenac or ibuprofen.

3.2. Franz cell studies

3.2.1. Surface and receptor compartment drug analysis

The amount of drug on the surface of the skin and that which had diffused through to the receptor compartment was determined 48 h after application of 1.75% w/w diclofenac or 20% w/w ibuprofen formulated in all the 1% w/w polysaccharide formulations. For diclofenac, none of the polysaccharides had a significant effect on the amount of drug remaining on the surface compared to the aqueous control (Table 1). However for ibuprofen, HA in contrast to the other polysaccharides, significantly reduced the amount of drug remaining ($P < 0.01$) on the surface, when compared to the aqueous control. In the receiver fluid (Table 2), significantly less diclofenac and ibuprofen ($P < 0.05$) were measured when the drugs were formulated in 1% w/w HA compared to the other polysaccharides. However, it was only in the case of ibuprofen with HA that a significantly lower amount of drug (< 0.01) was found in the receptor compartment compared to that diffusing from the aqueous control (Table 2).

Table 1
Effect of 1% w/w polysaccharide on the percentage of total drug remaining on the skin surface 48 h after application of 1.75% w/w diclofenac and 20% w/w ibuprofen formulations (mean ± SD, $n = 4$)

Formulation	% of applied dose	
	Diclofenac	Ibuprofen
DW	80.41 ± 3.73	89.37 ± 1.71
1% w/w HA	83.29 ± 0.82	84.47 ± 2.10*
1% w/w CS	76.43 ± 1.01	87.86 ± 2.63
1% w/w NaCMC	84.35 ± 0.93	89.94 ± 1.59
1% w/w PT	78.94 ± 1.09	88.50 ± 2.49

* Indicates significant difference from aqueous control.

3.2.2. Skin analysis

The amounts of diclofenac and ibuprofen in the skin 48 h after application of vehicles containing 1% w/w polysaccharide are shown in Figs. 3 and 4, respectively. In general, the amounts of either drug delivered into the skin were found to obey the following trend: HA > CS > NaCMC ≅ DW ⩾ pectin. Significantly, more diclofenac and ibuprofen was delivered into the skin when applied in HA or CS, when compared to the other polysaccharides and the aqueous control ($P < 0.01$). In addition, it was observed that after application of the vehicle containing HA significantly more drug was delivered to the skin when compared to that found after application of the CS formulation ($P < 0.05$). These results are in good agreement with those obtained for the skin

Table 2
Effect of 1% w/w polysaccharide on the percent of total applied drug found in the receiver fluid 48 h after application of 1.75% w/w diclofenac and 20% w/w ibuprofen formulations to the skin surface (mean ± SD, $n = 4$)

Formulation	% of applied dose	
	Diclofenac	Ibuprofen
DW	1.24 ± 0.09%	1.02 ± 0.11
1% w/w HA	1.97 ± 0.30%*	0.72 ± 0.04*
1% w/w CS	2.35 ± 0.10%*	0.92 ± 0.06
1% w/w NaCMC	2.64 ± 0.08%*	1.43 ± 0.11*
1% w/w PT	2.39 ± 0.09%*	1.14 ± 0.07

* Indicates significant difference from aqueous control.

Table 3
Effect of 1% w/w polysaccharide on percentage of total drug delivered to the skin 48 h after application of vehicles containing either diclofenac (DW: 2.5%; HA: 23% and CMC: 20% w/w) or ibuprofen (DW: 31%; HA: 2% and CMC: 1.75% w/w) at maximum drug solubility (mean ± SD, $n = 4$)

Formulation	% of applied dose	
	Diclofenac	Ibuprofen
DW	12.7 ± 1.97	6.62 ± 0.88
1% w/w HA	17.87 ± 1.93*	11.82 ± 1.6*
1% w/w NaCMC	14.06 ± 1.41	9.26 ± 0.63

* Indicates significant difference from aqueous control.

partition studies where similar trends were observed.

However, the results depicted in Fig. 3 and Fig. 4 were obtained at drug concentrations equivalent to the lowest maximum solubility in any of the vehicles i.e. the limit of solubility of either diclofenac or ibuprofen in 1% NaCMC. Thus, further studies were performed in which the drugs were formulated at their maximum solubility in HA, NaCMC and DW, i.e., at a thermodynamic activity close to unity. The results obtained (Table 3) showed that a significantly higher proportion of diclofenac and ibuprofen was delivered to the skin ($P < 0.05$) when applied in HA when compared to CMC and DW.

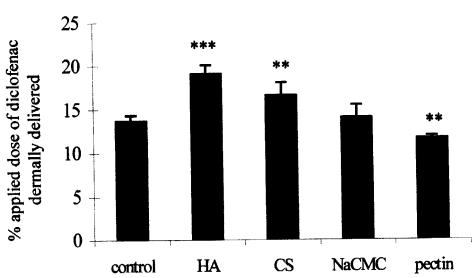

Fig. 3. The effect of 1% w/w polysaccharide on the percentage of the applied dose of diclofenac (1.75% w/w) remaining in the skin after 48 h, $n = 4$, mean ± SD (* represents $P \leqslant 0.05$, ** represents $P \leqslant 0.01$, *** represents $P \leqslant 0.001$, level of significant difference compared to aqueous control).

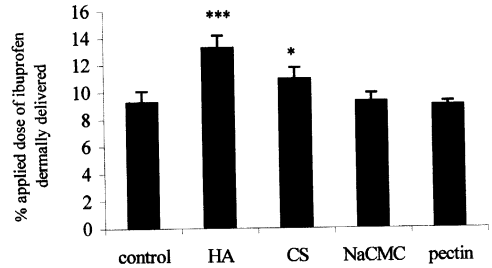

Fig. 4. The effect of 1% w/w polysaccharide on the percentage of the applied dose of ibuprofen (20% w/w) remaining in the skin after 48 h. $n = 4$, mean ± SD (* represents $P \leqslant 0.05$, ** represents $P \leqslant 0.01$, *** represents $P \leqslant 0.001$, level of significant difference compared to aqueous control).

4. Discussion

The skin absorption of drugs is a partition–diffusion process. First the drug partitions from the formulation into the stratum corneum and then, after diffusing across the stratum corneum, it partitions into and diffuses across the epidermis and the processes are repeated as the drug moves to deeper skin layers (Barry, 1988). The ability of a drug to partition into the skin, as a critical requisite for topical delivery is dependent on a number of physicochemical properties including its solubility in the applied vehicle and its partition coefficient between vehicle and skin. Until recently in vitro models used to predict percutaneous penetration have primarily involved the measurement of the partition behaviour of the drug between two mutually saturated liquid phases e.g. octanol/water. However, the ability of a homogenous water-saturated octanol phase to adequately mimic uptake into a structured heterogeneous bilayer remains unclear, and the validity of this approach has been questioned (Cornwall and Barry, 1994; Abraham et al., 1995).

Improved methods for determining skin/water partition coefficients have therefore been developed (Ahmed et al., 1995 Cornwall and Barry, 1994; Maitani et al., 1995; Megrab et al., 1995; Walker et al., 1997) and despite differences in the source of membrane, most of the methodologies have primarily involved placement of full thickness or epidermal sections into an aqueous phase, allowing total surface exposure of both sides of

the membrane to the penetrating drug molecule. Such exposure obviously does not occur in vivo to a topically applied formulation. Hence for the current studies this methodology was modified to include the use of specially designed partition cells where only the stratum corneum was exposed to partitioning drug molecules.

The results showed that at least for diclofenac and ibuprofen, the presence of HA within the vehicle significantly enhanced the partitioning of drug into the skin and that the effect appeared to be concentration dependent up to 1% w/w polysaccharide (data not shown) (Hanpanitcharoen et al., 1998). It was not possible to study the effects of higher concentrations of HA in this system due to the occurrence of gelation. The results also illustrate that the effects of HA in promoting the dermal partitioning of diclofenac and ibuprofen are not unique in that two other glycosaminoglycans, CS and HP (which both contain sulphate groups, unlike HA), although less effective, also increased the sorption of both NSAIDs to the skin. In contrast to the effects of the three glycosaminoglycans, neither the inclusion of pectin or NaCMC within the aqueous media showed similar effects on the skin partitioning of the drugs. Such findings were supported by the results from the Franz cell studies where similar trends were observed (Figs. 3 and 4, Table 2). Significantly less diclofenac and ibuprofen was measured in the receiver fluid with significantly more being found in the skin when the drugs were applied in 1% w/w HA rather than the other polysaccharides. Such findings suggest that HA is promoting the retention of drugs in the skin for longer periods when compared to the other polysaccharides studied.

It is known that the driving force for passive transport through a membrane is the chemical potential gradient across the membrane (Hadgraft, 1996). To create the gradient necessary to deliver a drug across the skin, it is necessary to dissolve a drug in a solvent or vehicle to establish a certain concentration, and thus activity of the drug at the outer surface of the skin. Since different vehicles have different capacities to dissolve drug, at any fixed level of concentration, different levels of thermodynamic activity of the drug will be obtained. In principle, the simplest way to maximise delivery of a drug is to formulate it at maximum solubility to achieve the highest thermodynamic activity (Smith, 1990; Hadgraft, 1996) (e.g. saturated systems). Thus, in an effort to eliminate any thermodynamic influence, the drugs were formulated at their maximum solubility in HA, NaCMC and DW i.e. at a thermodynamic activity of unity. The results obtained (Table 3) showed that significantly more of either drug was delivered to the skin ($P < 0.05$) when applied in HA when compared to CMC and DW. Thus, it is too simplistic to explain such enhanced dermal delivery by a change in drug thermodynamic activity in the presence of HA.

It is known that the degree of hydration of the stratum corneum influences skin permeability. Increased hydration opens the compact substance of the stratum corneum by loosening the dense, closely packed cells thus increasing the permeability to many drugs (Bucks and Maibach, 1999). Hydrophobic patches and oleaginous formulations are based on such a principle in that they occlude the skin, inhibiting transepidermal water loss and increasing stratum corneum hydration resulting in enhanced percutaneous delivery of the formulated drug. Polysaccharides are regarded as moisture-control agents (Whistler, 1993) which is the reason for their incorporation in many topical and cosmetic preparations. However, the skin hydration properties of HA are considered to be much higher than other polysaccharides because of its considerable capacity to bind water (Cowman et al., 1998). Thus, the topical application of HA may result in increased hydration of the stratum corneum and enhanced dermal delivery of a concomitantly applied drug across the stratum corneum.

However, such properties do not account for drugs formulated in HA being retained in the skin with little systemic absorption, as demonstrated in previous studies in vitro (Lin and Maibach, 1996; Martin et al., 1999) and in vivo (Rivers and McLean, 1997). Brown et al. (1999) have already examined the movement of HA into the keratin, epidermal and dermal layers of mouse and human skin and surprisingly found that radiolabelled HA is apparently absorbed rapidly from the surface of

the skin and into the epidermis. Thus, underlying skin HA receptors (Gustafson, 1998) and the hydrophobic patch within the hydrated structure of HA which it is claimed, enables the macromolecule to be absorbed across membranes (Scott, 1989) may both play a role in the dermal drug delivery properties of HA. Alternatively, the increased hydration of the surface layers of the skin in the presence of HA may not only result in enhanced drug absorption across the stratum corneum but also a decrease in drug diffusion in the lower more viable skin layers as previously unexposed potential binding sites become available within the more hydrated epidermal layers. However, all such postulations obviously require a great deal of further investigation.

5. Conclusions

HA is a naturally occurring polysaccharide which is biocompatible and non-immunogenic, rendering it an ideal formulation aid. The results in this study demonstrate that the inclusion of HA as a vehicle excipient offers clear potential in the dermal delivery and localisation of drugs for conditions such as AKs, although the mode of action still remains to elucidated.

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