PHD

Iontophoretic drug delivery to the nail

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Award date: 2008

Awarding institution: University of Bath

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IONTOPHORETIC DRUG DELIVERY TO THE NAIL

JULIE DUTET

A thesis submitted for the degree of Doctor of Philosophy

University of Bath
Department of Pharmacy and Pharmacology
November 2008

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Acknowledgements

I would like to thank my supervisor Dr Begoña Delgado-Charro for her guidance and support throughout my doctoral studies.

I gratefully acknowledge Dr Michael Cork and Dr Stephen Moss for accepting to evaluate this work.

I thank Adrian Rogers for his technical help to use the Laser Scanning Confocal Microscope.

I thank all the volunteers for their participation in the experiments and for their patience.

I would like to thank my friends and colleagues, past and present, for their moral and technical support. I would like to especially thank Sarah Cordery for her invaluable moral and technical support.

I acknowledge the University of Bath for its financial support.

Finally, I would like to thank my family for their constant moral support.
Abstract

Basic information about nail behaviour, under passive and especially iontophoretic condition, lacks in the literature. Thus, this thesis aims to fill gaps in the nail understanding by studying the potential and feasibility of the application of iontophoresis to human nail.

The iontophoretic and passive delivery of Sodium Fluorescein (SF) and Nile Blue Chloride (NBC) were studied, in vitro, in order to determine their pathways as well as their depth and uniformity of penetration into the nail.

The permselective properties of the nail were investigated by characterizing the contribution of electroosmosis, using mannitol as a marker, and by studying the flux of two inorganic cations, sodium and lithium, during in vitro experiments.

Finally, the feasibility of transungual iontophoresis and the extraction of sodium and chloride ions from the body through the nail plate were performed on a group of human volunteers.

Iontophoresis led the fluorescent markers slightly deeper into the nail plate than passive diffusion. The delivery of the bianion and of the cation was not different. Both compounds mainly penetrated the nail via the transcellular pathway.

Electroosmosis resulted only in a slight enhancement of the mannitol fluxes compared to passive diffusion and the fluxes presented high variability, especially at pH 7.4 and when the current was applied in the anode-to-cathode direction.

The delivery of the two inorganic cations was significantly higher at pH 7.0 than at pH 4.0 and supported that nails hold a negative charge at physiological pH.

Ions were easily extractable through the nail plate during in vivo iontophoresis and all volunteers’ feedbacks supported iontophoresis as an acceptable technique.

This thesis demonstrated the feasibility and potential of in vivo transungual iontophoresis.
List of abbreviations

AFM    Atomic Force Microscopy
ALA    5-aminolevulinic acid
D      Depth of penetration on dorsal images
5-FU   5-Fluorouracil
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC   High Performance Liquid Chromatography
IC     Ion Chromatography
IRB    Institutional Review Board
LSC    Liquid Scintillation Counting
LSCM   Laser Scanning Confocal Microscopy
MIC    Minimum Inhibitory Concentration
MW     Molecular weight
NBC    Nile Blue Chloride
pI     Isoelectric point
SEM    Scanning Electron Microscopy
SF     Sodium Fluorescein
T      Transversal
TEWL   TransEpidermal Water loss
TOWL   TransOnychial Water Loss
%T    Percentage of transversal penetration
Chapter 1:
INTRODUCTION

Nails represent a small part of the body but have the structure of a very efficient barrier. Nails are made up of layers of dead, flattened, overlapping keratinised cells which form a 0.2–0.6 mm hard structure (Walters et al., 1983 b; Murdan, 2002; Hao et al., 2008).

Two main diseases affect the nail unit: onychomycosis and psoriasis. Treatments of these two diseases usually lead to poor patient compliance. Indeed, due to the lack of penetration of topically applied drugs into the nails, long term systemic treatments resulting in side effects or painful injections in the nail folds are the usual alternatives.

Iontophoresis consists in the application of a small electrical current and has proven to efficiently enhance drug delivery in various fields such as transdermal application (Kalia et al., 2004), orthopaedics (Day et al., 2005), ophthalmology (Eljarrat-Binstock et al., 2005) or dentistry (Singal et al., 2005).

Recent publications investigating the in vitro application of iontophoresis to human nail plate demonstrated an interesting potential for drug delivery (Murthy et al, 2007 a and b, Hao et al., 2008 a, b and c).

The aim of this chapter is 1) to describe the structure and properties of the human nail, 2) to present the main diseases that can affect nails, onychomycosis and psoriasis, and their treatments, 3) the formulations developed for transungual delivery, 4) to introduce the principles of iontophoresis and 5) to describe the application of iontophoresis to the nail and its potential.
Nail structure and properties

1. Structure and growth

The nail plate is constituted of “hard” hair-type (80%) and “soft” skin-type (20%) keratins (Lynch et al., 1986; De Berker et al., 2000). Sulfur and nitrogen are the most important elements in nail composition. Lipids (mainly cholesterol) represent 0.1 to 1% of the nail composition and small quantities of potassium, calcium, magnesium, sodium, copper, zinc and iron can be found (Walters et al., 1983a; Debruyne et al., 2001). Water is the main plasticiser in the nail and nail water content is usually 18-20% but can be as high as 25% for excised nail exposed to 100% RH (Gunt et al., 2007a).

In the past, the terms used to describe the structure of the nail were not shared by all authors which led to the use either of similar words to indicate different areas or of different words for similar area. This misunderstanding was partly due to a disagreement on the existence/absence of a ventral layer and thus, of an area in the nail matrix to produce it. Now, as authors agree on the existence of this layer, the situation is simpler.

Fig. 1 shows a lateral view and the different parts where the nail formation takes place.

![Fig. 1: Lateral view of a nail. Reproduced from Jiavuthisan et al. (2007)](image)

The proximal and lateral nail folds are the skin parts holding in place the nail plate. The eponychium is the thin cuticle sealing off the space between the proximal nail fold and the nail plate and the hyponychium is the skin under the free edge of the nail plate.

Keratinocytes are present in the nail matrix and differentiate to produce the nail plate cells, the onychocytes (Lowry et al., 1999). The nail matrix is subdivided in the dorsal, intermediate and ventral matrix. The ventral matrix, at the level of the lunula, connects with the nail bed. There is no granular layer in the epithelium of the matrix or in the nail.
bed. This means that, unlike skin keratinisation, nail keratinisation occurs without the formation of keratohyalin.

By a process of parakeratosis, the dorsal layer originates from the dorsal matrix, the intermediate layer from the intermediate matrix and the ventral layer from the ventral matrix (Reardon et al., 1999). Parakeratosis is defined as the flattening of the cells associated with a reduction in size and the persistence of the nuclei.

Some authors (Lewis, 1954; Reardon et al., 1999) suggested that the walls of the lateral nail folds and the ventral proximal nail fold could participate in the formation of the intermediate and dorsal layers, respectively.

It is thought that the nail bed participates all along the nail growth to an increase in thickness (Johnson et al., 1993). However, no increase in the number of nail cells has been observed and it has been suggested that the shape of the cells could become altered as the nail grows resulting in a reduction of the number of cells but as well in an increase in thickness (De Berker et al., 2007).

The growth of the nail is the consequence of the pressure of the expanding cells. As Fig. 1 shows, the nail plate follows an angled growth: newly formed cells delimit an almost horizontal band which, with the nail growth, will be pushed towards a vertical position (De Berker et al., 2007).

The average growth rate is 3 mm per month for healthy fingernails and 1 mm per month for toenails (Murdan, 2002; Geyer et al., 2004).

2. Composition and mechanical properties

The orientation of the keratin fibres and contents in calcium, phospholipids, disulphide bounds (S-S), sulphydryl groups (S-H) and acid phosphatase activity are specific to each layer (Table 1).
Table 1: Comparison of the nail layers composition and orientation of the keratin fibres

<table>
<thead>
<tr>
<th></th>
<th>Dorsal</th>
<th>Intermediate</th>
<th>Ventral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium$^a$</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Phospholipids$^a$</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Disulphide bounds$^a$</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Sulphhydryl groups$^a$</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Acid Phosphatase</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>activity$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keratin fibres</td>
<td>60% perpendicular</td>
<td>All perpendicular</td>
<td>Similar to dorsal</td>
</tr>
<tr>
<td>orientation to the</td>
<td>40% parallel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>growth axis$^b$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ data from Jarrett et al. (1966).
$^b$ data from Farren et al. (2004)

In terms of mechanical behaviour, the dorsal and ventral layers, by their keratin fibres orientation, are isotropic whereas the intermediate layer is anisotropic. Despite this “sandwich” structure, the nail mechanical properties are governed by the isotropic intermediate layer. This means that when a cut happens in the nail plate, it propagates perpendicularly to the growth axis in accordance with the keratin fibres orientation in the intermediate layer (Farren et al., 2004). It is to note that this property allows the protection of the finger: indeed, if the cut propagated parallel to the growth axis it would reach the sensitive nail bed and might cause damage to the finger.

All the keratins fibres are held together by disulfide bounds (S-S). These bounds are the usual target of penetration enhancers such as N-acetyl-L-cysteine (drug formulations for nail delivery are addressed in a section below).

3. Permeability properties

The effects of the vehicle, the lipophilicity, the ionisation and the MW on nail drug permeation have been subject to controversy.

It is accepted by several authors that the aqueous or lipophilic nature of vehicles do not change the drug permeation rate (Walters et al., 1985a; Mertin et al., 1997a). However, Walters et al. (1983b, 1985a) asserted the existence of a lipophilic pathway determining the nail penetration of homologous n-alcanols whereas Mertin et al. (1997b) and Kobayashi et al. (2004) respectively used homologous nicotinic acid esters of increasing carbonated chain length (and increasing octanol/water partition coefficient) and homologous p-hydroxybenzoic acid esters and proved that no lipophilic pathway existed. The former authors (Mertin et al., 1997b; Kobayashi et al., 2004) also showed that the partition coefficient of the molecule had little importance upon drug permeation.
The same authors disagreed as well on the effect of ionisation on drug permeation: Walters et al. (1985b) studied the effect of the pH on the nail permeation of miconazole and did not find any whereas Mertin et al. (1997b) and Kobayashi et al. (2004) both found that ionised molecules penetrated the nail plate less efficiently than their respective neutral form.

The MW cut-off of the membrane, probably partly because of the previously cited controversy, is not well determined: Kobayashi et al. (2004) were not able to measure any permeation rate for compounds presenting MW above 240 whereas Mertin et al. (1997c) studied the permeation of several antimycotics with MW ranging from 151.2 to 956.1, Walters et al. (1985b) studied the permeation of miconazole which has a MW of 416.1 and Gunt et al. (2007b) studied the permeation of ketoconazole which has a MW of 531.4 showing that compounds having a MW above 400 Da were still able to penetrate the nail plate. Despite the fact that no clear value of a MW cut-off was found, an inverse relationship between the log of the permeability coefficient and the MW has been established (Mertin et al., 1997c; Kobayashi et al., 2004) which suggests that the permeability coefficient decreases when the MW increases.

**Onychomycosis and psoriasis**

1. **Onychomycosis**

Onychomycosis affects 3 to 10 % of the European population (Murdan, 2002) and approximately 18 % of the world population (Repka et al., 2002). It is more likely to affect 1) toenails than fingernails (4:1) and 2) elderly, immunosuppressed or diabetics patients rather than healthy “young” people (Lowry et al., 1999; Roberts, 1999; Murdan, 2002; Repka et al., 2002).

90 to 95% of toenail onychomycosis are due to dermatophytes (mainly *Trichophyton rubrum*, *Trychophyton mentagrophytes*), 5 to 10% are due to yeasts or moulds (mainly *Candida Albicans* or *Aspergillus Versicolor*). Fingernail onychomycosis are caused by the same microorganisms as toenail affections but 50 % are due to dermatophytes (mainly *T. rubrum* and *T. mentagrophytes*) and the other 50 % are due to yeasts or moulds (Roberts, 1999; Murdan, 2002; Repka et al., 2002; Lever, 2005).

Dermatophytes are eukaryotic and form multicellular tubes called hyphae which, when they intertwine, form mycelia. Their reproduction is asexual and the arthrospores are formed by hyphal segmentation (Lever, 2005).

Yeasts are single-celled usually rounded fungi. The association of the cells can form pseudo-hyphae (*Candida sp*) and mycelia. They reproduce by budding forming blastospores (Lever, 2005).
Onychomycosis is classified in four clinical types (Lever, 2005):

- **DLSO**: distal and lateral subungual onychomycosis is the most common form and is usually due to *T. rubrum*.
- **SWO**: superficial white onychomycosis which is primarily caused by *T. mentagrophytes* and frequently affects HIV patients.
- **PSO**: proximal subungual onychomycosis which is typically caused by *T. rubrum* but is rare in the general population.
- **Candida infections**: there is no nail plate invasion (Unlike dermatophytes, *Candida* sp do not “feed” on keratin) and yeasts are seen along the undersurface of the nail plate.

Two types of treatment can be used to treat onychomycosis: avulsion treatments or drug based treatments. The drug based treatments will be more highlighted as usually avulsion treatments are not well accepted by patients and drugs active against dermatophytes will be privileged.

**a) Avulsion treatments**

Two kinds of avulsion treatments can be found in the literature (Lowry et al., 1999; Murdan, 2002): 1) surgical avulsion consists in the removal of the infected nails. This is a very traumatic operation which is now kept as a last resort. 2) chemical avulsion consists in the application, under occlusive dressings, of a formulation containing around 40% of urea during 5 to 10 days. This formulation softens the nail plate and provokes its separation from the nail bed. The application of a topical antifungal is usually coupled with this treatment in order to ensure the growth of healthy nails.

**b) Drug based treatments**

Drug treatments can be systemic or topical. Oral treatments are based on two “old” molecules, namely griseofulvin and ketoconazole, and three “new” molecules; itraconazole, terbinafine and fluconazole. Main topical treatments involve nail lacquers containing amorolfine or ciclopirox.

Terbinafine (allylamine), azoles and amorolfine (morpholine) are inhibitors of enzymes in the biosynthesis of ergosterol (Fig. 2). Ergosterol is the equivalent of mammalian cholesterol in the fungus cell membrane and therefore, inhibiting enzymes in its biosynthesis results in an increased permeability of the fungus membrane and may provoke the cell death.
Oral treatments:

- Griseofulvin is derived from *Penicillium griseofulvum*. It was the standard treatment of onychomycosis until the development of azoles and allylamines. It is fungistatic and its spectrum is limited to dermatophytes (Develoux, 2001). It inhibits the formation of intracellular microtubules, disrupts the mitotic spindle and thus, prevents cell division of the fungus (Gupta et al., 2003a). Treatments must be pursued until complete cure (12 months or more) and relapses are common (Lowry et al., 1999). It is roughly well tolerated (digestive side-effects) but teratogenic and potentially carcinogenic effects were shown in animals (Develoux, 2001).

- Ketoconazole is fungistatic, generally well tolerated and keratophilic. A rare side effect, a drug-induced hepatitis, imposes short term treatment and close monitoring of the liver function and thus, leads to its very limited use (De Doncker, 1999; Debruyne et al., 2001). The usual dose is 200 to 400 mg daily.

- Itraconazole is fungistatic. It can be taken continuously, 200 mg daily for 3 months, or be taken in pulse therapy based on the administration of 200 mg twice daily during 7 days with subsequent courses repeated after 21-day intervals. The treatment takes 2 courses for fingernails and 3 courses for toenails (BNF 56).

- Terbinafine is fungicidal and well-tolerated with usually only gastro-intestinal disturbances and cutaneous reactions. Roberts (1999) gathered clinical studies of these molecules and determined that terbinafine was the most cost-effectiveness and the most efficient drug with a cure rate of 70 to 80%. However, this conclusion
has to be taken cautiously as this author received fundings from Novartis Pharma (Lamisil®, Terbinafine), Janssen Pharmaceuticals (Sporanox®, Itraconazole) and Pfizer (Diflucan®, Fluconazole).

- Fluconazole is used at a posology of 150-300 mg weekly until complete cure (Gupta et al., 2003a) but this application is not licensed in UK.

The description of the possible drug interactions of these oral treatments have been addressed by Katz et al. (2003) and can be of two kinds:

1) Pharmacodynamic:
   a) Itraconazole is thought to have a negative inotropic effect and thus association with other drugs presenting the same effect such as calcium channel blockers (eg. nifedipine or verapamil) or its administration to patients presenting already heart failure is not recommended.
   b) All antifungal drugs present a risk of hepatotoxicity. Therefore administration to patients presenting already a liver dysfunction or concomitant administration with drugs extensively processed by the liver are not recommended.

2) Pharmacokinetic:
   a) The gastro-intestinal absorption and bioavailability of some antifungals such as griseofulvin, ketoconazole and itraconazole (capsules) is highly dependent on the gastric pH or meals composition. Thus, drugs able to modify the gastric pH such as divalent cationic antiacids or proton pump inhibitors have to be administered carefully.
   b) Itraconazole and ketoconazole are inhibitors of the P-glycoprotein which is a membrane transporter found on the apical surface of enterocytes in the small intestine and in the secretory cells of the renal tubules. Its inhibition can cause increased gastro-intestinal absorption or decreased renal excretion and may result in an increased bioavailability of substances depending on this transporter (digoxin).
   c) Ketoconazole, itraconazole and fluconazole (dose > 200 mg/d) are inhibitors of the CYP3A4, fluconazole inhibits the CYP2C9 as well and terbinafine inhibits CYP2D6. All these enzymes are part of the cytochrome P-450 which is involved in the biotransformation/metabolisation of numerous drugs. Therefore, depending on the therapeutic window of the drugs involved, side effects may result from this inhibition by increasing the level of un-metabolised drugs in the blood flow.
d) ketoconazole, itraconazole and, to a partial extent, fluconazole are substrate of CYP3A4. Thus, CYP3A4 inducers such as rifampicin, or carbamazepin may be responsible for a bioavailability decrease and, potentially, for a failure of the antifungal treatment.

**Topical treatments:**

Amorolfin and ciclopirox are used topically and therefore cause only local side effects (eg irritation or rash).

- Amorolfin is a fungicidal drug of the morpholine class. It must be applied one or two times weekly. The treatment last 6 months for fingernail and 9 to 12 months for toenails (BNF 56). It has two sites of actions in the ergosterol biosynthetic pathway: it inhibits the $\Delta14$-reductase and the $\Delta7$-8-isomerase (Gupta et al., 2003b).

- Ciclopirox is a hydroxypyridone derivative. It is fungicidal and must be applied once daily. It has at least three mechanisms of action. The main one relates to its high affinity for trivalent cations such as $\text{Fe}^{3+}$ and $\text{Al}^{3+}$. These ions are involved in mitochondrial electrotransport processes to produce energy and thus, by blocking these transports, ciclopirox provokes a lack of energy production and subsequently the cell death. The second is related to a strong decrease of catalase and peroxidase activity responsible for the degradation of toxic peroxides creating an accumulation of the toxic compounds in the cell. Finally the third mechanism is responsible for a decrease in the nutrient uptake creating an intracellular depletion of essential aminoacids which reduces the synthesis of proteins and nucleic acids (Gupta et al., 2003b).

The posology, MIC, plasma and nail concentrations of drugs used for onychomycosis systemic and topical treatments are presented in Table 2.
Table 2: Antifungal drugs used to treat onychomycosis. Appl. means application and MIC is the minimum inhibitory concentration.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Dose</th>
<th>MIC (ng/g)</th>
<th>Plasma concentration (mg/L)</th>
<th>Nail concentration c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Griseofulvin</td>
<td>500 mg to 1 g/d</td>
<td>94 – 6000</td>
<td>1.39</td>
<td>600 – 900 ng/g</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>200 to 400 mg/d</td>
<td>94 – 12000</td>
<td>5.5 – 6.5</td>
<td></td>
</tr>
<tr>
<td>Itraconazole</td>
<td>200 mg/d</td>
<td>11 – 1500</td>
<td>600 – 900 ng/g</td>
<td>0.272</td>
</tr>
<tr>
<td></td>
<td>400 mg/d during one week repeated after 21-day interval</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Terbinafine</td>
<td>250 mg/d</td>
<td>2 – 750</td>
<td>0.03 – 1.39</td>
<td>250 – 1000 ng/g</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>150 mg/week</td>
<td>750 – 48000</td>
<td>0.3 – 2.7</td>
<td>8.5 – 9.5 µg/g (peak)</td>
</tr>
<tr>
<td>Amorolfine</td>
<td>1 – 2 appl./week</td>
<td>300</td>
<td>3.9 – 24.6 mg/g</td>
<td></td>
</tr>
<tr>
<td>Ciclopirox</td>
<td>1 appl./d</td>
<td>11 – 94</td>
<td>5.91±2.42 mg/g</td>
<td></td>
</tr>
</tbody>
</table>

a doses from BNF except fluconazole from Gupta et al. (2003a)

b MIC from Perea et al. (2001) (griseofulvin and fluconazole) and Gupta et al. (2003c) (Ciclopirox, terbinafine, itraconazole and ketoconazole) concern T. mentagrophytes and T. rubrum. Amorolfin MIC concerns C. albicans (Debruyne et al., 1999).


Table 2 shows that antifungal drugs are usually at an efficient concentration in nails (however MICs were not determined in the nail tissue itself) and that topical application usually allows greater concentrations in nails than systemic treatments. However, both types of treatments are limited by their duration because 1) the nail concentrations presented in Table 2 are reached after, at least, a couple of weeks and 2) the nail cure is not obtained before months. Further, relapses or reinfections (the distinction is not always obvious) are frequent whichever the molecule used. It is interesting to note that systemic drugs are not only incorporated in the nail in formation via the three matrices but that they diffuse as well into the whole nail plate via the nail bed (Palmeri et al., 2000).

Combination therapies coupling an oral and a topical drug such as terbinafine+ciclopirox or +amorolfin and itraconazole+amorolfin have been used for severe onychomycosis: they usually increase the cure rate significantly but the duration of the treatment remains unchanged.
Psoriasis

Psoriasis is a chronic, incurable disease which affects 1.5 – 3 % of the population. Up to 90 % of psoriatic people have their nails as well as their skin affected by the disease (Jiaravuthisan et al., 2007). The disease is related to an immunologic T-cells activation and skin manifestations are characterized by epidermal thickening and scaling.

To name a few symptoms, nail psoriasis appears under the form of pitting, onycholysis, subungual hyperkeratosis or splinter haemorrhages (De Berker, 2004).

Although several therapeutic options exist, there is a paucity of information concerning their outcomes on nail psoriasis and no reference treatment is established.

Treatments consist in drugs administrated orally, topically, by local injection or by radiation. The systemic treatments are only indicated for severe, complicated psoriasis. The use of drugs such as methotrexate, cyclosporine or acitretin (to name a few) has been reported to treat skin psoriasis and to have effects on nail psoriasis (De Berker, 2004; Jiaravuthisan et al., 2007). However cyclosporine and methotrexate are immunomodulators and acitretin is a retinoid (teratogenic side effect) and therefore the use of systemic treatments for nail psoriasis is usually not recommended (De Berker, 2004).

Topical therapies (ointment, cream, solutions and gels) must be applied at least once a day. It is to note that a single therapy is not efficient against all the manifestations of nail psoriasis. Topical therapies are based on:

- Corticosteroids and vitamin D₃ analogs. Both compounds have an anti-inflammatory effect but long-term corticotherapy can be responsible for skin atrophy in the nail folds. This treatment is efficient on hyperkeratosis but has not been studied for any other nail dystrophies.
- 5-Fluorouracil (5-FU) is an inhibitor of the nucleic acids synthesis. It has been reported that 5-FU could either improve or worsen the symptoms of nail psoriasis (Jiaravuthisan et al., 2007). 5-FU is efficient on nail pitting and hyperkeratosis but is not recommended for onycholysis.
- Anthralin (also known as dithranol) is classified as an antipsoriatic agent. It is effective on hyperkeratosis and onycholysis but has no affect on nail pitting. Anthralin is a safe treatment which acts by decreasing inflammation and cellular proliferation and by increasing cellular differentiation. However this drug colours skin and clothing and can cause skin irritation.
- Tazarotene is from the retinoid family. Its acts by normalising cell differentiation, reducing cell proliferation and decreasing inflammation. Tazarotene is efficient on
nail pitting and onycholysis but has little effect on hyperkeratosis. This treatment can be responsible for erythema, irritation or even peeling of the nail folds.

- Cyclosporine is an immunosuppressive agent. Cyclosporine is efficient on nail pitting and onycholysis. It is thought that topical applications of tacrolimus and pimecrolimus (immunosuppressive agents) might be of interest for future treatments of nail psoriasis (Jiaravuthisan et al., 2007).

Intralesional therapies consist in the injections of corticosteroids in the nail folds. Triamcinolone acetonide is the most frequently used drug. Although it is an efficient treatment, the injections are painful and can be responsible for hematomas. Further, the steroid itself can be responsible for skin atrophy. It has been suggested that this technique should be limited to severe nail psoriasis and to two or three injections per year (De Berker, 2004).

Radiation therapies such as phototherapy and photochemotherapy (PUVA therapy: Psoralene + UVA) are thought to lead to an improvement of nail psoriasis. However these therapies mostly have been investigated for their skin effects. Superficial radiotherapy and other types of radiations have been used in the past but their use is declining. The main reason for this decline is the possible effects of the radiations on the body after the treatment which can be as serious as a risk of carcinomas or sarcomas.

**Drugs formulations for transungual delivery**

As the previous sections of this chapter have shown, nails are characterized by a poor permeability which makes the treatment of nail diseases difficult. Therefore, efforts have been orientated towards the development of formulations to lengthen drug-nail contact and in the testing of substances to enhance drug delivery.
1. Formulations prolonging the contact of drugs with the nail surface:

To ensure a prolonged release of drugs, it is important that the formulations adhere to the nail surface and therefore, lotions or solutions are of little use as these formulations do not remain long on the nail surface.

Nail lacquers allow the drugs to remain in contact with the nail plate for long times. The applications can be performed either by brushing a new layer on top of the previous one or by cleaning off the nail from the previous application and applying a new layer of lacquer. The usual elements necessary in a nail lacquer formulation are: an organic solvent, a film forming polymer, some resins (to enhance the adherence of the lacquer) and plasticisers (to preserve the flexibility and enhance the durability of the film). Thus, nail lacquers are organic solutions of drug and polymer which form a water-insoluble film when the solvent evaporates. The drug diffuses from the film into the nail plate. The water-insoluble film is occlusive and leads to an increase of the hydration of the nail plate. It is to note that the hydration of the nail plate plays an essential role for drug permeability (Gunt et al., 2007b).

The delivery of the drug from the lacquer to the nail plate follows diffusion law (Eq. 1):

\[ J = \frac{D \cdot K}{h} \cdot C_h \]  

Eq. 1

Where J is the flux, D is the diffusion coefficient, K is the partition coefficient of the drug between the formulation and the membrane, h is the depth of penetration and \( C_h \) is the concentration of the drug at the depth h.

The essential factors affecting the drug delivery in nail lacquers are:

- The solubility of the drug in the polymer film
- The solubility of the drug in the nail
- The diffusion coefficient of the drug in the polymer film
- The diffusion coefficient of the drug in the nail
- The drug content in the polymer film

Amorolfine and ciclopirox described previously are commercialised as nail lacquers. Other types of formulation have been explored and some examples are given below.

Repka et al. (2004) described the formulation of a hot-melt extruded nail lacquer and of a carbopol gel of ketoconazole. Hot-melt extrusion has the advantage to avoid the use of organic solvent and to require fewer steps than the formulation of a classic nail lacquer.
The authors performed their study by applying these formulations on non-etched or etched nail samples. This etching step was performed by applying a 10% phosphoric acid solution on the surface of the nails during 60 seconds and aimed at improving the bioadhesion of the lacquer on the nail surface. Etching not only resulted in a better adhesion of the lacquer but also in a greater permeation of ketoconazole from both formulations compared to the non-etched nails (60% more and 6 fold higher than the non-etched nails for the gel and the lacquer, respectively).

Hui et al. (2004) compared the nail delivery of a ciclopirox gel formulation (2%) containing propylene glycol and urea with a commercialised gel (0.77%) and a commercialised lacquer (8%). The authors found that the commercialised gel, despite ciclopirox low concentration in the formulation, reached the highest concentration in the receptors of the diffusion cells. Their formulation reached higher concentration than the commercialised lacquer.

Some more unusual formulations have been described: Donelly et al. (2005) formulated a bioadhesive patch whereas Monti et al. (2005) tested a water-soluble lacquer. The bioadhesive patch formulated by Donelly et al. (2005) contained 5-aminolevulinic acid (ALA), a drug used in oncology, which has a bactericidal and fungicidal action and is activated by visible light (Photodynamic therapy). The formulation contained Gantrez® AN-139 (a copolymer of methylvinylether and maleic anhydride), tripropylene glycol methyl ether, ethanol and 50 mg/cm² of ALA. ALA penetrated well in the nail samples and microbiological tests on Sabouraud liquid medium showed that ALA associated with irradiation led to high percentages of kill for *C. albicans* and *T. interdigitale*. However, 100 mM of ALA applied for 30 min for *C. albicans* and 6 hours for *T. interdigitale* + irradiation were necessary to kill 90% of the microorganisms. This concentration was far away from what was reached in nail samples (6.9 mM after 72 h exposure to the patch) and moreover this concentration is probably as toxic for microorganisms as it is for human cells. Therefore ALA associated with photodynamic therapy did not seem to be applicable in practice.

The formulation of ciclopirox in a water-soluble nail lacquer containing hydroxypropylchitosan was described by Monti et al. (2005). Thanks to their formulation, ciclopirox achieved much shorter lag time than Penlac® (3.36h versus 12.48h) whereas both formulations contained 8% of the drug. Although the authors justified this water-soluble nail lacquer by its short lag time and thus, by the possibility of applying it before a night of sleep, the usefulness of such a lacquer which can be so easily removed remains arguable.
2. Formulations interacting with the nail structure:

Some chemical compounds are able to soften the nail plate or to break covalent bonds in the nail structure.

Urea and salicylic acid have been typically used in skin applications to soften and remove the external layers of the stratum corneum. Although these two substances have an effect on the nail aspect (Quintanar-Guerrero et al., 1998), this did not lead to enhanced nail permeations for Kobayashi et al. (1998) (5-FU), for Quintanar-Guerrero et al. (1998) (miconazole, ketoconazole and itraconazole) or for Malhotra et al. (2002) (water).

Repka et al. (2002) studied by SEM (Scanning Electron Microscopy) and AFM (Atomic Force Microscopy) the effect of urea, tartaric acid and phosphoric acid on the dorsal layer of the nail. Scanning electron micrographs of urea did not reveal any effect of the chemical on the nail whereas tartaric acid and phosphoric acid provoked significant etching and roughness visible on SEM and AFM images. Application times of tartaric acid and phosphoric acid were 90 and 30 seconds, respectively. As mentioned above, Repka et al. (2004) compared the permeation of two formulations with and without phosphoric acid pre-treatment and found that the pretreatment with phosphoric acid had an enhancing effect.

Nail swelling is thought to be a good indicator for formulations potential (Gunt et al., 2007b), it has therefore been used to screen penetration enhancers (Khengar et al., 2007). It is to note that nail swelling can be obtained, to a certain extent, by simply increasing nail hydration (Gunt et al., 2006).

Khengar et al. (2007) described the screening of twenty compounds by comparing their nail swelling effect (weight gain) and found that the four best compounds for this criteria were thioglycolic acid, glycolic acid, urea mixed with hydrogen peroxide ($H_2O_2$) and hydrogen peroxide alone. Further, they studied the effect of thioglycolic acid pre-treatment (20 h) on caffeine permeation and found that the steady flux of caffeine was 3.8-fold higher than the control.

Malhotra et al. (2002) tested twenty-two potential penetration enhancer formulations. They screened compounds coming from different classes: mercaptans which are known for breaking disulphide bounds, sulfites, keratolytics and surfactants. They ranked their chemical candidates by measuring their enhancing effect on water permeation. They concluded that the best enhancers were both mercaptans: N-(2-mercaptopropionyl) glycine (synergistic effect with urea) and pyrithione (non synergistic effect with urea).
Kobayashi et al. (1998) showed that N-acetyl-L-cysteine and 2-mercaptoethanol (both able to break disulphide bounds) significantly increased the *in vitro* permeation of 5-fluorouracil and tolnaftate. Hui et al. (2002, 2003) showed that the penetration enhancer contained in Econail®, 2-n-nonyl-1,3-dioxolane (SEPA: Soft Enhancement of Percutaneous Absorption), and a penetration enhancer formulation (containing DMSO, dimethylsulfoxide, and other excipients) were able to enhance the penetration of 1) econazole permeation with Econail® and 2) urea, salicylic acid and ketoconazole with their penetration enhancer formulation. To summarise, most of the nail penetration enhancers are molecules able to break the keratin disulphide bounds (2-mercaptoethanol, N-(2-mercaptopropionyl) glycine, pyrithione, N-acetyl-L-cysteine or thioglycolic acid) and thus, to provoke nail swelling and an increase in the water uptake. Keratolytics such as urea and salicylic acid are inefficient in enhancing drug permeation when used on their own. Sometimes, penetration enhancers efficient for transdermal applications are efficient as well for nail applications (e.g. 2-n-nonyl-1,3-dioxolane).

**Principles of iontophoresis**

Iontophoresis is a minimally invasive technique which has been extensively studied for topical and transdermal delivery (Kalia et al., 2004) or drug monitoring (Leboulanger et al., 2004a). It consists in the application of a small electrical current to a membrane in order to provoke the migration of molecules according to their charge and/or polarity. Two main mechanisms of transport exist: electromigration and electrosmosis (known as well as convective solvent flow). Fig. 3 is a scheme of an iontophoretic circuit and shows the electrochemical reactions happening at each electrode.
Figure 3: Scheme of an iontophoretic circuit. Only electromigration is shown: sodium ions are repelled from the anode and attracted by the cathode whereas chloride ions are repelled by the cathode and attracted by the anode.

In a simple way, electromigration is the repulsion of a charged molecule from the electrode presenting the same charge towards the electrode of the opposite charge. More precisely, when a current is applied, it produces electrochemical reactions at the surface of the electrodes resulting in ion fluxes. The ionic transports exist because the different compartments have to keep their electroneutrality (Fig. 3). The consumption of chloride at the anode provokes a depletion in anions in the anodal compartment and therefore, to keep the electroneutrality, the attraction of anions. At the cathode, the production of chloride ions provokes the repulsion of anions and the attraction of cations to keep the charge balance. As chloride carries only one charge, the reaction happens mole to mole (Fig.3) and the theoretical amount of chloride ions consumed/produced at the electrodes follows the Eq. 1:

\[ I.t/F = \text{mol Cl} \quad \text{Eq. 1} \]

where I is the intensity applied during the experiment in amperes, t is the length of the experiment in seconds and F is the Faraday’s constant (96500 coulombs/mol). This equation allows the determination of the amount of chloride salt to add to the anodal solution and how much chloride ions accumulate at the cathode.

During iontophoresis, each ion only transports a fraction of the current applied and the sum of the current carried by each ion is equal to the total current applied. For each ion, this fraction, called transport number, depends on its physicochemical properties and on the electrochemical composition of the milieu. The transport number varies accordingly with the different species present in the milieu which means that ions are competing for current charge transport. The transport number, \( t_d \), of an ionic drug can be expressed as:
where \( c, z \) and \( \mu \) refer to concentration, valence and mobility inside the membrane of either a drug \( d \) or an ion \( i \) (Phipps et al., 1992). This equation means that 1) the “single-ion” is the best situation and results in the highest \( t_d \) and 2) ions present in large concentrations with a high electrical mobility will carry most of the charge. However, in practice, it is difficult to develop a pharmaceutical formulation containing only the drug without adding excipients and therefore, the drug delivery will depend on its ability to be an efficient charge-carrier in the presence of competing co- and counter-ions. Co-ions are the ions possessing the same sign and present in the same compartment as the drug. In case of iontophoretic drug delivery, the effect of co-ions in the drug formulation can be minimised by limiting the amount of highly mobile co-ions, by using only co-ions presenting very low mobility or by increasing the concentration (molar fraction) of the drug. The counter-ions are the ions of opposite sign to the drug present on the other side of the membrane. In vivo, because of their concentration and electrical mobility, the physiological counter-ions are sodium and chloride. Obviously, their competition cannot be avoided.

As ions concentration and mobility inside a membrane cannot be predicted and are difficult to measure accurately, Eq. 2 has little practical application. Experimentally, Faraday’s equation allows the deduction of the transport number from the molecule flux, \( J_d \):

\[
J_d = \frac{I \cdot t_d}{F \cdot z_d} \quad \text{Eq.3}
\]

where \( I \) is the electrical current passed, \( F \), the Faraday’s constant and \( z_d \), the valence of the ion (Phipps et al., 1992). This equation demonstrates that the iontophoretic flux is proportional to the applied current. This is of great importance as it allows the control of the amount of drug delivered and offers the possibility to adapt the treatment to individuals.

In addition, a charged membrane shows permselectivity which means that it allows the transport of counter-ions preferentially. For example, the skin is negatively charged which results in a higher transport number for cations than for anions.

Electroosmosis is a consequence of the membrane permselectivity. In skin, electroosmosis happens in the anode to cathode direction because cations carry preferentially the current and, therefore, provoke a transport of solvent in the same direction (Burnette et al., 1987; Pikal et al., 1990). However, this net charge can be modulated by changing the pH of the...
solution in contact with the membrane. For example, keratin, the main constituent of skin, presents a pI (isoelectric point) close to 5 and therefore at physiological pH, skin is negatively charge, but, at pH below 5, skin’s permselectivity is reversed allowing preferential transport to anions and exhibiting an electroosmotic flow in the cathode to anode direction.

The transport of neutral compounds from the anode can be affected by the ionic strength of the donor by reducing the solvent flow (Santi et al., 1996).

It is important to note that transport of molecules through a charged membrane is complex: transport of a molecule is normally due to the contribution of both mechanisms and not only one of them. The electromigration contribution is the most important for small and charged molecules whereas the electroosmotic contribution is increased for big, polar but uncharged molecules. When a molecule shows a low mobility (and thus a low transport number) both transports can have a role to its transport (Guy et al., 2000; Abla et al., 2005; Schuetz et al., 2005).

**Application of iontophoresis to the human nail plate**

*In vitro* nail iontophoresis has been recently under the scope of two teams who investigated nail charge and its permselectivity (Hao et al., 2008a and c; Murthy et al., 2007a and b). They studied the electroosmotic transport of glucose, griseofulvin (Murthy et al., 2007a), mannitol and urea (Hao et al., 2008a and c). Electromigration was investigated using tetraethylammonium (Hao et al., 2008a and b) and salicylic acid (Hao et al., 2008a; Murthy et al., 2007b). During the last month, Nair et al. (2008, epub) published an article concerning the delivery of terbinafine (pKₐ ~7).

Hao et al. used mannitol whereas Murthy et al. used glucose to determine the nail permselectivity. They both found that the transport of their permeants was maximal in the anode to cathode direction when the pH was above 5 and in the cathode to anode direction when the pH was below this value. This demonstrated that nail holds a net negative charge at physiological pH.

Electromigration had a clear enhancing effect on the fluxes of the molecules previously cited when compared to passive diffusion.

The effect of penetration enhancers on iontophoretic fluxes has been investigated by Hao et al. (2008b): thioglycolic acid was found to increase the transport number of tetraethylammonium but not to affect electroosmosis.

To date, only one *in vivo* iontophoretic application has been performed by James et al. (1986) by investigating the plasma concentration of prednisolone after transdermal and
transungual iontophoresis. Unfortunately, the authors did not focus on the iontophoretic mechanisms involved during transungual iontophoresis. Nevertheless, this study proved the feasibility of in vivo nail iontophoresis.

Clearly, the in vitro characterization of the mechanisms involved in nail iontophoresis just started being addressed and information about its in vivo application remains anecdotal. This thesis addresses different important points to clarify the mechanisms of iontophoretic nail delivery.

The first chapter investigates the pathways used by two fluorescent markers as well as their depth of penetration in the nail layers under iontophoretic and passive conditions using confocal microscopy. This study was motivated by the fact that the main pathway of charged molecules during transdermal delivery is appendageal whereas this route does not exist in the nail plate. Thus it was of interest to determine if compounds were more likely to use a transcellular or a paracellular pathway. Further, the depth of penetration allows the evaluation of the effect of iontophoresis on compounds presenting different physicochemical properties (hydrophilic, lipophilic and anionic, cationic).

The second and third chapter investigate electroosmosis and electromigration by studying the flux of mannitol and of two cations. Indeed, the result of the first chapter showed no differences in the pathways or in the penetration depth of the two ionic fluorescent markers and thus, it was important to design experiments to study the two forces involved during iontophoresis to determine the nail permselectivity.

As the results obtained during the electromigration studies were extremely encouraging, chapter four investigates 1) the feasibility of an iontophoretic in vivo application in terms of voltage and participant comfort and 2) the extraction of sodium and chloride ions from the nail bed through the nail plate.
Chapter 2:
Visualisation of ungual penetration by laser scanning confocal microscopy.

Overview
The *in vitro* ungual penetration of sodium fluorescein (SF) and nile blue chloride (NBC), an anionic and a cationic fluorescent marker respectively, has been examined by Laser Scanning Confocal Microscopy (LSCM). The depth, uniformity and pathways of penetration of both markers into the human nail plate were compared after 18 hours of (a) iontophoresis at 0.1 and 0.4 mA (0.5 and 2 mA/cm² respectively) and (b) passive diffusion. SF was also used to investigate the role played by the dorsal, ventral and intermediate layers of the nail as permeation barriers. The possible binding of SF to the nail material was studied over a two week experiment. Two types of confocal images were used to assess the depth of penetration. In the first type, transversal images, the penetration of the marker was directly estimated (T) and compared to the whole thickness of the nail. This allowed for determination of the percentage of penetration (%T). In the second type, dorsal images, the penetration (D) was estimated taking xy-plans following the z axis into the nail. Iontophoresis resulted in deeper delivery of NBC and SF into the nail plate than passive diffusion, although the differences were quite moderate. Unexpectedly, an increase in current intensity from 0.1 to 0.4 mA did not modify ungual penetration. The two fluorescent markers typically penetrated 7-12 % of the nail thickness. SF permeation was independent of the nail layer exposed to the donor solution and showed no evidence of binding to the nail material. Finally, the images suggest that SF and NBC mainly penetrate into the nail following the transcellular pathway during both passive and iontophoretic experiments.
**Introduction**

Laser Scanning Confocal microscopy (LSCM) allies the advantages of being non invasive and of creating high resolution images. It allows the visualization, at multiple depths and without mechanical sectioning, of images parallel to the surface of the sample. The main limitations are 1) the range of lasers available which limit the number of usable fluorophores and 2) the intensity of the laser which can be responsible for photobleaching (Alvarez-Román et al., 2004). Further auto-fluorescence of biological samples can interfere with fluorescence emission of the markers. It should be noticed that LSCM is a semi-quantitative technique and that attempts made to calibrate the fluorescence intensities (Turner et al., 1998) did not warrant the use of LSCM as a quantitative method.

The main difference with conventional microscopy lies in the focus of the sample illumination. In conventional microscopy, most of the sample is simultaneously and uniformly illuminated so the areas above and below the plan of interest can be responsible for out-of-focus blurs. In contrast, during confocal microscopy, illumination is focused only on one spot at a time: the laser beam is focused via a scanning system on a spot of the sample, the whole sample emits fluorescence which is scattered in all the directions but only the fluorescence coming from the focal plan is collected by the objective and the scanning system and is then reflected off a dichroic mirror and focused onto the detector. This ability to focus on one plan at a time allows the acquisition of individual \( xy \)-plan images which can be subsequently used to build three-dimensional images following the \( z \)-axis.

LSCM has been previously used to visualize the skin structure and penetration pathways of fluorescent markers into the skin after passive and iontophoretic delivery. During transdermal passive diffusion of ionic fluorescent markers, the pathways have been shown to be paracellular and through the skin “pores”. Indeed, it has been established by Cullander (1992) and Alvarez-Román et al. (2004) that no transcellular route existed whereas the existence of a paracellular pathway via the lipid lamellae has been demonstrated by Cullander (1992) and Turner et al. (1998). Finally, the existence of a skin “pores” pathway has been described by Cullander (1992). It is to note that “pores” have been described to be not only and necessarily skin appendages (e.g. sweat glands and hair follicles) but as well to be skin imperfections.

It has been shown (Cullander et al., 1992; Scott et al., 1992; Bath et al., 2001) that the main pathway followed by ionic fluorescent markers during transdermal iontophoresis was that of lower electrical resistance constituted by the “pores” and especially the hair follicles. No evidence has been found for the existence of a transcellular pathway but Cullander (1992) and Turner et al. (1997) observed evidence of a non follicular, paracelluar pathway.
Turner et al. (1997) showed using calcein that iontophoresis led this compound into the skin primarily via the hair follicles but as well via a non follicular pathway. The same author (Turner et al., 1998) confirmed the coexistence of the two pathways during the iontophoresis of poly-L-lysines of different molecular weight. Bright fluorescent signal was emitted from the hair follicles whereas the signal was more diffuse for non follicular areas: the quantification and normalisation by the surface area showed that the transport through the “pores” was much greater than the non follicular transport.

Pathways of drug permeation into the nail layers have not been studied until now. Obviously, because of the structural differences between the skin and the nail, only the transcellular and the paracellular pathways are possible routes for transungual permeation. Excluding their keratinous nature, skin and nail are very different in terms of composition and thickness. Indeed, unlike skin, the nail is considered to behave like a hydrophilic gel membrane (Mertin et al., 1997b) and has low lipid content (< 5 %). These lipids are known to be in the cytoplasm membrane of the onychocytes, in the space between the cells, and are thought to fill the ampular dilatations which link cells in the dorsal layer (Garson et al., 2000). Lipids are more abundant in the dorsal and ventral layer than in the intermediate layer (Kobayashi et al., 1999).

The nail is not a homogenous keratin block. It is commonly accepted that three layers – namely, dorsal, intermediate and ventral – contribute to the total thickness in a 3:5:2 ratio (Lewis, 1954) and differ in composition and other properties. According to Jarret and Spearman (1966), the dorsal layer has high calcium, phospholipid and sulfydryl group content and little acid phosphatase activity. The corneocytes are 2.2 µm thick (Garson et al., 2000). It was determined using synchrotron X-ray microdiffraction (Garson et al., 2000) that nail is constituted of amorphous α-keratin (hair type) whose fibres were 60% perpendicularly and 40% parallel orientated to the growth axis in the dorsal and ventral layers.

The intermediate layer shows high acid phosphatase activity, a high number of disulphide bonds and a low content of disulfydryl groups, phospholipid and calcium (Jarret and Spearman, 1966). The corneocytes are ~5.5 µm thick and all the keratin fibres are orientated perpendicularly to the growth axis (Garson, 2000; Farren, 2004).

The ventral layer has a similar composition to the dorsal layer; high calcium, phospholipid and sulfydryl group content. As a difference with the dorsal layer, the ventral layer has high acid phosphatase activity and a high number of disulphide bonds.

It has been suggested that this structure composed of an anisotropic layer sandwiched in two isotropic layers is responsible for the nail mechanical rigidity (Farren, 2004).
Some studies suggest that drug permeability differs across these three layers. For example, Kobayashi et al. (1999) studied the permeation of 5-fluorouracil (water soluble) and flurbiprofen (poorly water soluble) across the dorsal, ventral and intermediate layers isolated by filing human nail clippings. The authors showed that the dorsal layer was the less permeable for the two drugs whereas the intermediate was more permeable to the hydrophilic drug, 5-fluorouracil, and the ventral more permeable to the lipophilic drug, flurbiprofen. These results probably reflect the difference in composition of the three layers.

There is, in conclusion, little information about the physicochemical properties of a drug determining nail permeation and the pathways followed during passive diffusion. Much less is known in the case of iontophoresis. LSCM has been used to study nail structure (Kaufman et al., 1995) and to assist on the diagnosis of onychomycosis (Hongsharu et al., 2000; Arrese et al., 2003). In this thesis, LSCM was used, for the first time, to investigate the pathways, depth and the uniformity of the passive and iontophoretic penetration of two model fluorescent markers into human nail plate. The two markers were sodium fluorescein (Fig. 1), a negatively charged, acidic hydrophilic fluorescent marker and nile blue chloride (Fig. 2), a positively charged, basic lipophilic marker.

![Molecular structure of sodium fluorescein](image1.png)

Fig. 1: Sodium fluorescein (SF) molecular structure. MW 376.28; pKa$_1$ ~ 4.5 and pKa$_2$ ~ 6.5. The calculated Log P ( LogKow from Syracuse Research Corporation) is -0.67.

![Molecular structure of nile blue chloride](image2.png)

Fig. 2: Nile blue chloride (NBC) molecular structure. MW 353.85; pKa ~ 9.5. Calculated Log P (software LogKow from Syracuse Research Corporation) is 4.99.
Materials and methods

1. Materials
Sodium fluorescein, nile blue chloride, sodium chloride and acetonitrile (HPLC grade) were obtained from Sigma Aldrich Co. (Gillingham, UK). Silver wire, silver chloride powder and platinum wire used to prepare the electrodes had a minimum purity of 99.99% and were obtained from Sigma Aldrich Co. (Gillingham, UK). All aqueous solutions were prepared using high purity deionised water (18.2 MΩ.cm, Barnstead Nanopure Diamond™, Dubuque, IA, USA).

2. Nails
Ethical approval was granted by the Bath Local IRB (Institutional Review Board) and finger nail clippings were obtained from fourteen healthy volunteers who gave their written informed consent (Forms in the Appendix 3). The harvested nails were washed with deionised water and kept at room temperature in desiccators until use. Prior to an experiment, the nail’s thickness was measured with a point micrometer (Point Anvil Micrometer, Mitutoyo, Andover, UK) on the portion of the nail which was the closest to the hyponychium. Some nails were filed in order to expose the intermediate layer to the donor solution. In this case the initial thickness was measured at the centre of the nail. Next, the dorsal layer of the nail was manually filed using a manicure metallic file until 30% of the initial thickness had been removed.

Each nail was soaked in deionised water during 10-15 minutes prior to the experiments in order to recover some flexibility, and then placed in 5 mm (0.20 cm² transport area) nail adapters (PermeGear Inc., Bethlehem, PA, USA).

The presence and possible interference of auto-fluorescence was investigated by LSCM examination of 3 nails which had been soaked in 154 mM NaCl for 18 hours. This control showed that the intensity of the laser used during the experiments was too low to cause any auto-fluorescence that could interfere with the images.

3. Nile Blue Chloride experiments
Nile Blue Chloride (NBC) is positively charged and was delivered by anodal iontophoresis. The three experiments performed with this marker, passive, 0.1 mA and 0.4 mA iontophoresis, used a (3.2-3.6 mL) donor solution containing 1% (28 mM) NBC and 50 mM NaCl. The receptor solution (700 µL) consisted of 0.9% (154 mM) NaCl. The donor concentration of NBC corresponds to its limit of solubility in water. The 50 mM sodium chloride present at the anodal donor solution provided enough chloride ions for the
electrode reaction but to avoid any depletion during the 0.4 mA experiments, the whole donor was replaced at 4 h during all the NBC experiments. The pH of the donor solution was ~3 which allowed NBC to be totally ionised (pKa ~9.5). The dorsal layer of the nail faced the donor anodal chamber in the three experiments indicated as NBC.Passive, NBC.0.1 and NBC.0.4 in Table 1.

4. Sodium Fluorescein experiments
Likewise NBC, experiments were performed on whole nails at 0.1 and 0.4 mA and passive diffusion was explored as well. Sodium fluorescein (SF) is negatively charged and was delivered by cathodal delivery. All the experiments used a donor solution (3.2-3.6 mL) containing 375 mM of SF in deionized water. The pH of the donor solution was ~8 therefore SF (pK_{a1} ~4.5 and pK_{a2} ~6.5) was predominantly (97 %) in its di-anionic form. The anodal receptor solution (700 µL) consisted of 0.9 % (154 mM) NaCl in the passive and the 0.1 mA experiments. The 0.4 mA experiments used a 8 % (1.37 M) NaCl solution in order to provide enough chloride ions for the anodal reaction. The experimental conditions are listed in Table 1. Due to the cathode electrochemical reaction, the whole donor was replaced at 4 hours to minimize the competition of the chloride ions released by the cathode with SF (See Introduction chapter).

The dorsal layer of the nail faced the donor cathodal chamber in the experiments coded as SF.Passive, SF.0.1 and SF.0.4 in Table 1. The ventral layer faced the cathodal donor solution in the experiments listed as SF.V.Passive and SF.V.0.1. Finally, the experiment listed as SF.I.0.1 was performed with nails whose dorsal layer had been removed by filing so the intermediate layer of the nail faced the donor solution.

Table 1: Experiments performed with SF and NBC. n indicates the number of replicates.

<table>
<thead>
<tr>
<th>Experiment (n)</th>
<th>Anode (Ag/AgCl)</th>
<th>Cathode (Ag/AgCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF.Passive (3)</td>
<td>154 mM NaCl</td>
<td></td>
</tr>
<tr>
<td>SF.0.1 (4)</td>
<td>154 mM NaCl</td>
<td></td>
</tr>
<tr>
<td>SF.0.4 (3)</td>
<td>1.37 M NaCl</td>
<td></td>
</tr>
<tr>
<td>SF.V.Passive (3)</td>
<td>154 mM NaCl</td>
<td>375 mM SF</td>
</tr>
<tr>
<td>SF.V.0.1 (4)</td>
<td>154 mM NaCl</td>
<td></td>
</tr>
<tr>
<td>SF.I.0.1 (3)</td>
<td>154 mM NaCl</td>
<td></td>
</tr>
<tr>
<td>NBC.Passive (3)</td>
<td>28 mM NBC + 50 mM NaCl</td>
<td>154 mM NaCl</td>
</tr>
<tr>
<td>NBC.0.1 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NBC.0.4 (3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5. Iontophoretic set up
Experiments were performed in Franz vertical diffusion cells (PermeGear Inc., Bethlehem, PA, USA) presenting a transport area of 0.20 cm^2. To facilitate the sampling of the receptor chamber, the bottom chamber was used to hold the donor solution while the
receptor was placed on the top. The nail adapter was placed so the nail’s dorsal layer faced the donor solution (Fig. 3). All experiments were performed at room temperature with a minimum of three replicates.

![Experimental Setup](image)

**Fig. 3: Picture of the experimental set up**

The current was applied by a power supply (Kepco APH 1000M, Flushing, NY) via homemade Ag/AgCl electrodes (Green et al., 1991).

During constant, direct current iontophoresis, the power supply modifies the voltage imposed depending on the resistance of the electrical circuit (as indicated by Ohm’s law; $V=I.R$) so a constant current passes through the circuit. It was observed, that the voltage required for imposing 0.1 or 0.4 mA from time zero was unacceptable as it would have a deleterious effect on the nails. Thus, a ~30 minutes “pre-treatment” period was applied to each nail during which a maximum voltage output (200 V) was set on the power supply (Kepco APH 1000M, Flushing, NY, USA). Voltage was monitored during the first two hours of each iontophoretic experiment.

### 6. Passive experiments

Passive controls (NBC.Passive, SF.Passive and SF.V.Passive) were performed as the 0.1 mA iontophoretic experiments except that no current was applied. The donor solutions were changed at 4 hours and the complete receptor compartments were sampled and replaced with fresh sodium chloride solution at 2, 4 and 18 hours.

### 7. Binding study

The possible binding of SF to the nail components was investigated in the following experiment. Three nails (D, E and F) were cut in three pieces (D1, D2, D3; E1, E2, E3 and F1, F2, F3). Each piece was placed in a vial containing 2 mL of 375 mM aqueous SF for 24 h. After 24 h, each piece was rinsed twice in 5 mL of NaCl 0.9% and one piece of each nail (D1, E1 and F1) was placed in a dish containing NaCl 0.9% and prepared for confocal
microscopy (as described beneath). The remaining pieces (D2, D3, E2, E3, F2 and F3) were placed in vials containing 5 mL of NaCl 0.9% for a week, with the 5 mL of sodium chloride being replaced at the middle of the week. After a week, one piece of each nail (D2, E2 and F2) was prepared for confocal microscopy whereas the three remaining pieces (D3, E3 and F3) were placed in a fresh 0.9% NaCl solution for one more week, renewing NaCl solution at the middle of the week. Finally, two weeks after the beginning of the experiments the last three pieces of nails (D3, E3 and F3) were prepared for confocal microscopy. The NaCl solutions were assessed for SF by HPLC.

![Diagram](image)

Fig. 4: Diagram of the steps followed in the SF binding study (nail D in the example).

8. Confocal microscopy analysis

Table 1 indicates that several (3-4) replicates were performed for each condition. However, each replicate was performed in a different day so each nail could be prepared and examined by LSCM as soon as the experiment was finished.

SF presents an excitation maximum $\lambda_{ex}$ at 494 nm and an emission maximum $\lambda_{em}$ at 521 nm, therefore samples were analysed using an argon laser ($\lambda_{ex} = 488$ nm) and two different filters: a NFT 545 (wavelengths above 545 nm are going through, wavelengths below are reflected 90°) and a LP 505 (wavelengths higher than 505 nm pass to the detector).

NBC has an excitation maximum $\lambda_{ex}$ of 630-640 nm and an emission maximum $\lambda_{em}$ of 660 nm, therefore samples were studied using a helium neon laser ($\lambda_{ex} = 633$ nm) with a dichroic beam splitter HFT 488/543/633, a LP 650 (wavelengths higher than 650 nm pass to the detector) and a NFT 490 (wavelengths above 490 nm are going through, wavelengths below are reflected 90°). A plan objective of 10x or 20x was used.
At the end of the experiments, the surface of the nail which faced the donor solution was rinsed under a thin dribble of running deionised water (the nail was still clamped in the nail adapter) and patted dry. Then, the nail was removed from the nail adapter and washed successively in three Petri dishes containing deionised water. The nail was patted dry between each wash. In the third Petri dish, the nail was cut with a scalpel in both polygonal (typically triangular) pieces and thin slices used for dorsal and transversal observations respectively (Fig. 5). The cuts were performed in the opposite direction to the penetration to avoid pushing artificially deeper the fluorescent with the blade. For example, the cut started at the ventral side when the dorsal layer of the nail had faced the donor solution. A minimum of four polygonal pieces and four thin slices pieces were prepared for each nail and analysed by confocal microscopy (LSM Meta 510 microscope, Zeiss, Oberkochen, Germany). The images obtained were treated using Zeiss LSM image browser version 4.0.0.157.

![Fig. 5: Scheme of the mechanical cuts prepared for confocal microscopy analysis](image)

Each piece was then patted dry and glued on a microscope slide to hold it in the right position; dorsal/ventral/intermediate layers facing upwards for dorsal views, and the side facing upwards for transversal views (Figure 5). Please note that the term “dorsal” view is used to indicate how the confocal examination took place: looking on top of the surface of the nail that had been in contact with the donor solution, and independently of the histological layer of the nail (dorsal, ventral, intermediate) that had been exposed to the donor.
Dorsal images:
Dorsal observations at x10 objective were made on a black background and dorsal at x20 objective observations were made under halogen light.
Images were xy-planar images acquired at different depths following the z axis (z-stack). To examine the distribution of the fluorescent marker through the samples and its penetration, xy-planar images taken at x10 objective were used to obtain two kinds of images: a) a series of xy-plans from top to deeper parts of the sample separated by 2 (NBC) or 5 µm (SF) and b) a reconstruction of all the images of the z-stack to give a three dimensional image. The depth of penetration (D) was measured on the 3-D images.
Because the separation between the xy plans was 2 or 5 µm, these values indicate the precision of the measurements. However, to simplify the treatment of the data, the analyses rely on the measurements performed with the software tool (step of 1.76 µm). For space reasons, the series of xy-planar images are shown only every 10 µm.
Dorsal images taken at x20 objectives were used to follow the pathways of the fluorescent into the nail. They were xy-planar images taken from surface to deeper parts of the sample separated by 1 or 2 µm (NBC). For space reasons, the series of xy-planar images are shown only every 2 µm.

Transversal images:
Transversal images at x10 objective were taken under halogen light to allow the view of the fluorescent penetration and the complete nail section. The Zeiss software was used to measure the thickness of the nails and the penetration of the fluorescent marker in the images. Images were taken as cross-sectional images: by browsing the sequences of xy-plans taken every 5 (NBC) or 10 µm (SF), the mechanically cut surface was localized and avoided for measurements. This was done because the scalpel could have transferred fluorescence at the cut surface, creating an artefact and invalidating the penetration measurement.
Two types of measurements were performed on the transversal images: first a direct measurement of the marker penetration depth (T) was taken, next the fluorescent penetration depth (T) was normalised by the whole thickness of the nail measured on the same image and expressed as percentage (%T).

9. HPLC analysis
A high performance liquid chromatograph (Dionex Corporation, Sunnyvale, CA, USA) coupled with a fluorescence detector RF 2000 and equipped with a P680 pump, an ASI-100 autosampler and a TCC-100 column oven thermostated at 65°C was used to measure
SF concentration in all samples. A mixture of water and acetonitrile 70:30 was pumped under isocratic conditions (1 mL/min) through a C18 column (Acclaim 120, 5 µm, 120 Å, 4.6x150 mm, Dionex). The wavelengths used were 495 nm for the excitation and 518 nm for the emission. Concentrations of SF were measured against calibration curves obtained from standard solutions (at least seven different concentrations) prepared in ultrapure water and ranging from 5 to 50 nM. The measurement error was below 5 %.

No SF was found in any receptor sample. Further, the confocal images indicated that neither marker reached the receptor solution. Therefore no analytical method was developed to measure NBC concentration in receptor samples.

10. Data analysis and statistics
All data are presented as mean ± SD unless otherwise indicated. The means and SD for each treatment were calculated using all the measurements taken for all the nails used in the respective condition (as opposed to obtaining a first average /per nail and then a second average for the 3-4 nails). Data analysis was performed with Graph Pad Prism version 5.00 (Graph Pad Software, San Diego California USA). Non-parametric Kruskal-Wallis followed by a Dunns’ post test was used to compare the depth of penetration measured under different experimental conditions (passive, 0.1 and 0.4 mA) via the three type of images previously described (D, T and %T). When only two sets of data were compared, for example to compare the permeation of the two fluorescents, a non-parametric unpaired Mann Whitney t-test was performed. The level for statistical significance was set to $p < 0.05$.

Results and discussion
Confocal microscopy examination on nails after passive and iontophoretic transport experiments was done to determine how deeply and uniformly fluorescent markers penetrate the nail plate. The potential different SF penetration into the ventral and the intermediate layers, the effect of current intensity and a possible binding of SF to the nail components were also investigated. To date, this is the first study investigating the pathways involved in transungual penetration under passive and iontophoretic conditions via confocal microscopy.

To validate the LSCM observations about depth of penetration, a considerable number of images were generated during this study. Only a representative selection is presented in this chapter. The complete collection of images can be found in the Appendix 1.

It is pertinent to discuss the different methods used to measure penetration depth given they do not always result in equivalent results. When xy-plans are taken, one would expect
the fluorescence to be intense in superficial layers and progressively disappear in deeper layers. However, fluorescence was observed in deeper xy planes of some images when it had been absent at more superficial levels (see Fig. 14 for an illustrative example). This can be explained by the nail curvature; in fact the xy-plan at 0 µm only captures a fraction of the nail outmost layer and the rest is progressively captured in deeper pictures. Thus, the depth of fluorescence inside the nail cannot be unequivocally determined. Further, images were taken at 2 to 5 µm intervals limiting the precision of the measurement. D measurements were made on the B reconstructions in which the depth had a continuous dimension; however, it is difficult to accept that the precision of these D measurements could be more than that of the starting images.

A T measurement seems to offer a better solution, the nail is looked at transversely, and all its width is included in the same xz-plan. The resolution for these measurements is 1.76 µm in a continuous scale, an improvement with respect to the 2-5 microns intervals. The measurement can take place at a certain depth of the specimen so the surface at which the cut took place is avoided. However, it is difficult to ensure a mechanical cut which is perfectly perpendicular to the nail. Thus, the measurements of depth and thickness could be slightly overestimated. This prompted the idea of the third parameter, %T, which would minimize any potential overestimation in the measurement by normalizing the depth of penetration by the total thickness of the nail. %T or percentage of penetration seem a very useful parameter from a drug delivery point of view, providing a quick appreciation of the efficiency of delivery achieved by different formulations. On the other hand, differences in %T can be equally caused by a real difference in the depth of penetration or in the thickness of the nails used.

1. NBC experiments

Table 2 shows the characteristics of the nails (participant’s gender and age, finger and hand of origin, and plate thickness) used in the NBC experiments. The 12 nails were donated by 6 participants aged between 21 and 52 years old. The average thickness was 290 ± 30 µm for NBC.Passive, 300 ± 80 µm for NBC.0.1, and 290 ± 40 µm for NBC.0.4.
Table 2: Nails used in NBC experiments.

<table>
<thead>
<tr>
<th>Experiment code</th>
<th>Nail code *</th>
<th>Gender</th>
<th>Age (year)</th>
<th>Thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBC.Passive</td>
<td>4RF2</td>
<td>Female</td>
<td>44</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>12LF5</td>
<td>Female</td>
<td>22</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>14RF4</td>
<td>Female</td>
<td>52</td>
<td>320</td>
</tr>
<tr>
<td>NBC.0.1</td>
<td>2LF5</td>
<td>Female</td>
<td>26</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>12RF1</td>
<td>Female</td>
<td>22</td>
<td>390</td>
</tr>
<tr>
<td></td>
<td>7LF4</td>
<td>Female</td>
<td>28</td>
<td>260</td>
</tr>
<tr>
<td>NBC.0.4</td>
<td>4LF4</td>
<td>Female</td>
<td>44</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>12LF3</td>
<td>Female</td>
<td>22</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td>13LF3</td>
<td>Female</td>
<td>21</td>
<td>330</td>
</tr>
</tbody>
</table>

* The first digit in the nail code indicates the participant number, L or R indicates left or right hand and F+digit indicates the finger where F1 = thumb.

The voltage of the electrical circuit was monitored during the two first hours of each experiment (Fig. 6, Table 3).

Table 3: Evolution of the voltage during the first two hours of NBC.0.1 and NBC.0.4 experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Nail code</th>
<th>Nail thickness (µm)</th>
<th>1 min</th>
<th>15 min</th>
<th>30 min</th>
<th>1 hour</th>
<th>2 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBC.0.1</td>
<td>2LF5</td>
<td>250</td>
<td>200</td>
<td>33</td>
<td>14</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>12RF1</td>
<td>390</td>
<td>200</td>
<td>200</td>
<td>125</td>
<td>29</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>7LF4</td>
<td>260</td>
<td>200</td>
<td>117</td>
<td>40</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>NBC.0.4</td>
<td>4LF4</td>
<td>260</td>
<td>200</td>
<td>62</td>
<td>37</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>12LF3</td>
<td>270</td>
<td>200</td>
<td>56</td>
<td>36</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>13LF3</td>
<td>330</td>
<td>200</td>
<td>200</td>
<td>101</td>
<td>38</td>
<td>24</td>
</tr>
</tbody>
</table>

Fig. 6: Evolution of voltage during the first two hours of the NBC.0.1 (filled symbols) and NBC.0.4 (open symbols) experiments.
With two exceptions the voltages were lower than the limit (200 V) in 15 minutes, indicating a relatively quick decline of the nail resistance. After 30 minutes all the voltages were less than 200 V, thus the required intensity of current was effectively passed across the circuit. After 1 hour all the values were lower than 40 V. The voltages of the electrical circuits involving the thickest nails (12RF1 at 0.1 mA and 13LF3 at 0.4 mA) were slightly higher than the others. After two hours, the voltage reached values between 5 and 12 V for 0.1 mA and between 16 and 24 V for 0.4 mA.

The depth of penetration of NBC into the nail was examined at the end of the experiments as described in Materials and Methods. The following figures illustrate NBC penetration after 18 hours of passive diffusion (Fig.7), 0.1 mA iontophoresis (Fig.8) and 0.4 mA iontophoresis (Fig.9).
Fig. 7: Representative confocal images illustrating NBC passive penetration into the nail plate (NBC.Passive). Panel A shows transversal cuts allowing direct measurement of the marker penetration depth T and %T. Panel B shows 3-D reconstructions of xy-planar images of dorsal images obtained at different depths, the nail surface in contact with the donor solution (dorsal layer in this case) faces the right of the image. The depth of penetration (D) was taken from B. Panel C shows xy-planar images of dorsal images taken at 2 µm intervals (but only shown at 10 µm) which were used to construct the 3-D images shown in Panel B. All the pictures were obtained at 10x magnification.
Fig. 8: Representative confocal images illustrating NBC iontophoretic penetration into the nail plate during the 0.1 mA experiment (NBC.0.1). Panel A shows transversal cuts allowing direct measurement of the marker penetration depth T and %T. Panel B shows 3-D reconstructions of xy-planar images of dorsal images obtained at different depths, the nail surface in contact with the donor solution (dorsal layer in this case) faces the left of the image. The depth of penetration (D) was taken from B. Panel C shows xy-planar images of dorsal images taken at 2 µm intervals (but only shown at 10 µm) which were used to construct the 3-D images shown in Panel B. All the pictures were obtained at 10x magnification.
Fig. 9: Representative confocal images illustrating NBC iontophoretic penetration into the nail plate during the 0.4 mA experiment (NBC.0.4). Panel A shows transversal cuts allowing direct measurement of the marker penetration depth T and %T. Panel B shows 3-D reconstructions of xy-planar images of dorsal images obtained at different depths, the nail surface in contact with the donor solution (dorsal layer in this case) faces the right of the image. The depth of penetration (D) was taken from B. Panel C shows xy-planar images of dorsal images taken at 2 µm intervals (but only shown at 10 µm) which were used to construct the 3-D images shown in Panel B. All the pictures were obtained at 10x magnification.
The following figure (Fig. 10) summarizes the depth of penetration estimated for each nail, type of experiment and measurement technique as well as the average values for each experimental condition.

Fig. 10: NBC delivery into the nail plate by passive diffusion and iontophoresis (NBC.Passive, NBC.0.1 and NBC.0.4). The two top panels and bottom-left panel show the depth of penetration (expressed as T, D and %T) measured for each nail (Mean ± SD of 5-8 images). The bottom-right panel shows the average depth of penetration for each condition studied (Mean ± SD). The symbols * and ** indicate significant (p = 0.01 to 0.05) and very significant (p = 0.001 to 0.01) differences between pairs of bars.

Taking multiple images and corresponding measurements is a laborious procedure, however it is necessary to validate results obtained from microscopic examination. Further, it allows investigating inter-nail variability and comparing the three different methods used to report depth of penetration. For example, the depth of passive penetration reported as D, was significantly greater for nail 14RF4 than for 4RF2 (Fig.10). The iontophoretic penetration (NBC.0.1) reported as %T was significantly lower for nail 12RF1. However, these differences are probably explained by the greater thickness of the nail 12RF1 used as there were no differences in the value of T.

When the three delivery conditions were compared (Figure 10, panel right-bottom, Table 4) it was shown that the depth of penetration reported as T and %T was greater after iontophoresis than after passive diffusion. However, NBC penetration was not significantly improved when the intensity was increased to 0.4 mA which was a surprising observation.
Table 4: NBC penetration as reported by T, %T and D, into the nail plate after 18h of iontophoretic or passive delivery. All values are given as mean ± SD of 16-27 measurements (n) taken on 3 nails.

<table>
<thead>
<tr>
<th></th>
<th>T (µm)</th>
<th>n^a</th>
<th>D (µm)</th>
<th>n^a</th>
<th>%T</th>
<th>n^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBC.Passive</td>
<td>22 ± 5</td>
<td>19</td>
<td>25 ± 11</td>
<td>17</td>
<td>6.7 ± 1.6</td>
<td>19</td>
</tr>
<tr>
<td>NBC.0.1</td>
<td>28 ± 6*</td>
<td>20</td>
<td>33 ± 11</td>
<td>16</td>
<td>8.3 ± 2.3*</td>
<td>27</td>
</tr>
<tr>
<td>NBC.0.4</td>
<td>30 ± 7*</td>
<td>20</td>
<td>34 ± 9</td>
<td>19</td>
<td>8.0 ± 1.6*</td>
<td>20</td>
</tr>
</tbody>
</table>

^a n, number of measurements performed
* Significantly different to passive at p < 0.05

Comparison of T and %T indicated that iontophoresis at 0.1 and 0.4 mA delivered NBC to ~ 8% of the nail thickness, only ~ 1-2% more of what was achieved with passive diffusion. No differences were found when D was compared, probably due to the bigger variability in D values. It is not known at the moment how predictive this data is with respect to therapeutic drugs. In fact, NBC (log P = 4.99 MW = 353.85) can not be considered as an ideal candidate for iontophoretic delivery. Further, NBC was delivered from a donor solution containing 50 mM NaCl, and a significant ion competition is expected from the highly mobile sodium ions, resulting in a low NBC transport number. Because NBC is delivered from the anode, electroosmosis also contributes to the transport. Thus the physicochemical properties of the drugs and the composition of the formulation must be seriously considered to achieve useful iontophoretic deliveries. Otherwise, one could argue that a moderate enhancement such that here described would not justify the development of a complicate and expensive iontophoretic device.

2. **SF dorsal penetration experiments**

Table 5 shows the characteristics of the nails (participant’s gender and age, finger and hand of origin, and plate thickness) used during these experiments. The 13 nails were donated by 4 participants aged between 22 and 44 years old. The average thickness was 240 ± 25 µm for SF.Passive, 265 ± 50 µm for SF.0.1, and 250 ± 20 µm for SF.0.4.
Table 5: Nails used for SF dorsal penetration experiments.

<table>
<thead>
<tr>
<th>Experiment code</th>
<th>Nail code</th>
<th>Gender</th>
<th>Age (year)</th>
<th>Thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF.Passive</td>
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<td>26</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>4R</td>
<td>Female</td>
<td>44</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>12LF2</td>
<td>Female</td>
<td>22</td>
<td>260</td>
</tr>
<tr>
<td>SF.0.1</td>
<td>2LF4</td>
<td>Female</td>
<td>26</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>12LF1</td>
<td>Female</td>
<td>22</td>
<td>330</td>
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<td></td>
<td>4LF5</td>
<td>Female</td>
<td>44</td>
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<td>28</td>
<td>270</td>
</tr>
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<td></td>
<td>4LF5</td>
<td>Female</td>
<td>44</td>
<td>230</td>
</tr>
</tbody>
</table>

* The first digit in the nail code indicates the participant number, L or R indicates left or right hand and F+digit indicates the finger where F1 = thumb.

Voltage measurements were taken during the first two hours of the experiments with the following results (Table 6, Fig. 11).

Table 6: Evolution of voltage during the first two hours of SF.0.1 and SF.0.4 experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Nail code</th>
<th>Nail thickness (µm)</th>
<th>Voltage (V)</th>
<th>1 min</th>
<th>15 min</th>
<th>30 min</th>
<th>1 hour</th>
<th>2 hours</th>
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<tbody>
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<td>120</td>
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<td>62</td>
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<td></td>
<td>4LF5</td>
<td>230</td>
<td></td>
<td>154</td>
<td>57</td>
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<td>20</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>7LF4</td>
<td>270</td>
<td></td>
<td>200</td>
<td>185</td>
<td>98</td>
<td>39</td>
<td>11</td>
</tr>
<tr>
<td>SF.0.4</td>
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<td>200</td>
<td>134</td>
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<td>200</td>
<td>168</td>
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</tbody>
</table>
During two of the 0.1 mA iontophoretic experiments performed (2LF4 and 12LF1) the limiting voltage was set on 120 V instead of 200 V as shown in the Fig. 11. As previously observed the voltage decreased quickly being equal to or less than 62 V after an hour of current application in all cases. After two hours, the voltage dropped to 11-19 V (0.1 mA) and 14-34 V (0.4 mA) reflecting the different intensities applied. There was no obvious relation between the voltage of the electrical circuit and the thickness of the nails.

The depth of penetration of SF into the nail was examined at the end of the experiments as described in Materials and Methods. The following figures illustrate SF penetration after 18 hours of passive diffusion (Fig.12), 0.1 mA iontophoresis (Fig.13) and 0.4 mA iontophoresis (Fig.14).
Fig. 12: Representative confocal images illustrating SF passive penetration into the nail plate (SF.Passive). Panel A shows transversal cuts allowing direct measurement of the marker penetration depth T and %T. Panel B shows 3-D reconstructions of $xy$-planar images of dorsal images obtained at different depths, the nail surface in contact with the donor solution (dorsal layer in this case) faces the right of the image. The depth of penetration (D) was taken from B. Panel C shows $xy$-planar images of dorsal images taken at 5 µm intervals (Only shown every 10 µm) which were used to construct the 3-D images shown in Panel B. All the pictures were obtained at 10x magnification.
Fig. 13: Representative confocal images illustrating SF iontophoretic penetration into the nail plate during the 0.1 mA experiment (SF.0.1). Panel A shows transversal cuts allowing direct measurement of the marker penetration depth T and %T. Panel B shows 3-D reconstructions of xy-planar images of dorsal images obtained at different depths, the nail surface in contact with the donor solution (dorsal layer in this case) faces the right of the image. The depth of penetration (D) was taken from B. Panel C shows xy-planar images of dorsal images taken at 5 µm intervals (Only shown every 10 µm) which were used to construct the 3-D images shown in Panel B. All the pictures were obtained at 10x magnification.
Fig. 14: Representative confocal images illustrating SF iontophoretic penetration into the nail plate during the 0.4 mA experiment (SF.0.4). Panel A shows transversal cuts allowing direct measurement of the marker penetration depth $T$ and $\%T$. Panel B shows 3-D reconstructions of $xy$-planar images of dorsal images obtained at different depths, the nail surface in contact with the donor solution (dorsal layer in this case) faces the right of the image. The depth of penetration (D) was taken from B. Panel C shows $xy$-planar images of dorsal images taken at 5 $\mu$m intervals (Only shown every 10 $\mu$m) which were used to construct the 3-D images shown in Panel B. All the pictures were obtained at 10x magnification.
The following figure (Fig. 15) summarizes the depth of penetration estimated for each nail, type of experiment and measurement technique as well as the average values for each experimental condition.

![Graphs showing the depth of penetration for different conditions.]

Fig. 15: SF delivery into the nail plate by passive diffusion and iontophoresis (SF.Passive, SF.0.1 and SF.0.4). The two top panels and bottom-left panel show the depth of penetration (expressed as T, D and %T) measured for each nail (Mean ± SD of 4-8 images). The bottom-right panel shows the average depth of penetration for each condition (Mean ± SD). The symbols *, **, and *** indicate significant (p = 0.01 to 0.05), very significant (p = 0.001 to 0.01) and extremely significant (p < 0.001) differences between pairs of bars.

As observed for NBC, some inter-nail variability was observed and the use of T, %T and D resulted in slightly different outcomes. For example, SF passive penetration into the nail 2LF5 as reported by D was significantly greater than into nail 12LF2. During SF.0.1 experiments and according to the T depth values, SF penetration was deeper into nail 12LF1 than into nail 7LF4. No statistical differences were found between the images obtained from the nails of SF.0.4. On the whole these differences are quite small and lack any practical relevance. However they suggest that the technique here developed is robust and able to find differences caused by formulation or physiological reasons and can have applications in future studies.

The three parameters (T, %T and D) indicate that iontophoresis (either 0.1 or 0.4 mA) enhances SF penetration with respect to passive diffusion. Moreover, the difference was clearly noticeable in the confocal images which showed a more uniform and intense band.
of fluorescence in the nails when SF was delivered via iontophoresis. However, the depth of SF penetration (T, %T and D) was the same for the 0.1 mA and 0.4 mA experiments.

Table 7: SF penetration as reported by T, %T and D, into the nail plate after 18h of iontophoretic or passive delivery. All values are given as mean ± SD of 15-27 measurements (n) taken on 3-4 nails.

<table>
<thead>
<tr>
<th></th>
<th>T (µm)</th>
<th>n⁴</th>
<th>D (µm)</th>
<th>n⁴</th>
<th>%T</th>
<th>n⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF.Passive</td>
<td>26 ± 4</td>
<td>17</td>
<td>19 ± 4</td>
<td>15</td>
<td>7.8 ± 1.5</td>
<td>17</td>
</tr>
<tr>
<td>SF.0.1</td>
<td>34 ± 7*</td>
<td>27</td>
<td>34 ± 9*</td>
<td>25</td>
<td>9.4 ± 2.2*</td>
<td>27</td>
</tr>
<tr>
<td>SF.0.4</td>
<td>39 ± 8*</td>
<td>21</td>
<td>37 ± 6*</td>
<td>19</td>
<td>11.3 ± 2.6*</td>
<td>21</td>
</tr>
</tbody>
</table>

*⁴n, number of measurements performed
* Significantly different to passive at p < 0.05

In conclusion SF was delivered to 9-11 % of the whole thickness of the nail with iontophoresis, whereas passive diffusion delivered the marker to ~8%. The little efficiency of SF iontophoretic delivery was unexpected; first of all SF is more hydrophilic (log P= -0.67) than NBC and was introduced in the donor chamber as a single ion (i.e., without competition), which would suggest SF has a higher transport number than NBC despite being anionic. One could suggest that chlorides released from the cathode could compete with the marker although the donor solution was refreshed at 4 hours as described in Materials and Methods. Further, recent work (Sylvestre et al., 2008) on chloride competition and transdermal iontophoresis of dexamethasone phosphate suggests this effect to be small in our experimental conditions.

3. SF ventral and intermediate layer penetration experiments

Table 8 shows the characteristics of the nails (participant’s gender and age, finger and hand of origin, and plate thickness) used during these experiments. The 10 nails were donated by 7 participants aged between 21 and 44 years old. The average thickness was 250 ± 30 µm for SF.V.Passive, 245 ± 35 µm for SF.V.0.1 and 200 ± 45 µm for SF.I.0.1.
Table 8: Nails used for SF ventral and intermediate layer experiments

<table>
<thead>
<tr>
<th>Experiment code</th>
<th>Nail code</th>
<th>Gender</th>
<th>Age (year)</th>
<th>Thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF.V.Passive</td>
<td>1RF5</td>
<td>Female</td>
<td>25</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td>2RF5</td>
<td>Female</td>
<td>26</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>12RF4</td>
<td>Female</td>
<td>22</td>
<td>270</td>
</tr>
<tr>
<td>SF.V.0.1</td>
<td>12RF3</td>
<td>Female</td>
<td>22</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>2LF3</td>
<td>Female</td>
<td>26</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>7LF5</td>
<td>Female</td>
<td>28</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>13RF4</td>
<td>Female</td>
<td>21</td>
<td>280</td>
</tr>
<tr>
<td>SF.I.0.1</td>
<td>12LF1</td>
<td>Female</td>
<td>22</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>8LF4</td>
<td>Female</td>
<td>31</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>4LF4</td>
<td>Female</td>
<td>44</td>
<td>150</td>
</tr>
</tbody>
</table>

* The first digit in the nail code indicates the participant number, L or R indicates left or right hand and F+digit indicates the finger where F1 = thumb.

Voltage measurements were taken during the first two hours of the experiments with the following results (Table 9, Fig. 16).

Table 9: Evolution of voltage during the first two hours of SF.V.0.1 and SF.I.0.1. The values in bracket indicate the thickness of the filed nails.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Nail code</th>
<th>Nail thickness (µm)</th>
<th>Voltage (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 min</td>
<td>15 min</td>
<td>30 min</td>
</tr>
<tr>
<td>SF.V.0.1</td>
<td>12RF3</td>
<td>240</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>2LF3</td>
<td>250</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>7LF5</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>13RF4</td>
<td>280</td>
<td>200</td>
</tr>
<tr>
<td>SF.I.0.1</td>
<td>12LF1</td>
<td>310 (240)</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>8LF4</td>
<td>300 (210)</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>4LF4</td>
<td>220 (150)</td>
<td>39</td>
</tr>
</tbody>
</table>
Fig. 16: Evolution of voltage during the first two hours of the SF.V.0.1 (left panel, filled symbols) and SF.I.0.1 (right panel, open symbols) experiments.

Voltage versus time profiles during SF.V.0.1 was similar to that described before for SF.0.1: the high values decreased and were \( \leq 52 \) V after 1 hour, and between 7-23 V after two hours. As expected, exposing the dorsal or the ventral face of the nail to the cathodal chamber had no effect on the voltage of the circuit.

Interestingly, the voltages measured with the filed nails, exposing the intermediate layer to the cathode (SF.I.0.1) were different from the previously described. In two out of the three replicates the initial voltages were less than 100 V, the values reached a peak in the three first minutes of current application and then decreased. After one hour, the voltage was between 9 and 18 V and after two hours, it had dropped to less than 7 V. It seems so, that filing the nail had a certain effect on the nail resistance which may be partially due to the decreased thickness.

The depth of penetration of SF into the nail was examined at the end of the experiments as described in Materials and Methods. The following figures illustrate SF penetration into the ventral layer after 18 hours of passive diffusion (Fig.17), 0.1 mA iontophoresis (Fig.18) and into the intermediate layer after 18 hour of 0.1 mA iontophoresis (Fig.19)
Fig. 17: Representative confocal images illustrating SF passive penetration into the ventral layer (SF.V.Passive). Panel A shows transversal cuts allowing direct measurement of the marker penetration depth T and %T. Panel B shows 3-D reconstructions of xy-planar images of dorsal images obtained at different depths, the nail surface in contact with the donor solution (ventral layer in this case) faces the left of the image. The depth of penetration (D) was taken from B. Panel C shows xy-planar images of dorsal images taken at 5 µm intervals (only shown every 10 µm) which were used to construct the 3-D images shown in Panel B. All the pictures were obtained at 10x magnification.
Fig. 18: Representative confocal images illustrating SF iontophoretic penetration into the ventral layer (SF.V.0.1). Panel A shows transversal cuts allowing direct measurement of the marker penetration depth $T$ and $%T$. Panel B shows 3-D reconstructions of $xy$-planar images of dorsal images obtained at different depths, the nail surface in contact with the donor solution (ventral layer in this case) faces the left of the image. The depth of penetration ($D$) was taken from B. Panel C shows $xy$-planar images of dorsal images taken at 5 µm intervals (only shown every 10 µm) which were used to construct the 3-D images shown in Panel B. All the pictures were obtained at 10x magnification.
Fig. 19: Representative confocal images illustrating SF iontophoretic penetration into the intermediate layer (SF.I.0.1). Panel A shows transversal cuts allowing direct measurement of the marker penetration depth T and %T. Panel B shows 3-D reconstructions of xy-planar images of dorsal images obtained at different depths, the nail surface in contact with the donor solution (ventral layer in this case) faces the right of the image. The depth of penetration (D) was taken from B. Panel C shows xy-planar images of dorsal images taken at 5 µm intervals (shown only every 10 µm) which were used to construct the 3-D images shown in Panel B. All the pictures were obtained at 10x magnification.
In agreement with previous results, the confocal images showed that the band of SF fluorescence was more uniform and intense after iontophoresis (SF.V.0.1, Fig. 18 B and C) than after passive diffusion (SF.V.Passive, Fig. 17 B and C). When the dorsal layer was filed the band of SF fluorescence obtained after iontophoresis was also strong and uniform (Fig. 19), however SF did not permeate deeper into filed nails than into full-thickness specimens.

The following figure (Fig. 20) summarizes the depth of penetration estimated for each nail, type of experiment and measurement.

As previously discussed, some inter-nail variability was observed and the use of T, %T and D resulted in slightly different outcomes. For example, passive penetration as %T into the nail 2RF5 was significantly greater than into 1RF5; but given there were no differences in T values this was probably a result of the different nail thicknesses. During ventral iontophoresis, the T values indicate that SF went significantly deeper into nail 2LF3 than into 12RF3 while %T values suggested a deeper penetration into 7LF5 than into 12RF3. Filing the nails to expose the intermediate layer did not increase variability, and only one significant difference was found between the relative penetration (% T) into 12LF1 and 4LF4.
Fig. 21 and Table 4 summarize the average depth of penetration estimated for each experimental condition and compare the effect of exposing dorsal, ventral or intermediate (filed nails) on SF penetration.

![Graph comparing T (µm) or D (µm) or %T for SF delivery into the nail plate by passive diffusion and iontophoresis (SF.Passive, SF.V.Passive, SF.0.1, SF.I.0.1 and SF.V.0.1).](image)

Comparison of the different type of measurements indicated that SF penetration (D, T and %T) into the ventral layer was significantly increased by iontophoresis compared to passive diffusion (Table 4).

Table 4: SF penetration (as reported by T, %T and D) into the ventral and intermediate layers of the nail plate after 18h of iontophoretic or passive delivery. All values are given as mean ± SD of 15-27 measurements (n) taken on 3-4 nails.

<table>
<thead>
<tr>
<th></th>
<th>T (µm)</th>
<th>n</th>
<th>D (µm)</th>
<th>n</th>
<th>%T</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF.V.Passive</td>
<td>26 ± 5</td>
<td>18</td>
<td>15 ± 4</td>
<td>15</td>
<td>7.5 ± 2.0</td>
<td>18</td>
</tr>
<tr>
<td>SF.V.0.1</td>
<td>31 ± 7*</td>
<td>27</td>
<td>21 ± 3*</td>
<td>24</td>
<td>9.3 ± 2.3*</td>
<td>27</td>
</tr>
<tr>
<td>SF.Passive</td>
<td>26 ± 4#</td>
<td>17</td>
<td>19 ± 4*</td>
<td>15</td>
<td>7.8 ± 1.5#</td>
<td>17</td>
</tr>
<tr>
<td>SF.0.1</td>
<td>34 ± 7</td>
<td>27</td>
<td>34 ± 9</td>
<td>25</td>
<td>9.4 ± 2.2</td>
<td>27</td>
</tr>
<tr>
<td>SF.I.0.1</td>
<td>30 ± 6</td>
<td>18</td>
<td>30 ± 6</td>
<td>19</td>
<td>11.2 ± 2.7</td>
<td>18</td>
</tr>
</tbody>
</table>

*a n, number of replicate measurements performed  
* Significantly different to SF.V.Passive at p < 0.05  
# Significantly different to SF.0.1 at p < 0.05  
¤ Significantly different to SF.I.0.1 at p < 0.05

As it is known that the dorsal layer contributes to 30% of the nail thickness and that it has been said that this layer was a limiting factor for drug permeation (Kobayashi et al., 1999 and 2004), one would therefore expect a deeper penetration of SF into the ventral and intermediate layers compared to the dorsal. However, this was not the case: during passive experiments (Fig. 21, Table 4), measurements of the penetration depth were either
equivalent (T and %T) or the dorsal penetration (D) was greater than the ventral and during iontophoretic experiments (Fig. 21, Table 4), the depth of penetration (D) in dorsal and intermediate layers were both significantly greater than in the ventral layer. This emphasizes the fact that the different types of measurements (D, T and %T) do not give equivalent results.

4. **Comparison between SF and NBC penetration**

Fig 22 shows penetration depth of the two fluorescent markers reported as T, D and %T after passive and iontophoretic experiments.

According to T values, SF permeated significantly deeper into the nail plate than NBC under all experimental conditions. D values, suggest on the contrary, a deeper passive penetration for NBC passive experiments but no differences in iontophoresis. Interestingly, none of the fluorescent markers went deeper than 11.2 % of the nail thickness whereas the dorsal layer represents 30 % of the thickness. This showed that both markers did not go further than the dorsal layer.

5. **SF binding study**

The little difference observed between SF permeation under different conditions was unexpected.
During transdermal experiments, it had been reported that keratin binds with antifungals such as omoconazole, bifonazole, clotrimazole (Hashiguchi et al., 1998) and cationic lipophilic peptides such as poly-L-lysines presenting MW greater than 2700 Da (Turner et al., 1997). For these cationic lipophilic peptides, it is thought that the positive charge of the peptide associates with fixed negative charge of the skin and that the lipophilic part of the molecule acts like an “anchor”. This phenomenon is believed to reduce the net negative charge of the skin and therefore to cancel its permselectivity and the electroosmotic flow. Similar binding phenomena have been observed during ungual experiments with salicylic acid (Hui et al. 2002; Murthy et al., 2007b) and griseofulvin (Murthy et al., 2007a). So, the possible binding of SF onto nail material which would stop the penetration of the marker into deeper layers was therefore investigated.

Three nails (D, E and F) were cut in three pieces (1, 2 and 3) and left in a 375 mM SF solution for 24 hours (see Materials and Methods). After this 24 hours exposure, one piece of each nail (D1, E1 and F1) was examined by confocal microscopy in order to determine the depth of penetration reached by the marker during the 24 hours period. The two other sets of samples were placed in sodium chloride solution for one (D2, E2, F2) and two weeks (D3, E3, F13), after which they were examined by confocal microscopy. The objective was to determine if the fluorescent band had or not been modified during these “washings”, indicating further diffusion of the marker into the nail or its release in the washing solution. Had the bands been very similar to those observed for the first set, it could be argued that SF was irreversibly bound to the nail material, and that this binding could be hindering its penetration into the nail. One transversal image illustrative of each piece is shown in Fig. 23 and Table 5 reports the average thickness of the ventral and dorsal bands measured for each nail piece.
Fig. 23: Top panel: Passive penetration of SF into human nails after 24 hours exposure. Middle and bottom panels: subsequent diffusion of SF into deeper section of the nail and/or into the washing solution. The nails were completely submerged, i.e., both the ventral and dorsal layers of the nail faced the exposure and washing solutions, which explains the appearance of two fluorescent bands.
Table 5: SF Depth of penetration in the ventral and dorsal layers of the nail pieces during the binding study.

<table>
<thead>
<tr>
<th>Penetration depth (µm)</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>E1</th>
<th>E2</th>
<th>E3</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ventral</strong></td>
<td>25 ± 3</td>
<td>80 ± 54</td>
<td>0</td>
<td>30 ± 3</td>
<td>66 ± 13</td>
<td>38 ± 23</td>
<td>24 ± 6</td>
<td>43 ± 6</td>
<td>0</td>
</tr>
<tr>
<td><strong>Dorsal</strong></td>
<td>36 ± 4</td>
<td>121 ± 42</td>
<td>66 ± 70</td>
<td>30 ± 6</td>
<td>87 ± 16</td>
<td>0</td>
<td>43 ± 5*</td>
<td>99 ± 41</td>
<td>0</td>
</tr>
<tr>
<td><strong>Thickness</strong></td>
<td>300 ± 35</td>
<td>300 ± 20</td>
<td>350 ± 25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* significantly different from its ventral depth of penetration (p < 0.05)

These images allow further comparison of the permeability of the ventral and dorsal layers, the advantage, in this case, is that paired comparisons can be made. Statistical tests (parametric paired t-test) deduced that the depth of penetration in the two layers was significantly different only for piece F1 (Table 5). The evolution of the fluorescence bands width between the pieces 1 (24 h) and 2 (one week) are shown in Fig. 24.

Fig 24: SF depth of penetration into the specimens D1, E1, F1 (24 h) and D2, E2, F2 (one week). Each panel shows the ventral and dorsal depths of penetration for each nail (mean±SD of 2-6 images). The symbols *, **, and *** mean a significant (p = 0.01 to 0.05), very significant (p = 0.001 to 0.01) and extremely significant (p < 0.001) difference between bars.

After one week, the following observations were made: (a) the fluorescent bands were less intense than at 24 hours but had broadened (Fig. 23) and an unpaired t-test showed that, for all the pieces (except the ventral diffusion of nail D), the depth of penetration in the ventral and dorsal layers was significantly greater at one week than at 24 h (Fig. 24) and (b) a thin dark layer, fluorescence depleted, wherever the nail had been in contact with the bathing solution (Fig. 23). These two observations indicate that SF had diffused both (a) into
deeper layers of the nails and (b) into the bathing solution. The confocal examination performed at two weeks found no fluorescence in specimen F3, a little fluorescence in a central area of specimen D3, and a ventral band, relatively weak and broad in specimen E3 (Fig. 23). These observations were in agreement with the analysis of the rinsing solutions performed (Fig. 25).

The amount of SF recovered in the rinsing solutions sequentially used in the study is shown in Fig. 25. The results indicate that SF effectively diffused from inside the nail into the solution. Two rinses were performed at 24 hours to remove SF from the surface of the nail and contained 662 ± 773 and 12.5 ± 9.8 nmoles of SF respectively. This important decline demonstrates the efficiency of the rinsing and that the SF recovered in subsequent washing solutions had been released from the inner part of the nail and was not originating from surface contamination.

![Graph showing SF recovery over time](image)

Fig. 25: SF (nmoles) recovered from the rinsing solutions used during the binding study. Note that two rinses were performed after the 24 hours exposure. The bars show the mean ± SD of nine (24 h), six (1 week) and three (2 weeks) replicates.

It was then investigated if the width of the fluorescence bands obtained on the 24 h images could be related to the cumulative amount of SF released during the two weeks of experiments (Fig. 26).
Fig. 26: Cumulative amount of SF released during the two weeks of experiment (excluding the 24 h rinses) in function of the average width of the fluorescence bands (ventral + dorsal band) is shown. The dotted line represents the regression line and was not significant.

Fig. 26 shows that the nail exhibiting the broader fluorescent bands did release more SF to the medium. The other two nails show similar bands and release. A regression line can be drawn through these points ($r^2 = 0.8$) but is not significant. More replicates are required to confirm this trend.

Finally, the cumulative amounts recovered into the rinsing solutions, 700 nmoles, represented only 0.09% of the total SF, $7.5 \times 10^5$ nmol, present in the donor (2 mL, 375 mM). These results reinforced the idea of the limited passive permeability. In summary, results show that little difference existed between dorsal and ventral passive penetrations which confirmed the results obtained in a previous section (Fig. 21, Table 4), and that SF was not bound to the nail material.

6. **Pathways of passive and iontophoretic transungual permeation**

This study used images obtained with a x20 magnification objective (Fig. 27) and involved 1) passive and iontophoretic penetration of NBC and SF into the dorsal layer, 2) passive and iontophoretic penetration of SF into the ventral layer and 3) iontophoretic SF penetration into the intermediate layer. The images of SF passive penetration onto the ventral layer are shown (Fig. 27D) but the little fluorescence present prevents a good comparison with the other images. Likewise, no fluorescence was observed in the images concerning SF passive penetration into the dorsal layer and thus those images are not shown.
Fig. 27: Panel A: NBC.Passive, Panel B: NBC.0.4. Black and white was used for both fluorescent markers to facilitate the comparison. All the pictures were obtained at x20 magnification.
Fig. 27 (continued): Panel C: SF.0.1. Panel D: SF.V.Passive, Panel E: SF.V.0.1. Panel F: SF.I.0.1. Black and white was used for both fluorescent markers to facilitate the comparison. Dark dots observed in images obtained after SF experiments are indicated by white arrows. All the pictures were obtained at x20 magnification.
No differences were observed between the images of passive and iontophoretic permeation of NBC (Fig. 27A and B). In general, the fluorescence was quite spread across the onychocytes suggesting a transcellular pathway in both cases. In some zones the intercellular pathway seemed slightly darker demarcating the cells.

In the case of SF (Fig. 27C, E and F) the images also support that the marker followed a transcellular pathway during iontophoresis independently of the layer (dorsal, ventral, and intermediate) which was exposed to the donor solution. However, in some images, the intercellular pathway was very bright and the transcellular was darker. As mentioned before, it has been shown that ionic compounds use both the appendegeal and intercellular pathways during transdermal iontophoresis (Cullander et al., 1992, Turner et al., 1998). Obviously, the appendegeal pathway is not possible for nails. More interestingly the distribution of SF and NBC fluorescence indicated a main role for the transcellular pathway which apparently would be that opposing the least resistance to the passage of current. This is probably the consequence of the different structure and chemical composition of the two membranes, but a clear discussion is difficult given how little is known about the nail. These results also reinforce the message that nails and skin are different and that specific studies on nail iontophoresis are required.

Cells of the ventral layer revealed by SF (Fig. 27E) showed a pitted and rough surface compared to the dorsal layer (Fig. 27C). This difference in the aspect of the ventral and dorsal layers had already been demonstrated by Scanning Electron Microscopy (Forslind et al., 1975), by light microscopy after dying the nail (Kobayashi et al., 1999) and by Atomic Force Microscopy (Repka et al., 2002).

The surface of the filed nails, i.e. the intermediate layer (Fig. 25F), was irregular which was probably due to the filing.

Finally, SF images often showed round regular dark intracellular spots while NBC stained the whole content of the cells. A NBC parent compound, nile blue sulphate (nile blue A, NBA), is commonly used as an intercalating dye because it interacts with nucleic acids (Huang et al., 1999). Nails are constituted of dead cells which still contain DNA fragments (which can be used for forensic investigations (Cline et al., 2003)), thus it was expected that NBC would colour the entire content of the corneocytes including the remnant nuclei if present. This could suggest that the small round dark areas which were not dyed by SF delimited the shape of the disintegrated nuclei (Germann et al., 1980; Kaufman et al., 1995).
Conclusions of chapter 2
A new method to estimate the depth of penetration into human nail plate via confocal microscopy has been developed and the value of three depth parameters compared. Despite their different physicochemical properties, NBC and SF presented similar depth of penetration under passive and iontophoretic conditions. Iontophoresis delivered the two markers slightly deeper into the nail than passive diffusion, but the level of enhancement was very moderate and independent of the intensity of current applied. SF passive and iontophoretic delivery into the dorsal, ventral or intermediate layer of the nail was very similar. SF did not irreversibly bind to keratin. Finally, the transcellular pathway was predominant for both markers and delivery methods.
Chapter 3:
Transungual iontophoresis of mannitol:
The electroosmotic contribution to transungual iontophoretic transport.

Overview

The application of an electrical current, namely – iontophoresis –, to biological membranes such as skin and cornea efficiently enhances drug transport into and across these membranes and therefore, this method recently attracted interest as a possible tool to improve nail drug delivery. However, a rational approach to this new opportunity requires the permselective properties of the nail to be characterized. The permselective properties of a charged membrane result in two phenomena: (a) preferential passage (higher transport numbers) of counter-ions (i.e., ions possessing a charge of opposite sign to that of the membrane) and (b) electroosmosis or convective solvent flow in the same direction of transport than that of the counter-ions. This solvent flow carries with it neutral polar molecules and assists in the transport of counter-ions. Electroosmosis is the scope of this chapter. The transungual fluxes of mannitol, a well established marker of electroosmosis, were determined during anodal and cathodal iontophoresis and performed with vehicles of different pH. The effect of inter-nail variability, and the passive contribution to the total transport was also investigated. The results showed evidence for nail cation-permselectivity at physiological pH (as such observed for the skin) i.e., preferential anode-to-cathode mannitol transport when a single-nail was considered. However, the wide inter-nail variability and the unexpectedly high passive transport of mannitol observed overwhelmed the results and did not allow a clear interpretation of the data. These results suggest that the electroosmotic contribution during transungual iontophoresis might be very variable and difficult to predict and thus, of limited use for practical applications. Future research should address the validity of the present observations in the in vivo conditions as well as the sources for the important variability observed.
**Introduction**

The human nail can be affected by two main diseases: onychomycosis and psoriasis (Murdan, 2002). The treatment of onychomycosis which represents 40-50% of all onychopathies (Gaburri et al., 2008) is long and difficult due to the adverse effects (mainly hepatic problems related to the insufficient specificity of the antifungals to target only the fungi cytochromes) and drug interactions (Debruyne et al., 2001). Topically applied therapies could be the answer but, in this case, drugs are facing the nail plate. High drug concentrations are easily achieved in the dorsal upper layers but reaching therapeutic levels at the nail bed, where the onychomycosis lie, is a challenge.

Different approaches have been considered to enhance ungual topical penetration: 1) chemical penetration enhancers (Hui et al., 2003 and Kobayashi et al, 1998) have been used with more or less success and 2) iontophoresis, a minimally invasive technique, has started being evaluated recently (Murthy et al, 2007a and b; Hao et al., 2008 a, b and c).

Murthy et al. (2007) performed the iontophoresis of salicylic acid, griseofulvin and glucose across human nail tips whereas Hao et al. (2008) studied the iontophoretic transport of mannitol, tetraethylammonium and urea, with and without chemical enhancers, across finger nail plates and nail tips. These two groups investigated the iontophoretic transport of both neutral and charged compounds at different pHs providing some information about the contribution of electrorepulsion and electroosmosis to transungual iontophoretic fluxes.

It is pertinent to quickly revise what is known about electroosmosis in transdermal drug delivery applications. However, precaution must be taken before applying this knowledge to the nail as these membranes are significantly different both in structure and in chemical composition. Pikal et al. (1990) demonstrated visually the electroosmotic flow occurring during transdermal iontophoresis: a piece of hairless skin mouse was fixed between the two halves of a diffusion cell which had capillary tubes fixed onto the cathodal and anodal chambers. The initial level of solution in both capillary tubes was registered and the current was started. The position of the meniscus in each tube was monitored during iontophoresis and, for example, a meniscus displacement of up to 4 cm indicating a 30 µL solvent flow could be measured. This data clearly showed the solvent movement between the two chambers during iontophoresis.

Pikal’s experiment was extremely laborious and, in practice, electroosmosis is usually determined indirectly by measuring the flow of an electroosmotic marker. The most frequently used marker is mannitol: a neutral, polar (logP -3.9) sugar with a similar molecular weight to glucose (182 and 180 for mannitol and glucose, respectively). Mannitol is preferred to glucose because it is an exogenous material which is not metabolized by cells. Thus, mannitol fluxes are neither confounded by metabolism or by
endogenous contributions (such as the skin glucose reservoir (Sieg et al., 2004a)). Further, despite of being neutral, it is an extremely hydrophilic compound which limits the contribution of passive diffusion to negligible levels during transdermal iontophoresis (Kim et al., 1993).

The electroosmotic flow is a less efficient mechanism of transport than electrorepulsion, however its contribution is the only possible for neutral, uncharged or zwitterionic, polar molecules. Further, it becomes progressively more important for charged molecules as their transport numbers decrease (typically as molecular weight increases and electrical mobility is reduced). Thus, its contribution could be critical in the case of onychomycosis; potent antifungal drugs have a relatively high molecular weight (207 to 706 Da) and are either neutral or partially ionised at physiological pH (Table 1).

<table>
<thead>
<tr>
<th>Administration</th>
<th>Molecules</th>
<th>MW</th>
<th>Acid/Base</th>
<th>pH&lt;sub&gt;a&lt;/sub&gt;</th>
<th>% ionisation at pH 7.4&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systemic</td>
<td>Itraconazole</td>
<td>706</td>
<td>Weak base</td>
<td>~6.5</td>
<td>~11</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole</td>
<td>531</td>
<td>Weak base</td>
<td>~6.9</td>
<td>~24</td>
</tr>
<tr>
<td></td>
<td>Griseofulvin</td>
<td>368</td>
<td>Weak acid</td>
<td>~11</td>
<td>~0.02</td>
</tr>
<tr>
<td></td>
<td>Fluconazole</td>
<td>306</td>
<td>Weak acid</td>
<td>~11</td>
<td>~0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Weak base</td>
<td>~3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Terbinafine</td>
<td>291</td>
<td>Weak base</td>
<td>~7</td>
<td>~28</td>
</tr>
<tr>
<td>Topical</td>
<td>Amorolfin</td>
<td>318</td>
<td>Weak base</td>
<td>~7.5</td>
<td>~56</td>
</tr>
<tr>
<td></td>
<td>Ciclopirox</td>
<td>207</td>
<td>Weak acid</td>
<td>~6.2</td>
<td>~94</td>
</tr>
</tbody>
</table>

<sup>a</sup> data taken from Scifinder Scholar and Medchem online.  
<sup>b</sup> data calculated from “Physicochemical principles of Pharmacy” from Florence and Attwood, 2<sup>nd</sup> edition (1988).

The direction of the electroosmotic flow is directly related to the membrane’s net charge. For example, skin and cornea membranes are negatively charged at physiological pH which induces an electroosmotic flux in the anode-to-cathode direction (Rojanasakul et al., 1989; Guy et al., 2000). In vitro experiments (Marro et al., 2001b) which gradually lowered the pH of the solutions bathing the skin, showed a progressive reduction of the anode-to-cathode mannitol flux suggesting a concomitant neutralization of the skin’s positive charges. Ultimately, at pHs below the apparent isoelectric point of the membrane, the skin becomes positively charged and mannitol net flow occurs in the cathode-to-anode direction. The characterization of the effect of pH on mannitol flux has become a standard tool on studies addressing the permselective properties of human, pig, mice and rabbit skin.
as well as rabbit cornea (Marro et al., 2001b; Kim et al., 1993; Nicoli et al., 2003, Li et al., 2005).

As previously discussed, the net negative charge of the skin at physiological pH also induces cation permselectivity (Burnette et al., 1987; Pikal et al., 1990). For example, transport numbers in a 0.1 M NaCl aqueous solution are 0.4 and 0.6 for Na\(^+\) and Cl\(^-\), respectively, but change to 0.6 and 0.3 during transdermal iontophoresis (Burnette et al., 1987). This indicates the preferential passage of cations (counter-ions as sodium) across the negatively charged membrane. Thus, the measurement of small inorganic transport numbers is another indirect evidence of the membrane charge and the existence of electroosmosis. However, electroosmosis and transport numbers are related but not interdependent phenomena, as evidenced by the next finding: the transport number of lidocaine (Marro et al., 2001a) in the single-ion situation (absence of competing co-ions in the donor solution) was 0.1 which indicated that the counter-ion (chloride) had a transport number close to 0.9. Despite the fact that most of the ionic charge was being transported by chloride ions in the cathode-to-anode direction, it was shown that the electroosmotic flow of mannitol occurred in the anode-to-cathode direction which demonstrated that electroosmosis is directly linked to the membrane charge and not necessarily associated to the ion with the highest transport number. Thus, our work has attempted a complete characterization of the nail permselective properties by investigating both phenomena: (a) the existence of an electroosmotic contribution during transungual iontophoresis as evidence by mannitol transport as a function of pH and (b) the preferential passage of cations/anions again as a function of pH (subject of the next chapter).

Keratin being the main component of skin and nail, nails are expected to be negatively charged at physiological pH. The strongest evidence supporting this hypothesis is the data reported by Murthy et al. (2007b) on the transungual iontophoresis of glucose and griseofulvin. These authors measured the anodal and cathodal electroosmotic fluxes of glucose as a function of pH across nail tips that had been hydrated for 6 hours before the experiments. The pattern (anodal and cathodal flux versus pH) observed was very similar to that demonstrated for the skin and suggested that (a) the isoelectric point of nail is close to 5 (human skin pI ~ 4.8 (Marro et al., 2001b)) and (b) human nails hold a net negative charge at physiological pH. The apparent solvent flux at pH 7 and a current intensity of 0.125 mA can be estimated from the data to be 0.3 µl/h, a value relatively high when compared to the 0.65 µl/h reported for human skin at pH 7.4 and 0.4 mA (Marro et al., 2001b). The iontophoretic transport of griseofulvin (weak acid, pKa~11) at pH 3, 5 and 7 also supported this evidence. For example, griseofulvin highest fluxes at pH 3 and 7 were obtained using cathodal and anodal iontophoresis respectively, again indicating...
electroosmosis occurring in the cathode-to-anode direction at pH 3 (nail positively charged) and in the anode-to-cathode at pH 7 (nail negatively charged). Griseofulvin fluxes were about a half of those reported by glucose; this could be due to the drug’s higher molecular weight (368 Da), increased lipophilicity (logP 2.2) or its extensive binding to keratin.

However, the 6-fold enhancement caused by iontophoresis at 0.125 mA on glucose fluxes with respect to passive conditions and the unambiguous distinction between anodal and cathodal fluxes is in contradiction with other data. Hao et al. (2008a) reported a very small enhancement (less than 3-fold) on the permeability coefficient of mannitol even when 0.1 and 0.3 mA iontophoresis had been used and that the nails had been hydrated for 24 hours prior the experiments. Unfortunately, Hao et al. (2008a, b and c) reported their results as permeability coefficients and Peclet numbers which limits their direct comparison with the fluxes reported by Murthy (2007a) and with those in this work.

It seems therefore that nail permselectivity has not been demonstrated unambiguously and the aim of the work reported in this chapter was to investigate this phenomenon. In line with Hao et al. (2008) and a multitude of skin and cornea studies (Marro et al., 2001b; Kim et al., 1993; Nicoli et al., 2003; Li et al., 2005) we have preferred the use of mannitol to avoid any confounding contribution to the fluxes originating from endogenous sources. The passive, anodal (anode-to-cathode) and cathodal (cathode-to-anode) electroosmotic flux of mannitol was measured at pH 4.0, 5.0 and 7.4. The significance of inter-nail variability was also considered and integrated in the experiment design. As a difference with other authors, we have limited prior hydration of the nail to a maximum of 15 minutes expecting this short hydration to mimic more closely a practical \textit{in vivo} application.

\section*{Materials and methods}

\subsection*{1. Materials}

Mannitol, sodium chloride, HEPES free acid, hydrochloric acid and sodium hydroxide were obtained from Sigma Aldrich Co. (Gillingham, UK). $^{14}$C-mannitol (200 $\mu$Ci/mL equivalent to 7.4 Bq/mL) was obtained from Amersham plc (Little Chalfont, UK). Ultima Gold XR, Hionic Fluor and Solvable were obtained from Perkin Elmer (Waltham, MA, USA). Silver wire, silver chloride powder and platinum wire used to prepare the electrodes had a minimum purity of 99.99\% and were obtained from Sigma Aldrich Co. (Gillingham, UK). All aqueous solutions were prepared using high purity deionised water (18.2 M$\Omega$.cm, Barnstead Nanopure Diamond\textsuperscript{TM}, Dubuque, IA, USA).
2. **Nails**

Ethical approval was granted by the Bath Local IRB and finger nail clippings were obtained from fourteen healthy volunteers who gave their written informed consent (Documents provided in the Appendix 3). The harvested nails were washed with deionised water and kept at room temperature in a dessicator until use. Prior to an experiment, the thickness of the nail in the site closest to the hyponychium was measured with a point micrometer (Point Anvil Micrometer, Mitutoyo, Andover, UK). Next, each nail was soaked in deionised water during 10-15 minutes in order to recover some flexibility, and placed in 5 mm (0.2 cm$^2$ area) nail adapters (PermeGear Inc., Bethlehem, PA, USA).

3. **Mannitol iontophoresis**

Mannitol experiments were performed in Franz vertical diffusion cells (PermeGear Inc., Bethlehem, PA, USA) presenting a transport area of 0.2 cm$^2$. To facilitate sampling of the receptor chamber, the bottom chamber was used to hold the donor solution while the receptor was placed on the top. The nail adapter was placed so the nail’s dorsal layer faced the donor solution (Fig. 1).

Experiments were performed with donor and receptor solutions adjusted to an identical pH: 4.0, 5.0 or 7.4.

Preliminary experiments indicated that nails exhibit a high resistance to the passage of current. During constant, direct current iontophoresis, the power supply modifies the voltage imposed depending on the resistance of the electrical circuit (as indicated by Ohm’s law; $V=I\cdot R$) so a constant current passes through the circuit. It was observed, that the voltage required for imposing 0.2 mA from time zero was unacceptable as it would have a deleterious effect on the nails. Thus, a ~30 minutes “pre-treatment” period was applied to each nail during which a maximum voltage output (200 V) was set on the power supply (Kepco APH 1000M, Flushing, NY, USA). During this time the current applied...
could be lower than 0.2 mA and varied with the resistance of each nail. Once the nail resistance drops enough, the voltage required to pass 0.2 mA is less than the limit imposed. Typically, the voltage required for passing 0.2 mA was 30-40 V and was reached in ~30 minutes. During this pre-treatment time the receptor and donor chamber were filled with HEPES buffer at the appropriate pH (containing no mannitol). Once this pre-treatment had been performed for the three nails, the donor and receptor chambers were filled with the appropriate mannitol donor and receptor solutions. The donor chamber (3.6 mL) was filled with a 10 mM mannitol solution containing 154 mM NaCl in 25 mM HEPES buffer. This solution was spiked with $^{14}$C-mannitol (200 µCi/mL equivalent to 7.4 Bq/mL) to get 2.5-3 µCi/cell (around 0.1 Bq/cell). The receptor chamber (700 µL) contained 154 mM NaCl in 25 mM HEPES buffer. The pH of the solutions was adjusted to 4, 5 and 7.4 by addition of sodium hydroxide or hydrochloric acid as appropriate.

The three cells were then connected in series and the experiment started. A constant current of 0.2 mA (1 mA/cm$^2$) was applied via homemade Ag/AgCl electrodes prepared as previously described (Green et al., 1991).

Four set of experiments were performed as described in Fig. 2. Set I comprised three independent experiments studying the effect of one factor (pH 4, 5 and 7.4). This experiment indicated that a wide inter-nail variability could confound the effect of the factors under investigation. Thus, the next Sets (II-IV) comprised only experiments that modified either pH or polarity sequentially. In experiments comprising several pH stages, the donor and receptor solutions were rinsed 3 times and filled with the new buffer solution before proceeding with the next stage. The donor and receptor were rinsed 3 times as well at the very end of each experiment with some receptor solution at the appropriate pH. In all cases three replicates were done.

Passive experiments were performed in the same way except that no current was applied and that the pre-treatment was not necessary.
4. Mannitol quantification

The whole content of the receptor chamber (700 µL) was sampled at the indicated times (Fig. 2) and the chamber refilled with fresh receptor buffer. Each receptor sample was mixed with 15 mL Ultima Gold XR before being assessed for its $^{14}$C-mannitol content on a Liquid Scintillation Analyzer Tri-Carb 2800TR equipped with the software QuantaSmart 4.01 (Perkin Elmer, Waltham, MA, USA). At the end of each experiment, nails were carefully removed from their adapters, placed in pre-weighed glass scintillation vials and weighed. 5 mL of Solvable were added to each nail and the vials were subsequently placed in an oven (Heraeus Function Line, Weiss-Gallenkamp, Loughborough, UK) at 60 ºC for nail digestion. The vials were taken out of the oven upon complete digestion of the nails (minimum 6 hours) and 10 mL Hionic Fluor scintillation cocktail were added once they were at room temperature. The nail content in mannitol was assessed by liquid scintillation counting (LSC) (Data shown in the Appendix 2).

Appropriate background samples were prepared for each experiment. Three 700 µL samples of buffer solution were mixed with 15 mL of Ultima Gold XR to obtain an average radioactivity background for the receptor samples. 5 mL of Solvable combined with 10 mL Hionic Fluor scintillation cocktail was used as background counting for digested nails.

Three reference samples were prepared by mixing 100 µL of the spiked donor solution with 15 mL of Ultima Gold XR. LSC of these references provided the ratio hot-to-cold.
mannitol in the donor solution and allowed estimation of the equivalent cold mannitol present in the samples.

5. Mannitol binding study
The possible binding of mannitol to the nail material was studied. Three nails (A, B and C) were cut in three pieces (A1, A2, A3; B1, B2, B3; C1, C2, C3). Each piece was weighed and placed for 24 h in a vial containing 2 mL of a 10 mM mannitol in a pH 7.4, 25 mM HEPES buffer spiked with $^{14}$C-mannitol (200 µCi/mL equivalent to 7.4 mBq/mL). After 24 h, all pieces were taken out of the mannitol solution and rinsed twice in 5 mL of a 25 mM HEPES buffer at pH 7.4. Samples A1, B1 and C1 were then mixed with Solvable and placed in the oven for nail digestion and subsequently 10 mL Hionic Fluor were added prior to LSC analysis. The remaining nail samples (A2, A3; B2, B3; C2, C3) were placed in individual vials containing 5 mL of HEPES 25 mM for one week. The buffer solution was completely refreshed in the middle of the week. At the end of the first week, the samples A2, B2 and C2 were prepared for digestion and LSC analysis as previously described. Samples A3, B3 and C3 were left in 5 mL of HEPES 25 mM for one more week. The buffer solution was replaced at the end of the first week and in the middle of week two. Finally, samples A3, B3 and C3, were prepared for digestion and LSC analysis at the end of the second week. The rinsing and buffer solution were also analyzed by LSC after addition of 15 mL Ultima Gold.

6. Data analysis and statistics
All data is presented as mean and standard deviation of at least three replicates. Linear regressions and statistics were performed using Graph Pad Prism V5.00 (Graph Pad Software Inc., San Diego, CA). When relevant, sets of data were compared by a one way ANOVA followed by a Tukey’s post-test and by paired t-test. Alternatively, non-parametric tests (Kruskall-Wallis followed by Dunn’s post-test and Wilcoxon paired t-test) were also used due to the small number of replicates. The slopes obtained from the mannitol amounts for each nail were compared at each pH and condition by using a comparison tool in the Graph Pad Prism V5.00 software. The level of statistical significance was fixed at p < 0.05. All linear regressions were significant (p < 0.05) unless otherwise indicated.
Results and discussion

1. Mannitol electroosmotic transport

Set I:
The nails used during these experiments came from female participants (21-44 years old). The average thickness of the nails used at pH 4, 5 and 7.4 was $290 \pm 20 \mu m$, $290 \pm 80 \mu m$ and $220 \pm 20 \mu m$, respectively (Table 2).

<table>
<thead>
<tr>
<th>pH of the experiment</th>
<th>Nail code*</th>
<th>Gender</th>
<th>Age (year)</th>
<th>Thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4.0</td>
<td>4LF3</td>
<td>Female</td>
<td>44</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td>12LF3</td>
<td>Female</td>
<td>22</td>
<td>290</td>
</tr>
<tr>
<td></td>
<td>13RF3</td>
<td>Female</td>
<td>21</td>
<td>310</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>2LF5</td>
<td>Female</td>
<td>26</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>7LF1</td>
<td>Female</td>
<td>28</td>
<td>360</td>
</tr>
<tr>
<td></td>
<td>13LF4</td>
<td>Female</td>
<td>21</td>
<td>290</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>2LF5</td>
<td>Female</td>
<td>25</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>7LF5</td>
<td>Female</td>
<td>28</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>9RF5</td>
<td>Female</td>
<td>24</td>
<td>230</td>
</tr>
</tbody>
</table>

*The first digit in the nail code indicates the participant number, L or R indicates left or right hand and F+digit indicates the finger where F1 = thumb

As described in Materials and Methods, Set I comprised three independent experiments which lasted for 52 h (Fig. 3). Mannitol was always delivered from the anode.

Fig. 3: Schematic diagram of the experimental conditions followed in Set I. Each line represents an independent experiment involving 3 nails. Each symbol represents a sampling time. The dotted line indicates that mannitol delivery occurred in the anode-to-cathode direction.

The cumulative amounts of mannitol delivered to the receptor solution for each nail and pH were plotted against time (Fig. 4). The total amount of mannitol delivered at each pH stage is shown in Table 3. The slopes of the linear regressions (Fig. 4) of each of these
lines were obtained using Graph Pad Prism V5.00 and used as an average flux for each nail and pH.

![Graph showing cumulative amounts of mannitol obtained for each nail at each pH. Dotted lines are linear regressions.](image)

**Fig. 4:** Cumulative amounts of mannitol obtained for each nail at each pH. Dotted lines are linear regressions.

**Table 3:** Total amount of mannitol delivered (nmoles) during set I. The order of the nail is the one indicated in Table 2.

<table>
<thead>
<tr>
<th></th>
<th>pH 4.0</th>
<th>pH 5.0</th>
<th>pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nail 1</td>
<td>192.5</td>
<td>323.6</td>
<td>34.4</td>
</tr>
<tr>
<td>Nail 2</td>
<td>327.8</td>
<td>101.2</td>
<td>11.7</td>
</tr>
<tr>
<td>Nail 3</td>
<td>231.1</td>
<td>182.4</td>
<td>14.4</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>250.5 ± 69.7</td>
<td>202.4 ± 112.5</td>
<td>20.2 ± 12.4</td>
</tr>
</tbody>
</table>

First of all, it was observed that the data was very variable (Fig. 4, Table 3). A simple test for comparison of the slopes (Zar, 1984) indicated that the three slopes (fluxes) obtained for each pH were significantly different (Fig. 4).

This variability was particularly manifest for the experiment performed at pH 5 and 7.4. The mean flux (± SD) was 5.48 ± 1.46, 4.39 ± 2.66 and 0.43 ± 0.26 nmol/h at pH 4.0, 5.0 and 7.4, respectively (Figure 5).

![Graph showing mannitol flux (nmol/h) as a function of pH.](image)

**Fig. 5:** Anodal delivery of mannitol as a function of pH.

Surprisingly, the fluxes measured at pH 7.4 were clearly the lowest of the set. The non-parametric Kruskall-Wallis could not demonstrate any significant difference between the data; however a parametric ANOVA indicated that the flux at pH 7.4 was statistically
different to that measured at pH 4.0 (Table 3, Fig. 5). In any case, the trend “anodal flux versus pH” was completely unexpected at it seemed to suggest that anodal electroosmosis decreased as pH increased, in other words that the nail was negatively charged at pH 4 and became progressively uncharged or positively charged at pH 7.4. This is in complete contradiction with previous data and with the physicochemical properties of keratin (Marshall, 1983; Murthy et al., 2007a; Hao et al., 2008). A wide inter-nail variability has been mentioned previously (Hao et al., 2008a) although not quantitatively reported. It was then hypothesised that experiments should be implemented in such a way that inter-nail variability could be separated from the experimental error, thus allowing an improved investigation of the effects of pH and polarity on the delivery. This justified the experimental approach followed in all the in vitro subsequent experiments of this thesis.

Set II:

All the nails used in this experiment came from the middle finger of female participants (26-44 years old). Their average thickness was 270 ± 10 µm (Table 4).

<table>
<thead>
<tr>
<th>Nail code*</th>
<th>Gender</th>
<th>Age (year)</th>
<th>Thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2LF3</td>
<td>Female</td>
<td>26</td>
<td>270</td>
</tr>
<tr>
<td>4RF3</td>
<td>Female</td>
<td>44</td>
<td>280</td>
</tr>
<tr>
<td>7LF3</td>
<td>Female</td>
<td>28</td>
<td>260</td>
</tr>
</tbody>
</table>

*The first digit in the nail code indicates the participant number, L or R indicates left or right hand and F+digit indicates the finger where F1 = thumb

In this experiment, mannitol was delivered from the anode at pH 7.4 during 28 hours after which the donor and receptor solutions were replaced by equivalent solutions at pH 4. The anodal iontophoresis of mannitol at pH 4 was then studied for another 24 h (28-52h) (Fig. 6).
Fig. 6: Schematic diagram of the experimental conditions applied in Set II. Each symbol represents a sampling time. The dotted line means that mannitol was delivered in the anode-to-cathode direction. The pH of both the donor and receptor solutions was changed from 7.4 to 4.0 after 28 h.

As previously, the cumulative mannitol delivered for each pH stage was represented as a function of time (Figure 7) and the fluxes for each nail/pH stage obtained from the slope of the respective linear regression. The total amount of mannitol delivered at each pH stage is shown in Table 5.

![Graph](image_url)

Fig. 7: Mannitol delivered by anodal iontophoresis during the pH 7.4 and pH 4.0 stages. Dotted lines are linear regressions. The linear regression of the nail 2LF3 at pH 4.0 was not significant.

<table>
<thead>
<tr>
<th></th>
<th>pH 7.4</th>
<th>pH 4.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>2LF3</td>
<td>44.7</td>
<td>20.7</td>
</tr>
<tr>
<td>4RF3</td>
<td>82.5</td>
<td>19.4</td>
</tr>
<tr>
<td>7LF3</td>
<td>24.0</td>
<td>12.3</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>50.4 ± 29.7</td>
<td>17.5 ± 4.5</td>
</tr>
</tbody>
</table>

Again, an important variability was observed at pH 7.4 were the three slopes were significantly different; no differences were found between the slopes corresponding to the pH 4.0 stage (Fig. 7). The average fluxes (±SD) measured at each pH are shown in Figure 8.
Fig. 8: Anodal delivery of mannitol as a function of pH. The arrow indicates that the first stage of the experiment was performed at pH 7.4 and the second at pH 4.0.

Table 5 clearly indicates that, if each nail is taken separately, changing the pH from 7.4 to 4.0 results in cutting the total amount of mannitol delivered by a factor 2 to 4. Mannitol flux at pH 7.4 (1.91 ± 1.04 nmol/h) was higher than at pH 4.0 (0.44 ± 0.13 nmol/h) but there were not statistically significant differences among the two values or between the mean total mannitol delivery of Table 5 (Wilcoxon paired test). However, the trend here observed was more consistent with a net negative charge for nail at pH 7.4 and with what is known about keratin (pI ~ 5) and the literature (Murthy et al., 2007; Hao et al., 2008). Indeed, anodal mannitol fluxes slightly decrease (although not to the level of statistical significance) as the bathing solutions become more acidic indicating the loss of negative charges in the nail. The variability observed at pH 7.4 suggests that the factors determining the magnitude of the convective flow (Hao et al., 2008c) (for example, the amount of fixed charge in the membrane and the geometry of the pathway of penetration) may vary considerably among different nails. In any case, it seemed a sensible step to perform subsequent experiments in the same fashion, i.e., use the same nails and sequentially modify the factor (pH or the current polarity) investigated.

Set III:
The nails used during this experiment came from the little finger of female participants (21-44 years old) and their average thickness was 230 ± 10 µm (Table 6).
Table 6: Nails used in set III.

<table>
<thead>
<tr>
<th>Nail code*</th>
<th>Gender</th>
<th>Age (year)</th>
<th>Thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2RF5</td>
<td>Female</td>
<td>26</td>
<td>220</td>
</tr>
<tr>
<td>4LF5</td>
<td>Female</td>
<td>44</td>
<td>240</td>
</tr>
<tr>
<td>13LF5</td>
<td>Female</td>
<td>21</td>
<td>240</td>
</tr>
</tbody>
</table>

*The first digit in the nail code indicates the participant number, L or R indicates left or right hand and F+digit indicates the finger where F1 = thumb.

This experiment comprised four stages (Fig. 9). Mannitol was delivered from the anode at pH 4.0 during the first stage (0 to 28 h) and at pH 7.4 (28 to 52 h) during the second one. The polarity of mannitol delivery was switched at 52 hours and mannitol was subsequently delivered from the cathode at pH 7.4 (52 to 76 h) in a third stage and at pH 4.0 (76 to 100 h) in a final fourth stage.

Fig. 9: Schematic diagram of the experimental conditions applied in Set III. Each symbol represents a sampling time. The dotted line means that mannitol was delivered in the anode-to-cathode direction, the black line means it was in the cathode-to-anode direction.

The amount of mannitol delivered for each pH/polarity stage was represented as a function of time (Fig. 10) and the fluxes for each nail/stage were obtained from the slope of the respective linear regressions.
Fig. 10: Effect of pH and polarity on mannitol transport across the nail. The following regressions were not significant: (a) anodal delivery: 13LF5 at pH 4.0 (B) cathodal delivery, 2RF5 at pH 7.4, 4LF5 at both pHs and 13LF5 at both pHs.

As before it seemed that the variability observed was higher for anodal delivery at pH 7.4 but formal comparisons were not performed in this case because many slopes were not significant (Fig. 10).

The average fluxes obtained from the slopes of the linear regressions have been plotted against the pH and according to the electrode of delivery (Fig. 11).

Fig. 11: Anodal and cathodal mannitol fluxes measured at pH 4 and 7.4. A dotted line means that mannitol was delivered in the anode-to-cathode direction whereas a black line indicates that it was in the cathode-to-anode direction. The arrows indicate in which way the pH was changed.

In this experiment, the results show the expected trend: anodal delivery increases from 0.02 ± 0.01 nmol/h at pH 4 to 0.11 ± 0.07 nmol/h at pH 7.4 and cathodal delivery decreased from 0.15 ± 0.02 nmol/h at pH 4 to 0.06 ± 0.01 nmol/h at pH 7.4. However, a non parametric paired t-test (Wilcoxon t-test) could only show statistically significant differences for the cathodal fluxes.

As indicated in Fig. 10, half of the fluxes used in the previous analysis originated from linear regression that were not significant, it could be argued that these fluxes had an
unacceptable error associated. Therefore, it was decided to analyse the total amount of mannitol delivered at each pH/polarity stage (Table 7).

Table 7: Total amount of mannitol delivered (nmoles) during set III.

<table>
<thead>
<tr>
<th></th>
<th>Anode to cathode</th>
<th>Cathode to anode</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 4</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>2RF5</td>
<td>0.96</td>
<td>2.51</td>
</tr>
<tr>
<td>4LF5</td>
<td>0.59</td>
<td>0.96</td>
</tr>
<tr>
<td>13LF5</td>
<td>0.45</td>
<td>0.89</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.67 ± 0.27</td>
<td>1.45 ± 0.91</td>
</tr>
</tbody>
</table>

Table 7 shows that, for each nail: 1) in the anode to cathode direction, the total amount of mannitol delivered at pH 7.4 was systematically higher than at pH 4 (factor 2.6, 1.6 and 2.0 for 2RF5, 4LF5 and 13LF5, respectively) and 2) in the cathode to anode direction, the total amount of mannitol delivered at pH 4.0 was higher than at pH 7.4 (factor 1.4, 1.8 and 1.8 for 2RF5, 4LF5 and 13LF5, respectively). However, these observations did not attain the level of statistical significance (Wilcoxon paired t-test).

Passive diffusion:

The nails used during this experiment came from the right middle finger of female donors (22-44 years old). Their average thickness was 240 ± 40 µm (Table 8).

Table 8: Nails used in Set IV - passive experiment.

<table>
<thead>
<tr>
<th>Nail code*</th>
<th>Gender</th>
<th>Age (year)</th>
<th>Thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2RF4</td>
<td>Female</td>
<td>26</td>
<td>200</td>
</tr>
<tr>
<td>4RF4</td>
<td>Female</td>
<td>44</td>
<td>240</td>
</tr>
<tr>
<td>12RF4</td>
<td>Female</td>
<td>22</td>
<td>270</td>
</tr>
</tbody>
</table>

*The first digit in the nail code indicates the participant number, L or R indicates left or right hand and F+digit indicates the finger where F1 = thumb.

As described in the introduction there are contradictory reports on the degree of enhancement caused by the iontophoresis of glucose and mannitol (Murthy et al., 2007a; Hao et al., 2008a). Thus it was decided to perform a passive control in our experimental conditions. The passive diffusion experiment had a total duration of 96 h and comprised a first stage at pH 4.0 (0 to 48 h) and a second stage at pH 7.4 (48 to 96 h) (Fig. 12).
The cumulative mannitol delivered for each pH stage was represented as a function of time (Fig. 13) and the fluxes for each nail/stage were obtained from the slope of the respective linear regressions. In this case the inter-nail variability was greater at pH 4 as the slopes at this pH were significantly different; there were no differences observed among the slopes corresponding to pH 7.4.

The average fluxes are plotted against the pH in Fig. 14.
The passive fluxes were 0.03 ± 0.02 and 0.17 ± 0.02 nmol/h at pH 4.0 and 7.4, respectively and were significantly higher (paired t-test) at pH 7.4.

The total amount of mannitol delivered during the passive experiments is reported in Table 9.

<table>
<thead>
<tr>
<th></th>
<th>pH 4.0</th>
<th>pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>2RF4</td>
<td>1.92</td>
<td>8.43</td>
</tr>
<tr>
<td>4RF4</td>
<td>0.67</td>
<td>6.45</td>
</tr>
<tr>
<td>12RF4</td>
<td>1.89</td>
<td>8.22</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>1.49 ± 0.71</td>
<td>7.70 ± 1.08</td>
</tr>
</tbody>
</table>

Surprisingly, the cumulative amount of mannitol obtained at pH 7.4, for each nail, was higher than at pH 4.0 (by a factor 4.4, 9.6 and 4.3 for 2RF4, 4RF4 and 12RF4, respectively). However, a Wilcoxon paired t-test did not reveal any statistical differences between the two means.

Comparison of all results:

Fig. 15 shows the passive and iontophoretic mannitol fluxes measured during the four sets of experiments and according to the pH and polarity of delivery.

It was pertinent to examine all the data gathered in this work (Fig. 15) and to compare it with the starting hypothesis and previous data. On the one hand, (Murthy et al., 2007a), data suggests that the nail behaves like the skin (Kim et al. 1993) and that the following should be observed: (1) a passive flux which is always smaller than the iontophoretic
fluxes at any pH, (2) a cathodal flux greater than the anodal at pH 4.0 and (3) an anodal flux greater than the cathodal at pH 7.4. On the other hand, Hao et al (2008a and c) observed an extremely low enhancement and a practically negligible effect of pH and polarity of delivery on mannitol transport.

Individual experiments performed with the same nails (Set II and III) indicates that the nails behave as a cation permselective membrane at physiological pH, however, Fig. 15 suggests that this fact might be overwhelmed by other factors when a bigger population of nails is considered. Thus, while cathodal electroosmosis at pH 4 is higher for a given set (Set III), it is clear that for other sets anodal electroosmosis is even higher (set I and II). Similarly, while set III shows that anodal iontophoresis at pH 7.4 is the best condition of delivery for a given set, it is also true that higher fluxes can be obtained for other sets with cathodal delivery at pH 4 (Set I and II). Moreover, even when the nails come from the same finger of different volunteers, this does not make the results more comparable: at pH 4.0, nails in set I and II were all from F3 and at pH 7.4, nails in set I and III came from F5 but no clear similarities could be noticed. On the whole, these data suggest that nails are possibly negatively charged at physiological pH, but that there is a huge inter-nail variability in the magnitude of the electroosmotic fluxes caused by iontophoresis. It can also be deduced the need of considering inter-nail variability in future studies and more crucially, of studying electroosmosis across the nail plate *in vivo*. If these findings about nail clippings *in vitro* are representative of the *in vivo* situation it will be extremely difficult to predict transungual fluxes of a drug which is only transported by electroosmosis.

A final point can be made on the degree of enhancement caused by iontophoresis. It is easily seen (Figure 14) that the level of enhancement varies from zero to 100 fold depending on the pair of experiments compared. Unfortunately, passive controls were done in a separate study and the effect of nail variability can not be separated. However, these results could help to understand the contradictory observations previously reported by different groups. Hao et al. used the same nails for passive, anodal and cathodal and observed a very moderate enhancement caused by iontophoresis; it seems that Murthy used different nails for each conditions and it could be possible that, by chance, the least permeable nails were used as passive controls. Unfortunately, there is not enough information on Murthy’s published work to discuss more deeply this detail. In any case, if the results here reported are predictive of the *in vivo* situation, one could difficulty support the use of iontophoresis, a more complex and expensive technique, with respect to passive diffusion delivery systems.
Comparison with other works:
The anodal and cathodal mannitol fluxes measured at pH 7.4 have been summarised in Table 10 for comparison with the glucose nail results of Murthy et al. (2007a) and with the mannitol skin results of Marro et al. (2001b).

Table 10: Comparison of skin and nail iontophoretic fluxes (nmol/h) and convective solvent flow normalized by the current intensity, $J_s$ (µL/h/mA), of mannitol or glucose at pH 7 and 7.4.

<table>
<thead>
<tr>
<th>Current (mA)</th>
<th>Fluxes (nmol/h)</th>
<th>$J_s$ (µL/h/mA)</th>
<th>Fluxes (nmol/h)</th>
<th>$J_s$ (µL/h/mA)</th>
<th>Fluxes (nmol/h)</th>
<th>$J_s$ (µL/h/mA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Set I</td>
<td>0.43 ± 0.26</td>
<td>0.21 ± 0.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anodal</td>
<td>Set II</td>
<td>1.91 ± 1.04</td>
<td>0.95 ± 0.52</td>
<td>3.10 ± 0.32</td>
<td>2.48 ± 0.26</td>
<td>0.66 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>Set III</td>
<td>0.11 ± 0.07</td>
<td>0.05 ± 0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cathodal</td>
<td>Set III</td>
<td>0.06 ± 0.01</td>
<td>0.03</td>
<td>0.28 ± 0.05</td>
<td>0.22 ± 0.04</td>
<td>0.04 ± 0.03</td>
</tr>
</tbody>
</table>

Murthy et al. (2007a) worked on the transport of a 10 mM solution of glucose across nail by anodal and cathodal delivery at 0.125 mA. Marro et al. (2001b) studied the transport of a 1 mM mannitol solution across human skin by anodal and cathodal delivery at 0.4 mA. Murthy et al. (2007a) were using glucose and so they performed a 10 h control experiment to check that no glucose was released from nails and did not find any. This statement is in accordance with a preliminary experiment that we performed and revealed the absence of glucose in nails. Briefly, we dissolved three nails over night in NaOH 2 M and analysed them by amperometry on ion chromatography using NaOH as the eluent (Data not reported).

Murthy et al. (2007a) obtained results extremely consistent with a negative charge held by nails at pH 7 but, interestingly, the convective solvent flows that they obtained, 2.48 and 0.22 µL/h/mA for anodal and cathodal delivery respectively, were higher than what Marro et al. (2001b) obtained with human skin, 1.65 and 0.10 µL/h/mA for anodal and cathodal delivery respectively.

The anodal convective solvent flows obtained during our work were higher than the cathodal flow (0.03 µL/h/mA) and this latter flow was fairly similar with the one obtained in skin (0.10 µL/h/mA). Again, these findings suggest that nails hold a net negative charge at physiological pH. The anodal convective solvent flows that we found here are between 0.05 and 0.95 µL/h/mA. These values are smaller than the one found in skin, 1.65
µL/h/mA (Marro et al., 2001b), and much smaller than the one found in nails, 2.48 µL/h/mA (Murthy et al., 2007a). It is surprising to observe that Murthy et al. (2007a) could find anodal convective solvent flow 1.5 times higher than what Marro et al. (2001b) measured in skin.

Variability in nail permeation:
It is well known that membrane thickness plays an important role during passive diffusion via the permeability coefficient as defined in Eq. 1.

\[ P = \frac{K \cdot D}{h} \quad \text{Eq. 1} \quad (\text{Walters et al. 1981}) \]

where \( P \) is the permeability coefficient in cm/h, \( K \) is the membrane-solution partition coefficient, \( D \) is the diffusion constant of the permeant within the membrane and \( h \) is the membrane thickness. However, we could not find any relation between the thickness of the three nails used in passive diffusion and the fluxes measured. This may be due to the limited number of replicates available. For example, Walters et al. (1981) demonstrated that a nonlinear relationship existed between methanol and ethanol permeability and the nail thickness.

In the case of iontophoresis, transport by electrorepulsion has been shown to be mostly controlled by the relative concentration and mobilities of the competing ions, and to be much less dependent on the skin animal model (Phipps et al., 1989; Mudry et al., 2006b) or even on the skin damage (Sekkat et al., 2002; Abla et al., 2005) than passive diffusion. However, a comparable information concerning transport by electroosmosis is not available, although it could be anticipated to be probably more dependent on the skin properties as it relies on the membrane charge. However, previous studies by Hao and Li (2008) and Murthy et al. (2007) did not report any effect of nail thickness in the fluxes. Similarly our data seems to suggest the same, for example, if one compares the three sets of experiments performed by anodal iontophoresis at pH 7.4: Set II performed with the thickest group of nails (270 ± 10µm) provided the highest flux while Set I (220 ± 20µm) and III (230 ± 10µm) which were very similar in thickness provided very different mannitol fluxes.

It could be hypothesized that nail permeability is related to their use or exposure to external factors. As an example, in every day life activities, the thumb and the forefinger are almost permanently under stress and their nails could either (a) have adapted to these aggressions by presenting a higher barrier to exogenous compounds (like the skin of the foot sole) or (b) be more damaged. Because nail tips have no mechanisms for “recovery”
this damage may result in a higher permeability. Thus, it was interesting to compare the results obtained with nail tips originating in different fingers. For example, Set II used only nails from the middle finger and Set III from the little finger. Fig. 15 shows that the results in Set III were consistently lower than those in Set II. There is not sufficient data at this point to be sure of the meaning of this observation which, ideally, should be confirmed in the future. Unfortunately, nails are very difficult to collect, which makes very hard the development of these systematic studies.

Another important factor suggested by Hao et al. (2008) and by Gunt et al. (2006) is the effect of nail hydration over permeability. Nail hydration has been related to nail swelling which is considered to be a prerequisite for enhanced drug penetration (Khengar et al., 2007) and Gunt et al. (2006) have related passive permeability across the nail with nail hydration. However, one would expect that all nails were similarly hydrated in our studies, and that would not be a cause of variability. On the other hand, it is interesting to notice that the level of hydration in previous studies have been much longer than in our work which obviously had an impact on the nails electrical resistance. Hao et al. (2008) soaked nails in PBS for 24 h prior to an experiment and Murthy et al. (2007) for 6 h in buffer. The increased hydration decreased the nail resistance dramatically so an Iomed Phoresor II (battery operated device) could be used for iontophoresis application. In this work, the nails were soaked for only 10-15 min in deionised water and therefore the resistance of the nails implied the use of a mains operated power supply able to maintain higher voltages. The use of this short time was based on a work from Wessel et al. (1999) who studied the nail hydration by NIR-FT-Raman spectroscopy and stated that “the water uptake of the nail showed a saturating effect after around 10 min” and on preliminary studies (not reported). It was also considered to represent a closer approach to a practical in vivo application. In any case, the different hydration times could cause inter-study differences but they cannot explain the inter-nail differences observed in our case.

Finally, it has been suggested that some drugs such as salicylic acid, griseofulvin, terbinafine or itraconazole (Hui et al., 2002; Murthy et al., 2007a; Leyden, 1998) extensively bind to keratin in skin and nails. Drug binding can considerably delay transport across the membrane as the drug accumulates into the skin or the nail rather than diffuse to the receptor. Thus a final experiment was performed to examine whether mannitol binds to nails and if its binding could be a factor contributing to variability. The results are shown in the following section.
2. Mannitol binding

Three nails cut in three pieces were placed in a 10 mM radioactive mannitol solution for 24 h (see Materials and Methods) and the amount of mannitol accumulated into the nail during this time was determined with samples A1, B1 and C1. The remaining samples were left in buffer for one (A2, B2 and C2) or two weeks (A3, B3 and C3). This allowed for determination of mannitol released from the nail. It was expected that any significant binding to keratin would result in a considerable amount of mannitol remaining in the nails after one or two weeks.

Table 11 and Fig. 16 show the amount and concentration of mannitol remaining in the nail at the end of each stage.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mannitol amount (nmol)</th>
<th>Sample dry mass (mg)</th>
<th>Mannitol concentration (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>13.60</td>
<td>5.89</td>
<td>2.31</td>
</tr>
<tr>
<td>A2</td>
<td>5.71</td>
<td>8.15</td>
<td>0.70</td>
</tr>
<tr>
<td>A3</td>
<td>0.89</td>
<td>6.51</td>
<td>0.14</td>
</tr>
<tr>
<td>B1</td>
<td>18.56</td>
<td>12.70</td>
<td>1.46</td>
</tr>
<tr>
<td>B2</td>
<td>2.91</td>
<td>7.55</td>
<td>0.39</td>
</tr>
<tr>
<td>B3</td>
<td>1.06</td>
<td>12.86</td>
<td>0.08</td>
</tr>
<tr>
<td>C1</td>
<td>10.40</td>
<td>6.74</td>
<td>1.54</td>
</tr>
<tr>
<td>C2</td>
<td>2.00</td>
<td>7.15</td>
<td>0.28</td>
</tr>
<tr>
<td>C3</td>
<td>1.15</td>
<td>6.82</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Table 11. Mannitol recovered in each nail sample.

The average nail content was $1.77 \pm 0.47$, $0.46 \pm 0.22$ and $0.13 \pm 0.04$ nmol/mg at 24 h, one week and two weeks, respectively. The content of mannitol in nails A, B and C decreased to 30, 28 and 18 % of the initial value after 1 week and to 6, 5 and 11 % after two weeks. The value show little inter-nail variability.

The amount of mannitol release to the rinsing solutions was also measured (Fig. 17).
The amount of mannitol recovered from the second rinses performed at 24 h was much smaller than the amount recovered from the first rinse or from the rinse at the middle of the first week. This showed that the mannitol recovered from the rinses after 24h was effectively coming from inside the nail and not from the surface.

After one week, 0.46 ± 0.22 nmol of mannitol per mg remained in the nails (Fig. 16) and 2.55 ± 4.61 nmol was found in the rinsing solutions (Fig. 17). By the end of the second week, only 0.13 ± 0.05 nmol of mannitol per mg remained in the nails and no mannitol was detectable in the rinsing solutions. On the whole these results showed that mannitol does not irreversibly bind to nail material.

**Conclusions of chapter 3**

The electroosmotic flux resulting from the application of iontophoresis to human nail tips has been studied using mannitol as a marker. It has been shown that mannitol transport was not hindered by binding to keratin.

Due to the important inter-nail variability observed in the Set I, experiments keeping the same nail clippings all along the different stages were performed in order to minimize this phenomenon. Subsequent sets of experiments showed results which were in favour of a negative charge held by nails at physiological pH. However, values obtained during the three sets of experiments remained very different suggesting that the transport of drugs by electroosmosis may not be easily predictable.
Chapter 4:

Transungual iontophoresis of lithium and sodium:
Electromigration and transport numbers.

Overview

The use of iontophoresis to enhance drug penetration into and across the nail has been suggested. However, basic knowledge about the permselective properties of human nails and the behaviour of transungual iontophoretic fluxes are still missing. The previous chapter studied the iontophoretic transport of mannitol, a neutral and polar compound, as a marker of electroosmosis and nail permselectivity. This chapter investigates the transungual fluxes of sodium and lithium, two small inorganic cations. The objective was to investigate whether nails show cation permselectivity at physiological pH and to improve our understanding of transport numbers during transungual iontophoresis. The donor solutions comprised single ion and binary mixtures of the two cations at different pHs. Cationic transport numbers showed, at all the ratios studied, a clear increase when the pH was sequentially changed from 4.0 to 7.0 suggesting that human nails carry a net negative charge at physiological pH. Iontophoretic fluxes showed very low inter-nail variability and were clearly superior to passive fluxes, suggesting key differences between iontophoresis delivery governed by the electroosmotic and the electromigrative mechanisms of transport. Sodium transport number (i.e., the fraction of charge transported by this ion) was maximal when the ion was formulated as a single ion (no presence of competing co-ions) and decreased as the molar fraction of lithium was increased in the vehicle. The magnitude of the transport numbers measured and their response to changes in the cations’ molar fraction and pH in the donor solution were remarkably similar to those observed during transdermal iontophoresis. Another interesting similarity was the existence of a cationic transport number threshold. On the whole, this work provided some key information about nail permselectivity and transungual transport numbers which will help in developing this technique to efficiently deliver therapeutic compounds into the nail plate.
Introduction

As discussed in the introduction chapter, iontophoresis is driven by electrorepulsion and electroosmosis. It has been demonstrated, at least in the case of transdermal iontophoresis (Guy et al., 2000), that electrorepulsion (or electromigration) is the most efficient of both mechanisms. The magnitude of the electromigrative fluxes resulting from the direct interaction of charged molecules with the electric field is given by Faraday’s law (Eq. 1):

\[ J_i = t_i I / Z_i F \]  \hspace{1cm} \text{Eq. 1 (Phipps et al. 1992)}

where \( J_i \) is the flux of the ion “i” in mol/sec, \( t_i \) is the transport number of the ion “i”, \( I \) is the current intensity in coulombs/sec, \( Z_i \) is the valence of the ion “i” and \( F \) is the Faraday constant (~ 96500 coulomb/mol).

When the electrical circuit is started, ions at both sides of the biological membrane compete for transporting the charge; and ions present in large concentrations and possessing high electrical mobilities will carry most of it. The transport number corresponds to the fraction of the total charge carried by a specific ion and is one of the key parameters that determine the feasibility of drug delivery by iontophoresis. The transport number of a given ion (drug) depends on the ability of the ion (drug) to be a charge carrier (electrical mobility) in the presence (relative concentration and electrical mobilities) of competing co- and counter-ions. Co-ions are the ions in the formulation presenting a charge of the same sign than the ion (drug) of interest and transporting charge from the vehicle into the body across the biological membrane. The presence of co-ions can be minimized with a judicious formulation. Counter-ions have an electrical charge of opposite sign than the ion (drug) of interest and carry charge from underneath the skin or nail into the formulation. The most important competing counter-ions are sodium and chloride due to their high mobility and concentration in the physiological fluids. Obviously, competition by counter-ions can not be avoided in an in vivo application and drug transport numbers are typically optimized by minimizing the sources of co-ions in the vehicle. It has been shown that maximum transport numbers during transdermal iontophoresis are measured (Luzardo-Alvarez et al., 2003; Mudry et al., 2006a) in the “single-ion” situation where competition only comes from the counter-ions (for example chloride ions in the case of cationic drug) arriving from the inner side of the membrane (Mudry et al., 2007). Further, it has been predicted by Kasting et al. (1989) and shown experimentally (Marro et al., 2001a) that single-ion fluxes (Eq. 1) are independent of the drug concentration in the vehicle being solely controlled by the relative diffusivities of the two ions involved (positively charged drug and chloride ions present in the subdermal compartment):
\[ t_M = \frac{D_M}{D_M + D_{Cl^-}} \]  \hspace{1cm} \text{Eq. 2}

where \( t_M \) is the transport number of the drug, \( D_M \) and \( D_{Cl^-} \) are the diffusion coefficient of the ionic drug M and chloride ion, respectively.

A “single ion” formulation provides the best transport numbers for a given drug however; practical issues (stability, pH, etc) usually require the introduction of additional components in a formulation. If possible, preference should be given to uncharged or low mobile species added at the lowest possible concentration so competition with the drug is minimized. Because of these practical issues it is fundamental to understand how transport numbers respond to the introduction of competing ions. Phipps et al (1992) proposed an equation linking concentration, mobility and valence of the ions (Eq. 3):

\[ t_d = \frac{c_d z_d \mu_d}{\sum_i c_i z_i \mu_i} \]  \hspace{1cm} \text{Eq. 3}

where \( t_d, c_d, \mu_d \) and \( z_d \) represent transport number, concentration, mobility and valence of the ionic drug, \( "d" \), within the membrane, respectively. Eq. 3 emphasizes the importance of the ions mobility and concentration which are going to determine the extent of competition involved by the introduction of co-ions.

Mudry et al (2006a and b) showed that the relative concentration of the drug with respect to that of competing co-ions (as expressed by the drug’s molar fraction) is a better predictor of the transport number than the drug’s nominal concentration in the formulation. For example, the transport number of lithium delivered from two binary solutions of sodium and lithium 20:20 and 100:100 mM was 0.21 ± 0.03 and 0.26 ± 0.05, this is due to the fact that lithium had the same molar fraction in both vehicles (Mudry et al., 2006b). A good linear relationship has been observed between the transdermal transport number of small ions and lidocaine (Marro et al., 2001a) and their molar fraction in the vehicle. Further, the transport number in the single ion situation was reasonably well predicted from the regression equation (Mudry et al., 2006a).

The measurement of transport numbers during transdermal iontophoresis is standard; this is not the case in transungual iontophoresis. There are no systematic investigations on this matter and transport numbers of drugs have not been reported. For example, Hao et al., (2008a) have studied the anodal iontophoresis of tetraethylammonium which enhanced transport ~29 fold as compared to passive conditions but did not explore the effect of competing ions. Further, because the authors reported the results as permeability.
coefficients and Peclet numbers, the available data does not provide any information about fluxes or transport numbers. Murthy et al (2007b) studied the cathodal delivery of salicylic acid across nail tips and included in their study the effect of drug concentration and of the ionic strength of the buffer. Salicylic acid fluxes increased with the drug concentration (0.2 -2 mg/mL) as expected. In another series of experiments different amounts of sodium chloride (0 to 140 mM) were added to the 10 mM phosphate buffer saline containing 2 mg/mL of the drug. The effect of the ionic strength of the donor solution on the drug flux was not clear, the drug flux unexpectedly increased (10-50 mM ionic strength) and then plateau/decreased when additional sodium chloride was introduced in the buffer (100-200 mM ionic strength). According to the authors the results indicate the requirement of a minimal ionic strength for iontophoresis delivery. However, it should be noticed that the concentration of sodium chloride in the receiver (anodal) buffer was modified in parallel with the cathodal buffer. It is known that iontophoresis with anodal buffers containing none or little sodium chloride results in anomalous anodal electrochemistry at the Ag/AgCl anode (water electrolysis) and that the pH quickly turns very acidic in these conditions. Thus, a pH shift could also have modified the drug fluxes. In conclusion, the available literature offers very little information about ionic competition during nail iontophoresis and we addressed this lack of knowledge to facilitate a rational development of transungual iontophoretic drug delivery systems.

The aims of this work were 1) to investigate the rules that govern ion competition and transport numbers during transungual iontophoresis and 2) to measure the transport numbers of sodium and lithium across the nail as an additional proof of nail permselectivity.

Materials and Methods

1. Materials

Lithium chloride, sodium chloride, HEPES free acid (MW 238.3), magnesium chloride, hydrochloric acid, methanesulfonic acid, sodium hydroxide 50 % (Ion chromatography grade) and potassium hydroxide were obtained from Sigma Aldrich Co. (Gillingham, UK). Silver wire, silver chloride powder and platinum wire used to prepare the electrodes had a minimum purity of 99.99 % and were obtained from Sigma Aldrich Co. (Gillingham, UK). All aqueous solutions were prepared using high purity deionised water (18.2 MΩ.cm, Barnstead Nanopure Diamond™, Dubuque, IA, USA).
2. Nails

Ethical approval was granted by the Bath Local IRB and finger nail clippings were obtained from fourteen healthy volunteers who gave their written informed consent (see Appendix 3). The harvested nails were washed with deionised water and kept at room temperature in a dessicator until use. Prior to an experiment, the thickness of each nail was measured with a point micrometer (Point Anvil Micrometer, Mitutoyo, Andover, UK) as close as possible to the hyponychium. Each nail was then soaked in deionised water during 10-15 minutes in order to recover some flexibility, and finally placed in 5 mm nail adapters (PermeGear Inc., Bethlehem, PA, USA).

3. Sodium and lithium iontophoresis

The nail adapter containing the fingernail clipping was clamped between the two halves (3.5 mL donor, 2 mL receptor, transport area of 0.2 cm$^2$) of a side-by-side diffusion cell (PermeGear Inc., Bethlehem, PA, USA) with the nail dorsal surface facing the anodal chamber. Three replicates were performed for each experiment. The anodal chamber was filled with the respective donor solution (Table 1) containing either sodium as a single cation or a binary mixture of sodium and lithium ions. Both donor and receptor solutions were prepared in HEPES 5 mM and the pH was adjusted either with hydrochloric acid or with potassium hydroxide.

<table>
<thead>
<tr>
<th>Experiment code</th>
<th>Donor (HEPES 5 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100 mM NaCl</td>
</tr>
<tr>
<td>B</td>
<td>50 mM NaCl and 25 mM LiCl</td>
</tr>
<tr>
<td>C</td>
<td>50 mM NaCl and 50 mM LiCl</td>
</tr>
<tr>
<td>D</td>
<td>50 mM NaCl and 250 mM LiCl</td>
</tr>
<tr>
<td>E</td>
<td>50 mM NaCl and 500 mM LiCl</td>
</tr>
</tbody>
</table>

The halide salts and their concentration in the anodal solution provided the necessary chloride ions for the anodal electrochemical reaction. 5 mM of MgCl$_2$ was added to the receptor buffer to provide a source of chloride which represented the main endogenous competing ion and to avoid analytical interferences between the sodium and lithium peaks of the chromatogram. KOH was preferred for correcting the pH of the buffer solution to avoid the introduction of sodium ions. The HEPES concentration was reduced to the minimum that would keep a constant pH throughout the experiments. HEPES is a switterion and has a pKa$_1$ ~3 and a pKa$_2$ ~7.55. Therefore, it is expected that HEPES will
be mainly transported by electroosmosis at both pH experimented and will not compete with lithium and sodium.

Following the observations on inter-nail variability reported in the previous chapter the parameters investigated (pH and molar fraction) were compared in the same nail. The pH of the HEPES buffered solutions was identical on both sides of the nail and was sequentially modified from 4.0 to 5.0 and then from 5.0 to 7.0. Each pH stage, started with 16 hours of passive diffusion followed by 8 hour of 0.2 mA direct constant current iontophoresis applied via homemade Ag/AgCl electrodes (Green et al., 1991) and a power supply (Kepco APH 1000M, Flushing, NY, USA). All the experiments started by passive diffusion and it was possible to connect all the nails in series without involving any “pre-treatment” (at the opposite of the confocal microscopy and mannitol experiments in the previous chapters). Figure 1 represents schematically the experimental design followed.

![Figure 1: Schematic diagram of the experimental design. A discontinued line indicates passive diffusion while the continuous line represents the iontophoretic stage.](image)

The whole content of the receptor chamber (2 mL) was sampled and refilled with fresh receptor buffer at the end of the passive diffusion step (16 hours) and every 2 hours during iontophoresis. At the end of each pH stage, both chambers were rinsed at least 3 times with the respective donor and receptor solutions at the pH subsequently tested.

4. **Cation quantification**

Ion chromatography (IC) with suppressed conductivity detection (AS50 autosampler and thermal compartment, GP50 gradient pump, ED50 electrochemical detector, Chromeleon software, Dionex, Sunnyvale, CA) was used to measure the concentrations of sodium and lithium in all samples. 25 mM methanesulfonic acid eluent was pumped under isocratic conditions (1 mL/min) through an IonPac™ CS12A column (Dionex, 250 x 4 mm) thermostated at 30°C and a CSRS ULTRA II suppressor (Dionex, 4 mm) set at a current of 80 mA. The concentrations of Na⁺ and Li⁺ in the samples were measured against
calibration curves obtained at the adequate pH. Calibration curves were prepared at pH 4.0, 5.0 and 7.0 with at least seven standard solutions, ranging from 50 µM to 5 mM, prepared in 5 mM HEPES buffer containing 5 mM MgCl₂. Standard solutions contained both NaCl and LiCl at the same concentration at the pH 4.0, 5.0 and 7.0. For both cations, the measurement error was below 5%.

5. Data analysis and statistics
Cationic fluxes for each sampling period were directly determined by dividing the amount of ion delivered to the receptor solution (as determined by IC) by the corresponding time. The results showed that iontophoretic fluxes were not stable by the end of the experiments. Therefore, the use of an average 0-8 hours iontophoretic flux would not be appropriate and the 6-8 hours iontophoretic flux, expected to be the closest to the steady value, was used for all comparisons.

Cationic transport numbers were calculated using the previously described Faraday’s law (Eq. 1).

Linear regressions and statistics were performed using Graph Pad Prism V5.00 (Graph Pad Software Inc., San Diego, CA).

Sodium and lithium fluxes and their transport numbers measured at different pH were compared by one way ANOVA followed by a Tukey’s post-test. The level of statistical significance was fixed at $p < 0.05$. All linear regression presented were significant ($p < 0.05$) unless otherwise indicated. All data are presented as mean and standard deviation unless otherwise stated.

Results and discussion

1. Sodium and lithium iontophoresis
Table 2 shows the characteristics of the nails used according to the donor composition: origin – namely, participant’s gender and age – and thickness that were used for each experiment. The nail average thickness was 240 ± 10 µm for experiment A, 340 ± 50 µm for B, 280 ± 40 µm for C, 380 ± 80 µm for D and 270 ± 20 µm for E. On the whole, the 15 nails used came from 8 participants. The age of the participants ranged from 21 to 52 years old, only one of whom was a male.
Table 2: Nails used for lithium and sodium experiments.

<table>
<thead>
<tr>
<th>Experiment code</th>
<th>NaCl:LiCl concentration (mM) [XNa⁺ : XLi⁺ #]</th>
<th>Nail code *</th>
<th>Gender</th>
<th>Age (year)</th>
<th>Thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100:0 [1:0]</td>
<td>2LF4 Female</td>
<td>26</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>7LF4 Female</td>
<td>28</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12LF4 Female</td>
<td>22</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>50:25 [0.67:0.33]</td>
<td>2RF1 Female</td>
<td>26</td>
<td>340</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4LF1 Female</td>
<td>44</td>
<td>290</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>14LF1 Female</td>
<td>52</td>
<td>380</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>50:50 [0.5:0.5]</td>
<td>7LF3 Female</td>
<td>28</td>
<td>280</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12LF3 Female</td>
<td>22</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>14LF3 Female</td>
<td>52</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>50:250 [0.17:0.83]</td>
<td>5LF2 Male</td>
<td>33</td>
<td>460</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12LF2 Female</td>
<td>22</td>
<td>310</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>13LF2 Female</td>
<td>21</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>50:500 [0.09:0.91]</td>
<td>4LF4 Female</td>
<td>44</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10RF4 Female</td>
<td>25</td>
<td>290</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12RF4 Female</td>
<td>22</td>
<td>250</td>
<td></td>
</tr>
</tbody>
</table>

* X stands for molar fraction

* The first digit in the nail code indicates the participant number, L or R indicates left or right hand and F+digit indicates the finger where F1 = thumb.

As a first clear difference with the previous chapter, all the experiments on cation iontophoresis indicated that current application caused a clear enhancement of lithium and sodium transport as compared to passive diffusion.

A second difference was the reduced variability observed in cation electromigration as compared with mannitol electroosmosis.

A third difference lies on the voltage measured for the three cells in series. At the beginning of each iontophoretic period, the voltage was between 11 and 130 V (usually 30-60 V). At 2 hours and at 8 hours, the voltage ranged between 14 and 47 V and 11 and 31 V, respectively. It is assumed that the application of the current without pre-treatment was possible thanks to the 16-hour passive stage applied before each iontophoretic period resulting in an increase of the nails hydration and a decrease of the nails resistance.

A considerable amount of data was generated during these experiments which are organized in the following manner:

- Figures 2 to 6 show the sodium and lithium fluxes measured for each nail, donor composition, pH and sampling time. Tables 3 to 11 show the corresponding average (± SD) cationic fluxes and transport numbers.
Figures 7 to 11 show the 8 hours sodium and lithium iontophoretic fluxes (mean ± SD) as a function of pH. Statistical differences found are shown as appropriate.

Figures 12 to 16 show the 8 hours sodium and lithium transport numbers (mean ± SD) as a function of pH. Statistical differences found are shown as appropriate.

Fig. 2: Passive and iontophoretic sodium fluxes measured for each nail during experiment A (100:0).

Fig. 3: Passive and iontophoretic sodium and lithium fluxes measured for each nail during experiment B (50:25)
Fig. 4: Passive and iontophoretic sodium and lithium fluxes measured for each nail during experiment C (50:50).

Fig. 5: Passive and iontophoretic sodium and lithium fluxes measured for each nail during experiment D (50:250).
Fig. 6: Passive and iontophoretic sodium and lithium fluxes measured for each nail during experiment E (50:500).

Table 3: Passive and iontophoretic fluxes (µmol/h) and transport numbers of sodium (t_{Na+}) during experiment A (100:0). The data shows the mean ± the standard deviation of three replicates.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH 4.0</th>
<th>pH 5.0</th>
<th>pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flux</td>
<td>t_{Na+}</td>
<td>Flux</td>
</tr>
<tr>
<td>16</td>
<td>0.09 ± 0.11</td>
<td>passive</td>
<td>40</td>
</tr>
<tr>
<td>18</td>
<td>3.03 ± 0.38</td>
<td>0.41 ± 0.05</td>
<td>42</td>
</tr>
<tr>
<td>20</td>
<td>2.98 ± 0.52</td>
<td>0.40 ± 0.07</td>
<td>44</td>
</tr>
<tr>
<td>22</td>
<td>2.77 ± 0.27</td>
<td>0.37 ± 0.04</td>
<td>46</td>
</tr>
<tr>
<td>24</td>
<td>2.63 ± 0.21</td>
<td>0.35 ± 0.03</td>
<td>48</td>
</tr>
</tbody>
</table>
Table 4: Passive and iontophoretic fluxes (µmol/h) and transport numbers of sodium (t_{Na}+) during experiment B (50:25). The data shows the mean ± the standard deviation of three replicates.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH 4.0</th>
<th></th>
<th>Time (h)</th>
<th>pH 5.0</th>
<th></th>
<th>Time (h)</th>
<th>pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flux</td>
<td>t_{Na}+</td>
<td></td>
<td>Flux</td>
<td>t_{Na}+</td>
<td></td>
<td>Flux</td>
</tr>
<tr>
<td>16</td>
<td>0.03 ± 0.11 passive</td>
<td>40</td>
<td>0.03 ± 0.00 passive</td>
<td>64</td>
<td>0.05 ± 0.00 passive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>1.52 ± 0.20 0.20 ± 0.03</td>
<td>42</td>
<td>2.03 ± 0.20 0.27 ± 0.03</td>
<td>66</td>
<td>2.90 ± 0.11 0.39 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.81 ± 0.37 0.24 ± 0.05</td>
<td>44</td>
<td>2.60 ± 0.24 0.35 ± 0.03</td>
<td>68</td>
<td>3.09 ± 0.38 0.41 ± 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>2.03 ± 0.59 0.27 ± 0.08</td>
<td>46</td>
<td>2.69 ± 0.08 0.36 ± 0.01</td>
<td>70</td>
<td>3.37 ± 0.28 0.45 ± 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>2.07 ± 0.69 0.28 ± 0.09</td>
<td>48</td>
<td>3.04 ± 0.17 0.41 ± 0.02</td>
<td>72</td>
<td>3.54 ± 0.30 0.47 ± 0.04</td>
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<td></td>
</tr>
</tbody>
</table>

Table 5: Passive and iontophoretic fluxes (µmol/h) and transport numbers of lithium (t_{Li}+) during experiment B (50:25). The data shows the mean ± the standard deviation of three replicates.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH 4.0</th>
<th></th>
<th>Time (h)</th>
<th>pH 5.0</th>
<th></th>
<th>Time (h)</th>
<th>pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flux</td>
<td>t_{Li}+</td>
<td></td>
<td>Flux</td>
<td>t_{Li}+</td>
<td></td>
<td>Flux</td>
</tr>
<tr>
<td>16</td>
<td>0.00 passive</td>
<td>40</td>
<td>0.01 ± 0.00 passive</td>
<td>64</td>
<td>0.01 ± 0.00 passive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>0.49 ± 0.07 0.07 ± 0.01</td>
<td>42</td>
<td>0.67 ± 0.05 0.09 ± 0.01</td>
<td>66</td>
<td>1.08 ± 0.04 0.15 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.64 ± 0.15 0.09 ± 0.02</td>
<td>44</td>
<td>0.88 ± 0.08 0.12 ± 0.01</td>
<td>68</td>
<td>1.19 ± 0.16 0.16 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>0.72 ± 0.21 0.10 ± 0.03</td>
<td>46</td>
<td>0.92 ± 0.05 0.12 ± 0.01</td>
<td>70</td>
<td>1.31 ± 0.13 0.18 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.73 ± 0.23 0.10 ± 0.03</td>
<td>48</td>
<td>1.05 ± 0.02 0.14 ± 0.00</td>
<td>72</td>
<td>1.37 ± 0.14 0.18 ± 0.02</td>
<td></td>
<td></td>
</tr>
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</table>

Table 6: Passive and iontophoretic fluxes (µmol/h) and transport numbers of sodium (t_{Na}+) during experiment C (50:50).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH 4.0</th>
<th></th>
<th>Time (h)</th>
<th>pH 5.0</th>
<th></th>
<th>Time (h)</th>
<th>pH 7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flux</td>
<td>t_{Na}+</td>
<td></td>
<td>Flux</td>
<td>t_{Na}+</td>
<td></td>
<td>Flux</td>
</tr>
<tr>
<td>16</td>
<td>0.01 ± 2.10^{-3} passive</td>
<td>40</td>
<td>0.02 ± 0.01 passive</td>
<td>64</td>
<td>0.07 ± 0.08 passive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>1.09 ± 0.23 0.15 ± 0.03</td>
<td>42</td>
<td>1.43 ± 0.21 0.19 ± 0.03</td>
<td>66</td>
<td>2.34 ± 0.38 0.31 ± 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.65 ± 0.31 0.22 ± 0.04</td>
<td>44</td>
<td>1.84 ± 0.16 0.25 ± 0.02</td>
<td>68</td>
<td>2.48 ± 0.69 0.33 ± 0.09</td>
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</tr>
<tr>
<td>22</td>
<td>1.82 ± 0.16 0.24 ± 0.02</td>
<td>46</td>
<td>1.77 ± 0.35 0.24 ± 0.05</td>
<td>70</td>
<td>3.06 ± 0.40 0.41 ± 0.05</td>
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</tr>
<tr>
<td>24</td>
<td>1.79 ± 0.15 0.24 ± 0.02</td>
<td>48</td>
<td>1.91 ± 0.09 0.26 ± 0.01</td>
<td>72</td>
<td>3.14 ± 0.41 0.42 ± 0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 7: Passive and iontophoretic fluxes (µmol/h) and transport numbers of lithium ($t_{Li^+}$) during experiment C (50:50).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH 4.0</th>
<th></th>
<th>pH 5.0</th>
<th></th>
<th>pH 7.0</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flux</td>
<td>$t_{Li^+}$</td>
<td>Flux</td>
<td>$t_{Li^+}$</td>
<td>Flux</td>
<td>$t_{Li^+}$</td>
</tr>
<tr>
<td>16</td>
<td>$2.10^3 \pm 1.10^3$ passive</td>
<td></td>
<td>40</td>
<td>$0.02 \pm 3.10^3$ passive</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>18</td>
<td>$0.87 \pm 0.34$ 0.12 ± 0.05</td>
<td></td>
<td>42</td>
<td>$0.98 \pm 0.17$ 0.13 ± 0.02</td>
<td></td>
<td>66</td>
</tr>
<tr>
<td>20</td>
<td>$1.13 \pm 0.24$ 0.15 ± 0.03</td>
<td></td>
<td>44</td>
<td>$1.29 \pm 0.12$ 0.17 ± 0.02</td>
<td></td>
<td>68</td>
</tr>
<tr>
<td>22</td>
<td>$1.23 \pm 0.11$ 0.16 ± 0.01</td>
<td></td>
<td>46</td>
<td>$1.26 \pm 0.23$ 0.17 ± 0.03</td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>24</td>
<td>$1.23 \pm 0.11$ 0.17 ± 0.01</td>
<td></td>
<td>48</td>
<td>$1.35 \pm 0.07$ 0.18 ± 0.01</td>
<td></td>
<td>72</td>
</tr>
</tbody>
</table>

Table 8: Passive and iontophoretic fluxes (µmol/h) and transport numbers of sodium ($t_{Na^+}$) during experiment D(50:250).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH 4.0</th>
<th></th>
<th>pH 5.0</th>
<th></th>
<th>pH 7.0</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flux</td>
<td>$t_{Na^+}$</td>
<td>Flux</td>
<td>$t_{Na^+}$</td>
<td>Flux</td>
<td>$t_{Na^+}$</td>
</tr>
<tr>
<td>16</td>
<td>$2.10^3 \pm 1.10^3$ passive</td>
<td></td>
<td>40</td>
<td>$0.02 \pm 5.10^3$ passive</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>18</td>
<td>$0.78 \pm 0.09$ 0.10 ± 0.01</td>
<td></td>
<td>42</td>
<td>$0.71 \pm 0.06$ 0.09 ± 0.01</td>
<td></td>
<td>66</td>
</tr>
<tr>
<td>20</td>
<td>$0.82 \pm 0.14$ 0.11 ± 0.02</td>
<td></td>
<td>44</td>
<td>$0.80 \pm 0.12$ 0.11 ± 0.02</td>
<td></td>
<td>68</td>
</tr>
<tr>
<td>22</td>
<td>$0.86 \pm 0.15$ 0.12 ± 0.02</td>
<td></td>
<td>46</td>
<td>$0.80 \pm 0.04$ 0.11 ± 0.01</td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>24</td>
<td>$0.92 \pm 0.04$ 0.12 ± 0.01</td>
<td></td>
<td>48</td>
<td>$0.86 \pm 0.09$ 0.11 ± 0.01</td>
<td></td>
<td>72</td>
</tr>
</tbody>
</table>

Table 9: Passive and iontophoretic fluxes (µmol/h) and transport numbers of lithium ($t_{Li^+}$) during experiment D (50:250).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH 4.0</th>
<th></th>
<th>pH 5.0</th>
<th></th>
<th>pH 7.0</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flux</td>
<td>$t_{Li^+}$</td>
<td>Flux</td>
<td>$t_{Li^+}$</td>
<td>Flux</td>
<td>$t_{Li^+}$</td>
</tr>
<tr>
<td>16</td>
<td>$2.10^3 \pm 2.10^3$ passive</td>
<td></td>
<td>40</td>
<td>$0.04 \pm 4.10^3$ passive</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>18</td>
<td>$2.52 \pm 0.47$ 0.34 ± 0.06</td>
<td></td>
<td>42</td>
<td>$2.66 \pm 0.32$ 0.36 ± 0.04</td>
<td></td>
<td>66</td>
</tr>
<tr>
<td>20</td>
<td>$3.20 \pm 0.54$ 0.43 ± 0.07</td>
<td></td>
<td>44</td>
<td>$2.93 \pm 0.52$ 0.39 ± 0.07</td>
<td></td>
<td>68</td>
</tr>
<tr>
<td>22</td>
<td>$3.36 \pm 0.58$ 0.45 ± 0.08</td>
<td></td>
<td>46</td>
<td>$2.96 \pm 0.10$ 0.40 ± 0.01</td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>24</td>
<td>$3.51 \pm 0.25$ 0.47 ± 0.03</td>
<td></td>
<td>48</td>
<td>$3.30 \pm 0.46$ 0.44 ± 0.06</td>
<td></td>
<td>72</td>
</tr>
</tbody>
</table>
Table 10: Passive and iontophoretic fluxes (µmol/h) and transport numbers of sodium ($t_{Na^+}$) during experiment E (50:500).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH 4.0</th>
<th></th>
<th>pH 5.0</th>
<th></th>
<th>pH 7.0</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flux</td>
<td>$t_{Na^+}$</td>
<td>Flux</td>
<td>$t_{Na^+}$</td>
<td>Flux</td>
<td>$t_{Na^+}$</td>
</tr>
<tr>
<td>16</td>
<td>0.02 ± 6.10$^{-3}$</td>
<td>passive</td>
<td>40</td>
<td>0.02 ± 0.01</td>
<td>passive</td>
<td>64</td>
</tr>
<tr>
<td>18</td>
<td>0.25 ± 0.04</td>
<td>0.03 ± 0.01</td>
<td>42</td>
<td>0.33 ± 0.02</td>
<td>0.04 ± 0.00</td>
<td>66</td>
</tr>
<tr>
<td>20</td>
<td>0.33 ± 0.02</td>
<td>0.04 ± 0.00</td>
<td>44</td>
<td>0.37 ± 0.02</td>
<td>0.05 ± 0.00</td>
<td>68</td>
</tr>
<tr>
<td>22</td>
<td>0.35 ± 0.02</td>
<td>0.05 ± 0.00</td>
<td>46</td>
<td>0.37 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td>70</td>
</tr>
<tr>
<td>24</td>
<td>0.35 ± 0.02</td>
<td>0.05 ± 0.00</td>
<td>48</td>
<td>0.39 ± 0.02</td>
<td>0.05 ± 0.00</td>
<td>72</td>
</tr>
</tbody>
</table>

Table 11: Passive and iontophoretic fluxes (µmol/h) and transport numbers of lithium ($t_{Li^+}$) during experiment E (50:500)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH 4.0</th>
<th></th>
<th>pH 5.0</th>
<th></th>
<th>pH 7.0</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flux</td>
<td>$t_{Li^+}$</td>
<td>Flux</td>
<td>$t_{Li^+}$</td>
<td>Flux</td>
<td>$t_{Li^+}$</td>
</tr>
<tr>
<td>16</td>
<td>0.02 ± 0.00</td>
<td>passive</td>
<td>40</td>
<td>0.06 ± 3.10$^{-3}$</td>
<td>passive</td>
<td>64</td>
</tr>
<tr>
<td>18</td>
<td>1.73 ± 0.27</td>
<td>0.23 ± 0.04</td>
<td>42</td>
<td>2.22 ± 0.09</td>
<td>0.30 ± 0.01</td>
<td>66</td>
</tr>
<tr>
<td>20</td>
<td>2.25 ± 0.27</td>
<td>0.30 ± 0.04</td>
<td>44</td>
<td>2.39 ± 0.18</td>
<td>0.32 ± 0.02</td>
<td>68</td>
</tr>
<tr>
<td>22</td>
<td>2.41 ± 0.30</td>
<td>0.32 ± 0.04</td>
<td>46</td>
<td>2.48 ± 0.21</td>
<td>0.33 ± 0.03</td>
<td>70</td>
</tr>
<tr>
<td>24</td>
<td>2.40 ± 0.20</td>
<td>0.32 ± 0.03</td>
<td>48</td>
<td>2.62 ± 0.24</td>
<td>0.35 ± 0.03</td>
<td>72</td>
</tr>
</tbody>
</table>

To the best of our knowledge this is the first time that passive and iontophoretic fluxes of sodium and lithium across the nail have been reported. Both cations were easily measured in passive and iontophoretic samples.

Lithium was introduced as a competing ion in these experiments because of similar previous experiments done with skin and because it can be considered as an exogenous ion and thus, it can assess the effective transport of the two cations across the nail plate. Indeed, trace amounts of sodium (19 µmol/g of nail) have been found in nails (Walters et al., 1983a; Vellar, 1970) and thus, it seemed sensible to study sodium delivery along with lithium delivery to characterize the cations electrorepulsive flux. Therefore, lithium fluxes reported here originate not from the nail (as could be the case for the ubiquitous sodium) but from the donor solution and thus, showed that lithium has been transported across the complete nail thickness. It could be concluded that the iontophoretic transport of small inorganic ions is relatively fast.

On one hand, Murthy et al. (2007b) reported a lag time of ~8 h for salicylic acid during iontophoresis and passive diffusion and Hao et al. (2008a) found that the lag time for mannitol was around 12 h during passive diffusion. In our case, no conclusion can be
drawn on the passive lag time as only one sample was taken during this period. Although iontophoresis provoked a very quick response for both cations, iontophoretic fluxes had not completely stabilized after 8 hours of current application and this delay was more evident at pH 7.0. Hao et al. (2008a) reported that the iontophoretic fluxes of tetraethylammonium were not stable even after 36 h of current applications. However, comparison of the data is difficult given the different experimental conditions applied. It must be noticed that the concept of steady-state in passive diffusion is not applicable to iontophoresis. In fact, the factors determining the iontophoretic fluxes stability are not known: factors such as depletion of the membrane ion reservoir have been cited (Nixon et al., 2007; Wascotte et al., 2008) but the role played by the ion physicochemical properties has not been clarified.

The iontophoretic fluxes of both lithium and sodium were always greater than their respective passive controls. Iontophoretic fluxes were 13 to 615 fold more than passive diffusion. Another interesting observation is that passive fluxes fall to pre-iontophoresis values upon current termination suggesting that the nail permeability had not been permanently damaged (increased) by current application.

![Fig. 7: Sodium flux (Mean ± SD) measured after 8 hours of iontophoresis during experiment A (100:0). ** and *** denote a very significant (p = 0.001 to 0.01) and an extremely significant (p < 0.001) difference respectively.](image)

![Fig. 8: Sodium and lithium fluxes (Mean ± SD) measured after 8 hours of iontophoresis during experiment B (50:25). * indicates a significant (p = 0.01 to 0.05) difference.](image)
Fig. 9: Sodium and lithium fluxes (Mean ± SD) measured after 8 hours of iontophoresis during experiment C (50:50). ** indicates a very significant (p = 0.001 to 0.01) difference.

Fig. 10: Sodium and lithium fluxes (Mean ± SD) measured after 8 hours of iontophoresis during experiment D (50:250). * indicates a significant (p = 0.01 to 0.05) difference.

Fig. 11: Sodium and lithium fluxes (Mean ± SD) measured after 8 hours of iontophoresis during experiment E (50:500). ** indicates a very significant (p = 0.001 to 0.01) difference.
Fig. 12: Sodium transport number (Mean ± SD) after 8 hours of iontophoresis during experiment A (100:0). ** and *** denote a very significant (p = 0.001 to 0.01) and an extremely significant (p < 0.001) difference respectively.

Fig. 13: Sodium and lithium transport numbers (Mean ± SD) after 8 hours of iontophoresis during experiment B (50:25). * indicates a significant (p = 0.01 to 0.05) difference.

Fig. 14: Sodium and lithium transport numbers (Mean ± SD) after 8 hours of iontophoresis during experiment C (50:50). ** indicates a very significant (p = 0.001 to 0.01) difference.

Fig. 15: Sodium and lithium transport numbers (Mean ± SD) after 8 hours of iontophoresis during experiment D (50:250). * indicates a significant (p = 0.01 to 0.05) difference.
Fig. 16: Sodium and lithium transport numbers (Mean ± SD) after 8 hours of iontophoresis during experiment E (50:500). ** indicates a very significant (p = 0.001 to 0.01) difference.

Two main observations are easily drawn from these data:
1. Sodium and lithium fluxes and transport numbers (Figs 7 to 16, Tables 3 to 11) increased with the pH of the bathing solutions. The effect was more evident and often statistically significant when the pH was raised to 7.0. The results suggest that the nail net charge is positive at pH 4 and negative at pH 7.0. It follows that the nail would exhibit anion permselectivity at low pH and cation permselectivity (shown in cationic transport numbers > 0.5) at pH 7.0.

2. Sodium and lithium fluxes and transport numbers (Figs 7 to 16, Tables 3 to 11) were clearly related to the molar fraction and mobility of each cation. $t_{Na^+}$ decreases progressively as the concentration of competing lithium is increased. $t_{Li^+}$ continuously increased as the donor solution became richer in this ion. The effect of molar fraction and mobility on $t_{Na^+}$ and $t_{Li^+}$ is very similar to that observed during transdermal iontophoresis (Mudry et al., 2006 a). The results are next discussed in detail.

2. Effect of cation mobility and concentration ratio on cationic transport numbers

Phipps and Gyory (1992) proposed a model to predict transport numbers during transdermal iontophoresis (Eq. 3). The model assumes: (1) the biological membrane to be homogenous and uncharged, (2) constant current application and (3) the total cation concentration at the anodal chamber to be the same as the anion concentration at the cathodal solution.

$$t_d = \frac{c_d z_d \mu_d}{\sum_i c_i z_i \mu_i}$$

Eq. 3

where $t_i$, $c_i$, $\mu_i$ and $z_i$ represent transport number, concentration, mobility and valence of the ion, “i”, within the membrane, respectively. The sum term includes all ions on both sides.
of the membrane, thus chloride, lithium and sodium (considering negligible the contribution of the HEPES charged species).

The above equation indicates that both concentration and mobility are equally important in determining the magnitude of transport numbers. If lithium and sodium (having the same valence) were present at equal concentration in the donor solution, their transport numbers would be different unless their respective mobilities in the membrane were the same. In fact, it has been shown for the skin (Mudry et al., 2006a), and now for the nail (Experiment C, Figs 9 and 14, Tables 6 and 7) that \( t_{Li^+} \) was lower than \( t_{Na^+} \) in these conditions. This suggests the mobility of lithium in the nail and the skin to be lower than that of sodium, which is in agreement with their respective aqueous mobilities of 4.01 and 5.19 \( \times 10^{-4} \) cm\(^2\).s\(^{-1}\).V\(^{-1}\) for lithium and sodium respectively (Brett et al., 1993).

It was interesting to test the validity of Eq. 3 even when most of the assumptions are clearly not respected in skin and nail iontophoresis. Further, the values of ion mobility and concentration in the membrane are not necessarily the same as the aqueous mobility and concentration in the donor formulation are those usually known.

Eq. 3 can be rewritten for sodium and lithium transport numbers. Chloride is the counter-ion and all ions are monovalent \((z =1)\). This gives Eq. 4 and 5:

\[ t_{Na^+} = \frac{(C_{Na^+} \cdot \mu_{Na^+})}{(C_{Li^+} \cdot \mu_{Li^+} + C_{Na^+} \cdot \mu_{Na^+} + C_{Cl^-} \cdot \mu_{Cl^-})} \quad \text{Eq. 4} \]

\[ t_{Li^+} = \frac{(C_{Li^+} \cdot \mu_{Li^+})}{(C_{Li^+} \cdot \mu_{Li^+} + C_{Na^+} \cdot \mu_{Na^+} + C_{Cl^-} \cdot \mu_{Cl^-})} \quad \text{Eq. 5} \]

By multiplying numerator and denominator in Eq. 5 by \((C_{Na^+} \cdot \mu_{Na^+})\) the following expression (Eq. 6) is obtained:

\[ t_{Li^+} = \frac{(C_{Li^+} \cdot \mu_{Li^+}) (C_{Na^+} \cdot \mu_{Na^+})}{(C_{Na^+} \cdot \mu_{Na^+})(C_{Li^+} \cdot \mu_{Li^+} + C_{Na^+} \cdot \mu_{Na^+} + C_{Cl^-} \cdot \mu_{Cl^-})} \quad \text{Eq. 6} \]

which can be rearranged by use of Eq. 4 to give Eq. 7:

\[ t_{Li^+} = t_{Na^+} \cdot \frac{(C_{Li^+} \cdot \mu_{Li^+})}{(C_{Na^+} \cdot \mu_{Na^+})} \quad \text{Eq. 7} \]

and Eq. 8:

\[ \frac{t_{Li^+}}{t_{Na^+}} = \frac{(C_{Li^+} / C_{Na^+}) \cdot (\mu_{Li^+} / \mu_{Na^+})}{\mu_{Na^+}} \quad \text{Eq. 8} \]
Eq. 8 predicts that the ratio of lithium and sodium transport numbers is directly related to their relative concentration and mobility inside the membrane.

If ion mobilities were independent of ion concentration and pH, the ratio \( \mu_{Li^+} / \mu_{Na^+} \) would be constant for each series of experiments performed at a given pH. If this is true, the ratio \( t_{Li^+} / t_{Na^+} \) would be linearly dependent on the cations concentration ratio \( C_{Li^+} / C_{Na^+} \). The validity of this relationship has been investigated and the results are shown in Fig. 17 for the nail experiments at pH 4, 5 and 7. The same approach (Fig. 18) was used for transdermal iontophoresis plotting data previously reported (Mudry et al., 2006a). The results of the linear regression analysis are presented in Table 12.

![Fig. 17: Relationship between the ratio of lithium and sodium transport numbers (\( t_{Li^+}/t_{Na^+} \)) calculated from the average transport number values) measured during transungual iontophoresis at three different pHs and the ratio of cation concentration in the donor solution (\([Li^+]/[Na^+]\)). All linear regressions were significant (p < 0.05) with \( r^2 > 0.98 \).](image1)

![Fig. 18: Relationship between the ratio of lithium and sodium transport numbers (\( t_{Li^+}/t_{Na^+} \)) measured during transdermal iontophoresis and the ratio of cation concentration in the donor solution (\([Li^+]/[Na^+]\)). The linear regression was significant (p < 0.05) with \( r^2 > 0.9 \) (Data taken from Mudry et al., 2006a).](image2)
Table 12: Linear regression analysis examining the dependence of the ratio of cation transport number ($t_{Li^+}/t_{Na^+}$) on the ratio of cation concentration ($C_{Li^+}/C_{Na^+}$) according to Eq. 8: $t_{Li^+}/t_{Na^+} = (C_{Li^+}/C_{Na^+}) \cdot (\mu_{Li^+}/\mu_{Na^+})$. The values of the slope ($\mu_{Li^+}/\mu_{Na^+}$) and Y-intercept are reported as estimate $\pm$ SE.

<table>
<thead>
<tr>
<th>pH</th>
<th>Y intercept $\pm$ SE</th>
<th>Slope ($\mu_{Li^+}/\mu_{Na^+}$) $\pm$ SE</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>$0.19 \pm 0.31$</td>
<td>$0.65 \pm 0.05$</td>
<td>0.99</td>
</tr>
<tr>
<td>5.0</td>
<td>$0.06 \pm 0.23$</td>
<td>$0.71 \pm 0.04$</td>
<td>0.99</td>
</tr>
<tr>
<td>7.0</td>
<td>$0.28 \pm 0.39$</td>
<td>$0.67 \pm 0.07$</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Pig skin$^{(1)}$

<table>
<thead>
<tr>
<th>pH</th>
<th>Y intercept $\pm$ SE</th>
<th>Slope ($\mu_{Li^+}/\mu_{Na^+}$) $\pm$ SE</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-6</td>
<td>$-0.01 \pm 0.02$</td>
<td>$0.79 \pm 0.03$</td>
<td>0.99</td>
</tr>
</tbody>
</table>

(1) Data taken from Mudry et al., 2006a.

Figures 17-18 and Table 12 show that equation 8 fits well the data; a very good linear relationship between the ratio of cation transport numbers and the ratio of cation concentration was observed in nail and skin. The lithium-to-sodium mobility ratio ranged between 0.65 and 0.71 in nail experiments and was 0.79 for the skin. These values are remarkably similar among them. Further, the ratio of the aqueous mobility of lithium and sodium is 0.77 ($4.01 \times 10^{-4}$ for lithium and $5.19 \times 10^{-4}$ cm$^2$.s$^{-1}$.V$^{-1}$ for sodium (Brett et al., 1993)). It seems that the mobility ratios ($\mu_{Li^+}/\mu_{Na^+}$) in nails, skin and water are fairly similar. This suggests that the ionic pathway followed by small inorganic ions across nail and skin has a certain hydrophilic nature.

3. **Effect of cation molar fraction on cationic transport number**

It has been demonstrated that cations transport number in *in vivo* and *in vitro* transdermal iontophoresis are linearly dependent to their molar fraction in the vehicle (Mudry et al. 2006b). The relationship is described by Eq.(9):

$$t_{C^+} = a.X_{C^+} + b \quad \text{Eq. 9}$$

where $t_{C^+}$ is the transport number of the cation $C^+$, “a” is the slope of the linear regression, $X_{C^+}$ is the molar fraction of cation $C^+$ in the donor aqueous solution and “b” is the intercept of the linear regression with the Y axis.

Further, it has been shown that by substitution of $X_{C^+} = 1$ in the above equation, the value of the transport number in the “single-ion” situation ($t_{C^+,SI}$) could be reasonably well predicted. Finally the slope of the linear regression was directly related to the aqueous mobility of the ion considered. For example, when the aqueous mobilities of lidocaine, lithium and sodium were 1.5, 4.01 and $5.19 \times 10^{-4}$ cm$^2$.s$^{-1}$.V$^{-1}$ respectively, the values of the slopes were 0.18, 0.54 and 0.56 for lidocaine, lithium and sodium, respectively (Mudry et al., 2006b). It seemed interesting to examine the validity of this relationship during
transungual iontophoresis and for this, sodium and lithium transport numbers were plotted against their respective molar fraction (Fig. 19) in the donor solution. The analysis of regression was done for each pH and cation and the results are summarised in Table 13.

![Fig. 19: Linear relationships between cation transport number \(t_{C^+}\) and molar fraction \(X_{C^+}\). The transport numbers were determined in experiments involving binary ion donor solutions. The symbols represent experimental values and the lines are linear regressions. All \(r^2 > 0.78\). All regressions were significant (p<0.05) except for \(t_{Li^+}\) versus \(X_{Li^+}\) at pH 4 and 5.](image)

Table 13: Linear regression analysis examining the dependence of cation transport number \(t_{C^+}\) on molar fraction \(X_{C^+}\): \(t_{C^+} = a X_{C^+} + b\). The values of the slope (a) and Y-intercept (b) are reported as estimate ± SE. The experimental transport numbers in the “single-ion” situation \(t_{C^+, SI}\) for sodium are also presented.

<table>
<thead>
<tr>
<th></th>
<th>Lithium</th>
<th>Sodium</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
<td>(a)</td>
</tr>
<tr>
<td>Nail</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 4.0</td>
<td>0.54 ± 0.20</td>
<td>-0.08 ± 0.10</td>
<td>0.38 ± 0.06</td>
</tr>
<tr>
<td>pH 5.0</td>
<td>0.48 ± 0.15</td>
<td>-0.03 ± 0.10</td>
<td>0.57 ± 0.06</td>
</tr>
<tr>
<td>pH 7.0</td>
<td>0.84 ± 0.16</td>
<td>-0.10 ± 0.11</td>
<td>0.61 ± 0.12</td>
</tr>
<tr>
<td>Skin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 5-6(1)</td>
<td>0.54 ± 0.01</td>
<td>-0.01 ± 1.10^{-3}</td>
<td>0.56 ± 0.02</td>
</tr>
</tbody>
</table>

(1) Mudry et al. (2006 b), pig skin.

The slopes of the regressions obtained with nail data increased clearly with the pH. As previously discussed the value of the slope is an excellent predictor of the transport number in the single ion situation and is directly related to the ion aqueous mobility. Thus the data in Table 13 indicates that sodium and lithium transport numbers in the single ion situation increase with pH. This agrees with the experimental data. We believe the increase in the values to reflect the switch from anion to cation permselectivity in the nail which translates into higher transport numbers at pH 7.

The transport numbers measured during transdermal and transungual iontophoresis are also in a relatively good agreement. It should be noticed that the values in Table 13 were obtained with dermatomed frozen pig skin and were used in the Table because of the very similar experimental conditions followed in both this and Mudry’s work. However, the
variation of sodium transport number with pH has also been reported for human skin. For example, Kasting et al. (1990) used frozen dermatomed (0.25 µm) human skin and PBS buffer finding that $t_{Na^+}$ was 0.24 and 0.51 at pH 4.0 and pH 7.4, respectively. Burnette et al. (1987) used freshly excised dermatomed (0.8 mm) human skin and pH 7.4 HEPES buffer finding that $t_{Na^+}$ was 0.62. Given the variability observed for the same skin model and for different skin models (pig versus human skin) it could be considered that the difference between nails and skin is relatively low.

It remains to be seen if the relationship here observed between the transungual transport number of an ion and its molar fraction in the vehicle can be extended to therapeutic molecules. If that is the case, drug transport numbers in the “single-ion” situation could be predicted from experiments performed in binary mixtures, as it has been shown for lidocaine and lithium in transdermal iontophoresis (Mudry et al., 2006b). This is handy in the case of cationic drug not available as chlorhydrate salts or possessing limited solubility that must be combined with chloride salts in the anodal formulation.

4. *Total cationic transport number* ($\sum t_{C^+} = t_{Na^+} + t_{Li^+}$).

Mudry et al. (2006a) measured the transport numbers of small inorganic ions (lithium, sodium, ammonium and potassium), in “single-ion” formulation and in binary and quaternary vehicles during transdermal iontophoresis. It was observed that the total cationic transport number ($\sum t_{C^+}$), defined as the sum of the transport number of the cations present in solution, was remarkably similar despite of the big variation in total cation concentration used in these experiments. This suggests that it is not possible to eliminate completely chloride competition from underneath the skin, and the existence of a cationic threshold ranging between 0.65 and 0.85 for a total ionic concentration of 125 to 200 mM. Thus, unless extreme differences are introduced in the overall mobility of a donor (anodal) formulation, changes in the vehicle composition will result in a re-arrangement of the cations transport numbers but chloride transport number will remain unchanged. For example, if a drug is present in a buffer, additional amounts of sodium chloride to the buffer will reduce the transport number of the drug, but will not decrease that of chloride.

Based on these observations, we have calculated the total cation transport number in all our experiments as defined by $\sum t_{C^+} = t_{Na^+} + t_{Li^+}$. The values are shown in Fig. 20.
Fig 20 shows that $\Sigma t_{C+}$ increased with pH, pointing towards a modification in nail permselectivity. It must be noticed that the experiments involved very different total cation ionic concentration in the donor solution, which ranged from 75 mM (Expt B) to 550 mM (Expt. E). However there was no sign that total molarity had an effect on $\Sigma t_{C+}$.

- At pH 4.0, $\Sigma t_{C+}$ ranged between 0.35 and 0.59, experiments A and D, respectively.
- At pH 5.0, $\Sigma t_{C+}$ ranged between 0.40 and 0.56, experiments E and D, respectively.
- At pH 7.0, $\Sigma t_{C+}$ ranged between 0.58 and 0.88, experiments B and A, respectively.

During skin experiments at pH 5-6, $\Sigma t_{C+}$ ranged between 0.65 and 0.85 (Murthy et al., 2006a).

There are some differences between the values reported between nail and skin which probably reside in the slightly different experimental approach. Mudry et al. (2006a) did not use any buffer or pH adjustment to perform their experiments to avoid the presence of any ion other than the cations investigated. In the present work, buffered solutions were required to investigate the effect of pH. HEPES was incorporated as the free acid and the pH adjusted either with hydrochloric acid or potassium hydroxide. It was considered that the competition induced by these co and counter-ions (0.6-0.8 mM HCl at pH 4.0, 0.07-0.08 mM HCl at pH 5.0 and 0.9-1.4 mM KOH at pH 7.0) was negligible. Indeed, the decrease in the cation transport numbers of sodium and lithium (at least 75 mM) by a maximum 1.4 mM KOH at pH 7.0 would be certainly small. Another difference between the two studies is the total cation concentration employed: 125 to 200 mM for Mudry et al. (2006a) and 75 to 550 mM for our work.

On the whole these results suggest that despite huge variations in the total cation concentration (75-550 mM) in the anodal chamber, chloride being present at only 10 mM keeps a minimum transport number of ~0.12. This agrees with the threshold observed in skin experiments.
Conclusions of chapter 4

This work has demonstrated that several principles concerning transdermal iontophoresis were also applicable to transungual iontophoresis. We have demonstrated (a) a relationship between the ratio of $t_{Li^+}/t_{Na^+}$ and the ratio $[Li^+]/[Na^+]$ for the first time, (b) a linear relationship between a cation transport number and its molar fraction, (c) the existence of a threshold or maximal total cation transport number achievable by manipulation of the formulation and (d) evidence of nail cation permselectivity at pH 7.
Chapter 5:

*In vivo* transungual iontophoresis: Effect of current application on ion transport and on transonychial water loss

Overview

The *in vitro* nail iontophoretic experiments described in the previous chapters and elsewhere in the literature indicate the potential of using iontophoresis to improve drug penetration into the nail. However, there is almost no information concerning transungual iontophoresis *in vivo*. Nothing is known about key issues such as the effect of current application on ionic transport and on transonychial water loss (TOWL) and the magnitude of the voltages required for a practical use of the technique. This work describes the application of transungual iontophoresis to six healthy human volunteers. Each volunteer participated in three experiments: passive control, 0.2 mA anodal transungual iontophoresis and 0.2 mA cathodal transungual iontophoresis. A commercial electrode on a skin site was used to complete the electrical circuit. The outward extraction of sodium and chloride ions by passive diffusion and iontophoresis was also quantified. Iontophoresis enhanced chloride and sodium transport ~8 and 27 folds respectively compared to passive diffusion. Only sodium transport numbers could be measured with certainty to be $t_{Na^+} = 0.51 \pm 0.11$. TOWL was used as a potential marker of nail damage and hydration. Basal TOWL was measured before each experiment, and the return to baseline values was monitored for one hour after the treatment (passive or iontophoresis application) was finished. TOWL was increased after both iontophoretic and passive experiments and typically returned to baseline values in one hour post-treatment. The voltages of the nail-skin circuits were monitored during iontophoresis and the values compared to a skin-skin circuit. Nail-skin circuit voltages were generally ~50 V when the current was started and dropped fast to 20-30 V, a value comparable to that observed in the skin-skin circuit. On the whole, the clear enhancement of ionic transport observed, the feedback from volunteers, the small effects in TOWL, and the magnitude of voltages indicate that nail iontophoresis is feasible and probably a safe technique.
Introduction
The treatment of nail diseases such as onychomycosis is far from satisfactory. Oral treatments are efficient in delivering the drug but cause numerous side effects and interactions. Given the prevalence and cost of the disease (Elewski, 2000; Einarson et al., 1996) it is not surprising that the development of new topical treatments has received increased interest. However, research in nail drug delivery is usually limited by the difficulties associated with nail sourcing. Human nails consists of three layers (Dawber, 1980; Kobayashi et al., 1999) which are different in composition and keratin fibres orientation as described in the first two chapters of this thesis, although their respective roles in drug permeation have not been completely characterized (Kobayashi et al., 1999). Hooves and keratin disks have been used to screen topical formulations but their usefulness as a predictive tool for human nail permeation has not been established. Nail plates are scarce, expensive and difficult to obtain as they are typically sourced from cadaver or amputations. Thus, the use of nail clippings or nail tips as a model to study transungual drug delivery seems the most sensible approach. However, the nail plate is firmly attached to the nail bed, while nail clippings are obtained from the free edge. It follows that the hydration of the free edge and of the attached nail plate must be significantly different. No information is available about differences in the structure of these two parts of the nail except for an increase in the nail thickness during its growth which has been reported by Johnson et al. (1994). At the present time, it is not known if any of these differences could be significant in drug permeation in general and even less in iontophoresis.

In vivo transungual iontophoresis has hardly been reported. James et al. (1986) measured plasma levels of prednisolone after anodal iontophoresis applied with a metal electrode. The thumbnails were first soaked in soapy water for 5 min to decrease their electrical resistance and then, a current density of 0.63 mA/cm$^2$ was applied for 10 minutes. Prednisolone plasma concentrations were ~50 ng/mL after only 15 minutes of iontophoresis and remained detectable for 15 days. This study was the first to show the feasibility of transungual iontophoresis. However the amount of drug delivered is very surprising. For example, taken an average volume of distribution for the central compartment of 36 L (Magee et al., 2001), it follows that ~1.8 mg (~5 µmoles) of drug were delivered in only 10 minutes. The actual size of the patch used on the nail is not given but one could assume an average thumb nail to be a 2 cm$^2$ square, so the current effectively passed was 1.3 mA, or in 10 minutes, ~8 µmoles of electrons. This means that a charged drug would have transported ~60% of the total charge (i.e., 0.6 transport number) a value extremely high. These results are further problematic because prednisolone (MW 360) is a
neutral drug (Vogt et al., 2007). Thus, prednisolone could have only been transported by electroosmosis a much weaker mechanism of transport (as seen in Chapter 3). Finally, the patch would have become very acidic because metal electrodes that hydrolyze water were used. This acidic pH could be related to the different tolerance to current by the four individuals. Thus this study was not performed in the best conditions for iontophoresis such as the use of reversible electrodes (such as Ag/AgCl) which do not acidify the pH.

The second article refers to a case study (Sakata et al., 2007). Iontophoresis of dexamethasone was used to treat nail psoriasis. However the iontophoretic application had actually occurred on the skin. No information is provided about the outcome of the treatment. Finally none of these two reports show unequivocally the usefulness of iontophoresis as none of them included passive controls.

There is very little known about the feasibility of applying iontophoresis in vivo. Transdermal iontophoresis is considered a minimally invasive and safe technique. Indeed several iontophoretic devices (Glucowatch®, LidoSite®, Ionsys®) have been approved by the regulatory agencies. However, the thickness and the structure of nail and skin are very different. First of all, the stratum corneum, or main barrier to skin permeation has an average thickness of 10-20 µm (Pailler-Mattei et al., 2007) while the nail thickness varied from 200 µm to more than 1 mm. Second, the contribution of the appendegeal (hair follicles and sweat glands) pathways to both electromigration and electroosmotic transport during transdermal iontophoresis (Cullander et al., 1992; Scott et al., 1992; Bath et al., 2001) is not possible for the nail plate. Thus, one could expect the nails to offer a greater electrical resistance to the passage of current, and that the high voltages required for current application could damage the nail. Indeed, the previous data in this thesis and the literature have shown that high voltages (> 200 V) are usually observed in vitro when iontophoresis is started unless the nails are hydrated for relatively long times. These observations challenged the future of transungual iontophoresis as a drug delivery technique. Neither the voltages observed in vitro without extensive hydration (as those measured in previous chapters) are acceptable, nor are the extensive hydration times applied in other studies (6 and 24 h for Murthy et al. (2007) and Hao et al. (2008), respectively). However, James’ publication and preliminary experiments (unpublished) in our laboratory indicated that transungual iontophoresis is feasible in vivo after a short period of hydration. Therefore, one main objective of this chapter was to determine if iontophoresis is an acceptable procedure in vivo.

The skin barrier integrity after iontophoresis has been investigated using impedance spectroscopy (Curdy et al., 2002), infrared spectroscopy (Brand et al., 1997) TEWL (TransEpidermal Water Loss), and laser doppler velocimetry (Thysman et al., 1995; Van
der Geest et al., 1996; Anigbogu et al., 2000). TEWL is the insensible loss of water from the body through the skin and it is considered a good marker of the skin barrier. The term TOWL or TransOnychial Water Loss simply indicates that the measurement takes place on the surface of the nail plate. In comparison to TEWL, TOWL has been very rarely reported (Spruit, 1971; Jemec et al., 1989; Kröner et al., 2001; Murdan et al., 2008). It is not clear yet if TOWL can be used as a marker for nail damage in the same way as TEWL is routinely applied. The skin has a crucial barrier function, most importantly to limit water loss from the body and the increased TEWL, consequence of barrier perturbation, acts as a “danger” signal to induce barrier repair (Fluhr et al., 2006). The thick nail plate structure also limits passage of water and it is expected that a damaged nail would be more permeable to water and show higher values of TOWL. However the nail function is not as strongly associated to water loss regulation as the skin is; and, for example, to the best of our knowledge, no studies have been done in which nails were damaged at different degree, and the recovery was monitored by TOWL.

This work has investigated the in vivo nail application of iontophoresis in six healthy volunteers. The objectives were to 1) measure and compare the transungual sodium and chloride transport during passive and iontophoretic conditions, 2) investigate the effect of iontophoresis on TOWL as a possible marker of the nail barrier, and 3) decide on the feasibility of iontophoresis as a drug delivery technique.

**Materials and methods**

1. **Materials**

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) free acid, magnesium chloride, potassium hydroxide 1.0 N in water, sodium hydroxide 50% (ion chromatography eluent grade), sodium chloride and methanesulfonic acid were obtained from Sigma Aldrich Co. (Gillingham, UK). All aqueous solutions were prepared using high purity deionised water (18.2 MΩ·cm, Barnstead Nanopure Diamond™, Dubuque, IA, USA). Silver wire, silver chloride powder and platinum wire used to prepare the electrodes had a minimum purity of 99.99% and were obtained from Sigma Aldrich Co. (Gillingham, UK).

2. **Volunteers**

Ethical approval was granted by the Salisbury Local IRB (UK). All volunteers gave their written informed consent prior to any experiment (See Appendix 4).

A first study to collect TOWL values was performed on six healthy volunteers (A to F, 1 male and 5 females) aged between 21 and 43 years old.
Six healthy volunteers (V1 to V6, 3 females and 3 males aged between 25 and 44 years old) participated in the transport/TOWL experiments. Six volunteers participated in three experiments (nail anodal iontophoresis, nail cathodal iontophoresis and passive) performed on the same nail in different days separated at least by 24 h. Five of the volunteers participated also in a skin-to-skin iontophoresic experiment.

3. Methods

Basal TOWL:
Six volunteers participated in the first basal TOWL study (A to F). The TOWL was measured in six replicates on each fingernail of both hands.
The TOWL measurements were performed using a condenser-chamber AquaFlux evaporimeter from Biox Systems Ltd (London, UK). A nail adapter and corresponding correction factor were kindly supplied by Biox Systems Ltd. The probe was placed on the nail; TOWL measurements started after 80 seconds of equilibration (Fig. 1). The final value was the mean of 15 measurements taken between 80-240 seconds of contact and with a CV target precision of 1%. The relative humidity and temperature of the room ranged between 26 and 44 % and between 20 and 26 °C.

Fig. 1: TOWL measurement.

Nail iontophoresis:
All nail-to-skin experiments were performed on different days separated at least by 24 h. After giving their informed consent, the volunteers were asked to indicate on which hand they would prefer the experiments to take place. Being right-handed, they all opted for their left hand which was subsequently used for all experiments.
The thickness of the thumbnail was measured before the first experiment. The measurement took place on the free edge of the nail using a point micrometer (Point Anvil Micrometer, Mitutoyo, Andover, UK). Due to the shortness of certain nails, some measurements lacked precision and are given as a range.
Next, the baseline TOWL was measured, as described previously, in three replicates prior to experiment.

Some preliminary work was done to overcome several challenges offered by nail iontophoresis. First of all, the nail electrode chamber had to adapt to the curved surface of the nail plate, and had to be fixed using an acceptable glue for human use. A home made high-grade polypropylene chamber was used for the thumbnail site to contain the buffer and the electrode. The chamber (~0.40 cm$^2$ contact surface area and ~ 2.5 cm high) was fixed to the thumbnail using MD 7-4602 silicone kindly provided by Dow Corning Europe SA (Seneffe, Belgium).

A dispersive pad (rectangular shape with round corners, 4.6 cm x 5.6 cm, ~25 cm$^2$) from Iogel gelsponge electrodes’ kits (Balego & Associates, Inc., Saint Paul, MN, USA) was placed on the dorsal face of the hand to close the electrical circuit. In the nail anodal experiments (chloride extraction), a conditioned Ag/AgCl electrode (Green et al., 1991) was placed in the nail chamber and connected to the positive pole of a Phoresor II power supply (Iomed Inc., Salt Lake City, UT, USA); the cathodal skin dispersive pad was connected to the negative pole. In the nail cathodal experiment (sodium extraction), the Ag/AgCl electrode was placed in the nail chamber and connected to the negative pole of the Phoresor II whereas the anodal skin dispersive pad was connected to the positive pole (Fig. 2).

Fig. 2: In vivo experimental set-up. The picture above shows a nail cathodal experiment (negative pole applied on the nail site, positive pole applied on the skin site).

The nail chamber was filled with 1 mL of a pH 7.0 buffer (HEPES 5 mM added with MgCl$_2$ 5 mM) for (at least) three minutes to facilitate the nail hydration, before current application. The volunteers usually reported an uncomfortable “tingling effect” on the skin when current was applied directly after filling the nail chamber. After this short hydration
time (usually 3 to 10 min) current was started and four 30 minute periods of a 0.2 mA direct constant current were applied.

At the end of each iontophoretic period, the whole content of the nail chamber was sampled, filtered with 0.22 µm filters (Millipore, Sigma Aldrich Co., UK) and frozen at –20 ºC until analysis. The samples were analyzed for sodium (nail cathodal experiments) and for chloride (nail anodal experiments) as described below. The nail chamber was then filled again with 1 mL of fresh buffer and the following iontophoretic period started.

The voltage of the nail-skin circuit was monitored during the whole experiment with a voltmeter as follows: the voltage was recorded every minute for the first 10 minutes of the first iontophoretic period, then every 5 minutes and finally 1 min before the end. For each subsequent iontophoretic period, the voltage was taken every 5 min and in addition, 1 min after the beginning and 1 min before the end.

At the very end of each experiment, the electrode, dispersive pad and nail chamber were removed and the silicone was wiped off with isopropyl alcohol obtained from Sigma Aldrich Co (UK). 5 minutes after wiping with isopropyl alcohol, the TOWL was monitored during one hour (at 0, 5, 10, 15, 20, 30, 45 and 60 min).

Despite MD 7-4602 silicone being very effective glue, leaks were observed at the fourth period of current application of nail cathodal iontophoresis for V5 and of nail anodal iontophoresis for V3. In the case of V4, the nail cathodal iontophoresis experiment was not possible probably due to the high electrical resistance presented by this thumbnail.

The volunteers were asked to fill a form (see Appendices for forms) at the end of each iontophoretic experiment to score the discomfort experienced at the nail site, the skin site and overall by using a scale from 0 to 5 (5 being a very high discomfort).

Passive experiments:
A passive diffusion experiment was performed on each volunteer. Briefly, three repeated measurements of the TOWL were taken prior to the fixation of the plastic chamber with MD 7-4602 silicone to the left thumbnail which was subsequently filled with 1 mL buffer for 4 periods of 30 min. The sampling and the TOWL monitoring at the end of the experiment were performed in the same manner as during the nail iontophoretic experiment. Each sample obtained was filtered (0.22 µm), stored frozen and assayed for sodium and chloride ions as described below.

Sodium and chloride analysis:
Ion chromatography with suppressed conductivity detection (AS50 autosampler and thermal compartment, GP50 gradient pump, ED50 electrochemical detector, Chromeleon
software, Dionex, Sunnyvale, CA) was used to measure the concentrations of sodium and chloride. For the quantification of sodium, the 25 mM methanesulfonic acid eluent was pumped under isocratic conditions (1 mL/min) through an IonPac™ CS12A column (Dionex, 250 x 4 mm) thermostated at 30ºC and a CSRS ULTRA II suppressor (Dionex, 4 mm) set at a current of 80 mA. Concentration of Na+ was measured against calibration curves obtained from standard solutions ranging from 0.05 to 15 mM (at least five different concentrations) of its chloride salt in a pH 7.0 buffer (HEPES 5 mM added with 5 mM MgCl₂). For the quantification of chloride, the 35 mM NaOH mobile phase was pumped through an IonPac™ AS16 column (Dionex, 250 x 4 mm) thermostated at 30ºC and the ASRS ULTRA II suppressor (Dionex, 4 mm) set a current of 90 mA. The calibration curves were obtained from at least five chloride solutions ranging from 0.05 to 15 mM prepared in a pH 7.0 buffer (HEPES 5 mM alone). For chloride and sodium analysis, the measurement error was below 5 %.

Skin-to-skin iontophoretic experiment:
To approximately estimate the contribution of the nail plate to the voltage in the nail-to-skin previously described experiments, a skin-to-skin experiment was performed on 5 volunteers (one volunteer was not available). A dispersive pad was cut into a 4 cm² square, placed on the thumb as close as possible to the thumbnail and used as a cathode. A second dispersive pad (4.6 cm x 5.6 cm) was placed on the dorsal face of the hand as in the previous experiments and used as an anode (Fig. 3). A 0.2 mA current was passed during 30 min and the voltage monitored as in the first iontophoretic period of the nail-to-skin experiments.

![Skin dispersive pads](image)

**Fig. 3: Skin-to-skin experimental set-up.**

Silicone content in sodium and chloride:
Sodium and chloride ions are ubiquitous so their presence in the silicone adhesive was assessed in the following way: an amount of silicone equivalent to that used to fix the nail...
chamber in place (5 – 15 mg) was placed in 1 mL of deionised water and shaken for 2 hours. The liquid in contact with the silicone was then passed through using 0.44 µm filters (Millipore, Sigma Aldrich Co., UK) and analysed for sodium and chloride by ion chromatography. A total 0.09 µmoles of chloride and 0.07 µmoles of sodium were extracted in this 2 hours extraction process. The contact between buffer and silicone during the in vivo experiments is much reduced compared to this extraction procedure, so it was considered that the silicone would release a negligible amount of ions to each 30 minutes sampling period (less than 0.02 µmoles of chloride and sodium ions).

Fluxes and transport numbers:

Chloride iontophoretic flux and transport number:
The anodal chamber extracts chloride ions from inside or underneath the nail plate as it contains the positive electrode. However, the electrochemical reaction at the anode consumes chloride ions which react with the oxidized silver to precipitate at the electrode surface (Eq. 1).

\[
\text{Ag(s)} + \text{Cl}^- \rightarrow \text{AgCl(s)} + e^- \quad \text{Eq. 1}
\]

The theoretical amount of chloride consumed by the anode during each of the 30 minutes iontophoretic periods in the experiments (Q\(_E\)) can be calculated using Eq. 2.

\[
Q_E = I \cdot t / F \quad \text{Eq. 2}
\]

where \(I\) is the intensity applied during the experiment in amperes (here, 0.2 \times 10^{-3} \text{ A})\), \(t\) is the length of one experimental period in seconds (here, 30 min = 1800 s) and \(F\) is the Faraday constant which value was approximated to 96500 coulombs/mol. In these conditions, the theoretical amount of chloride ions consumed by the anode for each 30 min iontophoretic period was 3.73 µmol.
The anodal buffer solution contained 5 mM HEPES and 5 mM MgCl\(_2\). Thus, to determine the chloride amount effectively driven across the nails by iontophoresis, Eq. 3 (Marro et al., 2001c) was used:

\[
Q_S = Q_0 + Q_I - Q_E \quad \text{Eq. 3}
\]

where, for each iontophoretic period, \(Q_S\) is the chloride amount in the sample, \(Q_0\) is the initial chloride amount in the buffer before experiment (typically between 8.7 and 10.7
mM), $Q_E$ is the amount of chloride consumed electrochemically at the anode and $Q_J$ is the amount of chloride driven across the nail. $Q_E$ is determined theoretically using Eq. 2 and $Q_S$ and $Q_0$ are measured by ion chromatography. $Q_J$ or amount of chloride reaching the anodal solution from inside or underneath the nail for each 30 min iontophoretic period was estimated using Eq. 3. Fluxes were directly deducted (multiplying by 2) and expressed in µmol/h. Chloride transport number was then calculated using Eq. 4:

$$J_i = t_i I / Z_i F$$

Eq. 4  (Phipps et al., 1992)

where $J_i$ is the flux of the ion “$i$” in mol/sec, $t_i$ is the transport number of the ion “$i$”, $I$ is the current intensity in coulombs/sec, $Z_i$ is the valence of the ion “$i$” and $F$ is the Faraday constant.

The transport number, $t_i$, is the fraction of the total charge transported by a specific ion during iontophoresis and the sum of all transport numbers must be equal to 1. If iontophoresis is the only mechanism contributing to chloride transungual transport and the assumptions leading to Eq. 3 are valid on our experimental conditions, chloride transport number should vary from a minimum 0.3 to a maximum 1. These transport numbers translate into fluxes of 2.24 and 7.46 µmol/h respectively.

**Sodium iontophoretic flux and transport number**

Sodium ions were extracted during cathodal nail experiments as this positive ion is attracted to the negative electrode. At the cathode, the electrochemical reaction:

$$\text{AgCl}_\text{(s)} + e^- \rightarrow \text{Ag}_\text{(s)} + \text{Cl}_\text{(aq)}$$

Eq. 5

does not require sodium participation and the cathodal buffer does not contain any sodium ions. The buffer was prepared with HEPES free acid instead of the routinely used HEPES sodium salt and the pH was adjusted with KOH instead of NaOH. IC analysis indicated that the traces of sodium in the HEPES buffer were negligible as compared to sodium fluxes. Thus, sodium fluxes from inside or underneath the nail plate were directly estimated from the sodium content in the cathodal samples. The sodium transport number was then calculated using Eq. 4.
Passive fluxes:
The amount of chloride and sodium ions extracted from inside or underneath the nail during passive experiments were calculated from the amount of ion in the receptor sample at the end of each 30 minutes period as measured by IC and by taking into account the background ion concentration present in the buffer. Fluxes were directly deducted as described for iontophoretic experiments.

Relative TOWL:
The relative TOWL was used to monitor the return to baseline values after a passive or an iontophoretic experiment and was calculated using the TOWL measured at a given post-treatment time normalized by the mean baseline TOWL measured for the respective volunteer before the treatment (iontophoresis or passive) was started. It follows that a relative TOWL of 1 indicates that the TOWL has returned to the baseline values.

4. Data analysis and statistics:
All the results are expressed as mean ± SD unless otherwise stated. Linear, non linear regressions and statistics were performed using Graph Pad Prism V5.00 (Graph Pad Software Inc., San Diego, CA). Statistical differences were assessed with a one way ANOVA followed by a Tukey post-test and a two way ANOVA as described in the text. The level of statistical significance was fixed at p < 0.05.
An empirical relation (one-phase exponential decay, Graph Pad Prism V5.00) between baseline TOWL and the thumbnail thickness was investigated (Eq. 6) by non linear regression analysis:

\[
\text{Baseline TOWL} = (Y_0 - \text{plateau}) \cdot e^{(-K \cdot \text{thickness})} + \text{plateau} \quad \text{Eq. 6}
\]

where \(Y_0\) is the TOWL value when the thickness is equal to 0, the plateau is the TOWL value when the nail thickness becomes infinite and \(K\) is a constant; all determined by the Graph Pad Prism software. “Baseline TOWL” is the average of the three TOWL measurements taken before each experiment and “Thickness” is the nail thickness measured for each volunteer. A linear relationship between baseline TOWL and nail thickness was investigated as well (Eq. 7).

\[
\text{Baseline TOWL} = \text{slope} \cdot \text{Thickness} + b \quad \text{Eq. 7}
\]

where \(b\) is the intercept with the Y-axis (TOWL value corresponding to a null thickness).
Results and discussion

This is the first time that passive and iontophoretic sodium and chloride transport across the nail have been measured and compared, demonstrating, *in vivo*, the potential of iontophoresis as an enhancing technique in the field of nail drug delivery. Also, we have investigated some key issues such as the voltages required for a practical application and started some preliminary safety evaluation of the technique via TOWL.

Basal TOWL

This first study was aimed to gather information about TOWL in order to plan the subsequent experiments (Fig. 4).
Fig. 4: Variation of TOWL in 6 volunteers and as a function of the finger and hand where the measurement took place. The last graph shows the average TOWL for the 6 volunteers. F1 indicates thumb, F2 forefinger, F3 middle finger, F4 ring finger and F5 little finger.

In this study, all volunteers except D were right-handed. For each volunteer, a two way ANOVA revealed significant statistical difference between the hands: all volunteers except A had significantly higher TOWL values for the fingernails of the left hand. When
volunteers were considered individually, no fingernail systematically showed higher TOWL values than others (paired one way ANOVA followed by a Tukey post-test performed for each hand).

On the graph presenting the average of the 6 volunteers, a two way ANOVA showed a statistical difference between the TOWL of the two hands. The mean TOWL for all the fingernails of the six volunteers was 38.0 ± 4.9 and 34.7 ± 6.3 g/(m²h) for the left and right hand respectively. For the average left hand, the TOWL of the thumb nail (F1) was significantly higher than the forefinger nail (F2) and for the right hand, the TOWL of the thumbnail (F1) was significantly lower than the ring finger nail (F4) and the little finger nail (F5) (paired one way ANOVA followed by a Tukey post-test performed for each hand).

This study was undertaken as, at the time, there was not sufficient information on how TOWL varied inter and intra-individually. It was then decided that the thumb nail offered little differences to the other nails, but being bigger would be more suitable for the iontophoretic study.

**Iontophoretic fluxes of sodium and chloride ions and their transport number:**
The iontophoretic sodium and chloride fluxes measured for each volunteer are reported in Table 1 and the chloride fluxes are shown in Fig. 5.
Table 1: Chloride and sodium iontophoretic fluxes (µmol/h) for each volunteer and sampling interval.

<table>
<thead>
<tr>
<th>Period (min)</th>
<th>Chloride flux</th>
<th>Chloride Average flux</th>
<th>Sodium flux</th>
<th>Sodium Average flux</th>
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<tr>
<td>V1</td>
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<tr>
<td>30</td>
<td>1.5</td>
<td>3.80 ± 2.89</td>
<td>3.45</td>
<td>4.16 ± 0.67</td>
</tr>
<tr>
<td>60</td>
<td>7.04</td>
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<td>3.93</td>
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<tr>
<td>90</td>
<td>2.86</td>
<td></td>
<td>4.21</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>8.88*</td>
<td></td>
<td>5.05</td>
<td></td>
</tr>
<tr>
<td>V2</td>
<td></td>
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</tr>
<tr>
<td>30</td>
<td>51.47*</td>
<td>5.77 ± 1.11</td>
<td>4.62</td>
<td>4.61 ± 0.18</td>
</tr>
<tr>
<td>60</td>
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<tr>
<td>120</td>
<td>5.37</td>
<td></td>
<td>4.86</td>
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<tr>
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<td>120.65*</td>
<td>3.15</td>
<td>3.01</td>
<td>3.74 ± 0.51</td>
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<tr>
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<td>9.05*</td>
<td></td>
<td>3.93</td>
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</tr>
<tr>
<td>90</td>
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<td>120</td>
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<td>3.87</td>
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<tr>
<td>V4</td>
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<td></td>
<td></td>
<td>ND</td>
</tr>
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<td>7.22</td>
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</tr>
<tr>
<td>60</td>
<td>35.56*</td>
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<tr>
<td>90</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>V5</td>
<td></td>
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</tr>
<tr>
<td>30</td>
<td>6.53</td>
<td>4.44 ± 1.81</td>
<td>2.38</td>
<td>2.51 ± 0.22</td>
</tr>
<tr>
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<td>2.38</td>
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<td>3.51</td>
<td></td>
<td>2.76</td>
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</tr>
<tr>
<td>120</td>
<td>11.37*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>2.83</td>
<td>4.48 ± 1.32</td>
<td>3.27</td>
<td>3.66 ± 0.31</td>
</tr>
<tr>
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<td>4.77</td>
<td></td>
<td>3.61</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>4.29</td>
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<tr>
<td>120</td>
<td>6.03</td>
<td></td>
<td>3.75</td>
<td></td>
</tr>
</tbody>
</table>

* values which were not included in the average chloride flux because they corresponded to a transport number > 1.
As discussed in Materials and methods, a chloride flux of 7.46 µmol/h indicates a chloride transport number equal to 1. This is an abnormal value, as one would expect sodium ions to carry some charge as well. There were several chloride fluxes even higher, suggesting chloride transport numbers higher than 1. These anomalous values indicate that the assumptions made in the calculation (see Materials and Methods) were not valid. An examination of Eq. 3 suggests two possible sources of error. First of all, the value of $Q_E$ or the chloride consumed at the electrode is calculated theoretically, that is, assuming that oxidation of silver as described in Eq. 1 is the only process occurring at the anode. However, endogenous substances released from the nail into the electrode chamber could also be oxidized at the anode without chloride consumption. In this case, the theoretical value of $Q_E$ is an overestimation of the amount of chloride consumed and, accordingly, of chloride flux. A second source of error could originate from the analytical methods. The buffer at the electrode contains some chloride (~10 mM or 10 µmoles in 1 mL), the amount of chloride arriving from beneath the nail is 3.73 µmol/h (~1.87 µmol/30 min) assuming a 0.5 transport number. It follows that reading this small amounts against a given chloride background may carry some error as the amount of chloride for each period corresponds to ~19 % of the amount in the buffer. An apparent transport number for chloride has been calculated after elimination of the fluxes corresponding to transport numbers higher than 1; thus the fluxes $3.8 \pm 2.9$, $5.8 \pm 1.1$, $3.1$, $7.2$, $4.4 \pm 1.8$ and $4.5 \pm 1.3$ µmol/h for V1, V2, V3, V4, V5 and V6 respectively were used (Fig. 6). However, these fluxes are subjected to the same errors discussed above and cannot be considered as valid measures.
The apparent transport numbers obtained for each volunteer were as follow: 0.51 ± 0.39 for V1, 0.77 ± 0.15 for V2, 0.42 for V3, 0.97 for V4, 0.59 ± 0.24 for V5 and 0.60 ± 0.18 for V6. The average apparent chloride transport number for all the volunteers was 0.63 ± 0.24. The same difficulties in estimating chloride fluxes have been described by Nixon et al. (2007). An accurate measure of chloride transport numbers would require the use of a different type of electrodes. However, the use of inert electrodes, which do not require chloride for functioning, results in pH variations that alter nail permselectivity (as seen in chapter 3). Moreover, the introduction of buffers to avoid pH fluctuations also complicates measurements by introducing competing ions.

On the other hand, the iontophoretic fluxes and transport number of sodium were easily measured and were obtained for each volunteer and period as shown in Fig. 7 and 8.

Fig. 6: Apparent chloride transport number for each volunteer.

Fig. 7: Iontophoretic sodium fluxes of extraction across the nail plate for each volunteer and sampling period. The dotted line indicates the flux corresponding to a transport number equal to 0.5.
The average iontophoretic sodium fluxes for each volunteer were: 4.2 ± 0.7, 4.6 ± 0.2, 3.7 ± 0.5, 2.5 ± 0.2, 3.7 ± 0.3 µmol/h for V1, V2, V3, V5 and V6 respectively. The average sodium flux was 3.8 ± 0.8 µmol/h.

![Fig. 8: Average (±SD) sodium transport number for each volunteer.](image)

The transport numbers obtained for the different volunteers were 0.56 ± 0.09, 0.63 ± 0.02, 0.50 ± 0.07, 0.34 ± 0.03 and 0.49 ± 0.04 for V1, V2, V3, V5 and V6 respectively. The average transport number for all the volunteers was 0.51 ± 0.11.

As a difference with chloride, sodium fluxes are directly deduced from the concentration of sodium ions in the buffer because they do not participate in the electrode reactions. Further, there is no sodium ions in the buffer, which facilitates a precise quantification of the amount of sodium arrived from underneath the nail. Thus, the transport numbers for each volunteer were very consistent and had a small CV (3 to 16%).

As previously discussed, this is the first time (to the authors’ knowledge) that in vivo sodium iontophoretic transungual fluxes and transport numbers have been measured. Thus, the closest comparisons can be done with the reverse iontophoretic transdermal extraction of sodium by Sieg et al. (2004b) and Leboulanger et al. (2004b) in human volunteers. Sieg et al. (2004b) extracted sodium in Tris buffer (10 mM, pH 8.5) by applying a 0.6 mA current (0.3 mA/cm²) for 5 hours and obtained a mean (±SD) of 0.55 ± 0.04 for sodium transport number (12 volunteers). Leboulanger et al. (2004b) used a 10 mM histidine solution (pH 7.47) and applied a 0.8 mA current (~0.25 mA/cm²) for four periods of 30 min; sodium transport number was 0.54 ± 0.04 (24 patients). Although our value presents a relatively high SD, our average value, 0.51, is fairly similar to those obtained by Sieg and Leboulanger (2004b). As this was an in vivo experiment, it is expected that other cations which were not quantified were extracted at the same time (e.g. potassium and calcium). This means that the total cation transport number is probably closer to 0.6 which is in favour of a net negative charge of the nail at physiological pH.

The smallest sodium transport number was obtained with V5 whose nail plate was the thickest (800 µm) of all used in the study. Thus, a potential relationship between sodium...
transport number and nail’s thickness was investigated. However, as Fig. 9 shows, no relation is observed when all the volunteers are considered.

![Graph showing sodium transport number against nail plate thickness](image)

**Fig. 9:** Sodium transport number and nail plate thickness determined for each volunteer. Sodium extraction took place either in the first experiment (open circles) or in the second experiment (closed square). Measurement of thickness was done before the first experiment.

**Passive diffusion:**

The chloride and sodium passive fluxes are shown in Table 2 and Fig. 10.

![Bar charts showing chloride and sodium fluxes](image)

**Fig. 10:** Sodium and chloride passive fluxes for each volunteer and sampling period. Missing columns indicate fluxes below the limit of detection.
Table 2: Chloride and sodium passive fluxes (µmol/h) for each volunteer and sampling interval.

<table>
<thead>
<tr>
<th>Period (min)</th>
<th>Chloride Passive flux</th>
<th>Chloride Average flux</th>
<th>Sodium Passive flux</th>
<th>Sodium Average flux</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.74 ± 0.48</td>
<td>0.16</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>V1 30</td>
<td>0.36</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>1.40</td>
<td>0.16</td>
<td>1.18 ± 1.90</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>90</td>
<td>0.80</td>
<td>0.16</td>
<td>0.12</td>
<td>0.04</td>
</tr>
<tr>
<td>120</td>
<td>0.40</td>
<td>0.16</td>
<td>0.14</td>
<td>0.02</td>
</tr>
<tr>
<td>V2 30</td>
<td>0.46</td>
<td>0.10 ± 0.12</td>
<td>0.14</td>
<td>0.10 ± 0.04</td>
</tr>
<tr>
<td>60</td>
<td>1.58</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>90</td>
<td>1.02</td>
<td>0.12</td>
<td>0.10</td>
<td>0.12</td>
</tr>
<tr>
<td>120</td>
<td>0.22</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>V3 30</td>
<td>0</td>
<td>0.10 ± 0.12</td>
<td>0.2</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>60</td>
<td>0.16</td>
<td>0.12</td>
<td>0.10</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>90</td>
<td>0</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>120</td>
<td>0.24</td>
<td>0.12</td>
<td>0.10</td>
<td>0.12</td>
</tr>
<tr>
<td>V4 30</td>
<td>0.82</td>
<td>0.10 ± 0.12</td>
<td>0.68</td>
<td>0.24 ± 0.29</td>
</tr>
<tr>
<td>60</td>
<td>1.08</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>90</td>
<td>0.22</td>
<td>0.10</td>
<td>0.08</td>
<td>0.10</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>0.10</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>V5 30</td>
<td>0.19</td>
<td>1.18 ± 1.90</td>
<td>0.10</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>60</td>
<td>0.53</td>
<td>0.10</td>
<td>0.06</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>90</td>
<td>0</td>
<td>0.10</td>
<td>0.04</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>120</td>
<td>4.01</td>
<td>0.10</td>
<td>0.06</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>V6 30</td>
<td>0</td>
<td>0.09 ± 0.16</td>
<td>0.12</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>60</td>
<td>0.04</td>
<td>0.10</td>
<td>0.10</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>90</td>
<td>0.34</td>
<td>0.10</td>
<td>0.08</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>0.10</td>
<td>0.08</td>
<td>0.09 ± 0.02</td>
</tr>
</tbody>
</table>

Passive fluxes of sodium were low and relatively constant (0.06 to 0.24 µmol/h) whereas chloride fluxes were highly variable (0.09 to 1.18 µmol/h). Average (±SD) passive fluxes were 0.58 ± 0.86 µmol/h and 0.14 ± 0.12 µmol/h for chloride and sodium respectively. The wide variation observed in the chloride fluxes could be explained by the chloride (10 mM) background in the buffer which probably interfered with the measurement of the very small change in chloride concentration due to the passive flux.

Sodium passive fluxes were significantly higher in vivo than in vitro (Mann Whitney non parametric t-test). For example, the average sodium flux at pH 7.0 in vitro was 0.06 ± 0.01µmol/h. The reason for this small difference is not clear, but this could be due to the different experimental conditions (2 hours in vivo compared to 16 hours in vitro). For example, the nail plate and the nail tips could have a slightly different content in sodium. The content in potassium, calcium and sodium of finger and toe nail clippings has been
reported to be 357, 368 and 440 µg per g of nail substance, respectively (Vellar, 1970) which correspond to 9, 9 and 19 µmol per g of nail substance, respectively. The portion of the nail exposed to iontophoresis corresponds to a weight of ~10-20 mg. Therefore, it is expected that the content in sodium of this portion will be ~190-380 nmoles. On the one hand, the in vivo and in vitro sodium passive fluxes are 140 and 60 nmol/h, respectively. On the other hand, the in vivo sodium iontophoretic flux is 3800 nmol/h. This shows that the sodium ions extracted by iontophoresis were rapidly coming from underneath the nail plate.

In any case, iontophoretic fluxes of the two ions were significantly higher than their respective passive fluxes (Mann Whitney non parametric t-test). Iontophoretic fluxes enhance chloride and sodium transungual transport by 8 and 27 fold, respectively, compared to passive. These results clearly demonstrate the ability of iontophoresis to enhance the in vivo transport of small charged molecules across the nail plate and thus, its potential for transungual drug delivery.

TOWL- iontophoresis:

The baseline TOWL was measured for each volunteer at the beginning of each experiment and is shown in Table 3 along with the information regarding the volunteer’s age, gender and the thumbnail thickness.

Table 3: Baseline TOWL (Mean ± SD, n=3) measured for each volunteer and participation.

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Gender</th>
<th>Age</th>
<th>Nail thickness (µm)</th>
<th>Baseline TOWL (g/(m²h))</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>Female</td>
<td>27</td>
<td>250-340</td>
<td>56.3 ± 1.4 (2)a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>51.4 ± 2.3 (1)a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>65.1 ± 1.8 (3)</td>
</tr>
<tr>
<td>V2</td>
<td>Female</td>
<td>32</td>
<td>390-440</td>
<td>42.2 ± 1.3 (1)ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>48.8 ± 2.4 (2)b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>52.6 ± 2.5 (3)</td>
</tr>
<tr>
<td>V3</td>
<td>Female</td>
<td>44</td>
<td>300</td>
<td>47.3 ± 1.1 (1)a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>51.0 ± 5.5 (2)a</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>63.6 ± 2.7 (3)</td>
</tr>
<tr>
<td>V4</td>
<td>Male</td>
<td>26</td>
<td>450-500</td>
<td>38.9 ± 2.9 (2)ab</td>
</tr>
<tr>
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<td>46.6 ± 2.5 (3)</td>
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<td>49.0 ± 1.3 (1)</td>
</tr>
<tr>
<td>V5</td>
<td>Male</td>
<td>25</td>
<td>800</td>
<td>38.8 ± 3.0 (2)</td>
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<td></td>
<td></td>
<td>44.5 ± 3.4 (3)</td>
</tr>
<tr>
<td>V6</td>
<td>Male</td>
<td>25</td>
<td>450</td>
<td>43.1 ± 1.1 (1)a</td>
</tr>
<tr>
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<td>47.5 ± 1.0 (2)a</td>
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<td></td>
<td></td>
<td></td>
<td>56.0 ± 4.0 (3)</td>
</tr>
</tbody>
</table>

\(^{(1a)}, (2), (3)\) indicate in which order the experiments were performed for each volunteer.

\(a\) baseline TOWL before iontophoretic experiments significantly different (p < 0.05) to their baseline TOWL before passive diffusion.

\(b\) baseline TOWL significantly different (p < 0.05) for the two iontophoretic experiments.

The baseline TOWL values (37.5 – 65.1 g/m²h) are consistent with those measured for the thumb nail of the left hand in the first study (32.0 – 48.5 g/m²h).

Previous values reported in the literature are 18.5-30.7 (Spruit, 1971), 11.7-33.5 (Jemec et al., 1989), 12.9 (Krönauer et al., 2001) and 28-75 g/(m²h) (Murdan et al., 2008). Spruit
(1971) used a home-made capsule to measure the vapour loss whereas Jemec et al. (1989) and Krönauer et al. (2001) used open chamber evaporimeters. Our values are higher than Spruit, Jemec and Krönauer but are consistent with Murdan et al. (2008) who used the same type of closed-chamber evaporimeter.

A curious observation was that the baseline TOWL for 5 of the 6 volunteers increased as the experiments took place (Table 2) as it is shown in Figure 11.

![Figure 11: Baseline TOWL for each volunteer and experiment.](image)

The difference in the baseline values between the first and the last experiments was +13.7 (+27%), +10.4 (+25%), +16.3 (+34%), -2.4 (-5%), +7 (+19%) and +12.9 g/(m²h) (+30%) for V1, V2, V3, V4, V5 and V6 respectively.

It is difficult to decide on the causes behind these differences. It would be premature to conclude that iontophoresis had an effect on the nail plate because the passive experiment was usually the last one performed. It would have been necessary to have the passive treatment done first in some volunteers to discard a possible effect of temperature, humidity or season. Further, this would be in contradiction with the prompt return to baseline values observed after each iontophoretic treatment. In the case of transdermal iontophoresis, TEWL is elevated for a transient period (~1-2 hours) before it returns to baseline values (Van Der Geest et al., 1996; Brand et al., 1997; Fouchard et al., 1997; Anigbogu et al., 2000). The purpose of the passive experiment was to hydrate the nail with the same buffer used during iontophoresis so the effects on TOWL due to hydration and passage of current could be separated. Figure 12 shows that TOWL was elevated both after iontophoresis and passive treatments and returns relatively fast to baseline values. Because the baseline values measured for each volunteer are different, it is easier to compare their relative values. A relative TOWL of 1 indicates a TOWL close to the baseline value.
The results indicate that TOWL was back to pre-experimental values one hour after iontophoresis. Further, the profiles observed after passive and iontophoretic treatments were very similar suggesting that the elevated TOWL was mostly caused by the increased nail hydration. Thus, it would seem that iontophoresis did not increase water permeability across the nail plate. While these results are encouraging, it must be noticed that TOWL has not been validated as a marker of nail damage, and that there could be other effects of the current in the nail structure which are not accompanied by an increment in water loss. Thus, a more exhaustive study is required before drawing any definitive conclusion on the safety of the technique.

A possible relation between TOWL and thickness of the nail plate was also investigated. Fig. 13 shows the baseline TOWL measured for each volunteer and experiment as a function of thickness of the nail.
Fig. 13: Relationship between nail thickness and baseline TOWL for each volunteer and experiment. The experimental data was fitted to the curve by non-linear (Eq. 6: $TOWL = (Y_0 - \text{plateau}) \cdot e^{(-K \cdot \text{thickness})} + \text{plateau}$) and linear relationship (Eq. 7: $TOWL = \text{slope} \cdot \text{thickness} + b$). The dotted and continuous lines indicate the linear and non-linear regression prediction curves, respectively. All linear regressions were significant and $r^2$ was 0.55, 0.53 and 0.63 whereas, for non-linear regressions, $r^2$ was 0.55, 0.64 and 0.79 for the 1st, 2nd and 3rd experiment, respectively.

Eq. 6 was used because it assumes, a fairly high value of TOWL for a nail of null thickness and second, a plateau for the TOWL, i.e. a threshold value of TOWL when the thickness increases (which can tend to 0). Linear regressions were calculated to compare both fitting methods (Eq. 7). The results of the non-linear and linear regressions are given in Table 4.
Table 4: Equation data for the non-linear and linear relationships between nail thickness and baseline TOWL. The results are expressed as estimate ± SE of the regressions. Exp. stands for experiment.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>(Y_0^a) ± SE</th>
<th>Plateau(^b) ± SE</th>
<th>(K) ± SE</th>
<th>Slope ± SE</th>
<th>(b^c) ± SE</th>
<th>(X)-intercept(^c) (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(^{st}) exp.</td>
<td>58.9 ± 12.0</td>
<td>22.1 ± 55.5</td>
<td>0.001 ± 0.003</td>
<td>-0.022 ± 0.005</td>
<td>55.1 ± 2.4</td>
<td>2505</td>
</tr>
<tr>
<td>2(^{nd}) exp.</td>
<td>113.8 ± 57.1</td>
<td>37.0 ± 4.6</td>
<td>0.005 ± 0.003</td>
<td>-0.030 ± 0.007</td>
<td>60.8 ± 3.4</td>
<td>1999</td>
</tr>
<tr>
<td>3(^{rd}) exp.</td>
<td>175.9 ± 78.9</td>
<td>43.3 ± 3.6</td>
<td>0.006 ± 0.002</td>
<td>-0.039 ± 0.007</td>
<td>72.6 ± 3.6</td>
<td>1858</td>
</tr>
</tbody>
</table>

\(a\) TOWL for null thickness \(b\) TOWL for infinite thickness \(c\) thickness for null TOWL

For the first experiment, both fitting models presented similar \(r^2\) (0.55) whereas for the two subsequent experiments the non-linear model fitted better the data (linear: \(r^2 = 0.53\) and 0.63 for 2\(^{nd}\) and 3\(^{rd}\) experiment, respectively. Non-linear: \(r^2 = 0.64\) and 0.79 for 2\(^{nd}\) and 3\(^{rd}\) experiment, respectively).

The inverse linear regressions suggest that a null TOWL can be achieved for nails presenting a thickness ranging from 1.8 to 2.5 mm whereas the non-linear curves suggest that a TOWL can not be null for any thickness and would rather tend to a plateau ranging between 22.1 to 43.3 g/m²h.

\(Y_0\) in the non linear relationship and \(b\) in the linear relationship represent the same parameter: the TOWL value for a null thickness of the nails. In both relationships, this value increases with the experiments. The meaning of this increase is not clear and it is hoped that future experiments will help to understand it.

Jemec et al. (1989) and Murdan et al. (2008) also studied the relationship between nail thickness and TOWL. The former did not find any dependence whereas, like in this study, the latter found one. Murdan et al. (2008) found an inverse linear relationship between the two variables for two individuals (mean ± SD for each fingernail of each volunteer) and found a Pearson correlation of -0.83 and -0.92 for the first and second individual. The relationship was stronger when each individual was considered separately than when the data from the two individuals were pooled together (Pearson correlation became -0.42).

Our study pools the thumb nail thickness and TOWL of six individuals (mean ± SD for the left thumb nail of each volunteer) and, in these conditions, the linear regressions offer a stronger relationship than Murdan’s findings. This could be related to the fact that we only used thumbnails whereas Murdan et al. (2008) pooled all the fingernails together. However, the non-linear model that we used showed that the relationship became stronger...
with the chronological order of the experiments; that was less apparent with the linear model. It is not clear which model's assumptions are the closest to reality but it is likely that other factors like Murdan et al. (2008) suggested (i.e., nail composition) interfere in the relationship between TOWL and nail thickness.

**Feasibility of iontophoresis:**

The important voltages observed at the beginning of iontophoresis application *in vitro* were worrying. In fact, they were high enough to question the feasibility of transungual iontophoresis as a drug delivery technique. Deciding on this feasibility was an important objective of this *in vivo* study. The power supply, Phoresor II, used in this chapter is approved for medical applications and offers the level of safety required for *in vivo* studies. The Kepco power supply used in the *in vitro* studies is connected to the mains and is capable of maintaining very high voltages whereas the Phoresor II is battery (9V) operated and is able to apply a maximum voltage of 80 V. If for any reason the maximum voltage is reached, the device stops passing the current and provides an error message.

The voltage measured during the nail-to-skin and the skin-to-skin experiments for each volunteer are shown in Fig. 14.
No difference in the voltage versus application time profile was apparent between nail anodal and cathodal experiments; usually the voltages declined in both cases becoming quite similar at the end of the experiments. For 2 male volunteers (V4 and V5), experiments started at voltage superior to 60 V whereas for V1, V2, V3 and V6 the voltage started around 50 V and dropped fast. It is known that the dorsal nail is less hydrated than the nail bed (Gunt et al., 2007b) and it probably contributes to a higher electrical resistance. As current is passed the nail hydration and ionic content is increased, which decreases the electrical resistance. The importance of hydration was clearly observed; hydrating the nail with the buffer for ~ 3-10 min usually helped the application of the current, diminished the voltage at the beginning of the experiment and the patients “tingling” feelings. However, the nail cathodal experiment was not possible for V4 even after a 30 minutes hydration time. The voltage reached 80 V after a few minutes of current application which caused the Phoresor to automatically stop the current passage. A possible explanation would be a very high resistance of the nail plate for this volunteer; nevertheless the same volunteer had previously participated in an anodal experiment carried out without complications. It must be noticed that the voltages here measured correspond to the complete electrical circuit; and while the assumption that the nail is a significant contributor to the resistance of the other circuit is correct, any other faulty component of the circuit could have caused the problem. Further investigation on the nail behaviour during current application is necessary to confirm these observations in a higher number of volunteers and also to understand what causes the \textit{in vivo} voltages to be lower than \textit{in vitro}.

The skin-to-skin experiments performed roughly estimate the nail contribution to the total voltage. The circuit used was similar to a nail cathodal experiment, but the cathode was placed on the skin of the thumb very close to the thumbnail, the anode was placed on the dorsal face of the hand as usual (Fig. 3). The voltages of the skin-to-skin circuits were...
clearly lower than those observed in the first 30 minutes of the nail-to-skin experiments, indicating a variable but sometimes high contribution of the nail to the resistance of the total circuit. The nail-to-skin voltages slowly decrease with time, reaching for all the volunteers, with the exception of V6, values similar to the skin-to-skin experiments. This happened during the third (V2 and V3) or the fourth (V1 and V4) period of current application and suggests that the resistance of the nail decreases greatly with the time of the application of the current.

In any case, the results indicate that iontophoresis is feasible. Further, as shown by Table 5, the discomfort experienced by the volunteers during the iontophoretic experiments was lower if not comparable to that reported during skin iontophoresis.

Table 5: Discomfort experienced during nail iontophoresis as reported by the participants. The discomfort was ranked on a 0 to 5 scale where 5 represented a very high discomfort.

<table>
<thead>
<tr>
<th>Site</th>
<th>Experiment</th>
<th>V1</th>
<th>V2</th>
<th>V3</th>
<th>V4</th>
<th>V5</th>
<th>V6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin site</td>
<td>Nail anodal</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Nail cathodal</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>ND</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Nail</td>
<td>Nail anodal</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Nail cathodal</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>General</td>
<td>Nail anodal</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Nail cathodal</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>ND</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

ND: Not determined.

The discomfort score was typically between 0 and 2, showing that the volunteers tolerated well nail iontophoresis. Two volunteers (V1 and V6) reported verbally a higher “tingling” sensation at the skin site during the nail cathodal experiment and indeed, it has been also reported for transdermal iontophoresis that volunteers usually expressed a greater discomfort at the anodal than at the cathodal site (Ledger, 1992; Brand et al., 1997; Leboulanger et al., 2004b; Sieg et al., 2004b). However non parametric t-tests performed between anodal and cathodal discomfort and between the skin and nail discomfort did not reveal any statistical differences.

Finally, a possible relationship between voltages and nail’s thickness was investigated (Fig. 15). This investigation did not reveal any relationship between the two parameters during nail cathodal experiment but a linear relationship presenting a $r^2$ equal to 0.76 was found during nail anodal experiment.

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Fig. 15: Voltage measured for each volunteer and type of experiment as a function the nail thickness. Open circles and closed squares indicate whether the experiment was the first (open circle) or the second (closed square) for the participant.

**Conclusions of chapter 5**

In summary, we have measured sodium and chloride fluxes across the nail during passive and iontophoretic transport. Iontophoresis clearly enhanced sodium and chloride fluxes demonstrating that iontophoresis efficiently improves ionic transport across the nail. Sodium transport numbers could be measured and were similar to those measured during transdermal iontophoresis.

Transungual iontophoresis was feasible and well tolerated by volunteers. A transient elevation in TOWL after iontophoresis was probably due to the increased hydration of the nail plate. Baseline TOWL values were typically recovered 1-2 hours post-treatment suggesting that iontophoresis had not altered irreversibly the permeability of water across the nail plate. These encouraging results indicate that transungual iontophoresis merits further investigations with regard to future applications in drug delivery.
GENERAL DISCUSSION AND PERSPECTIVES

The aims of this thesis were to investigate iontophoresis as a possible enhancing method for transungual delivery. As little information existed on the feasibility and outcomes of ungual iontophoresis, basic studies were designed to investigate: 1) the *in vitro* extent of ionic fluorescent markers penetration and to visualize the routes used to penetrate the nail, 2) the *in vitro* permselective properties of the nail plate 3) the relative enhancement of nail penetration induced by iontophoresis as compare to passive diffusion and 4) the feasibility of an *in vivo* application of iontophoresis.

General discussion

We developed a new method using confocal microscopy which gave information on the depth and pathways of penetration of two fluorescent markers under passive and iontophoretic conditions: SF, a hydrophilic di-anion, and NBC, a lipophilic cation. Both compounds use mainly the transcellular pathway (with a possible contribution of the paracellular pathway) during passive diffusion and iontophoresis. Given this is the first study of this kind and that it only investigated two compounds, it is not possible to draw conclusions about these being the pathways followed by most drugs. However, knowing the pathways of transport is a step towards a rational optimization of nail transport and this will be useful information on which future research can be based. Surprisingly, increasing the current intensity did not result in a proportional deeper penetration of the markers. Indeed, the iontophoretic delivery of ionised compound is governed by Faraday’s law (Eq. 1).

\[
J_d = \frac{I \cdot I_d}{F \cdot z_d} \quad \text{Eq. 1}
\]

This equation, as mentioned previously, shows the proportionality of the flux with the intensity of the current. It seemed clear from these results that other factors were causing these anomalous observations. Unfortunately, additional experiments performed, such as the binding study, did not provide explanation for these results. Previous works have demonstrated that salicylic acid, terbinafine, mannitol and urea (although only in the anode to cathode direction for mannitol and urea) transungual fluxes to be directly determine by the current applied (Murthy et al., 2007b; Hao et al., 2008a; Nair et al., 2008). Again, it
will be necessary to perform additional studies to establish if iontophoresis will be an efficient method to control drug delivery across the nail or not. Due to the molecular weight of the markers used (353.85 and 376.28 Da for NBC and SF, respectively), it seemed logical to think that their transport was not only the fact of electromigration but was also due to electroosmosis. We have used a cationic and a di-anionic compound, and assuming that nails are negatively charged at physiological pH, one could expect the cationic compound to be transported more efficiently than the di-anionic one. It was not the case. However, as mentioned in Chapter 2, the solution of NBC had a pH of 3 whereas SF solution had a pH of 8. A pH of 3 could have reversed the nail charged to be positive, and in this case, NBC would have been delivered against the electroosmotic flow, the same as SF. It follows that the iontophoretic transport of the two markers was not facilitated. Therefore, the contribution of electroosmotic and electrorepulsive forces needed to be investigated. Murthy et al. (2007a) and Hao et al. (2008a and b) both showed that the nail membrane presents a net negative charge at physiological pH. Therefore, it was expected that the results of our electroosmotic study support this finding. However, even if the results tend to show that the electroosmotic flux is in the anode to cathode direction at physiological pH, the variability of the mannitol fluxes measured in the different nails prevented to draw any clear conclusion. There is no clear reason for this variability which seems to be maximal at physiological pH and in the anode to cathode direction. This could be related to a difference in the nail swelling at different pH. However, no information is available about this subject. Another hypothesis could be related to the incorporation of drugs/elements into the nail plate during nail formation. Indeed, nails have been used in forensic investigations because drugs are incorporated in the nail in formation (Palmeri et al., 2000). Therefore, nail composition could vary between individuals and even in the same individual according to diet, drug treatment or the chemicals handled during work, house work or spare time activities. This variability in nail composition could thus be responsible for the inter- and intra-nail variability observed in the electroosmotic flux. Further, that would mean that for a same person, the nail composition may vary greatly with time and thus, even if the same experiment was performed on the tip coming from a same finger, results might differ and show variability. These unexpected results raised questions about the feasibility of a transungual iontophoretic application for big, polar, uncharged compounds (most of the criteria presented by antifungals) and made the study of the electrorepulsive force even more essential to characterize the nail behaviour. The iontophoresis of sodium and lithium ions revealed little variability and provided a clear evidence of a nail cation permselectivity at physiological pH. Moreover, nails
demonstrated behaviours similar to skin in term of evolution of transport numbers with the molar fraction and in term of a threshold in the total cation transport number. Considering the high variability in the mannitol fluxes and the weak penetration of SF and NBC, it is possible to hypothesize that the molecular size of these permeants may be too close to the mesh size of the nail. To a certain extent, this hypothesis could be supported by Khengar et al. (2007): these authors studied the passive mannitol transport after pre-treating the nail tips with penetration enhancers and they obtained extremely variable mannitol fluxes after each enhancer pre-treatment. It is possible that the nails did not react in the same manner to the pre-treatment and thus did not swell to the same extent resulting in different mesh size modifying drug penetration.

One could argue as well that this variability in the fluxes may demonstrate that the chemical enhancers and iontophoresis have an irreversible effect on the nail membrane. Indeed, Khengar et al. (2007) and Malhotra et al. (2002) noticed that when a chemical enhancer was efficient, this was associated with an irreversible change in the nail surface and properties. However, passive diffusion fluxes of sodium and lithium did not vary much during in vitro experiments whereas the same nails were used across three different pHs and were exposed three times to iontophoresis. If the passive fluxes had varied a lot, this would have demonstrated an irreversible effect of the current. As the in vitro cations fluxes were very encouraging, it was decided that the feasibility of an in vivo application should be explored.

The in vivo experiments showed that, indeed, it was possible to apply iontophoresis to human volunteers. The current application did not result in high voltages and was well accepted by the volunteers. This was demonstrated by evaluating the discomfort that volunteers experienced during the experiments. Due to the experimental setting, the transport number obtained for chloride ions was not exploitable but the transport number obtained for sodium ions was in favour of a negative charge hold by the nail in vivo at physiological pH. Interestingly, whereas during in vitro experiments, sodium fluxes were not stable even at the end of 8 hours of iontophoresis, in vivo, sodium fluxes seemed stable after 30 min/1 hour of current application. This finding might be related to a structural or hydration difference between the nail plate in vivo and the nail clippings in vitro. In vitro iontophoresis of terbinafine (Nair et al., 2008) has shown to lead to steady-state fluxes after only 15 min of current application whereas salicylic acid delivery needed ~8 hours to reach steady-state (Murthy et al., 2007b); and mannitol and tetraethylammonium (Hao et al., 2008a) needed less than 9 hours and ~15 hours, respectively. The reasons for these very different lag times are not clear. Although Hao et al. (2008a) specifically used mannitol and tetraethylammonium for their similar effective hydration radius, the two molecules
demonstrated very different lag time. Moreover, terbinafine, despite its MW (almost 300 Da), reached steady-state in less than 15 min whereas sodium (23 Da), in this thesis, needed more than 8 hours. Nair’s results are surprising but, to date, not enough information are available to discuss this particular point.

On the whole the in vivo study supports the practical use of iontophoresis as a drug delivery technique if justified by the enhancement provided for a given drug.

**Perspectives**

During confocal microscopy experiments, SF and NBC were used. As discussed previously, both compounds present a relatively high MW and therefore, it could be hypothesised that different results may be obtained if using fluorescent markers presenting a lower MW. However, the molecular weight of fluorescent markers is unlikely to be below mannitol’s molecular weight (182 Da) and thus, it might be easier to study the penetration of radioactive compounds of different MW and to estimate their penetration by counting the radioactivity in the successive layers of the nail. For this, a method to cut small slices of nail of constant thickness still needs to be developed. Nails are difficult to slice in a regular fashion due to their rigidity/elasticity properties. While methods such as the successive drilling (Hui et al., 2002) or slicing of nail layers by microtomes (Wegener et al., 1990) have been tried, there is not yet a technique available that can be considered equivalent to “tape stripping” in the skin.

Whole human nails or clippings of sufficient size for experiments are difficult to obtain. Some authors like Mertin et al. performed some of their studies on hooves and on human nail to determine if hooves could be a suitable model. However, due to their different structure, the hoof does not seem to behave exactly like the human nail and, therefore, it could be interesting to perform a study similar to what have been done in this thesis in order to measure the penetration depth and visualize the pathways of fluorescent markers. This could allow the identification of the different structural elements involved in nail and hoof permeability.

During the binding study, SF clearly diffused deeper in the nail at the end of the first week compared to its penetration at 24 hours. SF could be applied in iontophoretic pulse therapy for 3 or 4 hours (once, twice or three times a week. The regimen will probably depend on further studies) and its penetration followed by confocal microscopy after 2 or 3 weeks. The results of this study might give useful information and allow the design of a therapeutic procedure during which the nail plate could be loaded with the drug during short iontophoretic sessions applied by health personnel and using the subsequent diffusion
phenomenon previously observed to drive the drug sequentially deeper into the nail plate between the sessions.

The nail hydration has been said to be of great importance during passive diffusion study. It seems important to investigate this parameter by varying the hydration times and/or hydration medium used to soak the nails before experiments. This could allow the determination of how and if this factor contributes to iontophoretic transport.

The ionic strength in the donor chamber has been said to have a great influence over electroosmosis (Santi et al., 1996) and therefore reducing the amount of NaCl in the donor chamber containing the mannitol from 154 mM to smaller values may increase the fluxes and make more obvious the differences between anodal, cathodal and passive delivery.

As it has been previously highlighted, TOWL has not been validated yet as a tool to monitor the effects of iontophoresis on the nail. Therefore, this technique could be validated by monitoring the evolution of the TOWL with time on nails damaged at different degree. Damages could be caused by a chemical agent or by tape-stripping, for example.

Other methods such as SEM or AFM could be explored to monitor the effects of iontophoresis. SEM and AFM have been used to assess the effect of chemical enhancers on the nail surface (Repka et al., 2002) and could allow the visualization of the \textit{in vitro} effects of iontophoresis.

The characterization of the \textit{in vivo} nail permselectivity properties will be complete only by studying electroosmosis. This study may be performed by attempting to extract glucose through the nail. However, this study might be difficult if the mannitol fluxes measured \textit{in vitro} are predictive of reverse iontophoretic glucose fluxes.

In this thesis, iontophoresis did not show an interesting enhancing potential for compounds presenting MW around or above 200 Da whereas a recent article (Nair et al., 2008) showed that terbinafine (291 Da) could be efficiently delivered and detected in the receptor solutions after only 15 minutes of iontophoresis. This finding is extremely surprising and some light needs to be put on the role of the MW upon transungual iontophoretic delivery. This could be done by measuring the transport number of ionic compounds of increasing MW. Indeed, as controversy and doubts remained about a MW cut-off existence, nails are usually treated as a black box and most authors perform experiments with drugs because of their systemic potency and not according to their suitability for nail delivery. Obviously, it would be of little use to administrate a drug only because it penetrates well in the nail but, if a very potent drug hardly penetrated the nail, a drug of less potency with a good penetration profile would be a better option. The determination of the relationship between MW and transport number could be a useful tool to predict how efficiently a molecule will...
be delivered by iontophoresis and then, it could be used to screen potential drug candidates.

The association of chemical enhancers and iontophoresis has been recently investigated by Hao et al. (2008b). The authors (Hao et al., 2008b) found that thioglycolic acid was able to increase the transport number of tetraethylammonium but had no effect on electroosmosis. Although this finding certainly needs to be confirmed by further investigations, this could lead to an enhancement of the already enhanced delivery due to iontophoresis and therefore, maximize the possibility to treat effectively and quickly certain nail diseases.

The future of iontophoresis for nail application is not known but device to apply efficiently iontophoresis to several finger or toe nails could simplify treatments. Indeed, nail diseases usually affect several fingers or toes and thus topical treatments are individually administrated to each finger or toe. For iontophoresis, a device could be designed in the form of a glove or of a sock with little gel pads at each finger/toe extremity connected to electrodes; the skin electrodes could be placed on the dorsal face of the hand/foot and the treatment gel pads could be filled injecting the drug solution. The whole device could be simply pulled on the hand/foot, tighten to the limb with adequate elastics or bands and the iontophoretic device could be fixed along the wrist or the ankle. This could allow the simultaneous treatment of all the diseased area and each device could be adapted to individual.

Finally, this work has used nail plates from healthy volunteers only; one could expect the structure and properties of the diseased nails to be very different, so it would be very interesting to validate our findings by experimenting on diseased nail.
CONCLUSIONS

We developed a new technique to follow the \textit{in vitro} penetration of fluorescent markers into human nails using confocal microscopy. Iontophoresis of SF and NBC was performed during 18 hours and showed that the current intensity had no influence on the markers penetration depth. Under no conditions the markers went deeper than 12 \% of the whole nail thickness and the layer exposed to the fluorescent marker was not a discriminating factor. During passive diffusion and iontophoresis, both markers mainly followed the transcellular pathway.

The \textit{in vitro} experiment investigating the contribution of electroosmosis gave results stained with significant variability. However, results were in favour of a negative charge of the nail at physiological pH.

Iontophoresis enhanced only moderately the delivery of the fluorescent markers and mannitol compared to passive diffusion whereas a clear enhancement was noticeable for lithium and sodium ions.

The \textit{in vitro} study of cation electromigration revealed similarities between skin and nail iontophoresis and a cation permselectivity of the nail at pH 7.0. Unlike electroosmosis, the fluxes measured showed little variability.

The iontophoretic extraction of chloride and sodium ions was successful and ionic fluxes were measured \textit{in vivo} for the first time. The participants did not experience any major discomfort during the experiments and, as a general rule, the current application as well as the resulting voltage were well accepted by the participants.

On the whole, it has been proven that transungual iontophoresis enhanced \textit{in vitro} and \textit{in vivo} fluxes of small ions compared to passive diffusion. The feasibility of \textit{in vivo} iontophoresis was established and showed that further investigations were necessary to fully demonstrate the ability of this technique to enhance transungual drug delivery.
REFERENCES


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APPENDICES
Appendix 1: Confocal microscopy images
Fig. 1: Transversal measurements on NBC. Passive images. Panel A: images from nail 4RF2. Panel B: images from nail 12LF5. Images taken at objective x10.
Fig. 1 (continued): Transversal measurements on NBC. Passive images. Panel C: images from nail 14RF4. Images taken at objective x10.

DORSAL MEASUREMENTS

Fig. 2: Dorsal measurements on NBC. Passive 3-D reconstructions. Panel A: images from nail 4RF2. Images taken at objective x10.
Fig. 2(continued): Dorsal measurements on NBC. Passive 3-D reconstructions. Panel B: images from nail 12LF5. Panel C: images from nail 14RF4. Images taken at objective x10.
Fig. 3: Dorsal measurements on NBC. Passive z-stack. Panels A: images from nail 4RF2. Panel B: images from nail 12LF5. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on NBC. Passive z-stack. Panels B: images from nail 12LF5. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on NBC. Passive z-stack. Panel B: images from nail 12LF5. Panels C: images from nail 14RF4. Images taken at objective x10.
Fig. 3: Dorsal measurements on NBC. Passive z-stack. Panel A: images from nail 4RF2. Panel B: images from nail 12LF5. Panel C: images from nail 14RF4. Images taken at objective x10.
Fig. 4: NBC pathway during NBC.Passive. Panel A: images from nail 4RF2. Panel B: images from nail 12LF5. Panel C: images from nail 14RF4. Images taken at objective x20.
NBC.0.1
Fig. 1: Transversal measurements on NBC.0.1 images. Panel A: images from nail 2LF5. Panel B: images from nail 12RF1. Images taken at objective x10.
Fig. 1 (Continued): Transversal measurements on NBC.0.1 images. Panel C: images from nail 7LF4. Images taken at objective x10.

DORSAL MEASUREMENTS

Fig. 2: Dorsal measurements on NBC.0.1 3-D reconstructions. Panel A: images from nail 2LF5. Images taken at objective x10.
Fig. 2: Dorsal measurements on NBC.0.1 3-D reconstructions. Panel B: images from nail 12RF1. Panel C: images from nail 7LF4. Images taken at objective x10.

Fig. 3 Dorsal measurements on NBC.0.1 z-stack. Panel A: images from nail 2LF5. Images taken at objective x10.
Fig. 3 (continued) Dorsal measurements on NBC.0.1 z-stack. Panels A: images from nail 2LF5. Images taken at objective x10.
Fig. 3 (continued) Dorsal measurements on NBC.01 z-stack. Panels B: images from nail 12RF1. Images taken at objective x10.
Fig. 3 (continued) Dorsal measurements on NBC.01 z-stack. Panels B: images from nail 12RF1. Images taken at objective x10.
Fig. 3 (continued) Dorsal measurements on NBC.0.1 z-stack. Panels C: images from nail 7LF4. Images taken at objective x10.
Fig. 3 (continued) Dorsal measurements on NBC.0.1 z-stack. Panel C: images from nail 7LF4. Images taken at objective x10.
Fig. 1: Transversal measurements on NBC.0.4 images. Panel A: images from nail 4LF4. Panel B: images from nail 12LF3. Images taken at objective x10.
Fig. 1 (continued): Transversal measurements on NBC.0.4 images. Panel C: images from nail 13LF3. Images taken at objective x10.

Fig. 2: Dorsal measurements on NBC.0.4 3-D reconstructions. Panel A: images from nail 4LF4. Images taken at objective x10.
Fig. 2 (continued): Dorsal measurements on NBC.0.4 3-D reconstructions. Panel B: images from nail 12LF3. Panel C: images from nail 13LF3. Images taken at objective x10.
Fig. 3: Dorsal measurements on NBC.0.4 z-stack. Panel A: images from nail 4LF4. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on NBC.0.4 z-stack. Panels A: images from nail 4LF4. Panels B: images from nail 12LF3. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on NBC.0.4 z-stack. Panels B: images from nail 12LF3. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on NBC.0.4 z-stack. Panels C: images from nail 13LF3. Images taken at objective x10.
Fig. 3: Dorsal measurements on NBC.0.4 z-stack. Panel C: images from nail 13LF3. Images taken at objective x10.
Fig. 4: NBC pathway during NBC.0.4. Panels A: images from nail 4LF4. Images taken at objective x20
Fig. 4 (continued): NBC pathway during NBC.0.4. Panel A: images from nail 4LF4. Panels B: images from nail 12LF3. Images taken at objective x20.
Fig. 4 (continued): NBC pathway during NBC.0.4. Panel B: images from nail 12LF3. Panels C: images from nail 13LF3. Images taken at objective x20.
Fig. 4 (continued): NBC pathway during NBC.0.4. Panel B: images from nail 12LF3. Panels C: images from nail 13LF3. Images taken at objective x20.
TRANSVERSAL MEASUREMENTS

Fig. 1: Transversal measurements on SF. Passive images. Panel A: images from nail 2LF5. Panel B: images from nail 4R. Images taken at objective x10.
Fig. 1 (continued): Transversal measurements on SF. Passive images. Panel C: images from nail 12LF2. Images taken at objective x10.

DORSAL MEASUREMENTS

Fig. 2: Dorsal measurements on SF. Passive 3-D reconstructions. Panel A: images from nail 2LF5. Images taken at objective x10.
Fig. 2 (continued): Dorsal measurements on SF. Passive 3-D reconstructions. Panel B: images from nail 4R. Panel C: images from nail 12LF2. Images taken at objective x10.
Fig. 3: Dorsal measurements on SF.Pasive z-stack. Panel A: images from nail 2LF5. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.Passive z-stack. Panel B: images from nail 4R. Panel C: images from nail 12LF2. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.Passive z-stack. Panel C: images from nail 12LF2. Images taken at objective x10.
Fig. 1: Transversal measurements on SF.0.1 images. Panel A: images from nail 2LF4. Panel B: images from nail 12LF1. Images taken at objective x10.
Fig. 1: Transversal measurements on SF.0.1 images. Panel C: images from nail 4LF5. Panel D: images from nail 7LF4. Images taken at objective x10.
Fig. 2: Dorsal measurements on SF.0.1 3-D reconstructions. Panel A: images from nail 2LF4. Panel B: images from nail 12LF1. Images taken at objective x10.
Fig. 2: Dorsal measurements on SF.0.1 3-D reconstructions. Panel C: images from nail 4LF5. Panel D: images from nail 7LF4. Images taken at objective x10.
Fig. 3: Dorsal measurements on SF.0.1 z-stack. Panel A: images from nail 2LF4. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.0.1 z-stack. Panel A: images from nail 2LF4. Panel B: images from nail 12LF1. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.0.1 z-stack. Panel B: images from nail 12L1F1. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.0.1 z-stack. Panel C: images from nail 4LF5. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.0.1 z-stack. Panel C: images from nail 4LF5. Panel D: images from nail 7LF4. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.0.1 z-stack. Panel C: images from nail 4LF5. Images taken at objective x10.
Fig. 4 (continued): SF pathway during SF.0.1. Panel A: images from nail 2LF4. Panel B: images from nail 12LF1. Images taken at objective x20.
Fig. 4(continued): SF pathway during SF.0.1. Panel B: images from nail 12LF1. Panel C: images from nail 4LF5. Images taken at objective x20.
Fig. 4(continued): SF pathway during SF.0.1. Panel C: images from nail 4LF5. Images taken at objective x20.
Fig. 4(continued): SF pathway during SF.0.1. Panel D: images from nail 7LF4 Images taken at objective x20.
Fig. 1: Transversal measurements on SF.0.4 images. Panel A: images from nail 12RF5. Panel B: images from nail 2LF4. Images taken at objective x10.
Fig. 1 (continued): Transversal measurements on SF.0.4 images. Panel C: images from nail 4LF5. Images taken at objective x10.

**DORSAL MEASUREMENTS**

Fig. 2: Dorsal measurements on SF.0.4 3-D reconstructions. Panel A: images from nail 12RF5. Images taken at objective x10.
Fig. 2 (continued): Dorsal measurements on SF.0.4 3-D reconstructions. Panel B: images from nail 2LF4. Panel C: images from nail 4LF5. Images taken at objective x10.
Fig. 3: Dorsal measurements on SF.0.4 z-stack. Panel A: images from nail 12LF5. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.0.4 z-stack. Panel A: images from nail 12LF5. Panel B: images from nail 2LF4. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.0.4 z-stack. Panel B: images from nail 2LF4. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF:0.4 z-stack. Panel B: images from nail 2LF4. Panel C: images from nail 4LF5. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.0.4 z-stack. Panel C: images from nail 4LF5. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.0.4 z-stack. Panel C: images from nail 4LF5. Images taken at objective x10.
SF.V.Passive
TRANSVERSAL MEASUREMENTS

Fig. 1: Transversal measurements on SF.V.Passive images. Panel A: images from nail 1RF5. Panel B: images from nail 2RF5. Images taken at objective x10.
Fig. 1(Continued): Transversal measurements on SF.V.Passive images. Panel C: images from nail 12RF4. Images taken at objective x10.

**DORSAL MEASUREMENTS**

Fig. 2: Dorsal measurements on SF.V.Passive 3-D reconstructions. Panel A: images from nail 1RF5. Images taken at objective x10.
Fig. 2: Dorsal measurements on SF.V.Passive 3-D reconstructions. Panel B: images from nail 2RF5. Panel C:
images from nail 12RF4. Images taken at objective x10.

Fig. 3: Dorsal measurements on SF.V.Passive z-stack. Panel A: images from nail 1RF5. Images taken at
objective x10.
Fig. 3 (continued): Dorsal measurements on SF.V.Passive z-stack. Panel A: images from nail 1RF5. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.V.Passive z-stack. Panel B: images from nail 2RF5. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.V.Passive z-stack. Panel B: images from nail 2RF5. Panel C: images from nail 12RF4. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.V.Passive z-stack. Panel C: images from nail 12RF4. Images taken at objective x10.

Fig. 4: SF pathway during SF.V.Passive. Images taken at objective x20.
Fig. 1: Transversal measurements on SF.V.0.1 images. Panel A: images from nail 12RF3. Images taken at objective x10.
Fig. 1(continued): Transversal measurements on SF.V.0.1 images. Panel B: images from nail 2LF. Panel C: images from nail 7LF5. Images taken at objective x10.
Fig. 1(continued): Transversal measurements on SF.V.0.1 images. Panel D: images from nail 13RF4. Images taken at objective x10.

DORSAL MEASUREMENTS

Fig. 2: Dorsal measurements on SF.V.0.1 3-D reconstructions. Panel A: images from nail 12RF3. Images taken at objective x10.
Fig. 2 (continued): Dorsal measurements on SF.V.0.1 3-D reconstructions. Panel B: images from nail 2LF3. Panel C: images from nail 7LF5. Images taken at objective x10.
Fig. 2 (continued): Dorsal measurements on SF.V.0.1 3-D reconstructions. Panel D: images from nail 13RF4. Images taken at objective x10.

Fig. 3: Dorsal measurements on SF.V.0.1 z-stack. Panel A: images from nail 12RF3. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.V.0.1 z-stack. Panel A: images from nail 12RF3. Panel B: images from nail 2LF3. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.V.0.1 z-stack. Panel B: images from nail 2LF3. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.V.0.1 z-stack. Panel C: images from nail 7LF5. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.V.0.1 z-stack. Panel C: images from nail 7LF5. Panel D: images from nail 13RF4. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.V.0.1 z-stack. Panel D: images from nail 13RF4. Images taken at objective x10.

Fig. 4: SF pathway during SF.V.0.1. Panel A: images from nail 12RF3. Images taken at objective x20.
Fig. 4 (continued): SF pathway during SF.V.0.1. Panel A: images from nail 12RF3. Images taken at objective x20.
Fig. 4 (continued): SF pathway during SF.V.0.1. Panel B: images from nail 2LF3. Images taken at objective x20.
Fig. 4 (continued): SF pathway during SF.V.0.1. Panel B: images from nail 2LF3. Panel C: images from nail 7LF5. Images taken at objective x20.
Fig. 4 (continued): SF pathway during SF.V.0.1. Panel C: images from nail 7LF5. Images taken at objective x20.
Fig. 4 (continued): SF pathway during SF.V.0.1. Panel C: images from nail 7LF5. Panel D: images from nail 13RF4. Images taken at objective x20.
Fig. 4 (continued): SF pathway during SF.V.0.1. Panel D: images from nail 13RF4. Images taken at objective x20.
SF.I.0.1
TRANSVERSAL MEASUREMENTS

Fig. 1: Transversal measurements on SF.I.0.1 images. Panel A: images from nail 12LF1. Panel B: images from nail 8LF4. Images taken at objective x10.
Fig. 1(continued): Transversal measurements on SF.I.0.1 images. Panel C: images from nail 4LF4. Images taken at objective x10.

DORSAL MEASUREMENTS

Fig. 2: Dorsal measurements on SF.I.0.1 3-D reconstructions. Panel A: images from nail 12LF1. Images taken at objective x10.
Fig. 2 (continued): Dorsal measurements on SF.I.0.1 3-D reconstructions. Panel B: images from nail 8LF4. Panel C: images from nail 4LF4. Images taken at objective x10.

Fig. 3: Dorsal measurements on SF.I.0.1 z-stack. Panel A: images from nail 12LF1. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.1.0.1 z-stack. Panel A: images from nail 12LF1. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.I.0.1 z-stack. Panel A: images from nail 12LF1. Panel B: images from nail 8LF4. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.I.0.1 z-stack. Panel B: images from nail 8LF4. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.I.0.1 z-stack. Panel C: images from nail 4LF4. Images taken at objective x10.
Fig. 3 (continued): Dorsal measurements on SF.I.0.1 z-stack. Panel C: images from nail 4LF4. Images taken at objective x10.

Fig. 4: SF pathway during SF.I.0.1. Panel A: images from nail 12LF1. Images taken at objective x20.
Fig. 4 (continued): SF pathway during SF.I.0.1. Panel A: images from nail 12LF1. Images taken at objective x20.
Fig. 4 (continued): SF pathway during SF.I.0.1. Panel B: images from nail 8LF4. Images taken at objective x20.
Fig. 4 (continued): SF pathway during SF.1.0.1. Panel B: images from nail 8LF4. Panel C: images from nail 4LF4. Images taken at objective x20.
Fig. 4 (continued): SF pathway during SF.I.0.1. Panel C: images from nail 4LF4. Images taken at objective x20.
SF Binding Study
Fig. 1: SF binding study. Pieces D1, E1 and F1 at 24 hours.
Fig. 2: SF binding study. Pieces D2, E2 and F2 at one week.
Fig. 3: SF binding study. Pieces D3, E3 and F3 at two week.
Appendix 2: Mannitol recovered in the nails
### Set I

Mannitol content at pH 4.0:

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<td>Non exposed area</td>
<td></td>
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<tr>
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<td>1.69</td>
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Mannitol content at pH 5.0:

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<td>Mannitol content (nmol/mg)</td>
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Mannitol content at pH 7.4:

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<td>Non</td>
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Appendix 3: Ethical forms for nail collection
Participant information sheet

Collection of finger nails by nail clipping.

You are being invited to take part in a research study.
Before you decide on your participation, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.
Please, contact us if there is anything that is not clear or if you would like more information.
Take as much time as you want to decide whether or not you wish to take part in this study.

Thank you for reading this information sheet.

Purpose of the study:
Fungal infections of the nail are a common disease. Unfortunately, they are quite difficult to treat: local treatments have low efficiency because drugs penetrate poorly into the nail. The alternative is oral treatments (taken by mouth) but this exposes the whole body to the medicine which results in frequent adverse effects.
Our research team is looking into new ways of applying drugs to the nail. Specifically, we are investigating nail iontophoresis. This technique consists on the application of small electrical currents to the nail in order to improve drug penetration. Iontophoresis is currently used to increase the passage of drugs through the skin, and we think that it could also be used for delivering drugs to the nail.

This study wants to answer the following questions:
1. Does the application of small electrical currents (iontophoresis) improve the
penetration of antifungal drugs (such as terbinafine, griseofulvin or amorolfine) into the nail as compared with the standard topical treatments available?

And if the answer is yes:

2. Which are the most appropriate drugs; formulations and treatment conditions that should be used to treat nail fungal infections with this technique?

For doing these experiments we need human nails. Hooves from animal are different in their structure and composition to human nails, so we may get the wrong answers to the questions above if we use them.

This is the reason for performing this fingernail collection. We require healthy volunteers to donate their fingernails tips (8-10 mm length) to our research, so we can use the nails to do experiments that study antifungal drugs penetration.

Participants must be healthy and their nails should not present any disease, brittleness, infection or decolouration. This is important because diseased and healthy nails may behave differently.

The chief investigator teaches Pharmacy to undergraduate students. To avoid conflicts of interests, the undergraduate pharmacy students at the University of Bath are also excluded from this study.

**Your participation in the research:**

It is up to you to decide whether or not to take part in this study. If you agree to participate the following will happen:

1. You will be given a copy of this information sheet to keep for your records.

2. There will be some time (from 1 day minimum to 2-3 months) during which you will let your fingernails grow. Note that you can choose how many (1 to 10) nails, in which hand, and which nails you prefer to let grow. During all this time, you can change your mind about your participation; you are still free to withdraw at any time and without giving a reason.

3. Once the free edge of the nails has reached the right length, and if you still want to donate them to our research, you will contact the research team. You will be asked to come to our laboratories (9W 4.04 and 9W 4.06) at the University of Bath and you will be given a consent form to sign. After you have given your informed consent, a researcher will cut the free edge of your fingernails with a standard manicure scissors.

4. Your nail clippings will be made anonymous and identified with a code: Subject 1/nail 1, Subject 1/nail 2 and so on. The researcher will record the date of the
donation, your name, age and gender, the finger and hand where each nail was harvested from and the use of nail cosmetics (if relevant). All this information will be kept separately from the nails so it will not be possible to identify the donor of each sample. The researcher will keep this information securely.

5. Once, the nail collection is done, you will be allowed to leave the laboratories and your participation will be considered as finished. The total donation act will take a maximum time of 30 minutes. To acknowledge you for your time and contribution, we will pay you £3 per nail donated.

6. You may if you wish, participate more than once into the study.

**Lifestyle modifications:**
We would expect you to avoid the use of nail cosmetics (specially nail lacquers) while your nails are growing. Some people may find unpractical or unpleasant to have long nails. You may decide to stop your participation at any moment, or just grow the nails you think more convenient.

**What will happen to my nails?**
Your nail clippings will be used to investigate if the application of low electrical currents is likely to provide a better treatment of fungal infections. For this, we will study the penetration of different compounds (fluorescent markers and antifungal drugs) into the nail.

Your nail clippings will NOT be used for DNA analysis or any other test concerning your genetic inheritance.

At the end of the experiments the nail will be disposed of according to the regulations of the University of Bath concerning the use of human tissues.

**Can I get my nails back?**
If you agree to donate your nails to us, they will be considered as a gift and you will NOT retain any rights on them. For example, you can NOT claim them back. However, we will only use them for the research described in this form.

**Side effects:**
There are not side effects or risks expected from your participation into this study, other than those associated with nail clipping. Some people may find unpractical or unpleasant to have long nails. You may feel inconvenient to have very short nails just after the nail
clipping.

**Possible benefits of taking part:**
There is no direct benefit for you as a result of your participation. This is a preliminary study which will hopefully help us to develop more effective ways to treat nail fungal infections.

**If something goes wrong:**
The University of Bath has site specific insurance to cover research on healthy volunteers. Please note that this insurance does NOT cover any harm that you could cause with your nails to yourself, others or any items during the growing period.

**Confidentiality:**
All personal information collected during the course of this research will be kept strictly confidential. When the results are made public, these will not include any names, initials, or any type of information which could result in your identification.

**Results of the study:**
The results of this study will be published in scientific journals and/or presented during conferences, and/or internal reports. You will be allowed to have a copy of published articles upon request.

**Organisation and funding of the research:**
This research is funded by the Department of Pharmacy and Pharmacology. The protocol has been reviewed by the Bath Local Research Ethics Committee.

**Contact for further information:**
We will be more than happy to answer to all the questions you could have about this research and your participation. Please contact:

Ms Julie Dutet: jd251@bath.ac.uk  Phone: 01225 38 ext.4317 or 4313

Dr Begoña Delgado-Charro: prsbd@bath.ac.uk  Phone: 01225 383969

You will be given a copy of this information sheet and a signed consent form to keep.

Thank you for considering taking part in this study.
CONSENT FORM

Title: Collection of finger nails for iontophoretic in vitro study.

Researcher: Ms. Julie Dutet
Principal Investigator: Dr. M. Begoña Delgado-Charro

1. I confirm that I have read and that I understand the information sheet dated 07/07/2006 (version 2) for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I understand that the donation of my fingernails is a gift. If I donate my nails to this study I will not retain any rights on them.

4. I agree to take part in the above study.

Name of Participant: Date: Signature:

Researcher: Date: Signature:

1 copy for participant, 1 copy for researcher
Questionnaire

Date:

1. Name: …………………………………..

2. Subject code: ……………………………

3. Gender of the participant: …………………

4. Age of the participant: ……………………

5. The donation concerns (circle nail code):

   L.F.1       L.F.2       L.F.3       L.F.4       L.F.5

6. Please, tell us if you used any nail cosmetics during the time you let your nails grow.
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7. Let us know if you had any previous nails infections or problems (brittleness, fungal infection, psoriasis, decolouration, etc).
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Thank you for your participation!

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Appendix 4: Ethical forms for *in vivo* experiments
Participant information sheet

Application of iontophoresis to finger nails

You are being invited to take part in a research study.
Before you decide on your participation, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.
Please, contact us if there is anything that is not clear or if you would like more information.
Take as much time as you want to decide whether or not you wish to take part in this study.

Thank you for reading this information sheet.

Purpose of the study:
Fungal infections of the nail are a common disease. Unfortunately, they are quite difficult to treat: local treatments have low efficiency because drugs penetrate poorly into the nail. The alternative is oral treatments (taken by mouth) but this exposes the whole body to the medicine which results in frequent adverse effects.
Our research team is looking into new ways of applying drugs to the nail. Specifically, we are investigating nail iontophoresis. This technique consists on the application of small electrical currents to the nail in order to improve drug penetration. Iontophoresis is currently used to increase the passage of drugs through the skin, and we think that it could also be used for delivering drugs to the nail.
Before this technique can be developed, we need to understand how the nail behaves under the passage of a small electrical current (0.1-0.4 mA). This study wants to answer the following questions:

3. How are physiological molecules (such as glucose, chloride or sodium) transported through the nail? This will tell us about the electrical properties of the nail and help in identifying the best drugs and iontophoretic conditions that could be used in future studies.
4. How does the nail behave under the application of a small electrical current? We will compare the properties of the nail before and after iontophoresis. We will also ask you to describe your feelings during current application. This will help us in identifying the iontophoretic conditions which provide the least discomfort to future participants.
5. Which type of formulation (solutions, gels, creams) is better adapted for nail iontophoresis?

For this, we require healthy volunteers (a maximum of 100 participants) which will participate at different stages of the research project. The participation of each volunteer will take a maximum of 4-5 hours. Participants must be healthy and their nails should not present any disease, brittleness or decolouration. This is important because diseased and healthy nails may behave differently.

We will NOT apply any drugs to your nail or skin.

**Your participation in the research:**
It is up to you to decide whether or not to take part in this study. If you do decide to take part:

7. You will be given a copy of this information sheet to keep for your records, and
8. You will be asked to sign a consent form.
9. If you change your mind about it, you are still free to withdraw at any time and without giving a reason.

**What is going to happen to you during the study:**
You will be involved in this research for a maximum of 4-5 hours.
You will participate in one experiment which will take place at our research laboratories at the University of Bath (9.W 4.04 - 4.06).
1. We will choose either the left or right hand as the testing site. The nail of the thumb will be used as the nail iontophoresis site. A nearby skin site will be used as the skin iontophoresis site. This is required to close the electrical circuit. (Figure 1)

![Figure 1: Example of device for iontophoresis to the nail](image)

2. The initial values of the transepidermal water loss (TEWL) and the colour of both the skin and nail sites will be measured. These are completely non-invasive procedures standard in dermatological and cosmetic studies. (Figure 2)

![Figure 2: Device used to determine the TEWL](image)

3. A plastic or glass cylinder will be attached to the nail and to the skin sites. This will be used to hold a formulation consisting of aqueous solutions of physiological buffers or simply sodium chloride (salt). In some experiments, a gel formulation will be used. A gel formulation will also contain excipients (such as polymers) approved for topical medicines. These formulations will NOT contain any drug.

4. Iontophoresis will be applied using two silver/silver chloride electrodes which are the electrodes used in all the approved iontophoretic devices for skin applications. These electrodes will be placed inside the formulation, taking special care to avoid
any direct contact between the nail and/or the skin, and the electrode. In some experiments, a commercial Ag/AgCl electrode will be used for the skin site.

5. Current will be applied using the Phoresor Auto device which has the CE mark and is routinely used for iontophoretic skin applications. This device automatically stops passing current if the electrical circuit is faulty. The intensity of current will be fixed to a value comprised between 0.1 and 0.4 milliamperes (which is a very small current). In all cases, the area of the nail will be considered so the density of current does not exceed the limit of 0.5 milliamperes/square centimeter, which is considered the maximum value of current density well tolerated by the skin.

6. Four iontophoretic periods of 30 minutes each will be done. At the end of each iontophoretic period, the formulation will be removed and reserved for analysis. The plastic cylinder will be refilled with fresh formulation. Between two current periods, you will be allowed to have a comfort break.

7. During current application, you must remain seated. You will be able to perform some quiet activities like reading. The experiments will take place in a research laboratory, thus, accordingly to safety rules you will not be able to drink, eat or smoke during this time.

8. After 4 periods of 30 minutes (total 2 hours current) the iontophoresis will be stopped. The formulation will be taken for analysis and the plastic holder and commercial electrode removed.

9. Finally, the colour and TEWL values of the nail and skin application sites after current will be measured. We will do these measurements at the following times: just after iontophoresis, and 15, 30, 45, and 60 minutes after iontophoresis. In between these measurements, and in case of need, you will be allowed to have a comfort break out of the laboratory (eat, drink, etc).

10. At the end of the experiment, you will be asked to fill a questionnaire about the degree of feelings, sensations and/or discomfort you felt at the application sites during the experiment.
Lifestyle modifications:
We would expect you to avoid the use of nail cosmetics (creams, nail lacquers, for example) prior to the experiment.

Procedure which is being tested during the experiment:
Iontophoresis is a technique currently used to pass drugs across the skin. Some iontophoretic devices have been recently been marketed. There are not devices available for nail iontophoresis because this is a new idea.
The iontophoresis device Phoresor Auto is marketed and has a CE mark for skin applications.

Side effects of iontophoresis:
Iontophoresis is considered to be a safe technique. It can provoke light feelings of tingling or heat sensation when applied to skin. Some redness (erythema) is observed that usually resolves in several days. Several iontophoretic devices are approved in the market for delivering drugs to the skin.
There is not information on the effects of iontophoresis on the nail, but one would expect them to be similar to those observed with the skin.
If you experience unpleasant feelings or discomfort during the experiment, you should communicate it to the researcher who will stop the experiment at once at your request.

Possible benefits of taking part:
There is no direct benefit for you as a result of your participation. This is a preliminary study which will hopefully help us to develop more effective ways to treat nail fungal infections.

If something goes wrong:
The University of Bath has site specific insurance to cover research on healthy volunteers.

Acknowledgement:
To thank you for your participation and time we will pay you 15 pounds at the end of the experiment. You will receive this complete amount even if the experiment is stopped before completion, for example if you experienced strong discomfort during current application.
Confidentiality of taking part in this study:
All personal information collected during the course of this research will be kept strictly confidential. When the results are made public, these will not include any names, initials, or any type of information which could result in your identification.
The researcher may ask you for permission to take a picture of the experiment. If you agree, the photography will only include the treated skin or nail sites.

Results of the study:
The results of this study will be published in scientific journals and/or presented during conferences, and/or internal reports. You will be allowed to have a copy of published articles upon request.

Organisation and funding of the research:
This research is funded by the Department of Pharmacy and Pharmacology.
The protocol has been reviewed by the Salisbury and South Wiltshire Research Ethics Committee.

Contact for further information:
We will be more than happy to answer to all the questions you could have about this research and your participation.
Please contact:

Ms Julie Dutet: jd251@bath.ac.uk              Phone: 01225 38 ext.4317 or 4313
Dr Begoña Delgado-Charro: prsbd@bath.ac.uk       Phone: 01225 383969

You will be given a copy of this information sheet and a signed consent form to keep.

Thank you for taking part in this study.
CONSENT FORM

Title: Application of iontophoresis to finger nails.

Researcher: Ms. Julie Dutet
Principal Investigator: Dr. M. Begoña Delgado-Charro

1. I confirm that I have read and that I understand the information sheet dated 01/03/2006 (version 2) for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I allow the researcher to take photographs of the application sites during the experiment.

4. I agree to take part in the above study.

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Name of Participant:    Date:    Signature:

Researcher:    Date:    Signature:

1 copy for participant, 1 copy for researcher
Experiment Code:
Application sites:
Skin formulation:
Nail formulation:
Polarity of nail site:
Intensity of current applied:

**Questionnaire**

Express your degree of sensations and discomfort using the scale from 0 to 5. Please indicate 0 if you didn’t feel any discomfort, 5 if you felt a very strong discomfort.

1. Discomfort at the skin site: 0 1 2 3 4 5

2. Discomfort at the nail site: 0 1 2 3 4 5

3. General discomfort: 0 1 2 3 4 5

4. Please, describe your sensations and discomfort if you had any.
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5. Let us know if you have any comments about the experiment.
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Thank you for your participation!