Universidade de Lisboa

Faculdade de Farmácia

Topical Delivery of Hyaluronan Monomers: Optimisation Strategies

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Mestrado Integrado em Ciências Farmacêuticas

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Monografia de Mestrado Integrado em Ciências Farmacêuticas apresentada à Universidade de Lisboa através da Faculdade de Farmácia

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“Para ser grande, sê inteiro: nada
Teu exagera ou exclui.

Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.

Assim em cada lago a lua toda
Brilha, porque alta vive.”

Ricardo Reis, in "Odes"
Heterónimo de Fernando Pessoa

Resumo

A pele ou integumento é o maior órgão do corpo humano cobrindo uma área média de 1,7m^2 que atua como uma barreira, de forma a proteger o organismo de substâncias indesejáveis, ao mesmo tempo que impede a passagem de substâncias essenciais à vida para o exterior do corpo. É constituído por duas camadas principais, a epiderme e a derme, sendo a primeira a principal responsável por esta proteção, através do estrato córneo, o qual constitui a camada mais externa da epiderme, e que, com a sua composição única em corneócitos e uma camada dupla de lípidos, é o responsável maioritário por este controlo. O estrato córneo tem também como principal função o controlo da perda de água através da pele, de forma a evitar a desidratação do corpo humano, mas que também é um fator indicativo de problemas dermatológicos e de comprometimento da função de barreira da pele.

Os glicosaminoglicanos são polissacáridos complexos formados através da combinação de vários dissacáridos, de forma a formar longas cadeias. Estes glicosaminoglicanos são carregados negativamente, permitindo que se repelem e deslizem uns sobre os outros. Além disso, têm a capacidade de sofrer expansão ou compressão aquando da inserção ou remoção de água.

Dentro do grupo dos glicosaminoglicanos podem considerar-se seis diferentes tipos de glicosaminoglicanos, sendo eles a heparina, o heparan sulfato, as condroitinas 4 e 6-sulfato, o sulfato de dermatana, o ácido hialurónico e o sulfato de queratana.

Por sua vez, o ácido hialurónico, é um glicosaminoglicano cujas unidades básicas são o ácido glucurónico e a N-acetilglucosamina, sendo a ultima molécula o foco desta dissertação. O ácido hialurónico, enquanto molécula ativa, é um potencial agente terapêutico e cosmético de grande relevância e importância.

No que diz respeito à passagem de substâncias através da pele, esta pode ocorrer de três formas, seja por meio intracelular, por meio transcelular ou por meio de folículos, podendo uma ou mais destas vias ser utilizada em simultâneo.

Relativamente à passagem de moléculas através da pele, a utilização de “chemical penetration enhancers” tem sido uma técnica muito utilizada de forma a aumentar a quantidade de ativo que consegue passar através da pele, a partir de uma formulação, e assim exercer a sua função no local certo e na quantidade necessária. Vários compostos têm sido classificados como “chemical penetration enhancers”, no entanto, não existe nenhum que se aproxime da noção de

composto ideal para exercer esta função. Por isso, é comum associar mais que um destes compostos de forma a obter o efeito desejado e uma maior eficácia.
Alguns "chemical penetration enhancers" bastante utilizados nos dias de hoje são compostos como a água, glicóis, álcoois de cadeia curta, ácidos gordos e ésteres.

O principal objetivo deste estudo era perceber se a N-acetilglucosamina, a qual é uma molécula hidrofílica, teria bons resultados na cedência e permeação através da pele utilizando células de Franz para o efeito com duas membranas diferentes: Membranas Tuffryn® e Membranas Strat-M®.
Utilizando como base a teoria de "formulation for efficacy", baseada na capacidade de partição de um ativo desde o veículo até à sua entrada na pele, oito solventes foram testados como potenciais "chemical penetration enhancers", testando para isso a solubilidade do ativo, a N-acetilglucosamina, em cada um desses solventes. Dos oitos solventes testados, apenas três mostraram ter os requisitos necessários e foram selecionados para continuar em estudo. Foram eles a água, o glicerol e o propilenoglicol.
Para testar a cedência e permeação da N-acetilglucosamina, oito formulações foram elaboradas e analisadas. Estas formulações eram constituídas por um, dois ou três solventes em simultâneo em diferentes proporções de forma a perceber qual a influência que diferentes quantidades de cada solvente tinham no ativo.
A análise foi primeiramente efetuada utilizando as membranas Tuffryn® para os estudos de cedência, no qual os resultados foram promissores, com valores entre os 10-15% após 4h de ensaio, permitindo que o estudo prosseguisse para os ensaios de permeação.
Relativamente aos ensaios de permeação, estes foram então realizados utilizando as membranas Strat-M®. Nestes ensaios, como já era expectável, a percentagem de N-acetilglucosamina que sofreu permeação diminuiu comparativamente com os ensaios de cedência. No entanto, esta diminuição não foi muito acentuada, sendo que ao final de 4h de ensaio os valores de permeação se encontravam entre os 7-8%.
Através dos resultados foi também possível verificar que a formulação que apresentou melhores resultados para a permeação de N-acetilglucosamina foi a formulação constituída por água, glicerol e propilenoglicol numa proporção de solventes de 1:1:1.

Palavras-chave: Estrato córneo, Ácido hialurónico, N-acetilglucosamina, cedência *in vitro*, permeação *in vitro*.

Abstract

The skin or integument is the largest organ in the human body, covering an average area of 1.7 m^2, acts as a barrier to protect the human body against unwanted substances. It comprises two main layers: the epidermis, the outermost layer, followed by the dermis.

Stratum corneum, the most external layer in the epidermis, is the major responsible for everything that gets inside or outside of the skin due to its unique constitution.

Hyaluronan is a GAG present in the skin, composed of N-acetylglucosamine and Glucuronic Acid as subunits. It has a great potential as a therapeutic agent for osteoarthritis and in cosmetics.

The aim of this study was to understand N-acetylglucosamine, which is a hydrophilic molecule, is a suitable material for topical release and permeation by using Franz Cells and two different membranes: Tuffryn® Membrane Filters and Strat-M® Membrane Filters.

Using the formulation for efficacy theory, eight solvents were tested as CPEs in regard to the solubility of the N-acetylglucosamine in each one of them. From the eight solvents, three were selected to proceed with the study: water, glycerol and propylene glycol.

Different formulations using those solvents in different proportions were manufactured and analysed, firstly using the Tuffryn® Membrane Filters for the release study and then using the Strat-M® Membrane Filters for the permeation study.

The results obtained were promising; it was proved that the formulation comprised of the three solvents, water, glycerol, and propylene glycol in a proportion of 1:1:1 was the one where the release and permeation of N-acetylglucosamine had better results. Although the other formulations' results were not as good, all of them showed release and permeation rates that deserve further attention.

Keywords: Stratum Corneum, Hyaluronan, N-Acetylglucosamine, *In vitro* release, *In vitro* permeation

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List of abbreviations

CPE – Chemical Penetration Enhancer

DNS – 3,5-Dinitrosalicylic acid

GAGs – Glycosaminoglycans

GalNAc – N-acetylgalactosamine

GlcA – Glucuronic acid

GlcNac – N-acetylglucosamine

HAPLN – Hyaluronan and proteoglycan link proteins

HARE – Hyaluronan receptor for endocytosis

HAS – Hyaluronan synthases

LYVE-1 – Lymphatic vessel endothelial hyaluronan receptor

PBS – Phosphate buffer solution

SC – Stratum corneum

TEWL – Transepidermal Water Loss

UDP – Uridine Diphosphate

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1. Introduction

1.1 Skin Structure

The skin or integument is the largest organ in the human body covering an average area of 1.7 m^2 and contributing with more than 10% to the total body weight. It offers a wondrous protection to the body, acting as a barrier to keep unwanted substances outside the body and avoid the loss of endogenous substances, mainly water (1–4).

Structurally, the skin has two main layers, the epidermis and the dermis (1).

The first layer – epidermis – is less permeable and is comprised of four strata: stratum basale, stratum spinosum, stratum granulosum and stratum corneum (SC), from the inner to the outermost layer (1,3).

The epidermis has a major role in the physical protection of the skin. The SC, specifically, plays a significant role in drug delivery, since it is the outermost layer and it is the first barrier to the entry of xenobiotics (1,3,5).

The inner layers are composed mainly by keratinocytes, which are nucleated and metabolically active (5). These keratinocytes have their origin in progenitor cells from the stratum basale that undergo continuous differentiation and structural changes until they reach the upper layer – the SC (6).

The SC consists of cells that lost cytoplasmic organelles and nuclei, named corneocytes, which are enfolded by a lamellar lipid-rich matrix (intercellular space) (1,5).

The mechanism that originates corneocytes is the desquamation process (5). Keratinocytes become anucleated and flattened, containing only keratin filaments and enfolded by a lipid and cross-linked proteins envelope. During this process, desmosomes, which are fundamental in the nucleated layers, since they connect keratinocytes, are lost. On the other side, lamellar bodies secrete some glycoproteins, free sterols and sphingolipids, which attribute hydrophobicity and hydrophilicity to the SC (7)**.**

This is a process that contributes to the renewal of the skin and usually, in a healthy skin, there is a balance between desquamation and proliferation. These processes may be altered in some skin disorders (5).

The SC is often represented as a “brick and mortar” model, where the bricks are represented by the corneocytes, and the mortar is the lamellar lipid-rich extracellular matrix, with lipids

organised in a bilayer (7,8). This lamellar lipid-rich matrix is different from all the other lipid membranes (4) and it is mainly composed by free fatty acids, ceramides and cholesterol (2).
The SC's impermeability is due to this complex lipidic structure (1).
The skin, essentially the SC, is also responsible for the regulation of water loss. The lipids in its structure control the efflux of salts and water and the influx of water-soluble substances (5).
Different authors use the Transepidermal Water Loss (TEWL) as an assessment of the quality of the barrier function, since TEWL is proportional to the damage level of the skin. TEWL represents the water that effluxes form the SC to the environment and it differs from sweat since it is not visible or touchable (5,7).
TEWL is determined using an evaporimeter and can vary with different factors such as room temperature and humidity, anatomical site, or variations between individuals (9).
Variations of TEWL in different anatomic sites have been explained by *Machado M. et al* (10) in their *in vivo* study. TEWL was measured in different anatomic sites on ninety volunteers and it was observed that different anatomic sites showed a significant difference of TEWL. In the same study it was proved that TEWL and the pathlength that allows the movement of water in the skin are inversely correlated, what means that the sites with a bigger pathlength have a TEWL of approximately zero. This is important, since it can be used to understand the permeability variation in different anatomic sites (10).
The dermis, which is more permeable, can be divided in two layers: the uppermost is the papillary dermis and the deepest is the reticular dermis. The major function of the dermis is to protect the body from mechanical assaults and injury (1,7).
Most of the cells in the dermis are fibroblasts, macrophages and melanocytes (5,11). Fibroblasts are responsible for the production of elastin and collagen fibres (extracellular matrix) that provide flexibility and support to the skin, respectively (1,4,5).
The macrophages are responsible for the inflammatory and immune response, eliminating damaged tissue and foreign material (5,11).
The melanocytes' main function is to produce melanin, which provides pigmentation to the skin (11).
All these cells are involved by the ground substance of the dermis, mainly constituted by hygroscopic proteoglycans (1,11).
The dermis is a connective tissue comprising high vascularization, lymphatic vessels and specialised structures - the appendages: hair follicles, sebaceous glands and sweat glands – eccrine and apocrine (4,5).

1.2 Glycosaminoglycans (GAGs)

The dermis is mainly constituted by proteoglycans that are hygroscopic which means that they are able to attract and hold water molecules (4). The proteoglycans consist of GAG chains covalently attached to a core protein by peptide chains (11,12).

GAGs are complex polysaccharides whose basic units are disaccharides that combine to form long linear chains (7,12).

They are negatively charged, which is the main reason why they repel and glide over each other. GAGs can expand or contract by adding or removing water, respectively. When binding a high amount of water, they originate a gel-like matrix that is, for example, one of the constituents of the nasal secretions (7).

GAG's basic units, disaccharides, comprise uronic acid and amino sugar. The uronic acid is either iduronic or glucuronic acid (GlcA) (Carbonyl group replaced for a Carboxyl group) while the amino sugar has one Hydroxyl group replaced by an Amino group and it can be N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc) or a variety of N-substituted in Galactosamine (7,12).

There are several types of GAGs which are mainly hexuronic acids. Authors usually consider six different types of GAGs, five being hexuronic acids (7,13).

- Heparin is the unique intracellular GAG whose major role is being an anticoagulant (14);
- Heparan Sulphate is a GAG present on the cell surface since it belongs to the basement membrane and extracellular matrix (7,15);
- Chondroitin 4- and 6-sulphate are the prevalent GAGs in the human body and can be found in some arteries or cartilage tendons, for instance (16);
- Dermatan sulphate is a GAG present in the dermis and in cardiovascular tissues (17);
- Hyaluronan is the only GAG that does not have sulphate groups in its structure and that is not attached to a core protein, so it is not linked to proteoglycans by covalent bonds. Still, it is non-covalently bonded with proteoglycans by the hyaluronan-binding motifs (12,18).

  These motifs bind selectively to hyaluronan and are known as the link module of hyaladherines, expressed in different tissues and belong to the hyaluronan and proteoglycan link proteins (HAPLN) (12).

Hyaluronan is also the only GAG that besides being present in different sites of the human body, as shown in Table 1, it is also present in bacteria (7). Its key function is to protect the body, mainly joints, against mechanical shock and lack of lubrication (19).

Table 1. Normal concentrations (µg g-1) of Hyaluronan in different organs of the Human body.

Organ or Fluid	Man
Umbilical cord	4100
Synovial Fluid	1400-3600
Dermis	200
Vitreous body	140-338
Lung	-
Kidneys	-
Renal Papillae	-
Renal Cortex	-
Brain	35-115
Muscle	-
Intestine	-
Thoracic lymph	8.5-18
Liver	-
Aqueous humour	0.3-2.2
Urine	0.1-0.3
Lumbar CSF	0.02-0.32
Plasma (serum)	0.01-0.1

[Adapted from (20)]

- Keratan sulphate is the non-hexuronic acid GAG. Instead of the acidic residue, this GAG has galactose in its structure (21). Even if for years it was only considered a cellular connective molecule, nowadays, it is recognised as a GAG with an important role in hemostasis, tissue remodelling or even in disease progression (13).

As this project is focused on Hyaluronan and its monomers, this is the one that will be given more emphasis.

1.2.1 Hyaluronan

Hyaluronan, is a special GAG due to its particularities in terms of chemical structure. It is the only GAG synthesised in the plasma membrane, instead of the Golgi apparatus unlike all other GAGs (22,23).

Hyaladherins are responsible for the specific recognition of the structure of the hyaluronan, which allows the hyaluronan to bind to cell surfaces and adjust cell behaviour or to bind to other proteoglycans, increasing the stability of the extracellular matrix (20).

Hyaluronan is a water soluble molecule and is comprised of two sugars as basic units, GlcA and GlcNAc (18).

As a polymer, hyaluronan is a huge macromolecule which is the main reason why its molecular weight may easily reach the millions of g/mol, as *J. Fraser et al.* showed in their article: in synovial fluid, the average molecular weight is about 7 x 10^6 g/mol what means that when extended it could reach lengths up to 15 µm (20,24).

Hyaluronan synthases (HAS) are the enzymes that catalyse the biosynthesis of hyaluronan. These enzymes use UDP-GlcNAc and UDP-GlcA as substrates, and Uridine Diphosphate (UDP) is displaced when the following sugar is added to the chain, using M^{++} as a cofactor as shown in Figure 1 (22,25).

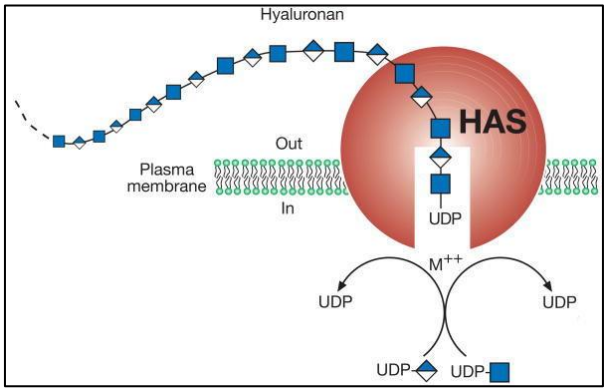

Figure 1. Hyaluronan biosynthesis. [From (22)]

Besides the role that hyaluronan exhibits in the extracellular matrix, this polymer has been used worldwide as a therapeutic agent to treat a variety of diseases (20).

Its anti-inflammatory function has been used to treat osteoarthritis, to reduce pain, as a lubricant and to decrease the level of cartilage damage. Furthermore, Hyaluronan can link to CD44 receptors supressing IL-1 production, what leads to an exacerbation of the synovial cells' proliferation as explained by *Altman RD et al* (26).

Other applications of Hyaluronan include ophthalmologic surgery due to the potential damage to the tissues during the procedure (24) and cosmetic use, proved by efficacy studies: *Yazdanparast T. et al* (27) discussed the efficacy and safety of a hyaluronic acid gel in the restoration of the fullness of the upper lip where it was shown that hyaluronic acid is effective for upper lip augmentation (27).

In oncology it also has a potential in altering signalling pathways that are selective since it inhibits tumour growth and induces apoptosis when *in vivo* (22).

With regard to the degradation of Hyaluronan, the tissue half-life of hyaluronan is short, from half a day to two or three days, after which turnover occurs. Hyaluronan's degradation can occur *in situ* if the tissues are densely structured, for example, in cartilage or bone (20). It can also occur after being transported by lymph to lymph nodes or by blood until it reaches the liver, where the major elimination occurs by receptor-facilitated uptake both in lymph nodes and liver sinusoids. The receptors present in the lymph nodes and liver sinusoids are the HARE (hyaluronan receptor for endocytosis) and the LYVE-1 (homolog of CD44) (20,22).

Hyaluronidases (HYALs) 1 and 2 are responsible for the degradation of the chains and the fragments of the Hyaluronan (28) that are afterwards internalised by cell-surface receptors following a lysosomal pathway that will turn the fragments into monosaccharides, which may involve enzymes as hyaluronidases, and the exoglycosidases β-N-acetylglucosaminidase and β-glucuronidase (22).

Hyaluronan plasma levels can increase due to several diseases, which contribute to an increased synthesis of hyaluronan: *Tang, S. et al* showed in their article that hyaluronan's levels in plasma increased significantly after patients suffered a stroke, and that lower levels of HA were a sign of a better prognosis when 48h from the stroke had passed (29)**.**

Endothelial cells and their functional capacity, as well as the cardiac output distribution through the sinusoids in the liver, are the most important factors concerning these elimination pathways (20).

1.3 Penetration Pathways

Nowadays, authors consider three main skin penetration pathways since a drug is applied on the skin surface until it reaches the systemic circulation: Intercellular pathway, Transcellular pathway and Appendageal pathway, represented in the Figure 2 (8,11). Some molecules can use the three pathways to cross the SC and permeate through the skin since these pathways are not necessarily independent (4).

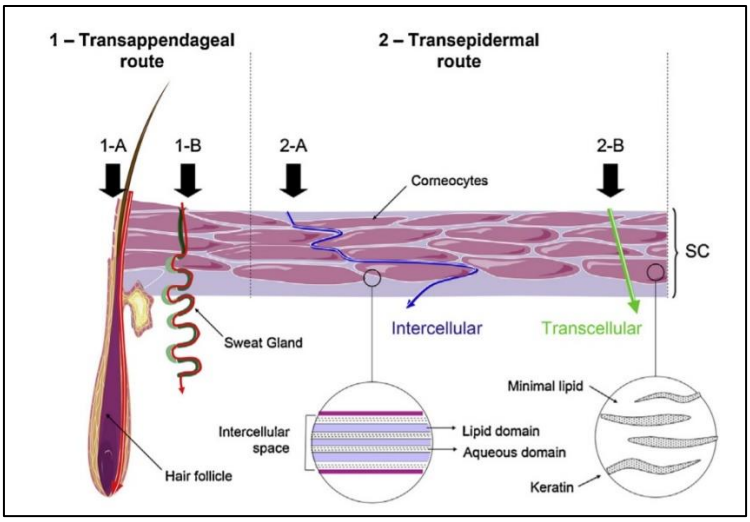

Figure 2. Penetration pathways through the SC. [From (30)]

1- Transappendageal route through (A) hair follicles and (B) sweat glands;
2- Transepidermal route across intact SC via (A) intercellular lipids and (B) via keratinised cells (or transcellular)

The intercellular pathway is considered the most important one in drug delivery (3). The molecules partition through the lipids bilayers (hydrophobic and hydrophilic domains) that surround the corneocytes (30,31). This pathway is much longer than the SC thickness since it is a tortuous route and not a direct one (3,4,8).

The transcellular pathway is known as the passage of the molecules through the lipid bilayers and the corneocytes (11), being the pathlength considered the same as the SC thickness (4). In order to pass the SC, molecules need to face different obstacles in a cyclic process. Even though the cellular components that compose the SC (with a high amount of keratin) can provide an aqueous environment to the solute, it is still a complex process, due to the presence of the lipid bilayers, so this is not a specific route just for hydrophilic or hydrophobic molecules.

With regard to the cyclic process, the solute partitions into the keratinocyte and diffuses through the keratin, followed by a partition to the lipid bilayer with further diffusion through the hydrophobic environment, until reaching the next keratinocyte and so on, until the solute reaches the viable epidermis (4,30).

The last pathway referred in the literature is the appendageal or shunt route. The appendages are specialised structures present in the dermis: hair follicles, sebaceous glands and sweat glands (7,32).

Although they represent only of 0.1% of the skin, this route is recognised as an important pathway in drug delivery (11,33), mostly in the early stages, before achieving the steady-state (8).

Using this route, charged molecules can pass through the skin by facilitated transport when an electric current is applied, like in iontophoresis. Nanoparticles can also be easily transported through this pathway; with their high molecular weights, they experience slow diffusion through the other pathways (8).

The importance of the appendageal pathway was also demonstrated by *Blume-Peytavi U. et al* (31) in their study using minoxidil foam to evaluate the follicular and percutaneous penetration pathways, where the detection of minoxidil occurred 25 min earlier when follicles were open (31).

1.4 Physicochemical parameters – Formulation for efficacy

The diffusion through the skin is characterised as the passage of molecules through a barrier from an area of high concentration to an area of lower concentration, being this passage a passive kinetic process (4,8).

Even with the complexity and heterogenicity of the skin and its role as a barrier, it is possible to describe dermal absorption and diffusion through the barrier by the Fick's law of diffusion (3).

The first equation that is important to refer is the one that describes the steady state flux or rate of transfer (J). It is represented by the Fick's first law (Equation 1) and gives the amount of permeant that is able to cross the skin or reach the circulation per unit area (34).

The permeant can be defined as the molecule that is in movement into or through the tissue (4).

$$\mathbf{J = KD(c_{app} - c_{rec})/h} \qquad \textbf{Equation (1)}$$

The flux is affected by different factors:

- K is the coefficient that represents the partitioning of the permeant between the vehicle and the membrane (3,4);
- D represents the Diffusion coefficient in the intercellular channels of the membrane thickness (h) (3,35);
- c_{app} and c_{rec} give the concentrations of the permeant in the vehicle and in the receptor phase, respectively (34).

KD/h = k_p represents the permeability coefficient which is the rate of permeant transport per unit of concentration. As usually $C_{rec} << C_{app}$, k_p can be estimated using the Potts and Guy equation (Equation 2) by an empirical relation from an aqueous solution through the SC (3,4,36):

$$\mathbf{\log[k_p/(cm\ h^{-1})] = -2.7 + 0.71 log K_{oct} - 0.061 MW} \qquad \textbf{Equation (2)}$$

- K_{oct} is the octanol water partition coefficient (3);
- MW is the molecular weight (3).

When looking at the previous equations, it is important to have in mind that to reach epidermis, dermis, or circulation, the permeant has to go through different processes that can be rate limiting (36):

- Diffusion from the formulation to the skin surface;
- Diffusion through the SC;
- Partitioning into the inner layers of epidermis and diffusion through it;
- Reaching the basal layer, partitioning and diffusion through the dermis;
- Partitioning into the lipidic deposits or reaching the circulation.

Analysing both equations and these steps, it is possible to conclude that the diffusion coefficient, the partition coefficient and the solubility are the most important properties to be considered (3,36).

Through the analysis of Eq. 2 it is possible to conclude that as molecules are larger and the MW increases, the diffusion will be lower and that an increased lipophilicity corresponds to an increased permeability, this phenomenon being represented by the K_{oct}.
In equation 2 is also important to note that the units (cm h^{-1}) do not reflect the amount of penetrant that passes through the SC, but the speed at which this movement occurs (36).

1.5 Penetration Enhancers

In the last years, scientists have tried hard to understand the skin as a barrier and to make it possible to control better the amount or velocity of substances that cross the SC until they reach the epidermis or the dermis and, in that way, clarify the complexity of transdermal drug delivery.
In order to achieve better results, Chemical Penetration Enhancers (CPEs) have been used as a strategy either to increase the flow of the permeant through the lipidic barrier by increasing the lipids' fluidity or to make the permeant more soluble in the SC (37).
CPEs are known as chemical compounds that are added to formulations in order to help the active to be delivered to and through the skin. Their use helps to increase the number of formulations that can be applied topically (38).
However, their use is still limited in the industry, since their mechanisms in the formulations are not completely understood and none of them have proved to be an ideal CPE.
A CPE is ideal if it combines at least seven main properties as described by *Williams A. et al* (39):

- Non-toxic, non-irritant and non-allergic;
- Works rapidly and the effect should be reproducible and predictable;
- Does not have pharmacological effects;
- Works unidirectionally by letting therapeutic agents enter the body and by preventing endogenous material to leave the body;
- Once it is removed, the properties of the skin barrier should return to the original fully and rapidly;
- Be compatible with actives and excipients;
- Be acceptable in regard to organoleptic characteristics.

Even though there are no CPEs considered ideal, a different range of compounds have been used as enhancers. Table 2 shows some of these compounds and their main mechanism of action (8,38,39).

Table 2. Examples of CPEs and respective mechanism of action.

Compound	Mechanism of action
Water	Increases the hydration of the tissue what increases transdermal delivery of lipophilic and hydrophilic actives.
Esters (e.g. isopropyl myristate)	Integrate the lipidic barrier disrupting it, increasing lipid fluidity.
Fatty Acids (e.g. oleic acid)	It causes disorder within the lipidic barrier increasing the diffusion of permeants through the skin.
Glycols (e.g. propylene glycol and dipropylene glycol)	PG is considered a carrier-solvent since its amount in the formulation is directly proportional to the amount of permeant that is delivered through the skin. Dipropylene glycol is considered a great CPE even though the mechanism of action is not yet explained.
Short chain alcohols (e.g. ethanol and isopropyl alcohol)	Ethanol and isopropyl alcohol are the short chain alcohols more commonly used as CPEs. They are able to modify the lipid bilayer organisation as a barrier allowing the permeant to cross the skin easily, for example, through lipid fluidisation.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Ultra-Pure Type 1 water was obtained from Merck Millipore equipment. N-acetyl-D-Glucosamine, D-(+)-Glucose, Phosphate buffer saline (PBS) tablets (pH 7.4), Potassium sodium tartrate tetra-hydrate and DNS (3,5-Dinitrosalicylic acid) were all supplied by Sigma-Aldrich (UK). Oleic acid was obtained by Benchmark Supplies (UK). Myritol® (Capric Caprylic Triglyceride) was purchased from BASF Australia Ltd. (Australia). Crodamol® IPM (Isopropyl Myristate) was supplied by Croda Europe Ltd (GB). Propylene Glycol (Propane 1-2-Diol) and Glycerol (Propane 1-2-3-Triol) were purchased from Scientific & Chemical Supplies Ltd (SciChem) (UK). Dipropylene Glycol was obtained from Aromatic Flavours & Fragrances Europe Ltd (AFF) (UK). Paraffin Liquid (light) and Sodium Hydroxide were supplied by Timstar Laboratory Supplies Ltd (UK). Carbopol® Ultrez 10 Polymer (Carbomer) was purchased from The Lubrizol Corporation (UK).

2.2 Methods

2.2.1 UV-Vis wavelength scanning

Each solvent was scanned using a Jenway 7315 Spectrophotometer using a wavelength range from 200 nm to 750 nm; all scanning was done in duplicate.
Peaks were obtained for all solvents and used for quantification purposes.

2.2.2 Quantitative Studies

2.2.2.1 Colourimetric assay for determination of reductive sugars

For the quantification of GlcNAc, a colourimetric quantification method based on the reaction between DNS and reducing sugars was used.

Glucose standards were made from a standard solution of 4 mg/mL, weighing 0.20 g onto a volumetric flask of 50 mL and completing the volume with ultrapure water. To create the calibration curve, serial dilutions were made from the standard solution: 3 mg/mL, 2 mg/mL, 1 mg/mL to a last one of 0.5 mg/mL.

The DNS reagent was prepared by mixing 0.160 g of sodium hydroxide (NaOH) in 2 mL of distilled water. 0.10 g of DNS and 5 mL of ultra-pure water were added. The components were dissolved completely with vigorous stirring and 3.0 g of potassium sodium tartrate were added, obtaining a final volume of 10 mL.

Ultrapure water was used as blank. 0.75 mL of the glucose standards were mixed with 0.75 mL of the DNS reagent and heated to 95 ºC for 5 min. These were left to cool down at room temperature for 15 min and 6 mL of ultrapure water were added. The 7.5 mL mixture was agitated gently, and 2.5 mL were transferred to the cuvette. Dilutions of 1:100 were made. All the measurements were done in duplicate and the absorbance was read at 540 nm.

2.2.2.2 GlcNAc Quantification in the Solvents

For each of the 8 solvents (Water, Glycerol, Propylene Glycol, Capric/Caprylic Triglycerides, Dipropylene Glycol, Paraffin, Isopropyl Myristate and Oleic Acid) measurements were performed at 217.0; 221.0; 219.0; 289.0; 221.0; 237.0; 271.0 and 312.0 nm respectively. Each solvent was used as blank and the respective sample was the same solvent and the active (GlcNAc) with a concentration of 0.1% (m/m).

All measurements were done in duplicate.

2.2.3 Solubility Tests

2.2.3.1 Saturation of solutions

For each one of the 8 solvents numbered above, a study of saturation was done.

Each solvent was saturated with GlcNAc; GlcNAc was added to each solvent until a precipitate was visible. The solutions were left stirring overnight to guarantee their saturation.

The following day, the solutions were centrifuged using the centrifuge Centurion scientific C2004.

Several dilutions were made to make it possible to read the absorbances for water, glycerol and propylene glycol.

Simultaneously, calibration curves were done to calculate the maximum concentration of GlcNAc that was diluted in each of the three solvents. Measurements were done in duplicate.

2.2.4 Formulations

Eight (8) gels with 2% of GlcNAc and a total of 50.0 g were prepared, according to the formulations in Table 3.

Table 3. Composition of the gels

	GlcNAc % (w/w)	Water % (w/w)	Carbomer % (w/w)	Glycerol % (w/w)	Propylene Glycol % (w/w)
Formulation 1 (F1)	2.00	97.5	0.50	-	-
Formulation 2 (F2)	2.00	48.75	0.50	48.75	-
Formulation 3 (F3)	2.00	32.50	0.50	65.00	-
Formulation 4 (F4)	2.00	65.00	0.50	32.50	-
Formulation 5 (F5)	2.00	48.75	0.50	-	48.75
Formulation 6 (F6)	2.00	65.00	0.50	-	32.50
Formulation 7 (F7)	2.00	32.50	0.50	-	65.00
Formulation 8 (F8)	2.00	32.50	0.50	32.50	32.50

In each formulation, the Carbomer was neutralized with a solution of Sodium Hydroxide (20%) (w/w) in order to form the gel structure ($6 > pH < 6.5$).

2.2.5 *In Vitro* Studies

2.2.5.1 Calibration curve

The Calibration curve for the gel formulations was obtained using PBS. A standard solution of 300 ppm was used, and serial dilutions were made to obtain concentrations of 200 ppm, 100 ppm and 50 ppm.

The absorbance was read at 202 nm considering the baseline of the spectra at 220 nm, since it was not settling at zero, as it was expected to.
It was necessary to correct all the values manually by subtracting the 220 nm absorbance value to the 202 nm absorbance value. The result would be the real absorbance if the spectra settled at zero.
All the measurements were performed with a NanoDrop 2000/2000c Spectrophotometer.

2.2.5.2 *In Vitro* Release Study

The method used was based on the Instruction Manual of Vertical Diffusion Cell Test System Model HDT1000 (40).
The Vertical Diffusion Cell is a reproducible and simple test that allows the measurement of the concentration of a drug released from a formulation through a synthetic, highly permeable and inert membrane.
In this test, Tuffryn® Membrane Filters were used to mimic the hydrophilic layer of the skin. These polymer-based membranes have Polysulfone as the main constituent and a pore size of 0.45 µm (41,42).
The Vertical Diffusion Cells or Franz Cells have two parts, the donor chamber where 0.1 g of the Formulation to be tested was applied and the receptor chamber containing the receptor medium which was 7 mL of PBS (pH 7.4) 0.01M (5 tablets in 1L of Ultrapure water).
The two parts were separated by a Tuffryn® Membrane – it allows the drug to diffuse and ensures that the sample is in contact with the receptor medium.
The membranes were soaked during 30 min in the same medium used in the receptor chamber. After assembling both parts as shown in Figure 3 two layers of Parafilm® were added around the connection between the two parts of the Franz cell and the loading of the sample to the donor chamber (0.1 g) was done using a five-decimal place analytical balance with a syringe. In the end, one layer of Parafilm® was added to the top of each Franz cell to minimise evaporation and a PVC cap sealed the sampling arm of the Franz Cells.

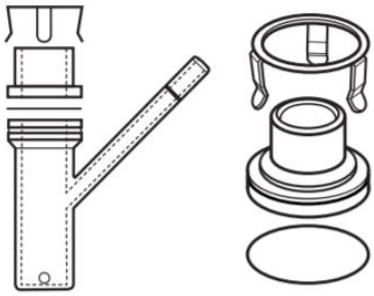

Figure 3. Schematic assembling of one Franz Cell. It comprises three parts: the membrane, the cell top and the clip that will fix the other two parts to the cell body.

In vitro release studies were performed at 32 ºC and a stirring speed of 600 rpm. Also, after collecting each sample from the Franz Cell, the volume collected was replaced with PBS to maintain the volume constant. These 3 parameters allowed to maintain sink conditions.

Each Formulation was analysed in 5 different Franz Cells in order to obtain reliable results and samples were collected after 0.5 h, 1 h, 2 h, 3 h and 4 h using an Eppendorf pipette. The sample volume was 50 µL. Each sample was stored in a labelled PCR tube.

After collecting each sample from the Franz Cells, in some of them it was necessary to add more PBS than the volume taken from the receptor chamber, since it was always necessary to refill the chamber and maintain the volume constant, meaning some degree of solvent evaporation was occurring.

All the samples were measured using NanoDrop 2000/2000c Spectrophotometer, using 2 µL of sample and 0.01 M of PBS as blank.

2.2.5.3 *In Vitro* Permeation Studies

The procedure was the same as the one used in the *In Vitro* Release Studies, except that each formulation was analysed in 3 different Franz Cells instead of 5.

The Strat-M® Membrane Filters are synthetic membranes of polyether sulphone. The fact that this membrane comprises different layers, it has a morphology closer the human skin's. The thickness and the porosity of the membrane will increase over the layers. Besides, the top layer is really tight as it happens with the SC of the human skin (43).

3. Results

3.1 UV-Vis Wavelength Scanning

This scanning showed that the chosen solvents had peaks at similar wavelengths: 217 nm for water, 221 nm for glycerol and 219 nm for propylene glycol (Table 4).

Table 4. Spectrum peaks obtained for each solvent.

Solvent	Peak (nm)
Water	217.0
Glycerol	221.0
Propylene Glycol	219.0
Capric/Caprylic Triglycerides	289.0
Dipropylene Glycol	221.0
Paraffin	237.0
Isopropyl Myristate	271.0
Oleic Acid	312.0

The peak wavelengths obtained in this scanning study, were used in the solubility studies to read the absorbance of the saturated solutions.

3.2 Quantitative Studies

3.2.1 Colourimetric assay for determination of reductive sugars

In the colourimetric assay, the results obtained were not the ones expected since the values for the peaks of the solvents were always the same, due to a change in the pH after the dilutions.

3.2.2 GlcNAc Quantification in the Solvents

Table 5. Absorbance values obtained for each solvent with GlcNAc.

Solvent + GlcNAc	Peak (nm)	Absorbance
Water	217.0	0.884
Glycerol	221.0	0.739
Propylene Glycol	219.0	0.840
Capric/Caprylic Triglycerides	289.0	0.080
Dipropylene Glycol	221.0	1.035
Paraffin	237.0	0.343
Isopropyl Myristate	271.0	0.109
Oleic Acid	312.0	0.101

Table 5 represents the maximum absorbance for GlcNAc with each solvent.

Some of the solutions needed to be filtered, such as Dipropylene Glycol and CCT.

3.3 Solubility Studies

3.3.1 Saturation of Solutions

When analysing all solutions that were saturated overnight, it was possible to conclude that GlcNAc is not soluble in all the seven solvents proposed initially.

That was the case of Dipropylene Glycol, Capric Caprylic Triglycerides, Oleic Acid, Paraffine Liquid and Isopropyl Myristate that after stirring overnight, and with concentrations of 50 ppm, did not dissolve GlcNAc, so they were deemed not fit to use.

After reading the absorbance of the solutions saturated with water, propylene glycol and glycerol it was possible to get the maximum amount of active that would be soluble in each solvent, according to the calibration curves presented in the figures below.

The concentrations obtained were 206.333 g/L for water, 7.390 g/L for propylene glycol and 40.375 g/L for glycerol.

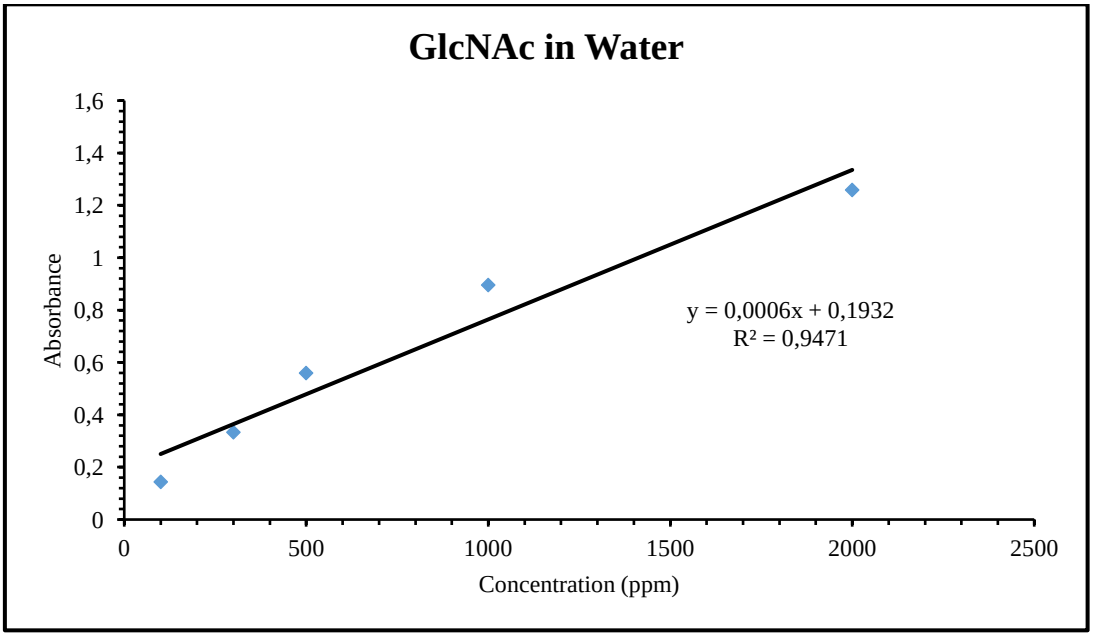

Figure 4. Calibration curve for GlcNAc in water.

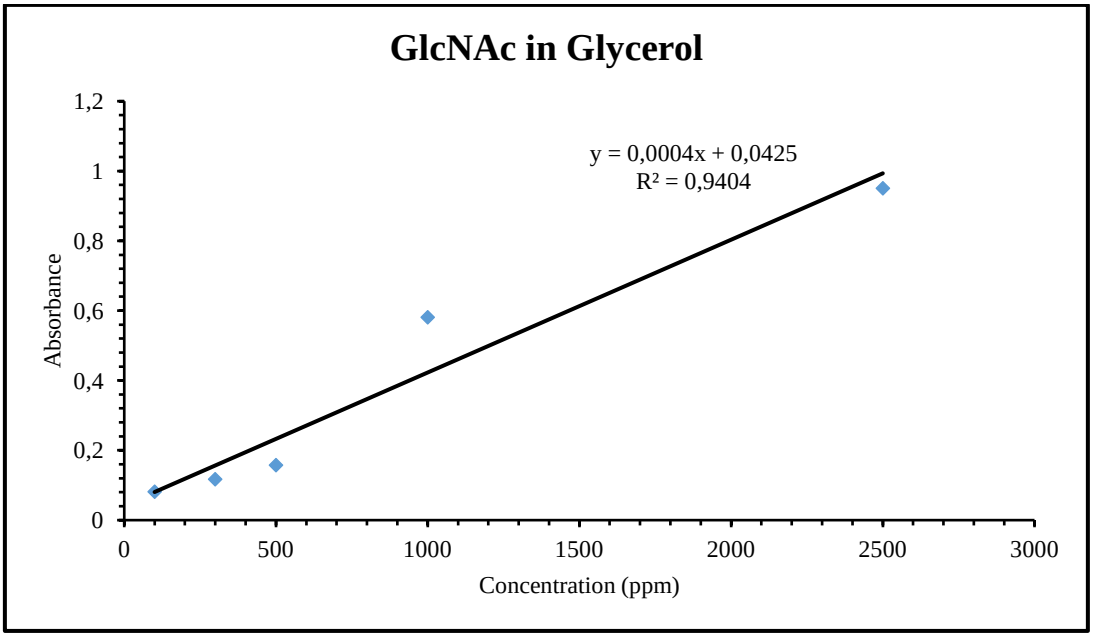

Figure 5. Calibration curve for GlcNAc in glycerol.

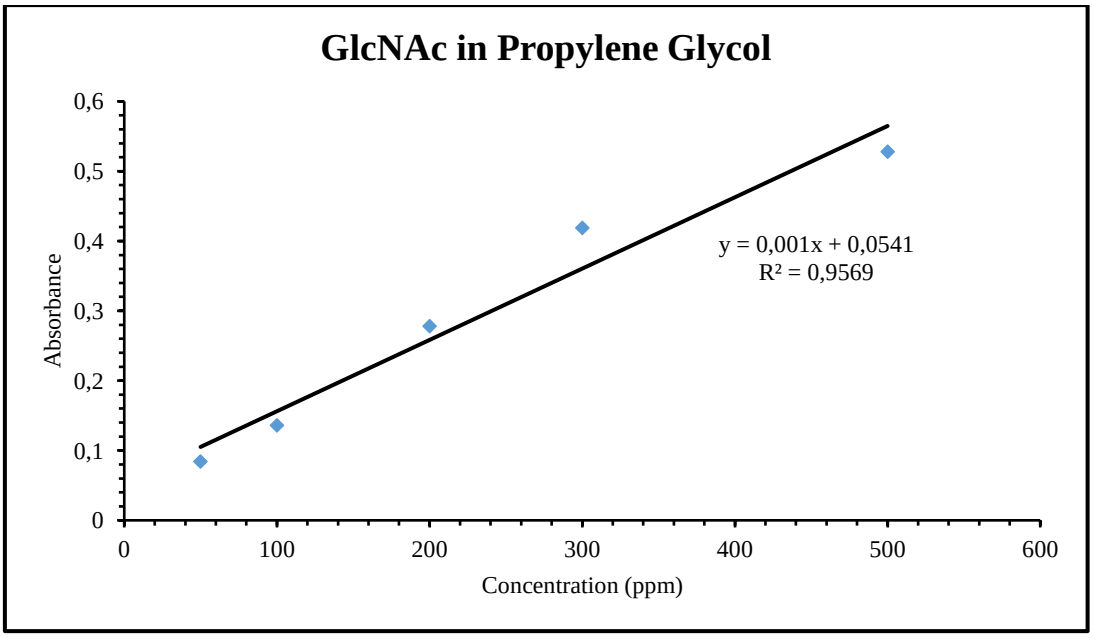

Figure 6. Calibration curve for GlcNAc in propylene glycol.

3.4 Formulations

The formulations were selected in order to evaluate if a difference in the proportion of the chosen solvents would have some difference in the release of the active.

Formulation 2, 3 and 4 did not take part in this work, since they were analysed in another report.

3.5 *In Vitro* Studies

3.5.1 Calibration curve

The final curve is represented in the Figure below.

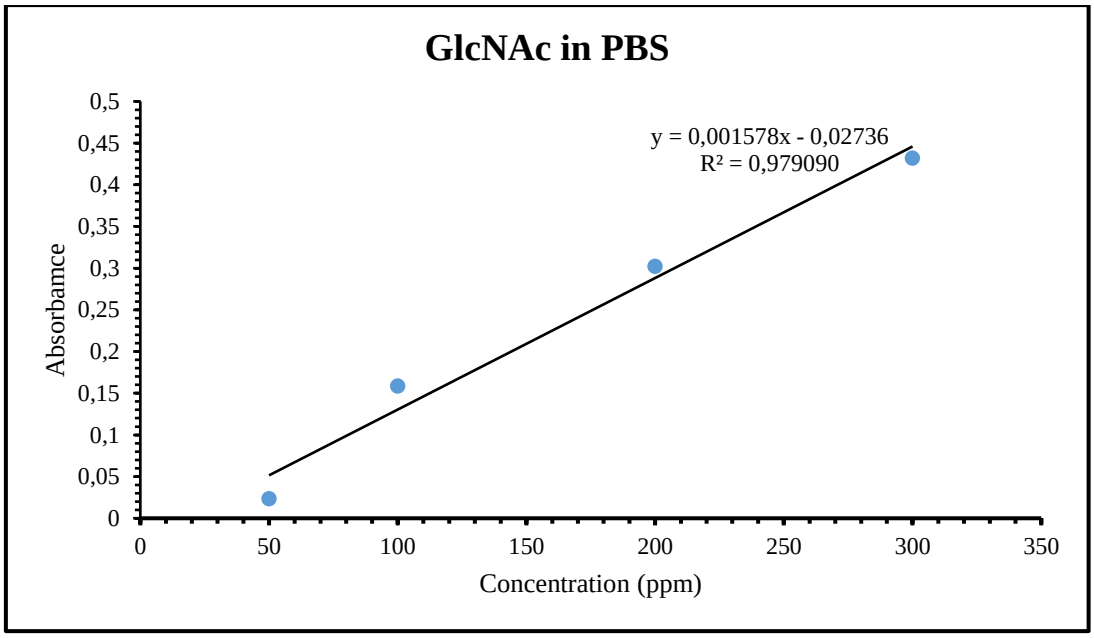

Figure 7. Calibration curve of GlcNAc in PBS

3.5.2 *In Vitro* Release Studies

The results obtained after analysing all the Formulations (1, 5, 6, 7 and 8) using the Tuffryn® Membranes are presented in the Figure below.

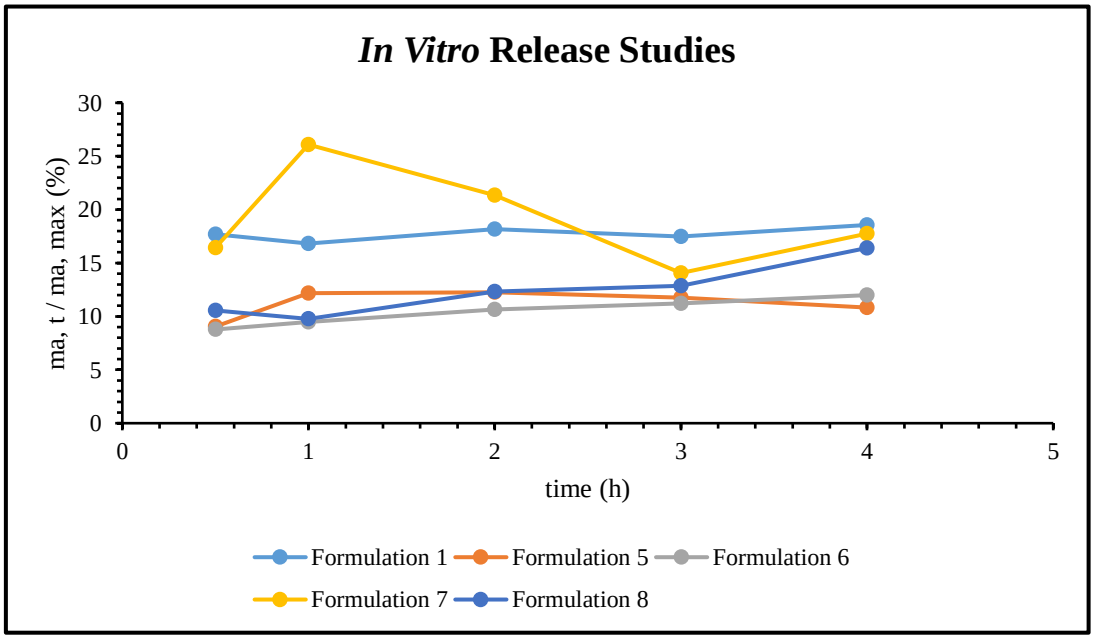

Figure 8. Release of active through Tuffryn® Membrane Filters during the 4 h of study from the five different Formulations.

The samples were taken at 0.5 h, 1 h, 2 h, 3 h and 4 h.

The Figure above represents the amount (%) of active that was released from each formulation from the maximum amount of active that could be released.

It is important to note that F1, just with water, is the one with a better release profile.

Among F5, F6 and F7, all with Propylene Glycol is important to note that the one with a higher release was F7 with a proportion water:propylene glycol of 1:2. F5 and F6 had similar profiles of release.

F8, the one with water:glycerol:propylene glycol in a ratio of 1:1:1 had a lower release during the first 2 h, with a growing release afterwards.

3.5.3 *In Vitro* Permeation Studies

The results obtained after analysing all the Formulations (1, 6, 7 and 8) using the Strat-M® Membrane Filters are represented in the Figure below.

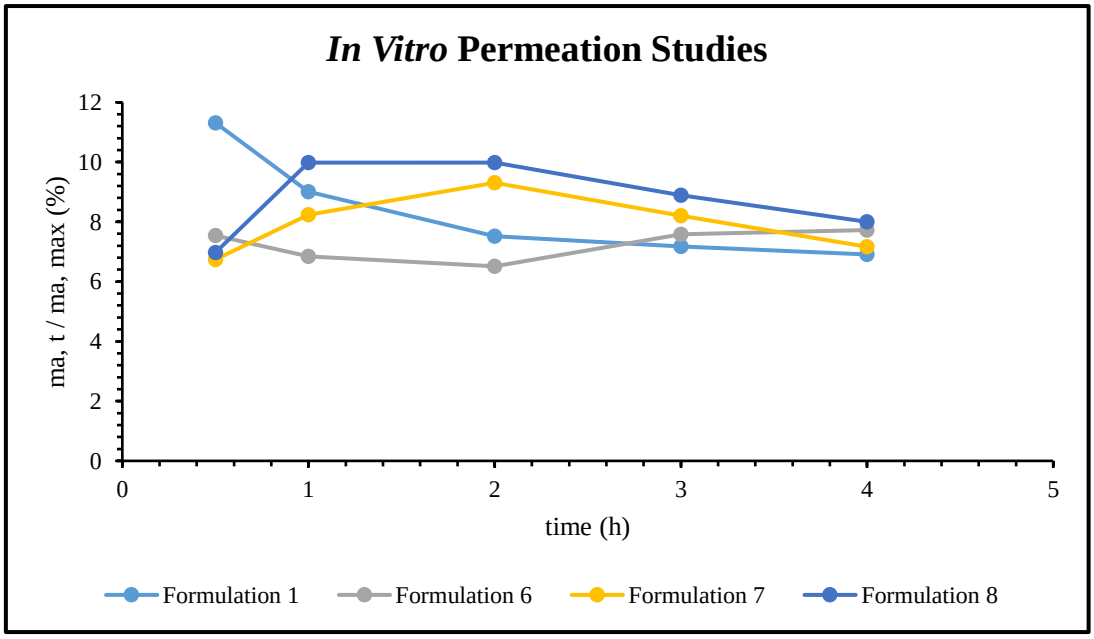

Figure 9. Permeation of active through Strat-M® Membrane Filters during the 4 h of study from the four different formulations.

The samples were taken at the exact same time as the *In Vitro* Release Studies.

Figure 9 shows the amount (%) of active that was permeated from each formulation, after the times labelled, from the maximum amount of active that could pass through the membrane.

In this Figure, it is possible to see that F7 and F8 had a very similar profile of permeation, being F8 the one with a higher permeation. From this, it is possible to conclude that when adding Glycerol to the gel, the permeation of GlcNAc increases.

Formulation 7 was originated only using the values of two cells. Cell number 3 gave nonsense results, that were ignored.

F6 showed low permeation values of GlcNAc when compared with the others, and F1, after 1h, showed decreasing values of permeation.

4. Discussion

4.1 UV-Vis wavelength scanning

In order to know which wavelength should be used in the quantification of the active with each solvent, a wavelength scanning was performed. Therefore, the main purpose of this method was to scan the spectra of all the solvents and determine their maximum absorbance peaks.

4.2 Quantitative Studies

4.2.1 Colourimetric assay for determination of reductive sugars

The DNS reagent was developed for the determination of reductive sugars and it is a complex reagent, comprised of several constituents. Alkaline conditions are necessary for the accurate determination of reductive sugars and thus, special care is required when handling this reagent. In fact, DNS that is yellow, is reduced to 3-Amino,5-Nitrosalicylic acid that turns into orange/red in the presence of reductive sugars, as the aldehyde groups of the sugars are oxidized into carboxyl groups (44).

Due to the limit of detection of the Jenway 7315 Spectrophotometer, the instrument could not read the colour of the samples, since they were too dark. In order to solve the problem, the samples were diluted to 1:10 and 1:100 until it was possible to measure.

This dilution caused colour reversion to yellow, as the pH became more acidic due to the addition of water.

For this reason, the values obtained were not representative of the concentration of reductive sugars and this method was deemed invalid.

Other problems that can occur with this reagent have been also reported by *Breuil C. and Saddler J.* (45) in which they explain that the Nelson-Somogyi method is more sensitive and easily detect lower concentrations of the sugar than when using DNS and that the latter is more affected by interferents than the first. *Gusakov A. et al* (46) in their studies of comparison of the determination of reductive sugars using the DNS method and another standard method (Nelson-Somogyi) proved that the first could give overestimations of the results up to 10 fold (45,46).

4.2.2 GlcNAc Quantification in the Solvents

Due to the difficulties and lack of validity of the colourimetric assay for the determination of reductive sugars, a direct UV quantification method was used as the alternative.

4.3 Solubility Tests

4.3.1 Saturation of Solutions

The main objective of this preliminary study was to verify which of the solvents could be chosen as proper vehicles for the release study of GlcNAc in each formulation as explained by *Ravula R. et al* (47).

Analysing the results of the solubility tests, it is possible to corroborate them with the fact that the GlcNAc is a hydrophilic molecule, that was shown to be very soluble in water, glycerol and propylene glycol.

50 ppm (50 mg/L) of GlcNAc was the lowest concentration assessed, since at lower concentrations it would not be reasonable to include it in topical formulations, as the amount of active that would reach the site of action would be very low.

4.4 Formulations

Hydrogels were the type of formulation chosen for this experiment. They are known to be an effective vehicle for the topical delivery of drugs due to their skin hydration potential, increasing the skin water content, leading to a more facilitated transport of the active and also because of the amount of water in their constitution, especially in hydrogels, that allows a high dissolution of hydrophilic actives like GlcNAc (48).

Besides, gels are simple formulations, easily formulated with few materials, what makes them easy formulations to work with.

4.5 *In Vitro* Studies

4.5.1 Calibration Curve

With regard to the Calibration Curve, the measurements were done at 202 nm, although this was not a well-defined peak, the results were more accurate when comparing with another peak at 195 nm.

As referred before, it was necessary to correct manually all the values, obtained from the NanoDrop 2000/2000c Spectrophotometer, due to a baseline shift that was occurring.

220 nm was chosen as the reference wavelength, since there was no interference of the active at this point, and 202 nm as the analytical wavelength.

The correction of the values was performed by subtracting the absorbance at the reference wavelength from the absorbance at the analytical wavelength, following the procedure of Internal Referencing referred by *Owen T.* (49). This correction allowed to shift the spectra back to a baseline of zero (49).

The same procedure was followed to obtain the absorbance values for each sample analysed for all formulations.

The shift is usually a result of unknown interfering errors that cannot be corrected, so it is necessary to use techniques that allow to correct these errors. Since the instrument is very sensitive, in this case, errors could have had their origin in a voltage disturbance, vibrations near the instrument, oscillations in the temperature or even due to an instrumental default.

4.5.2 *In Vitro* Release and Permeation Studies

The release and permeation of active from the gels was measured using the Vertical Diffusion Cell or Franz Cell.

As *Fox L. et al* (50) showed in their article, it would have been important to measure the solubility of the GlcNAc in the PBS, as well as the rate of permeation in this buffer, to verify if the active was an appropriate candidate for delivery through the skin (50). This procedure is also described in the OCED Guidelines for the testing of chemicals (51), so that the absorption is not inflected by the poor solubility of the active in the receptor fluid (51).

With regard to the experiments, the release study using the Tuffryn® Membrane Filters was used as a preliminary study to verify if it was worth it to proceed with the experiments with the Strat-M® Membrane Filters. If GlcNAc would not be released from the vehicle, permeation studies would not have been performed.

This was also the main reason why Formulation 5 was not tested in the *in vitro* permeation study.

Formulation 5 was the one with a proportion of solvents in the middle of Formulation 6 and Formulation 7. If a considerable difference between the proportions could be noticed, it would be easier to notice in the Formulations with a bigger variation between them, so Formulation 6 and 7. Formulation 5 was comprised of water:propylene glycol in a proportion of 1:1. In contrast, Formulation 6 had a proportion water:propylene glycol of 2:1 and Formulation 7, a proportion water:propylene glycol of 1:2.

One of the biggest challenges in topical drug delivery is to provide the correct dose of active to the right place for a reasonable amount of time, whilst reducing the secondary effects of the drugs in non-target places. In order to achieve these two goals, it is important to understand the mechanism of transport of the molecule (52).

Different mathematic models have been used so that the mechanism of release of the active becomes more predictable.

One of these models is Fick's first law of diffusion that allows to quantify the diffusion of the active through the skin, as shown before in Equation 1 (53).

Another one that can also be used, is the Higuchi model which also calculates de rate of release of a drug. This model was created as a basis for other mathematical models, as explained by *Paul D* (54).

Although these mathematic models are very useful, they were not applied in this report, since the results presented here are from initial studies that do not require this evaluation.

These models would be very convenient in a later phase of the studies, to optimise methods and for an easier understanding of the mechanism of release of the active from some specific vehicle (55).

Improvement of methods and vehicles might be the next step for this experiment, in order to get better results for the delivery of GlcNAc through the skin.

Experiments by *Garner S. et al* (56) showed that DMSO is an excellent skin penetration enhancer for this molecule, although just for veterinary use, since it is not approved for human use. Also, ethanol, was proved to be a good skin penetration enhancer, that can be used in humans, in a concentration range of 5-25% (56).

Lipid vesicular systems and nanoparticles are also under study for the transdermal delivery of GlcNAc. As *Shatalebi M. et al* (57) showed that niosomes, a lipid vesicular system, are a potential in these vesicles to deliver GlcNAc through the skin (57).

With regard to nanoparticles, *Marto J. et al* (58), have shown that Solid lipid nanoparticles are a great delivery system used with success in the cosmetics or pharmaceutical areas. According to this study, an emulgel with GlcNAc-loaded nanoparticles showed an improved permeation of the active through the skin, as the solid lipid nanoparticles acted as enhancers for the delivery of GlcNAc (58).

Israel B. et al (59) showed a potential in the transdermal delivery of GlcNAc when coupled with a non-steroidal anti-inflammatory drug as prodrugs, to treat osteoarthritis (59).

5. Conclusion and Perspectives

The transdermal delivery of molecules, is a more comfortable means of treatment, as opposed to other, more invasive administration routes, thus guaranteeing an improvement in patient compliance.

Important findings have been showed in this report with regard to the design of topical formulations with improved release and permeation.

The DNS method proved not to be appropriate for use with the instrument that was being used due to limit of detection issues, but it was replaced by the UV-Vis spectrophotometry that proved to be reliable and allowed to quantify GlcNAc in the different solvents.

These studies were very useful in terms of selecting the vehicles that can act as CPEs to GlcNAc, so that further studies can test new solvents and different vehicles for the transdermal delivery of this active.

Even though water, glycerol and propylene glycol proved to be promising CPEs that allowed a higher amount of GlcNAc to be released and permeated, with better results when the three were present in the same formulation, more CPEs should be tested.

It is important to note that future research should include different vehicles for this active, as well as more accurate detection methods. These two components might be the key to achieve more accurate results.

In conclusion, GlcNAc proved to be a molecule with a high potential in what concerns to transdermal delivery. Although, a lot of work and improvement of methods and techniques needs to be done in order to get better results.

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7. Annexes

Annexe I

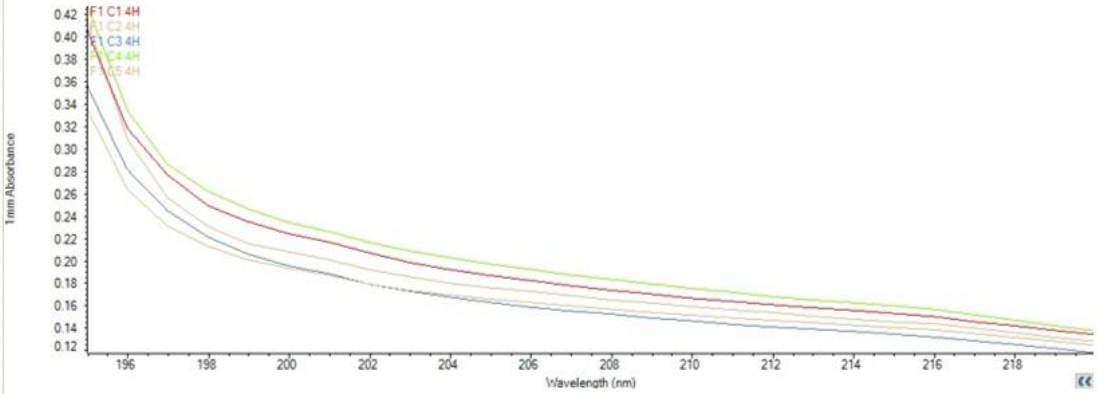
After 4h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.11161	0.10964	0.10987	0.10067	0.10911
$m_{a,max}$	0.002324	0.002283	0.002288	0.002097	0.002272
A_{bl} (220 nm)	0.134	0.127	0.117	0.137	0.124
$A_{max,a,t}$ (202 nm)	0.207	0.192	0.178	0.216	0.179
$c_{a,t}$	63.60	58.53	55.99	67.40	52.19
$m_{a,t}$	445.20	409.71	391.93	471.80	365.33
$m_{a,t}/m_{a,max}$	19.157	17.946	17.130	22.499	16.080
Spectra					

Figure 10. Spectra of the Formulation 1 after 4h of release of the GlcNAc.

Annexe II

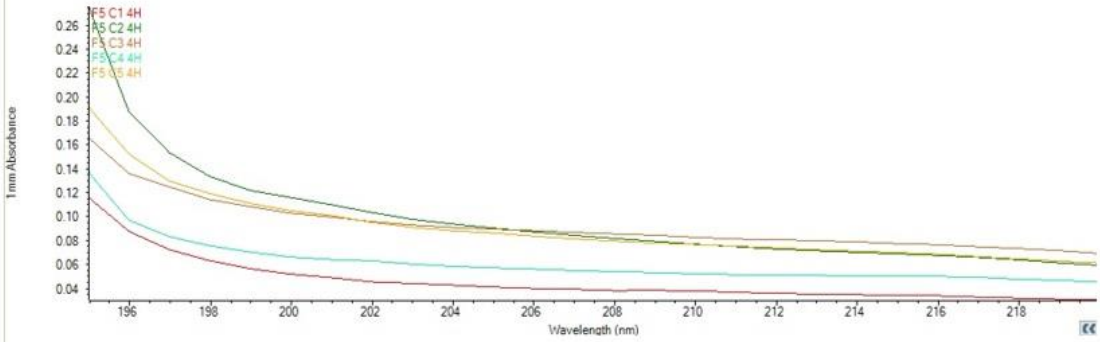
After 4h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.11822	0.10956	0.10341	0.12073	0.11632
$m_{a,max}$	0.002364	0.002191	0.002068	0.002415	0.002326
A_{bl} (220 nm)	0.029	0.059	0.068	0.045	0.061
$A_{max,a,t}$ (202 nm)	0.045	0.103	0.095	0.062	0.095
$c_{a,t}$	27.48	45.22	34.45	28.11	38.88
$m_{a,t}$	192.36	316.54	241.15	196.77	272.16
$m_{a,t}/m_{a,max}$	8.137	14.447	11.661	8.148	11.701
Spectra					

Figure 11. Spectra of the Formulation 5 after 4h of release of the GlcNAc.

Annexe III

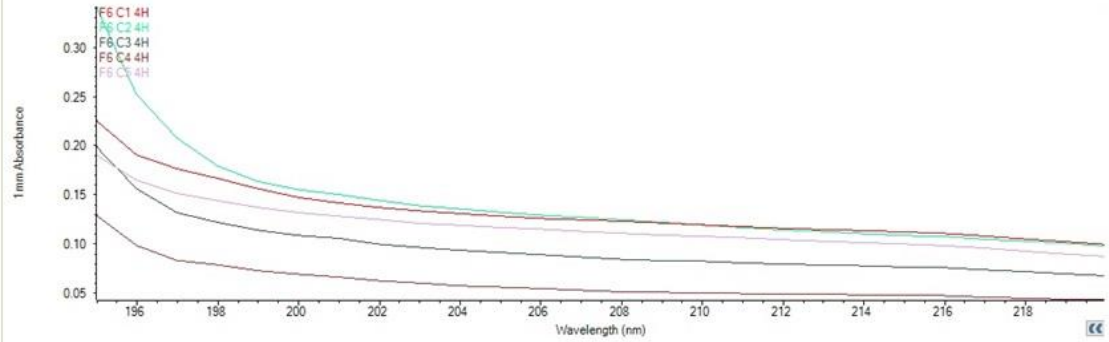
After 4h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.11423	0.10428	0.10247	0.10416	0.12213
$m_{a,max}$	0.002286	0.002087	0.002050	0.002084	0.002444
$A_{bl\ (220\ nm)}$	0.097	0.050	0.067	0.042	0.087
$A_{max,a,t\ (202\ nm)}$	0.144	0.074	0.099	0.062	0.124
$c_{a,t}$	47.12	32.55	37.62	30.01	40.79
$m_{a,t}$	329.84	227.85	263.34	210.07	285.53
$m_{a,t}/m_{a,max}$	14.429	10.918	12.846	10.080	11.683
Spectra					

Figure 12. Spectra of the Formulation 6 after 4h of release of the GlcNAc.

Annexe IV

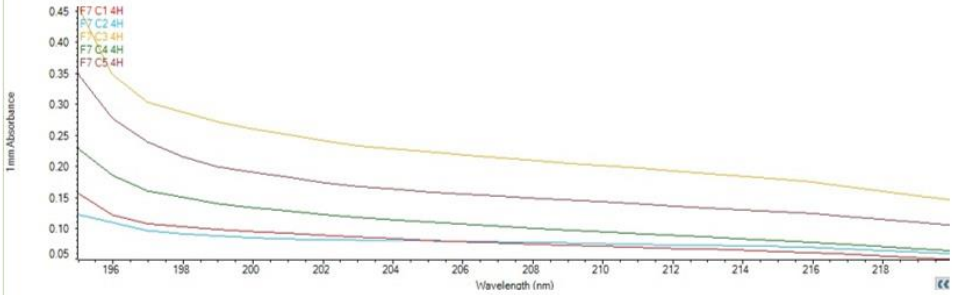
	After 4h				
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.10189	0.10794	0.10539	0.10077	0.10902
$m_{a,max}$	0.002046	0.002167	0.002116	0.002023	0.002189
A_{bl} (220 nm)	0.049	0.058	0.145	0.063	0.104
$A_{max,a,t}$ (202 nm)	0.088	0.081	0.241	0.121	0.173
$c_{a,t}$	42.05	31.91	78.17	54.09	61.06
$m_{a,t}$	294.35	223.37	547.19	378.63	427.42
$m_{a,t}/m_{a,max}$	14.387	10.308	25.860	18.716	19.526
Spectra					

Figure 13. Spectra of the Formulation 7 after 4h of release of the GlcNAc.

Annexe V

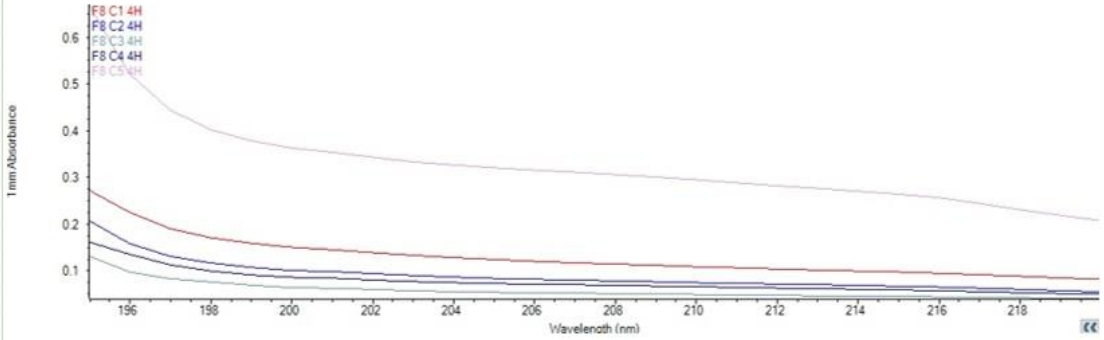
After 4h					
	Cell 1	**Cell 2**	**Cell 3**	**Cell 4**	**Cell 5**
m_{form}	0.10880	0.10675	0.10541	0.11908	0.11944
$m_{a,max}$	0.002176	0.002135	0.002109	0.002382	0.002389
A_{bl} (220 nm)	0.080	0.053	0.038	0.047	0.206
$A_{max,a,t}$ (202 nm)	0.137	0.092	0.058	0.078	0.342
$c_{a,t}$	53.46	42.05	30.01	36.98	103.52
$m_{a,t}$	372.22	294.35	210.07	258.86	724.64
$m_{a,t}/m_{a,max}$	17.106	13.787	9.961	10.867	30.332
Spectra					

Figure 14. Spectra of the Formulation 8 after 4h of release of the GlcNAc.