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INNOVATIVE DELIVERY SYSTEMS FOR THE LOCAL ADMINISTRATION OF IMMUNOMODULATORY DRUGS

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Summary

Immunomodulatory drugs are important tools for the treatment of numerous local diseases. Their topical administration can be particularly useful to obtain a therapeutic effect at the site of interest, limiting at the same time the systemic side effects.

Despite this great potential, the local administration is challenging for several reasons and, in particular, for the presence of biological barriers limiting drug penetration and accumulation in the target tissue.

The aim of this work was the development of innovative formulations for the local administration of drugs with immunomodulatory properties. Particular attention has been addressed to novel technologies, such as microemulsions and nanoparticles, which could be useful to optimize the administration, increasing the local delivery and/or reducing systemic absorption.

After a **general introduction** on the immune system, the drugs used and the local administration, the work was developed in two different parts.

In **PART 1** the dermal administration of an immunostimulant drug, imiquimod, was studied. Initial studies were performed in order to highlight the mechanisms involved in the skin accumulation, then attention was directed to the development of innovative formulations based on microemulsion technology to increase the efficiency of skin delivery.

In **PART 2** two immunosuppressive drugs (corticosteroids) were selected: triamcinolone acetonide (TA) and dexamethasone palmitate (DXP). In case of TA, microemulsions for the buccal delivery were developed, characterized and evaluated in vitro. For DXP, nanoparticles based on hyaluronic acid conjugate for the topical drug delivery were prepared.

General introduction

Table of contents

1 The immune system [1, 2] 9
1.1 The immune response 9
1.1.1 Innate immune system 9
1.1.2 Adaptive immune system 11
1.2 Diseases associated to immune response 11
2 Drugs acting on the immune system [3] 13
3 Imiquimod 14
3.1 Mechanism of action 14
3.2 Approved IMQ based therapies 16
3.2.1 Actinic Keratosis (AK) 16
3.2.2 Basal cells carcinoma (BCC) 17
3.2.3 Anogenital warts [13] 18
3.3 Off-label uses [6, 14] 19
3.3.1 Cutaneous infections 19
3.3.2 Neoplastic conditions 20
4 Corticosteroids [17-19] 22
4.1 Mechanism of action 22
4.2 Topical corticosteroids 23
4.2.1 Triamcinolone acetonide 23
4.2.2 Dexamethasone palmitate 24
5 Local administration 26
6 Bibliography 30

1 The immune system [1, 2]

The immune system is one of the most complex systems of our body and it acts in order to protect against exogenous harmful agents, usually named as non-self. Many kinds of organisms are potentially infective: other living things, non-living things such as virus and also molecules such as prions.

In mammals, the defensive mechanisms can be classified as passive or active. Passive are the natural barriers of our bodies, such as the skin, which is a physical barrier against the microbe penetration, or like the acid environment in the stomach which is able to kill many microbes ingested along with food. On the other side, active defence consists basically in the complex mechanism of the immune response.

1.1 The immune response

The immune response is a complex combination of events directed towards the elimination of potentially harmful non-self agents and it can be divided in two main components: innate and adaptive response.

The innate component of the immune system is less specific, it lacks of memory and it raises quickly, within minutes or hours from infection. The adaptive immunity, on the contrary, is specifically adapted for the inducing pathogens and improves with subsequent exposures to the same pathogen, due to the presence of memory cell line. The adaptive response is, however, less rapid and raises usually over a few days.

1.1.1 Innate immune system

The key for the recognition of infectious agents by the innate response is the expression of a family of receptors called pattern recognition receptors (PRRs).

PRRs are able to recognize: 1) conserved features of infectious agents which generally are shared by different classes of microbes called pathogen-associated

molecular patterns (PAMPs) and 2) specific patterns associated to cell components released during cell damage or death, called damage-associated molecular patterns (DAMPs). The cells responsible for the innate response are largely distributed into tissues and they express different types of PRR.

PRRs include different families of receptors. Among them, the most studied is surely the tool-like receptor family (TLRs).

The main cell types involved in the innate immune system are:

- macrophages: one of the two main specialized phagocytes; they are divided in two main types:
  - resident macrophages that are present in steady-state tissues and can detect the presence of microbes.
  - recruited macrophages that are not tissue resident cells, they develop from circulating precursors called monocytes.
- Mast Cells reside in the steady-state tissues and are characterized by the presence of internal granules which are externalized when stimulation occurs, they help in the development of local inflammation.
- Granulocytes are the major population of leukocytes (white blood cells) and they are characterized by the presence of cytoplasmatic granules. They are subdivided in three groups: neutrophils, eosinophils and basophils.
- Natural killer cells are present in tissues as resident cells but they can be recruited to inflammation sites as well. They can kill other cells and regulate immune response.

It is also important to underline the crucial role of the innate response in the development of the adaptive response.

1.1.2 Adaptive immune system

The adaptive immune response is based on lymphocytes presence. Lymphocytes are classified in:

- T cells, which can be further classified as
  - CD4 T cells, regulators in immune response;
  - CD8 T cells that can differentiate into cytotoxic cells with the ability to kill infected cells.
- B cells which are precursors of plasma cells and secrete soluble antibodies.

The recognition of molecules from infectious agents by lymphocytes is mediated by specialized antigen receptors present only on adaptive system immune cells.
In contrast with agonist of PRRs, antigens are usually highly specific towards a particular infectious agent.

1.2 Diseases associated to immune response

The immune response is a complex process that involves different components of the immune system: overall, the response is finely controlled by feedback mechanisms and cellular communication. A small alteration of this intricate machine can lead to pathological states.
The two main causes of immune diseases are linked to:

1. Self-non-self discrimination: autoimmune diseases are the most common class of diseases affecting the immune system. In this case the immune system develops a response against self-components. The effector mechanisms can cause tissue damage by the intervention of antibodies, large immune complex or T cells that aberrantly activate macrophages or cytotoxic T cells. The clinical pattern changes as a function of the distribution of the antigen: it could be localized in a specific organ or distributed into the body. In some cases, an antigen which is normally

innocuous, generates an intense immune response. The best example is represented by allergies.

2. Immunodeficiency diseases: they are divided in primary and secondary (or acquired) immunodeficiency. The primary ones are mainly due to a genetic defect that alters the function of a component that is normally involved in the immune response. The secondary ones are acquired during lifetime because of an external agent. The main example is HIV infection.

Moreover, it is important to underline that in some cases it is necessary to modulate the immune response, either enhancing or suppressing it, in order to overcome pathological conditions not directly associated to dysfunction of the immune system. For example, the normal immune response could lead to the appearance of unpleasant symptoms, like in the case of inflammatory response that is characterized by pain, heat, redness, swelling and loss of functionality.
In other cases, it could be useful to enhance the immune response to help the fight against some kind of infections or cancers.

2 Drugs acting on the immune system [3]

The term immunomodulation usually refers to all the interventions aimed at modifying the immune response. Drugs which are able to affect the immune response can be classified in:

-immunostimulants

-immunosuppressants

Sometime, it could be necessary to induce or suppress the immune response in order to manage different pathological conditions. Augmentation of the immune response is desirable in the case of immunodeficiency, in order to prevent infections or to fight cancer while, in the case of transplantation or autoimmune pathologies, drugs able to reduce the immune response are required.

Being such a complex system, when a disease is well-localized and in order to reduce possible side effects, a local application is preferred. There are many drugs currently employed for local application.

Concerning the immunosuppressive drugs, corticosteroids are probably the most common molecules used for local treatment. Interesting is also the family of macrolactums which includes macrolides such as tacrolimus and pimecrolimus, employed for the treatment of atopical dermatitis, and cyclosporine, which is a cyclic lipophilic polypeptide, presently commercialized as eye drops for the treatment of dry eye disease.

Regarding drugs with immunostimulant properties applied locally, the class of imidazoquinoles amines, represented by resiquimod and imiquimod, is particularly interesting. These molecules are mainly used to treat skin lesions induces by virus (herpes simplex virus) or neoplastic lesions.

3 Imiquimod

In 1995 a new class of immunomodulating agents called imidazoquinolineamines, was first synthetized to obtain analogues of nucleosides, guanosine in particular, with antiviral activity. However, preliminary studies showed that, when tested on culture cells system, no direct antiviral activity could be highlighted. Further studies on these compounds have shown that the efficacy against viral lesions could be attributed to the induction of cytokines production due to a pro-inflammatory effect.

Within this class, imiquimod (IMQ), became increasingly interesting because of its antitumoral activity, largely demonstrated in preclinical models [4], based on its effect on the innate immune system.

3.1 Mechanism of action

Many studies have demonstrated that IMQ exerts its primary effect on dendritic cells: in vitro studies demonstrate that at concentration up to 5 μg/ml it elicited a robust pro-inflammatory response. [4] IMQ acts via its binding to toll-like receptors family, in particular 7 and 8 [5]. These receptors are able to recognise specific molecular profiles associated to different pathogens: in particular they recognise specific lipopolysaccharides, proteoglycans, RNA, oligoxynucleotides [1]. From a structural point of view TLRs are glycoproteins with both extracellular and cytoplasmatic domain. They are expressed on dendritic cells, phagocytes, lymphocytes, epithelial and endothelial cells which are all involved in the primary defence of the organism. The bond activates a central transcription factor, NF-ƙβ, which is normally bound to an inhibitor ƙβ. When TLR 7 and 8 are activated, NF-ƙβ is dissociated from the inhibitor and it is able to enter to the nucleus where it promotes the transcription of different genes which codify for several pro-inflammatory mediators such as: tumor necrosis factor α (TNF-α), interferon α

(IFN-α), interleukin-1 (IL-1), (IL-6), (IL-8), (IL-10) and (IL-12). These cytokines up-regulate cell-mediated Th-1 responses, which have antitumoral and antiviral effects, while they down regulate Th-2 responses.

Moreover, it has been demonstrated that IMQ is able to interact with adenosine receptors A2A and down regulates adenylyl cyclase. The combination of these interactions brings to the suppression of a negative feedback mechanism which normally regulate the inflammatory response. [5, 6]

Finally, a study based on IMQ application for the treatment of basal cell carcinoma, demonstrated also a direct pro-apoptotic effect of IMQ via its activation of the caspase cascade, along with an activation of autophagy mechanism, in a dose dependent manner [7]. **Figure 1** resumes IMQ mechanisms of action [4]**.**

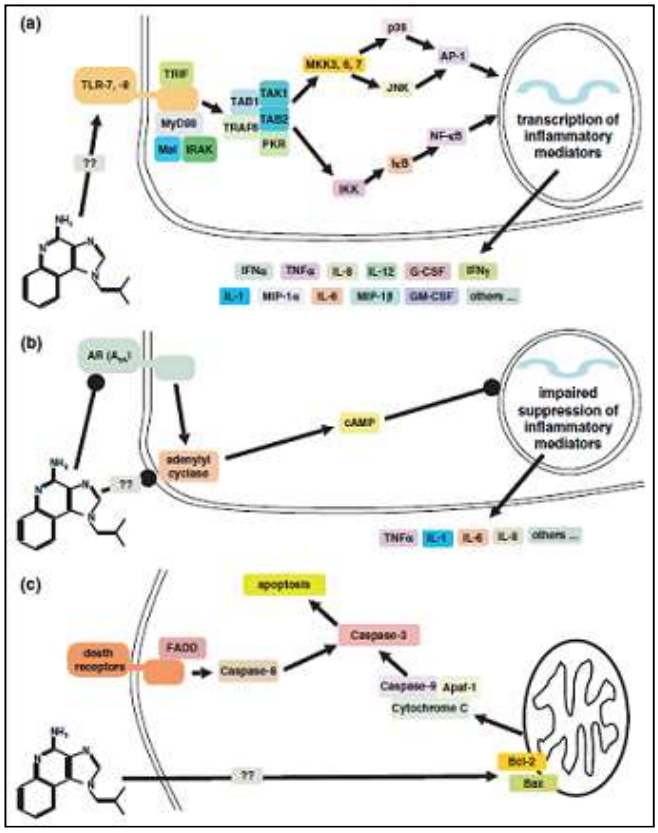

Figure 1. IMQ mechanisms of action.

3.2 Approved IMQ based therapies

IMQ was approved in 1997 by the U.S Food and Drugs Administration for treating external genital and perianal warts. In 2004 its use was extended to the treatment of actinic keratosis (AK) and superficial basal cell carcinoma (sBCC). For this indication, its efficacy resulted superior to topical fluorouracil and photodynamic therapy (PDT)[8, 9]. IMQ is present on the market as a semisolid cream formulation at 5 or 3.75% w/w.

Despite the high potential, IMQ is associated with a large number of local side effects. The most common local adverse reactions to IMQ, especially when applied at high dose are: erythema, itching, burning, irritation, pain, erosions and ulcerations. Cases of cutaneous side effects include also psoriasis onset, as well as eczema, vitiligo-like depigmentation, pemphigus and lichen planus.

Less frequent, but possible, are the systemic adverse reactions: headache, febrile seizure, influenza-like symptoms, as well as nausea, myalgias and arthalgias have been recorded, usually associated to the application of IMQ on large area or onto highly vascularized lesions.

3.2.1 Actinic Keratosis (AK)

Ak is a precancerous lesion characterised by a neoplastic proliferation of keratinocytes localized in the basal layer of the epidermis. The keratinocytes are abnormal, hyperplastic with atypical nucleus. The lesions are usually less than 1 cm of diameter and have a brownish/reddish coloration. The main risk factors for the development of this pathology are fairy skin, UV rays exposure and immunodeficiency.

Each single lesion can have three different outcomes:

- Spontaneous regression
- Remain stable, without further progression
- Evolve into invasive squamous cells carcinoma, which rarely metastasize.

In a study of 2009, conducted by Criscione and all, it has been underlined that the risk of evolution into cancerous cells is about 0.6% within one year and 2.57 % within 4 years from the development of the lesion [10]. Other studies found out a risk of progression into cancerous form between 0.025 and 16% of cases per year, per single lesion. Moreover the risk increases with the increase of the number of lesions [11]. Most of the lesions are completely asymptomatic and they frequently appear on areas of the body usually exposed to UV ray.

For AK treatment, imiquimod cream formulation at 5% has to be applied 3 times per week for 4 weeks on clean and dry skin for 6 to 10 hours, then, it has to be removed. The recommended dose is 250 mg per 20 cm^2 of lesional area. The 3.75% formulation has been approved in 2010 only for the treatment of actinic keratosis lesions on scalp, forehead and face in adults with normal immune system.

3.2.2 Basal cells carcinoma (BCC)

The most diffuse form of cutaneous cancer is the non-melanoma type (NMSC) which is represented for the 80% by basal cells carcinoma (BCC) and for the 20% by squamous cells carcinoma (SCC).

BCC originates from the basal cells, which are the most profound of epidermis, usually after UV-A exposure. IMQ therapy have been demonstrated to be useful: when applied from 5 to 7 times per week, for at least 6 weeks of treatment, it bring to complete clearance in case of single lesion for the 84% of cases, after 1 year from the beginning of treatment [12].

3.2.3 Anogenital warts [13]

Human papillomavirus (HPV) infection is one of the most common sexually transmitted viral diseases. The visible manifestation of the infection, in particular when the infection is caused by HPV types 6 and 11, is the appearance of warts in the genital area.

Imiquimod is considered to be among the first-line topical agents for the treatment of anogenital warts. Its efficacy and safety have been largely demonstrated. In randomized placebo controlled clinical trials, conducted in the US, about 50% of patients, treated 3 times per week for 16 weeks with 5% IMQ, showed complete clearance. In general, the treatment has shown to be more effective in females than males, probably because of a different localization of the warts: in females, lesions are usually located on the vulva, while for males on the penis. The skin in the two genders has a different keratinization level, higher in males than females.

3.3 Off-label uses [6, 14]

Along with FDA approved uses, IMQ is also employed off-label for the treatment of several cutaneous infections and of neoplastic conditions. Several clinical trials are now open for the evaluation of IMQ potential to treat different diseases like Cervical Intraepithelial Neoplasia and Vulvar Paget's Disease and also to assess its usefulness for some of the disease previously cited. It is however important to underline that in some cases there are contrasting evidences on its efficacy.

3.3.1 Cutaneous infections

Cutaneous non-anogenital warts

IMQ has been employed also for the treatment of common perungual and plantar warts with an efficacy that is apparentely lower than in case of anogenital ones. In one study, the use of IMQ 5% applied twice per days for more than 5 months, lead to complete resolution in 16 of 18 patients treated. Other studies are reported and in general, alone or in combination, the treatment with IMQ seems to be successfully. However, the main problem of these studies is that the number of patients is usually low and they are not controlled so further investigations have to be done.

Molluscum contagiosum (MC)

MS is a viral infection with characteristic skin lesions consisting of single or, more often, multiple, rounded, dome-shaped, pink, waxy papules 2-5 mm in diameter. The virus responsible for this infection is a non-classified member of Poxviridae family. Besides the appearance of papules, the main symptoms are inflammation and irritation. The results obtained in small randomized studies report benefits from the use of this drug both in adults and children. In contrast, other studies demonstrate that is not effective, especially in children. The possible reason of

inefficacy was attributed to the capacity of MC to induce the production of viral proteins able to inhibit the NF-ƙβ activation.

Cutaneous leishmaniasis

This infection, caused by a protozoan parasite, can usually have three outcomes: cutaneous, mucocutaneous or visceral. The lesions caused by this agent are very painful and can be so severe that they can be defined wounds instead of lesions. After healing, the signs of the infection remain visible because of the scars. The first-line treatment is presently represented by antimonials but interest has been focused on IMQ use alone or in combination with antimonials.
However, the literature reports contrasting results of its efficacy.

3.3.2 Neoplastic conditions

Squamous cell carcinoma in situ

A systematic review including 5 studies with a total of 98 in situ carcinoma cases, shows a clearance of about 70%. However, further investigations have to be performed since most of the evidences concern small studies. Additionally, in particular in case of treatment of invasive forms, the results are conflicting.

Melanoma in situ [15]

It is considered as the earliest histologically recognizable stage of malignant melanoma. The most frequent subtype of this skin cancer is represented by lentigo maligna (LM), specifically associated to chronic UV exposure. Again, many of the evidences are case report or short term follow-up studies. Evidences of the progression to more invasive form despite IMQ treatment are reported, so extreme caution has to be used.

Infantile hemangioma (IH)

IH is a benign vascular tumor, characterized by endothelial cells proliferation. It is the most common form of soft tumor of the childhood, with an incidence between 3-10% of population. Since 2002, IMQ at 5% has been reported to have beneficial effects on this neoplastic form in some case reports and small studies. In particular, IMQ efficacy was demonstrated for the treatment of superficial form of IH, with a remission in about 40%, while no significant effect was observed in deeper forms. Evidences of strong side inflammatory effects are reported, with the formation of permanent scars, so high attention has to be paid.

3.3.2.1 **IMQ as adjuvant in immunotherapy**

In recent years the addition of adjuvants able to strongly and safely increase the immune response after vaccination has been evaluated. IMQ has been studied for this particular purpose: experiments on animals have already demonstrated its effect when injected along with the vaccine. Particularly interesting is the study performed by Johnston and al.: they demonstrated the potential of IMQ as adjuvant when topically applied associated to a subcutaneous immunization. [16] Moreover, different trials on the employment of IMQ as adjuvant for vaccination against cancers, are under recruitment.

4 Corticosteroids [17-19]

The term “corticosteroids” refers to a class of steroid hormones produced by the adrenal cortex in vertebrates. They are divided in two main classes, glucocorticoids and mineralcorticoids. Glucocorticoids (GCs) in particular, are a class of molecules with anti-inflammatory, immunosuppressive, antiproliferative and vasoconstrictive effects.

After the discovery by Kendall and Hench in 1948, of the therapeutic effect of cortisol (**Figure 2**) in the treatment of rheumatoid arthritis, a large number of derivatives have been synthesized in order to increase the potency and the duration of action, as well as to obtain specificity towards anti-inflammatory or mineralocorticoid activity.

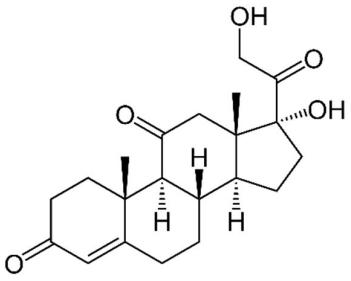

Figure 2. Cortisol structure

4.1 Mechanism of action

Corticosteroids inhibit many of the initial stages of the inflammatory response. Their effect is due to the binding to a nuclear receptor (GR) that modulates gene expression. In particular, the induction of lipocortin-1 (annexin-1) transcription takes place; lipocortin-1 exerts two main effects:

1. the inhibition of phospholipase A_2, enzyme responsible for the release of arachidonic acid (AA) from the cellular membrane. AA is a fundamental precursor for the production of important inflammatory mediators (prostaglandines, leukotriens and thromboxanes)
2. lipocortin-1 suppresses some crucial inflammatory events such as vasodilation, chemotaxis, phagocytosis and epithelial adhesion.

As previously mentioned, corticosteroids are able to induce immunosuppression. The major event beside this effect, is the inhibition of a transcription factor, NF-ƙβ, which is involved in the expression of different cytokines. The inhibition leads

to lower level of these mediators and consequently affects the T cells proliferation. Moreover, corticosteroids are able to induce apoptosis, especially in immature T cells.

4.2 Topical corticosteroids

Corticosteroids are used for the treatment of several diseases. Systemic corticosteroids are used for treatment rejections after organ transplantation and autoimmune disorders; unfortunately, they are associated to a large number of side effects such as osteoporosis, diabetes mellitus, glaucoma and, when high doses are used, increased susceptibility to infections. For this reason, when a well-localized disease has to be treated, local administration is preferred. The use of corticosteroids for topical application was introduced in 1952, when hydrocortisone was successfully employed for the treatment of dermatoses. After that, topical corticosteroids of varying strength have been synthetized.

Corticosteroids classified as low to medium potency, are generally employed to treat acute inflammatory states, whereas highly potent agents are required for chronic inflammations.

4.2.1 Triamcinolone acetonide

Triamcinolone has been introduced in therapy in 1958. The molecular structure (**Figure 3**), in comparison with cortisol (**Figure 2**), is characterized by the presence of: 1) F atom in 9α position and 2) –OH group in 16α position. The presence of a fluorine atom in 9α position leads to an enhancement of anti-inflammatory activity and the introduction of a hydroxyl group in 16α decreases the mineralocorticoid activity. However, although triamcinolone shows a definitely lower tendency to water and salts retention, it is usually associated to side effects like anorexia, weight loss, nausea and vertigo. Triamcinolone intramuscular

injection is an efficacious therapy for dermatosis treatment and, in combination with folic acid, for psoriasis [20].

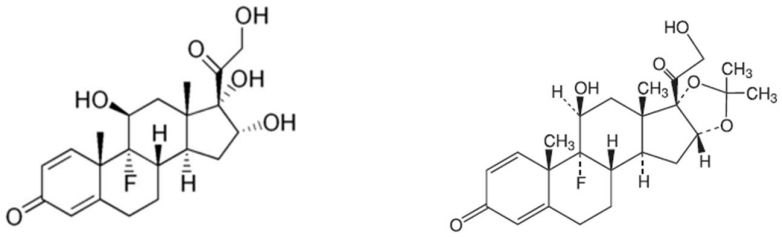

Figure 3. Triamcinolone and triamcinolone acetonide molecular structures.

Generally, triamcinolone it is used as 16α, 17α-ketal cycle called acetonide derivative (**Figure 3**), which is efficacious in the treatment of various dermatosis [20]. Triamcinolone acetonide is approved for the topical treatment of different pathological conditions like nasal congestion (Nasacort®), skin diseases characterized by inflammation such as eczema, dermatitis and allergies (for example Ledercort®). For buccal application it is approved for the treatment of recurrant aphtous stomatisis (RAS) and it is present on the Italian market in a mucoadhesive tablet formulation, Aftab®.

4.2.2 Dexamethasone palmitate

Further researches on GCs molecular structure highlighted that the substitution of the hydroxyl group in 16α with a methyl group lead to derivates with higher metabolic stability. Moreover, methyl group also increases the selectivity, reducing the mineralcorticoid activity. These studies brought to the synthesis of dexamethasone, having an anti-inflammatory activity 5-fold higher than prednisolone. Dexamethasone has been modified for the production of different prodrugs, forming esters on 17 or 21 position.

The main prodrugs of dexamethasone are:

- Dexamethasone valerate, employed for treatment of skin inflammation
- Dexamethasone phosphate and acetate

For the treatment of inflammatory states of the eyes it is formulated in eye drops suspension (Visumetazone); in other formulations, dexamethasone is used in conventional formulations not intended for topical use: is presented as aqueous solution (dexamethasone phosphate) or in crystalline form (dexamethasone acetate).

In 2001 Peter and Bias studied the efficacy of a prodrug of dexamethasone, dexamethasone palmitate (DXP) (**Figure 4**), for the treatment of osteoarthritis. DXP was formulated in an innovative carrier in which it is dissolved in soya bean oil vesicles, surrounded by lecithin coating. This formulation, Lipotalon®, is presently approved in Germany [21].

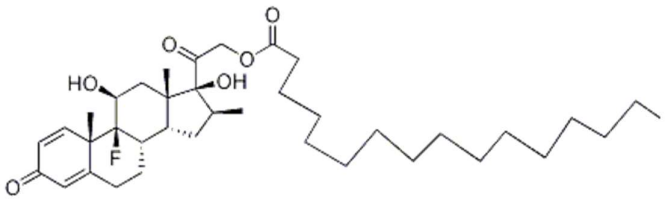

Figure 4. Dexamethasone palmitate structure

5 Local administration

The expression "local administration" means the application of a drug formulation directly at the site of action. The possibility to treat locally a pathological condition, instead of using a systemic therapy, is interesting for many reasons: it allows to avoid hepatic first-pass metabolism (in comparison with the oral route), to improve patient compliance and, at least in principle, to have easier access to the absorbing barrier. Above all, by directly administering the drug to the pathological site, adverse effects associated with systemic toxicity can be minimized.

Effective and target specific drug delivery is, however, one of the most challenging tasks in pharmaceutical field. Many limits are in fact associated with the local administration and some are specifically associated to the site of application.

In this thesis, we focused our attention on two routes of administration: the dermal and the buccal route.

Dermal administration

The skin is the largest organ of our body with a total area of approximately 2 m^2 and consists of three functional layers: epidermis, dermis and ipodermis.

The barrier function is mainly performed by the outermost layer of epidermis, the stratum corneum (SC). SC is, in fact, constituted by 15-20 layers of dead corneocytes, embedded in a lipid matrix. The barrier function is due to: 1) the content and composition of the lipid matrix (mainly ceramides, cholesterol and fatty acids) and 2) the structural arrangement of the intercellular lipids. Lipids, in fact, form bilayers surrounding the corneocytes, producing a "brick-and-mortar" structure, the corneocytes being the bricks and the intercellular lipids providing the mortar. Overcoming this lipophilic barrier is essential to reach therapeutic drug levels in viable skin.

The penetration of drugs across the skin can take place by diffusion across the intact epidermis (SC in particular) and/or through the skin appendages (hair follicles and sweat glands).

It is widely accepted that a drug can have a satisfying penetration when its molecular weight is lower than 500 Da and its log P about 2. Unfortunately, most of the drugs do not respond to these characteristics.

Buccal administration

The oral cavity is a challenging site for the administration of drugs. The oral mucosa is a pluri-stratified squamous epithelium, with a variable thickness depending on the region. Also for the buccal epithelium the main barrier is represented by the lipid content of the extracellular matrix, especially in the outermost layers. Moreover, the epithelial cells are surrounded by a mucus layer, mainly composed by proteins and carbohydrates, which favour the intercellular adhesion. Another important aspect of the buccal administration is represented by the presence of saliva, which could affect the residence time of formulation.

For these reasons, during the decades, research has found different strategies in order to ensure a satisfying penetration of drugs through the tissues.

- Chemical penetration enhancers

  A strategy to increase the permeability of the keratinized tissues is to affect its integrity. Several molecules have found to be effective on the skin: fatty acids, oleic acid in particular, has been demonstrate to induce the separation of the lipid domains, reducing the barrier function. Also dimethyl sulfoxide (DMSO), has been observed to increase the permeability because of its interactions with SC lipid alkyl chains.

  Another possible action of penetration enhancers is the capability to increase the solubility of the drug into the SC, increasing the partition

between the skin and the formulation. Chemicals that have been suggested to act in this way are ethanol, propylene glycol and Transcutol. These molecules, with relatively low molecular weight, penetrate well the skin and can increase the drug solubility into the tissue [22].

Because the buccal mucosa is a lipophilic tissue as well as the skin, chemical penetration enhancers can be effective also on this barrier [23].

- Physical penetration enhancement [24]

  During the last decades, different physical techniques, like iontophoresis, sonophoresis, electroporation and microneedles, have been investigated. Iontophoresis is a technique based on the application of an electrical potential across the tissue, able to enhance the penetration of both charged and neutral molecules. For the charged molecules the enhancement is due to electrorepulsion, while for neutral molecules to the movement of solvent, under the effect of electroosmosis.

  Microneedles is a minimally invasive technique which consist in piercing the SC to overcome its barrier. The diameter and length of needles is developed in order to be painless. Microneedles can be loaded with drugs or used as a device before applying a conventional formulation.

- In the case of buccal administration, it is essential to develop a formulation with mucoadhesive properties so as to be unaffected by the mechanical stress and the wash of saliva. The most commonly excipients used for dry or partially hydrated formulations (tables for example) include polyacrylic acid (PAA), polyvinyl alcohol (PVA), sodium carboxymethylcellulose (NaCMC), hydroxypropylmethyl cellulose (HPMC), hydroxyethyl cellulose (HEC), hydroxypropyl cellulose (HPC) and sodium alginate [23].

- The use of microemulsions has been studied especially for dermal drug delivery, with promising results. The enhancing properties of microemulsions (high solubilizing capability, presence of surfactants which could affect SC integrity and also the excellent wetting properties) were studied with large number of drugs, in particular corticosteroids and analgesics, because of their poor skin penetration properties [25]. On the contrary, only a few evidences are present in literature on the use of microemulsions for buccal application. However, promising results have been highlighted [26, 27]

- Nanoparticles

  Despite the controversial results obtained with the use of nanoparticles for dermal application, these systems are still widely studied. Their demonstrated capability to accumulate into the skin appendages and localize the drug in the upper skin layers make them suitable systems for dermal application [28].

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PART 1

Dermal administration of immunostimulants: Imiquimod

Table of contents

1 Introduction 37

2 Aim 41

3 Materials and methods 42

3.1 Materials 42

3.2 Methods 42

3.2.1 Analytical method 42

3.2.2 Solubility studies 43

3.2.3 Stratum corneum uptake experiments 43

3.2.4 Pseudo-ternary phase diagram construction 44

3.2.5 Blank microemulsion preparation 45

3.2.6 Blank microemulsion gelation 45

3.2.7 IMQ loaded formulations 46

3.2.8 Polarized optical microscopy 47

3.2.9 Reology studies 47

3.2.10 Accumulation and permeation experiments 47

3.2.11 Validation of the extraction procedure 48

3.2.12 Statistical analysis 48

4 Results and discussion 49

4.1 Validation of general analytical procedures 49

4.1.1 Validation of HPLC analytical method 49

4.1.2 Validation of extraction procedure 50

4.2 Mechanisms of imiquimod skin penetration 52

4.2.1 Solubility studies 52

4.2.2 IMQ skin delivery from saturated solutions..... 54

4.2.3 SC uptake experiments 58

4.3 Innovative formulations for IMQ skin delivery..... 62

4.3.1 Microemulsions 62

4.3.2 Microemulsion-based gels 66

4.3.3 Self-gelling microemulsions..... 72

5 Conclusion 86

6 Supplementary informations..... 88

7 Bibliography..... 90

1 Introduction

After its approval in 1997, IMQ have been widely studied because of its high potential. Together with the approved indications, in fact, several off-label applications have been reported [1]. The interest for this molecule is witnessed also by the large number of clinical trials (approx. 20 open/recruiting): some of them concern the treatment of neoplastic skin diseases, but great attention is also dedicated to the use of topical IMQ as adjuvant for vaccination [2, 3].

However, IMQ formulation and skin delivery is highly challenging because of its very low solubility in either hydrophilic or lipophilic vehicles. Additionally, despite the low molecular weight, IMQ has very poor skin penetration properties, probably due to the very low solubility in the stratum corneum (SC) and underlying tissues. After the commercialization of the innovator formulation, Aldara®, pharmaceutically equivalents products, having exactly the same composition, arrived on the market (Imunocare®, Zyclara®). Different IMQ creams at 5% have been introduced on the market in South America and in People's Republic of China, where they are considered as equivalents. However, in vitro release studies and physiochemical tests on these formulations, in comparison with the innovator formulation, have highlighted consistent differences. [4]. These results highlight the difficulty in IMQ formulation due to its low solubility.

In literature, efforts directed towards the development of alternative formulations containing IMQ are presents. Many of these works focused the attention on innovative formulations, like transfersomal vesicles, oleogel, transethosomes and patches, intended to be used for the treatment of different cutaneous cancer forms [5-8]. Besides its use for the treatment of neoplastic conditions, IMQ has been also evaluated as adjuvant in transcutaneous immunization. Efforts for the development of formulations intended for this

specific use are present in literature; in particular, solid nanoemulsion, nano-dispersed emulsion gel and nanoliposomes have been investigated [3, 9, 10].
Interestingly, in many studies, no comparison with the commercial formulation was performed.

During the last decades microemulsions have become increasingly interesting for dermal and transdermal application, because of their potential to enhance drug permeation and penetration in comparison with conventional formulations such as creams and gels [11]. One of the more interesting aspects is their capability to solubilize highly hydrophobic drugs increasing their concentration and enhancing skin penetration [12]. Microemulsions are thermodynamically stable colloidal dispersions. According to Danielsson and Lindman definition, microemulsions are systems composed by oil, water and amphiphile molecules, which are single phase and thermodynamically stable isotropic solutions. One of the major advantages of microemulsions, in comparison with emulsions, is the high stability, due to the presence of both surfactant and cosurfactant: normally, in fact, surfactant is not sufficient to reach a suitable value of interfacial tension, so crucial is the role of co-surfactant. This class of excipients, which is normally represented by short or long chain alcohols or polyglycerol derivatives, is able to decrease the value the interfacial tension, normally three or four orders of magnitude in comparison with the presence only of surfactant [12].

Despite the high potential, the low viscosity of these systems make them not suitable for skin application. For this reason, there are evidences in literature the use of microemulsions as a base for the development of semisolid formulations, gels in particular.

The preparation of gelled microemulsion can be performed by its inclusion in a already prepared gel, as described by Wang et al [13], or by addition of a gelling agent, for example like xantan gum [14] or hydropropylmethylcellulose [15].

Another interesting possibility is to take advantage of the capabilities of certain excipients, when combined in specific ratio, to give viscous microemulsions. There are evidences, in fact, of the formation of microemulsions which maintain the classical microemulsion properties, like the complete clearness, with a viscosity which is comparable to conventional gels.

These viscous microemulsions, that could be called "microgel", are particularly interesting since combine the advantages of both the enhancing properties of a microemulsion and the rheological properties of a gel.

Very few papers in the literature described this kind of systems. Ayannides and Ktistis [16], studied the formation of a microemulsion gel using water, isopropyl myristate, polysorbate 80 and glycerol. They found out that the glycerol to water ratio was able to influence the area of gel existence. They observed in particular, that for a fixed percentage of glycerol/water solution, the increase of glycerol and the consequent decrease of water leads to a decrease of viscosity. According to authors opinions, the water content is fundamental for polysorbate swelling.

Sheikh Shafiq-un-Nabi et all [17], described the formation of gel-like formulation mixing Sefsol 218, Cremophor, Carbitol and water, but they did not further investigate this system.

Ke and al [18], studied the formation of microemulsion systems for the oral delivery of poorly soluble drugs or protein using tocopheryl polyethylene glycol 1000 succinate, when combined with Captex 300 as oil phase, water and different surfactants and co-surfactants. They highlighted the existence of the gel-phase area and found that the gelification was dependent on the surfactant:cosurfactant ratio: for example, when they studied the combination TPGS/PEG600 the gel area was present only when the ratio was or 4/1 or 2/1. The formation of a gel systems is due, according to their explanation, to the water molecular adsorbed or intercalated between the hydrophilic polyoxyethylene

(POE) chains of TPGS via hydrogen bonding which resulted in less mobile and regular gel structure.

2 Aim

The aim of work has been divided in two different phases:

1. The comprehension of the mechanisms underlying imiquimod skin penetration and retention, so as to be able to formulate the drug in an efficient vehicle. Thus, the specific objectives of this part were: (1) to evaluate IMQ solubility in different solvents, vehicles and pharmaceutical excipients; (2) to evaluate IMQ skin retention after the application of simple saturated solutions; (3) to evaluate the role of stratum corneum and solvent uptake on IMQ skin retention.

2. The development of an innovative formulation based on microemulsion technology for IMQ topical application, in particular: (1) to formulate IMQ in microemulsions (prepared using previously investigated components) and compare them with the commercial formulation 2) to evaluate microemulsions gelled by direct addition of a thickening agent and 3) to evaluate vitamin E TPGS self-gelled microemulsions.

3 Materials and methods

3.1 Materials

IMQ (MW = 240.3 g/mol; pKa: 7.3) was purchased from TCI Europe N.V. (Zwijndrecht, Belgium) or Hangzhou Dayangchem, (Zhejiang, China). Oleic acid was purchased from Alfa Aesar (Karlsruhe, D), Transcutol and Capryol 90 were a gift from Gattefossè (Lyon, France). 70% perchloric acid solution, albumin from bovine serum and sodium alginate were purchased from Sigma Aldrich (St. Louis, MO, USA). PEG 400 and propylene glycol were obtained from A.C.E.F. (Fiorenzuola, Italy). Soluphor P (2-pyrrolidone), Lutrol F68, Lutrol F127, Lutrol L44, Pluronic P85, Kollidon 30 (PVP), Kolliphor, Cremophor ELP and Vitamin E TPGS (D-α-Tocopheryl polyethylene glycol 1000 succinate were obtained from BASF (Ludwigshafen, Germany). Aerosil® 200 was obtained from Evonik Corporation (Parsipanny, NJ, USA). Sodium hyaluronate 1000 kDa was a gift from IBSA Farmaceutici (Rome, Italy). Sorbitan monoleate 80 (Span80) was a gift from Croda Ibérica SA, Spain. For HPLC analysis, bidistilled water was used. Acetonitrile and methanol were of HPLC grade; all other reagents were of analytical grade.

3.2 Methods

3.2.1 Analytical method

Imiquimod quantification was performed by HPLC using a Flexar instrument (Perkin Elmer, Waltham, MA, USA) and a C18 column (Kinetex C18 2.6 m 100 Å 75 4.6 mm, Phenomenex, Torrance, CA, USA), equipped with column guard (Widepore C18 4-3 mm, Phenomenex). The mobile phase was CH_3OH:CH_3CN:H_2O:TEA (180:270:530:20) eluted at 0.5 ml/min. In case of samples from extraction and permeation experiments, fluorescence detection (λ_{exc} 260 nm, λ_{em} 340 nm) was used (injection volume: 1 µl), while solubility samples were

analysed by UV absorbance (λ 242 nm; injection volume: 10 µl). Stock solution was prepared by dissolving approx. 2 mg of imiquimod in 20 ml of HCl 0.1 M.

3.2.2 Solubility studies

2 mg of IMQ was added to 2 ml of the following vehicles: H_2O, PBS pH 7.4, PBS pH 4, propylene glycol, ethanol, octanol, Capryol 90, silicon oil, oleic acid, DMSO, Tween 80, PEG 200, PEG 400, PEG 600, miglyol, soybean oil, liquid paraffin, 2-pyrrolidone, Transcutol (diethylene glycol monoethyl ether), water solution of 1% w/v β-CD, 1% w/v albumin, 0.55% w/v Tween 20, 5% and 20% w/v TPGS, 0.5% w/v Lutrol F68, 1.5% w/v Lutrol F127, 1% w/v Brij 78, 4% w/v lauric acid in EtOH:H_2O (50:50). The samples were left overnight at room temperature, under magnetic stirring. The following morning, the vehicles in which IMQ was not dissolved (solubility < 1 mg/ml), were discarded. On the contrary, if IMQ was completely dissolved (solubility > 1 mg/ml), an excess amount of IMQ was added, and, after 24 hours mixing, the suspension was centrifuged and/or filtered (0.45µm), diluted and analysed by HPLC-UV for the accurate determination of the solubility.

3.2.3 Stratum corneum uptake experiments

In order to prepare SC sheets, isolated epidermal sheets were soaked at 4°C for 15 hours in 1% (w/v) trypsine in PBS pH 7.4. Epidermis was removed with a cotton swab and SC sheets obtained were individually rinsed 3 times with distilled water, dried in oven at 37°C for 1 h, and kept in dessiccator on $CaCl_2$ until use.

In order to probe any correlation between skin solvent uptake and the penetration of drug into the skin from specific vehicles, stratum corneum uptake experiments were performed.

SC sheets (≈1.6 mg/cm^2, area of approximately 2.5 cm^2) were first weighted (Mettler Toledo, sensibility 0.001 mg) and then individually soaked for 6h in 2 ml of vehicles in a temperature-controlled oven at 32±1°C. SC sheets were then

removed from the vehicles, carefully dried using filter paper and re-weighted. Solvent uptake was expressed both as moles/cm^2 and as percentage of weight increase using the following equation:

$$\%Weight\ increment = \left(\frac{Wf - Wi}{Wi}\right) \times 100 \qquad \textit{Equation 1}$$

Where W_f is final weight and W_i is initial weight of SC sheet.

SC uptake experiments were performed also using IMQ solutions in different vehicles, so as to measure both solvent uptake and IMQ accumulation. In this case, after blotting and re-weighting, IMQ was extracted from the SC using 1 ml of oleic acid: methanol mixture (1:3) overnight at room temperature.

3.2.4 Pseudo-ternary phase diagram construction

Pseudo ternary phase diagram allow to identify the region in which there is the existence of the microemulsion. Since a microemulsion is composed of 4 excipients, one axis usually reports a fixed mixture of surfactant:co-surfactant (smix), that in this case was either 1/1 (w/w) Tween 80/Transcutol or 1/1 (w/v) TPGS/Transcutol. The pseudo ternary phase diagrams were built using as oil phase oleic or isostearic acid, as surfactant TPGS or Cremophor, transcutol as co-surfactant and water.

The construction of the diagram is based on a quite simple procedure called acqueous titration method: for a fixed ratio oil/smix (1/9, 1.25/8.75, 2/8, 3/7, 4/6, 5/5, 6/4, 7/3, 8/2, 9/1) a known amount of water was added, in order to obtain concentrations between 5 and 95%. After each addition, the mixture was vortexed and left 1 minute to rest, then viscosity and clearness of the system were evaluated visually.

In fact, the mixture can result transparent, opalescent or milky as a function of dispersed phase size, and can have different rheological properties. The combination of the three phases that give systems clear with low viscosity define the microemulsion region; when the system is clear and viscous the microgel

region is defined. The system was considered gel when overturning the vial, the formulation didn't slide along the vial walls [19, 20]. The construction of the pseudo ternary diagram phase has been done using Origin 2016® software demo (Northampton, Massachusetts, USA).

3.2.5 Blank microemulsion preparation

Microemulsions were prepared by adding the different components into a glass vials, under magnetic stirring. The order of addition was the following: oil phase (oleic or isostearic acid), co-surfactant (transcutol), surfactant (tween 80 or vitamin E TPGS), water.

% w/w	ME 1	ME 2	ME 3	ME T 20	ME T 35	ME T 45	ME T 50	ME T 25 ISO
Oleic acid	70	50	10	9.1	7.5	6.2	5.7	
Isostearic acid	-	-	-					20.6
Transcutol	14	22.5	35	35.1	29.2	24.0	21.8	26.7
Tween 80	14	22.5	35					-
Vitamin E TPGS				35.5	29.6	24.3	22.1	27
Water	2	5	20	20.3	33.7	45.4	50.4	25.7

Table 1. Composition of microemulsions. For the determination of % w/w, the following values of density have been used: oleic acid 0.895 g/ml, isostearic acid 0.89 g/ml, transcutol 0.988 g/ml.

3.2.6 Blank microemulsion gelation

In order to increase the viscosity of microemulsions, different gelling agent were directly added: poloxamers (poloxamer 407, lutrol F68, pluronic P85), polyacrilates (carbopol 940), polyvinylpyrrolidone (kollidon 30), sodium alginate, sodium hyaluronate, fumed silica (Aerosil 200). All these thickening agents were added to microemulsions under magnetic stirring at different concentrations.

3.2.7 IMQ loaded formulations

3.2.7.1 Tween 80 based formulations

In order to load IMQ into the formulations, ME 1, ME 2 and ME 3 (composition in **Table 1**) were added of an excess of IMQ and left under magnetic stirring overnight. In order to remove the excess of IMQ, samples were then centrifuged for 10 minutes at 13000 rpm. IMQ-loaded gelled microemulsions were prepared by addition of silica to saturated ME 3. Since the addition of Carbopol to ME 3, at any concentration, induced IMQ precipitation, gelation was performed using ME 3 with a degree of saturation of 0.5, prepared diluting 1:1 the IMQ saturated ME 3 with a blank ME.

3.2.7.2 Vitamin E TPGS based formulations

To load IMQ into ME T 20 (composition reported in **Table 1**), an excess of drug was added, the suspension was left under magnetic stirring overnight and the obtained samples were centrifuged for 10 minutes at 13000 rpm in order to remove the excess of IMQ. In the case of the others vitamin E TPGS based microemulsions, this procedure was not possible due to the high viscosity that prevented the sedimentation of the undissolved IMQ. For this reason, the other TPGS based MEs (composition in **Table 1**) were prepared from IMQ saturated ME vit E TPGS 20 (IMQ concentration 4.35 mg/g) by adding known volumes of water, to reach the final water %. The system was vortexed to achieve homogeneity. No precipitation occured upon water addition.

In order to measure the IMQ concentration, about 10 mg of formulation accurately weighted were sampled, diluted 1:100 with methanol and analysed by HPLC.

3.2.8 Polarized optical microscopy

In order to characterize the formulations, images were taken using a polarized optical microscope (Nykon, Shinjuku, Japan). Samples were deposited on a slide plate with a spatula, then covered with a covering slide in order to prevent water evaporation. Samples were analysed at 4X, 10X and 20X magnification. Images were taken with a fixed integrated camera with 8megapixel resolution (IPhone 6, Apple, Cupertino CA, USA).

3.2.9 Reology studies

In order to compare the viscosity of the different systems, a modified Erramreddy and Ghosh method was employed [20]. A known volume of formulation (1ml) was placed into 8 ml polypropylene tube, then placed onto a wood support with a fixed angle of 10° compared to the table top. The time necessary to reach the equilibrium was measured. Measures were always performed at 23±1 °C.

3.2.10 Accumulation and permeation experiments

For permeation experiments, porcine skin was used. The skin was excised from the outer part of pig ears within 3 h from animal death, separated from the underlying cartilage with a scalpel and frozen at -20°C until use. All tissues were used within 3 months. The skin, once thawed, was mounted on vertical diffusion cells (DISA, Milano, Italy; 0.6 cm^2 surface area) with the stratum corneum facing the donor compartment. The receptor compartment was filled with 1% w/v albumin solution in PBS pH 7.4 (IMQ solubility: 143±3 µg/ml). Different formulations were tested: they were applied for 6 h at infinite dose (200 mg/cm^2, occluded) or finite dose (20 mg/cm^2). At the end of the experiments, the receptor solution was sampled, the donor formulation was removed, the tissue was rinsed with distilled water, blotted dry with filter paper and tape-stripped twice to remove possible traces of the formulation. Skin samples were then heated

(hairdryer for 60 s) and separated into epidermis and dermis with the help of a spatula. Extraction was performed overnight at room temperature using two different mixtures: epidermis was extracted with 1 ml of oleic acid: methanol (1:3 v:v), dermis with 1 ml of PEG 400: methanol: HCl 1 M (1:2:2 v:v:v). To measure IMQ permeation, 1 ml of the receptor solution was transferred in an Eppendorf tube, added of 50 ml of 70%v/v perchloric acid to precipitate albumin and centrifuged (12000 rpm, 15 min). Extraction and permeation samples were analysed by HPLC-fluorescence.

3.2.11 Validation of the extraction procedure

For the validation of IMQ extraction from skin, a different series of blank skin samples (which had not previously been in contact with IMQ) was cut and used in specificity and recovery determinations. Some of the blank skin samples were submitted to the assay procedure and the retention time of endogenous compounds was compared with that of IMQ. Then, a known amount of IMQ solution in oleic acid (5 µl; 0.2 mg/ml), were directly added to blank skin samples, which after 1 h of contact, were submitted to the above described extraction and analysis. The extraction recovery was determined by computing the ratio of the amount of IMQ extracted from spiked skin to the amount of IMQ added. The skin samples were from different donors.

3.2.12 Statistical analysis

The significance of the differences between conditions was assessed using Student's t-test. Differences were considered statistically significant when $p<0.05$. For sake of clarity, due to the high variability, IMQ accumulation data in the figures are reported as mean value ± standard error of the mean (sem), as indicated in the legend, while in tables are reported as mean value ± sd.

4 Results and discussion

4.1 Validation of general analytical procedures

4.1.1 Validation of HPLC analytical method

The analytical method has been validated. Calibration curves were prepared in the interval 0.05–3 µg/ml in the case of fluorescence and 5– 50 µg/ml for UV detection. The methods were validated for precision and accuracy. LOD and LOQ for fluorescence were respectively 0.01 and 0.05 µg/ml (RSD < 3 %; RE < 6 %) (**Figure 1** and **Table 2**).

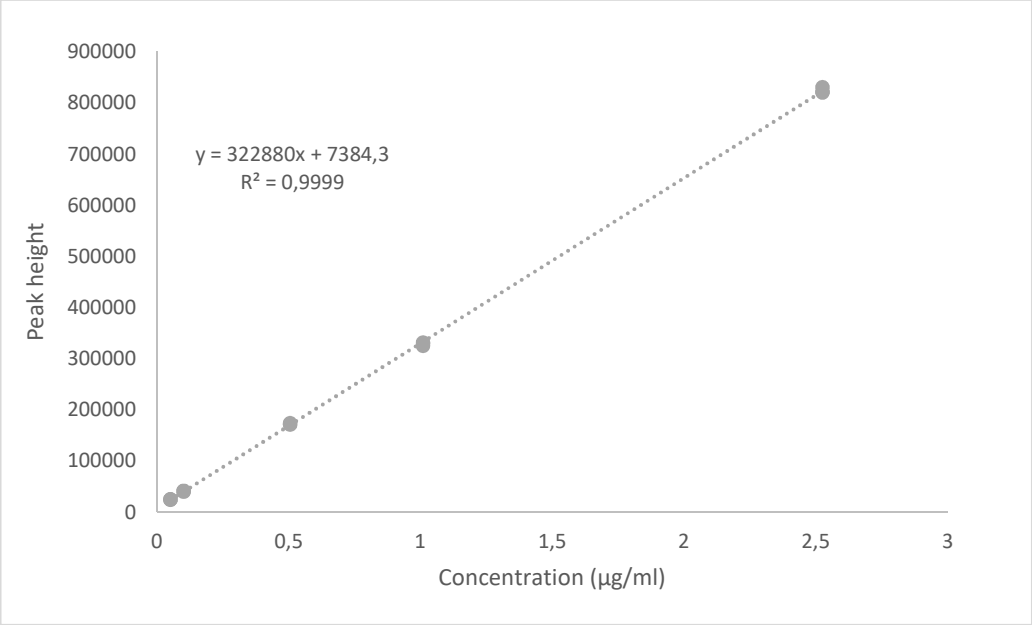

Figure 1. Calibration curve 0.05-2.5 µg/ml, fluorescence detection

Std concentration (µg/ml)	Height	Average	SD	rds%	Val. fitt.	ER%	Average	SD
2.525	830736.72	824272.29	5671.26	0.69	2.55	0.99	0.46	0.47
2.525	820133.65				2.52	0.31		
2.525	821946.51				2.52	0.09		
1.01	328905.36	328416.14	3037.37	0.92	1.00	1.41	1.56	0.93
1.01	331179.2				1.00	0.71		
1.01	325163.85				0.98	2.55		
0.505	171914.99	172264.95	1483.11	0.86	0.51	0.91	1.12	0.91
0.505	173891.74				0.52	2.12		
0.505	170988.11				0.51	0.34		
0.101	39817.8	40777.97	974.93	2.39	0.10	0.54	2.76	2.48
0.101	41767.02				0.11	5.43		
0.101	40749.1				0.10	2.31		
0.0505	23839.22	24540.11	725.05	2.95	0.05	0.92	5.22	4.45
0.0505	25287.11				0.06	9.80		
0.0505	24493.99				0.05	4.93		

Table 2. Validation of calibration curve, fluorescence detection.

4.1.2 Validation of extraction procedure

In order to validate a procedure for the extraction of IMQ from the different skin layers, different solvent mixtures were tested as well as different times and temperatures. Initial efforts were directed to quantify IMQ in stratum corneum (SC), viable epidermis (EP) and dermis (DER). However, the quantification in SC was affected by an analytical fluorescent interference given by the tape (different tapes were tested) used for SC separation. For this reason, the quantification was performed in all epidermis (SC+EP) after heat separation. Before the separation of epidermis and dermis, the skin was well-cleaned, wiped and tape-stripped twice in order to remove IMQ retained in the most superficial layers of SC, considered not available for absorption (OECD, 2011). This procedure is essential to avoid contamination of the deeper layers, in particular when the vehicles or formulations used have very high concentration.

As previously said different mixtures, time and temperatures were tested in order to reach a satisfactory recovery of IMQ.

The best results were obtained using a mixture of oleic acid: methanol (1:3) for epidermis (recovery 98±6%), and a mixture of PEG400: methanol: HCl 1M (1:2:2)

for dermis (recovery 97±6%). In both cases the extraction was performed overnight at room temperature. In order to assess the stability of IMQ in these conditions, also standard solutions were treated in this conditions and no degradation was observed. Also the procedure for IMQ recovery from the receptor samples was validated: the recovery after albumin precipitation in acid environment was 91±6%. The HPLC analysis was selective, allowing to clearly separate IMQ peak from the peaks deriving from the biological matrix.

4.2 Mechanisms of imiquimod skin penetration

4.2.1 Solubility studies

IMQ is a lipophilic molecule, with a calculated log D at pH 7.4 of 2.65, a molecular weight of 240.3 g/mol and a pKa of 7.3.

IMQ solubility has not been widely investigated; in literature a paper of Chollet et all. reports the solubility in some excipients employed in dermal formulations and found that a good solubility (≥ 17 mg/ml) was possible only with fatty acids (oleic acid, isostearic acid and linoleic acid). IMQ was insoluble in aqueous media and its solubility is a function of pH. At neutral pH the solubility is about 0.001 mg/ml, at pH of about 3, the solubility raises up to 1 mg/ml [21].

IMQ solubility was here evaluated on different pharmaceutical excipients and/or solutions. We found a value lower than 1 mg/ml

a. when solutions of surfactants or cyclodestrin were used (50 and 200 mg/ml TPGS, 1% w/v β-CD, 0.5% w/v Tween 20, 5 mg/ml lutrol F68, 15 mg/ml lutrol F127, 10 mg/ml Brij 78, 4% w/v lauric acid dissolved is 1/1 EtOH/water mixture)
b. in case of hydrophilic solvents such as PG, ethanol, Capryol 90 and Lutrol L44
c. with hydrophobic solvents such as silicon oil, octanol, paraffin oil, miglyol and soybean oil.

In the other cases (solubility > 1mg/ml) the solubility was accurately measured by HPLC after 24 h of equilibration in presence of an excess of drug. Results are reported in **Table 3** together with the MW and solubility parameters of solvents.

	MW	Solubility Parameter (δ) (cal $cm^{-3})^{1/2}$	Solubility (mg/ml)
ACIDO OLEICO	286.46	7.91[b]	73.86 ±14.2
TWEEN 80	1310[a]	9.74	0.66 ±0.02
TRANSCUTOL	134.17	10.9	1.11 ±0.07
PEG 600	600[a]	≈11 [b]	12.83 ±1.58
PEG 400	400[a]	≈11 [b]	7.3 ±1.84
PEG 200	200[a]	≈11 [b]	1.98 ±0.38
2-PYRROLIDONE	85.1	11.9	1.64 ±0.12
DMSO	78.13	13.4 [b]	1.29 ±0.13
PG	76.09	14 [b]	0.60 ±0.03

Table 3. Solubility of IMQ in the different excipients used (average± SD). [a]Average MW; [b][22]

Also the solubility in PG and Tween 80 (lower than 1 mg/ml) was measured because these excipients are frequently used in pharmaceutical preparations.
From the table is evident that IMQ solubility is not related to solubility parameter, even when the single contributions are considered (dispersion, polarity or hydrogen bonding) [23]. The highest solubility of this molecule was found in oleic acid: this could be ascribed to both the high lipophilicity and the possibility of hydrogen bond formation. The discrepancy between the solubility in oleic acid found by Chollet (20 mg/ml) and the one reported here (≈ 74 mg/ml), could be due to the different experimental set-up, in particular to a lower equilibration time (30 minutes) compared to the present work [21]. Interestingly, a linear correlation is noticed when IMQ solubility is related to PEG molecular weight (**Figure 2**) suggesting hydrophobic interactions with the ethyl groups and/or formation of hydrogen bonds.

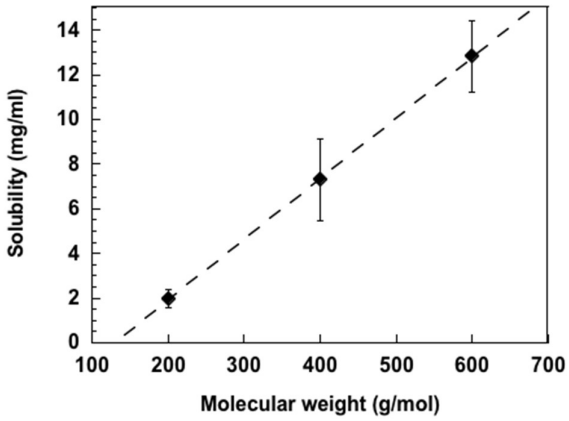

Figure 2. IMQ solubility as a function of the average PEG MW (average±SD).

4.2.2 IMQ skin delivery from saturated solutions

IMQ accumulation was evaluated starting from different solutions and using different barriers (full thickness porcine skin, stripped skin and isolated dermis). The results are overall collected in **Table 4** and expressed as amount accumulated per cm^2 after 6 h of skin contact.

FORMULATION	SKIN	TOTAL (µg/cm^2)	SD
TWEEN 80	FT	0.33	0.06
2-PYRROLIDONE	FT	0.69	0.48
PG	FT	0.19	0.06
TRANSCUTOL	FT	0.38	0.17
PEG 200	FT	0.17	0.19
PEG 400	FT	0.43	0.16
PEG 600	FT	1.14	0.81
	TS	1.79	0.98
	ID	61.9	20.79
OLEIC ACID	FT	1.62	0.4
	TS	4.03	1.2
	ID	2.8	2.44
DMSO	FT	1.02	0.27

Table 4. Amount of IMQ accumulated (epidermis+dermis) from saturated solutions applied for 6 h in infinite dose conditions (average± SD). FT= Full thickness skin, TS= tape stripped skin, ID= isolated dermis

General considerations can be done: despite the favourable log P (calculated log D pH 7.4: 2.65 [24]) and the low molecular weight, IMQ is characterised by very poor penetration properties. Moreover, the results are affected by high variability.

The addition of albumin in the receptor compartment ensured sink conditions, however the permeation was very low in all the conditions tested. Frequently the concentration measured in the receptor phase was between LOD and LOQ, so the amount permeated after 6 hours was between 0.08 and 0.3 μg/cm^2.

To elucidate the mechanisms underlying IMQ skin penetration, we evaluated IMQ skin retention from saturated solutions in pure solvents to keep the systems as simple as possible. The use of saturated solutions in fact, guarantees the same thermodynamic activity (equal to 1), so the same tendency of IMQ to "escape" from the vehicle. The total amount accumulated (epidermis+dermis) expressed as μg/cm^2, and the % accumulated with respect to the amount applied (volume 0.2 ml) are reported in **Figure 3**

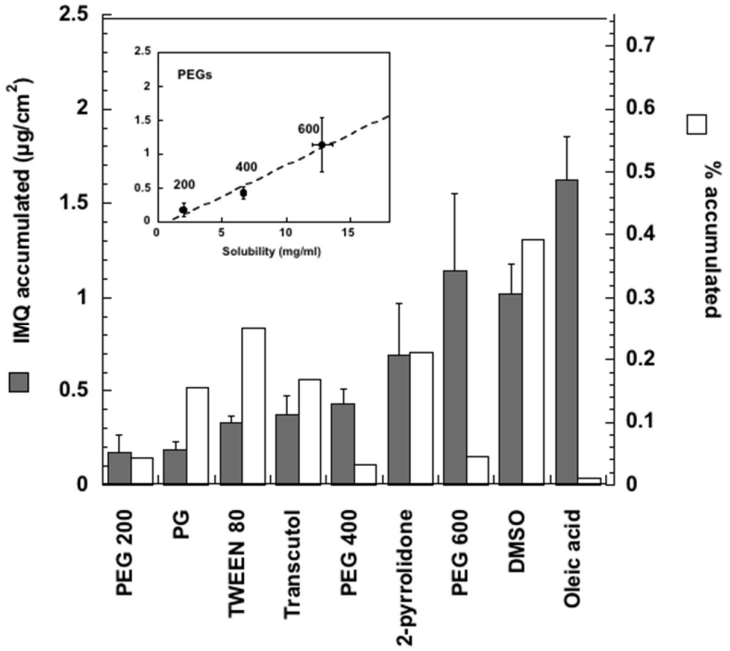

Figure 3. IMQ skin (epidermis+dermis) retention (μg/cm^2) and % of IMQ accumulated starting from saturated solutions. The data are represented as mean± sem. In the insert, IMQ skin accumulation is represented as a function of solubility in PEGs.

The vehicles used for these preliminary studies are very different in terms of IMQ solubility, physico-chemical characteristics and ability to affect the integrity of SC. However, it is possible to see that the amounts accumulated are not dramatically different, being, for example, 1.6 μg/cm^2 in case of oleic acid (solubility ≈ 74 mg/ml) and 0.18 μg/cm^2 in case of PG (solubility ≈ 0.6 mg/ml).

Some of the vehicles tested are known to modify the SC structure, increasing its permeability, however this effect is not evident in the data here collected, with the exception of DMSO, characterised by the highest accumulation.

Considering only PEGs, which are characterised by a similar repetitive structure and lipophilicity (solubility parameter about 11) a linear correlation is present when solubility and skin retention are related (see insert **Figure 3)**. This concentration-dependent skin accumulation is probably due to the fact that the thermodynamic activity and capability to interact with the tissue is the same for the three PEGs, and the concentration of the vehicle is the driving force for skin penetration.

In order to further clarify the role of SC on IMQ skin penetration, accumulation experiments were performed on tape stripped skin (skin without SC). The vehicles selected for these experiments were PEG 600 and oleic acid, characterised by a good IMQ solubility and very different physico-chemical properties. The results are reported in **Figure 4** expressed as total amount (Epidermis + Dermis) accumulated in the skin: no difference between TS and FT can be detected when PEG 600 is applied while a 2-fold difference is measured in the case of oleic acid.

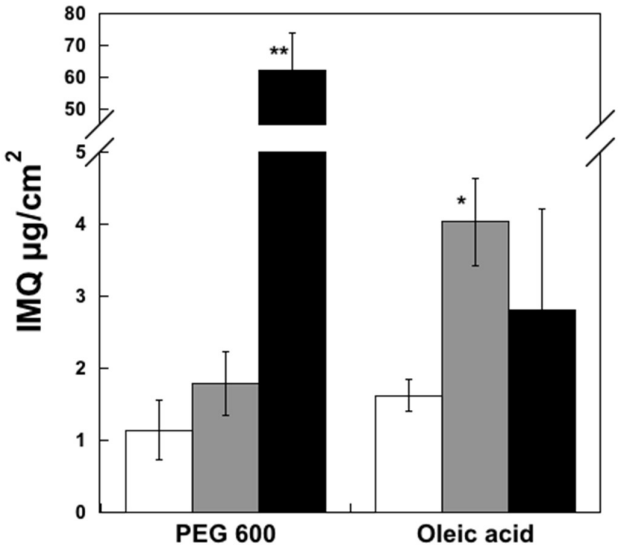

Figure 4. IMQ skin accumulation (mg/cm^2; average± sem) starting from saturated solutions in PEG (12.8 mg/ml) and oleic acid (73.8 mg/ml) following the application on intact skin (white bar), SC stripped skin (grey bar) and isolated dermis (black bar). Asterisks indicate that the accumulation is statistically higher with respect to full thickness skin *$p < 0.05$;**$p < 0.01$.

In order to understand also which is the role of viable epidermis, the same experiments were performed on isolated dermis. No further difference was found in the case of oleic acid while a dramatic increase was measured for PEG 600.

The results, illustrated in **Figure 4**, suggest that the fate of IMQ is linked to the affinity of the vehicle itself for the different barriers considered. PEG 600 is highly hydrophilic and has a very low tendency to permeate through lipophilic tissues, like SC and epidermis, while it penetrates easily though hydrophilic tissues like dermis. It is possible to affirm that the increase detected in the case of dermis is due to a solvent penetration, that "drag" IMQ. On the contrary, because oleic acid is more lipophilic, it penetrates better through lipophilic tissue and no further enhancement is detected when applied on dermis.

These results suggest that the transport of IMQ is, at least to a certain extent, linked to the capability of the solvent to diffuse into the tissue (solvent uptake). This can be due to two phenomena [25]: 1) the modification of IMQ solubility in the tissue that could increase the partition 2) the solvent "drag" effect, a non-specific mechanism where solute and solvent permeate simultaneously [26]

4.2.3 SC uptake experiments

In order to understand if the solvent capability to penetrate into the skin is critical for IMQ accumulation we decided to perform simple experiments based on soaking the tissue into either pure solvents or in IMQ solutions. In order to quantify the solvent uptake we decided to use a simple gravimetric method while for IMQ quantification extraction procedure and HPLC analysis were performed. Initial efforts were done using full-thickness skin, however the quantification of solvent uptake was a failure since the amount penetrated was not clearly detectable with gravimetric method. Since, as previously said, it has been widely demonstrated that SC is the main barrier for penetration into the skin, studies were then done using completely dehydrated SC-sheets. The results obtained are reported in

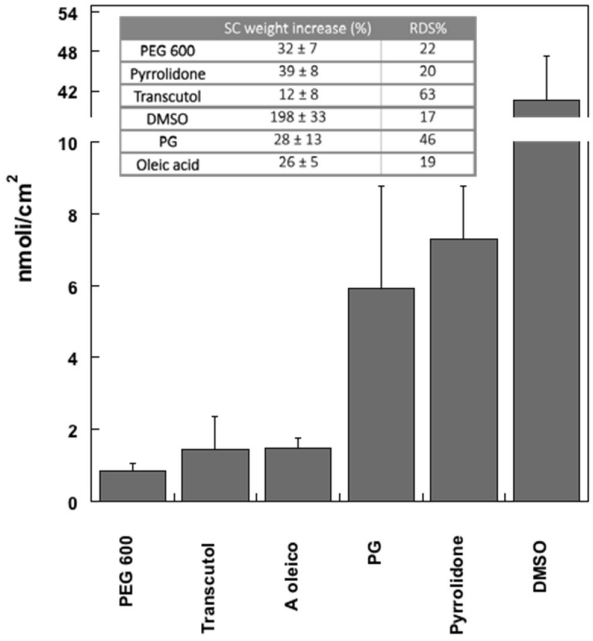

Figure 5. SC uptake of different vehicles obtained by soaking dehydrated SC sheets for 6 h at 37 °C in an excess of each solvent (nmoles/cm^2; average ±sd); in the insert, the SC weight increase (%) is presented.

Figure 5 and expressed as nmol/cm^2 and as % of stratum corneum weight increase.

Some general considerations can be done: even if the method used for the solvent uptake quantification is rough, the data variability is relatively small with the exception of Transcutol and propylene glycol. For Transcutol, however, the value obtained is reasonably in line with the one obtained by GC_MS by Oliviera and al. using human skin at 10 % hydration, after 24 h of equilibration [27].

The uptake is clearly related to solvent molecular weight except for DMSO. This vehicle in fact presents a MW and a solubility parameter similar to pyrrolidone and PG but a 6-fold higher uptake. However, it is reported in literature that DMSO interacts with both the lipid polar head groups causing expansion of the hydrophilic domains, and with keratin, displacing bound water [28-30]. The weight increase with DMSO was about 200%, while in case of other solvents, the value was about 30% with the exception of Transcutol, characterised by very low and variable uptake.

Then IMQ was added to vehicles and experiments were performed quantifying both the uptake of IMQ and the uptake of solvents. The paired data are reported in **Figure 6**: in this case the amount of IMQ experimentally measured, expressed as µg/cm^2, is plotted vs the theoretically one, expressed as µg/cm^2. This theoretical amount is calculated considering the concentration of IMQ in the vehicle (µg/ml) and the vehicle uptake (ml/cm^2).

The linear relation is clearly evident for all the vehicles tested with the exception of Transcutol, mainly because of the variability of the solvent uptake results.

A similar result has been observed, using human SC, in case of methyl paraben when dissolved in isopropylmiristate, methyl ether of isosorbide and Transcutol [27]. The linear correlation means that the uptake of solvent has dragged IMQ into the SC.

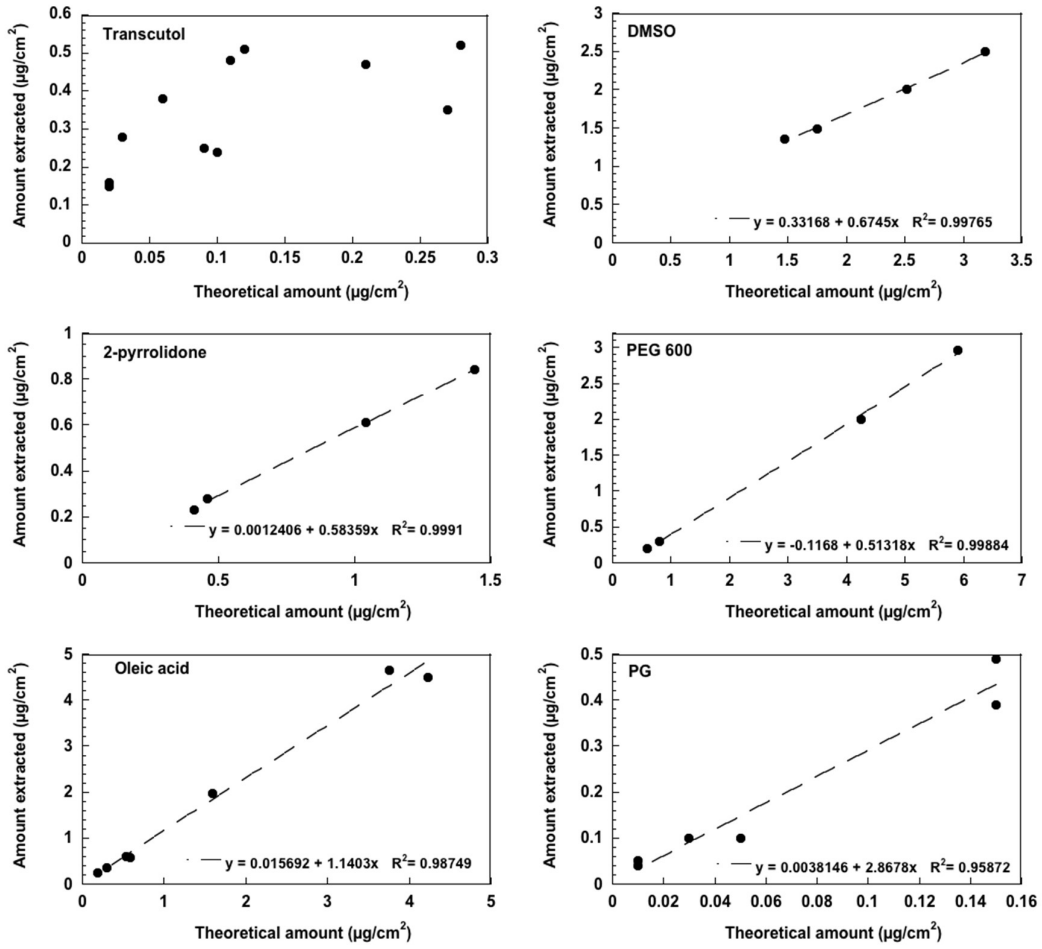

Figure 6. Correlation between the amount of IMQ extracted from the SC and the theoretical amount calculated considering the solvent uptake (ml/cm2) and the IMQ

Moreover, it is possible, from the graphs, to make others considerations: IMQ solubility into the SC containing the vehicle does not coincide with IMQ solubility in the vehicle alone, since the slope is not always 1. The presence of SC seems not to influence the IMQ solubility in case of oleic acid (slope=1), while is increased in case of PG (slope >1) and reduced in the case of DMSO, pyrrolidone and PEG 600 (slope <1). It is possible that the interaction between the solvent and stratum corneum affects the capability of solvent to interact with IMQ.

An attempt to correlate the results of SC uptake with the ones obtained from the accumulation experiments was done as well. In **Figure 7** the total amount of IMQ accumulated into the skin is related to the product between IMQ solubility and solvent uptake. The graph clearly shows that there is a correlation, even if it is not direct (log scale on x axis). This could be ascribed to the different condition of the tissue: dehydrated SC-sheets were used for SC uptake experiments while accumulation experiments were performed on full thickness skin. The SC normally present in the skin is characterised by a water gradient that goes from 15-20% at the surface, to 70% at the interface with the granular layer [31].

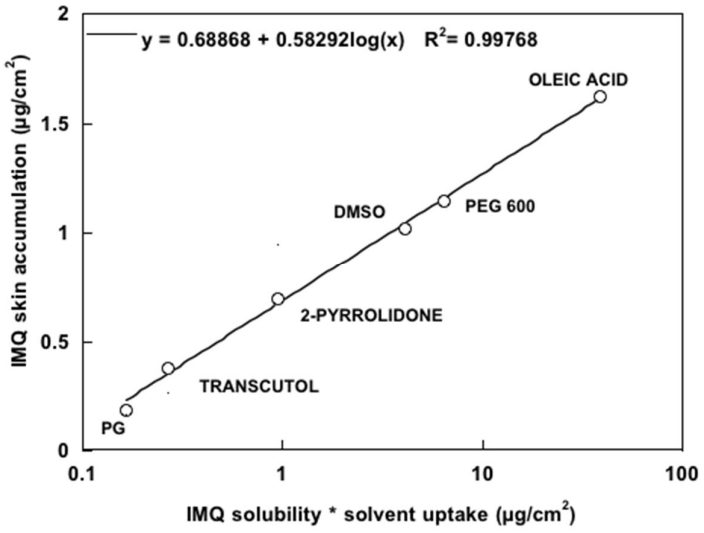

Figure 7. Correlation between the amount of IMQ accumulated into the skin during permeation experiments (data in **Table 4**) and the product of solvent uptake X solubility (log scale).

It is also important to underline that the experimental set-up for accumulation experiments contemplate the removal of 2 strips of SC, with a consequent reduction of IMQ accumulated.

4.3 Innovative formulations for IMQ skin delivery

Different formulations were prepared and evaluated, in particular microemulsions and gels based on microemulsion technology.

4.3.1 Microemulsions

For the development of an innovative formulation containing IMQ, we tested as reference the 5% w/w IMQ commercial cream formulation (Imunocare®). This semisolid formulation contains isostearic acid, benzyl alcohol, cetyl alcohol, stearyl alcohol, white soft paraffin, polysorbate 60, sorbitan stearate, glycerol, methyl hydroxybenzoate, xanthan gum and purified water.[32]

Preliminary studies have been directed to the evaluation of IMQ accumulation from the commercial formulation when applied at infinite dose, finite dose and finite dose on tape stripped skin. The results are reported in **Figure 8**, expressed as $\mu g/cm^2$.

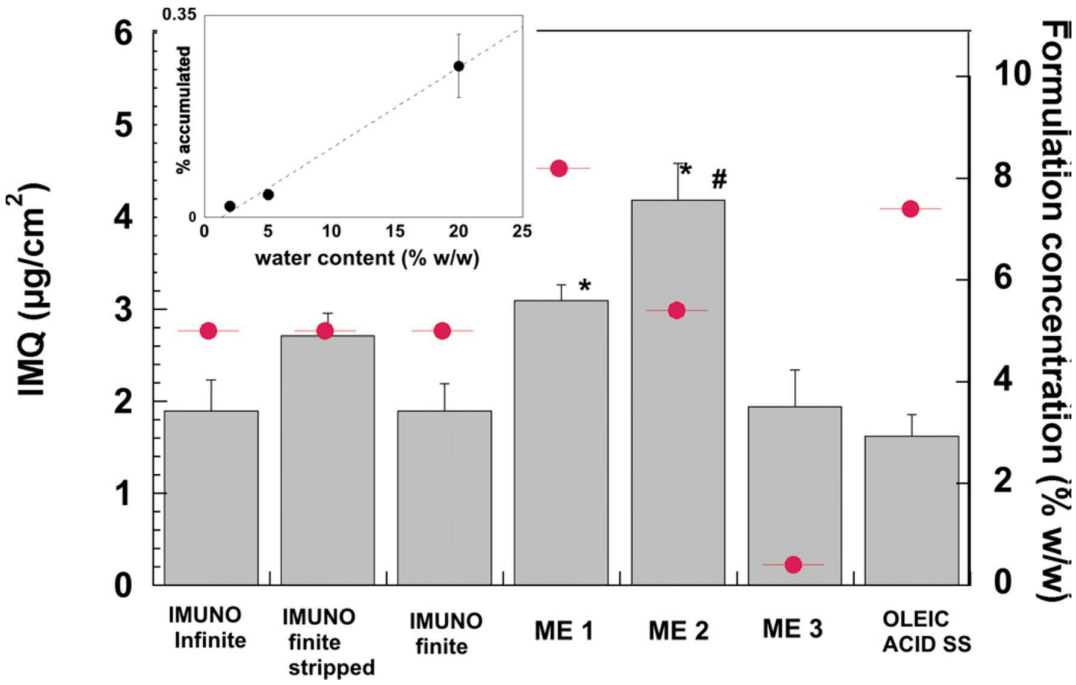

Figure 8. IMQ accumulation in the skin (mg/cm^2; average ±sem; gray bars) from the commercial formulation and from the saturated ME. As a comparison, the value obtained from the oleic acid saturated solution is also reported. *Significantly different form oleic acid saturated solution ($p < 0.01$); #significantly different from Imunocare ($p < 0.05$). Full circles indicate on Y2 axis the IMQ concentration (% w/w) in the formulations tested. The insert illustrates the accumulation efficiency (% accumulated) of MEs as a function of water content.

Some considerations can be done: 1) despite the high concentration, IMQ delivery is low 2) the amount of formulation applied does not influence the accumulation, suggesting that skin occlusion does not enhance the permeation 3) the removal of SC does not significantly increase the delivery confirming our previous observation on the role of SC barrier on IMQ accumulation (see **Figure 4**). We formulated IMQ in vehicles suitable for skin administration, taking into account the information collected until now on the role of solvent uptake. We decided to prepare microemulsions, since Hatout et al. have studied the penetration of ME components into the SC and the results suggest a more

efficient uptake of the components when they are applied as a ME, compared to their separate application [33].

The composition of microemulsions (reported in **Table 5**) was selected from the region of existence of ME individuated from Bhatia et all in their pseudo-ternary diagram phase [34]. Tween 80, previously tested as IMQ solvent, was employed as surfactant instead of Tween 20. Apparently, the different length and saturation of the hydrophobic chain does not affect the microemulsion formation since the system remains transparent.

IMQ was added to saturation to the selected MEs and the concentration obtained are reported in **Table 5**: the solubilities are roughly proportional to oleic acid content, suggesting that probably the drug is localized in the oil phase.

	% w/w	ME 1	ME 2	ME 3
COMPOSITION	OLEIC ACID	70	50	10
	TRANSCUTOL	14	22.5	35
	TWEEN	14	22.5	35
	WATER	2	5	20
	IMQ SOLUBILITY (mg/ml)	82 ± 9	54 ± 1	3.7 ± 0.7
IMQ SKIN RETENTION	TOT (µg/cm^2)	3.09	4.18	1.94
	SD	0.38	0.9	1.07

Table 5. Composition of the prepared microemulsions, solubility of IMQ and amount of IMQ accumulated in the skin (epidermis + dermis) after 6 h of application at infinite dose (average ± sd).

IMQ accumulation from these MEs is represented in **Figure 8**: the values obtained from the application of ME 1 (conc. 82 ± 9 mg/ml) and ME 2 (conc. 54 ± 1 mg/ml), containing respectively 70 and 50% of oleic acid, are significantly higher than those obtained with oleic acid saturated solution (conc. 73.86 ± 14.2). This effect cannot be attributed neither to the thermodynamic activity of the drug (both

solutions and MEs are saturated) nor to a direct effect of ME components on the SC permeability with a consequent higher diffusion of IMQ, as reported in case of many other drugs formulated in ME [35]. In fact, as demonstrated with the commercial cream (**Figure 8**) and with saturated solutions of PEG600 and oleic acid (**Figure 4**) the SC represents only a modest barrier for IMQ permeation, if any. Or better, it represents a barrier in so far as hinders the penetration of the solvent. We can hypothesize that in the case of MEs, a high penetration of the ME components takes place, thus "dragging" IMQ into the skin. This hypothesis is sustained by literature evidences: it has been demonstrated that the concentration profiles into the SC of oleic acid, Tween 20 and Transcutol are consistent with a deeper and faster penetration when applied as ME (oleic acid 15.4%, Tween 20 30.8%, Transcutol 30.8%, water 23%) than as pure solvents [33]. Additionally, Mahrhauser et al. have studied the penetration of both the drug (ibuprofen) and the surfactant from a microemulsion (oleic acid 10%, fluorosurfactant 32.5%, isopropanol 32.5% and water 25%) and demonstrated their simultaneous penetration into SC, suggesting a "drag" effect [36].

The comparison of the different MEs allows to appreciate that the water content plays an important role, in fact the accumulation efficiency resulted proportional to the water % in the ME (see insert in **Figure 8 and Table 5**). Similar results were obtained by from Lopes et al. for other permeants like sucrose, ketoprofen, lidocaine and alpha-tocopherol [35]. It has been suggested that the content of water influences the ME internal structure, affecting drug mobility [37, 38].

Finally, it is important to highlight that ME3, containing only 0.37% IMQ, gives an accumulation which is comparable with the commercial formulation (5% w/w) and can represent a starting point for a more suitable and efficient dermal formulation.

4.3.2 Microemulsion-based gels

One possible problem of microemulsion administration is their low viscosity, not suitable for skin application. For this reason, and due to their great potential, microemulsions have been largely studied as a basis for the preparation of gel-like formulations. Microemulsion-based gels are prepared either by directly adding the thickening agent to the ME [13] or by mixing the ME with an already prepared gel. In the latter, among others, Carbopol 940 [39], xantan gum [14] or hydroxyproprylmethylcellulose [15] gels have been used.

It is important to underline that the addition of the gelling agent could be problematic, since it could modify the microemulsion internal structure which seems to be fundamental for the enhancing properties [35].

4.3.2.1 Thickening of blank ME

Since the highest IMQ delivery efficiency was found for ME 3 (**Figure 8**), we selected this formulation for the thickening studies. In order to increase ME 3 viscosity, different compounds, characterised by different MW and properties, were directly added to the microemulsion; the thickener concentration and the result obtained are reported in **Table 6**.

THICKENER	% w/v	SOLUBLE IN in ME 3	ME 3 THICKENING
Poloxamer 407	20-25	yes	no
Lutrol F68	2-4	yes	no
Pluronic P85	4	yes	no
Sodium hyaluronate	0.05-2	partially	low
Kollidon 30 (PVP)	20	yes	no
Sodium alginate	20	no	no
Carbopol 940	0.05-0.1	yes	no
	0.5-1	yes	yes
Fumed silica	0.5-6	yes	no
	7-10	yes	yes

Table 6. Gelling agents used for ME 3 thickening, concentration tested and results.

When poloxamers (Poloxamer 407, Lutrol F68, Pluronic P85) were added, they dissolved into the microemulsion without inducing any viscosity increase: it is possible to hypothesize that the presence of ME prevents the formation of micelles, that are the structures underlying poloxamers-induced gelation.

The same behaviour was seen for PVP and can be explained considering that it is reported to be a stabilizer in microemulsion formulation [40]. A different behaviour characterised the natural hydrocolloids: sodium alginate did not dissolve in the microemulsion, while in case of sodium hyaluronate, a slight increase of viscosity took place along with increase in ME opalescence, that suggests a change in the internal structure, probably due to subtraction of water. Good results have been obtained with Carbopol 940 at 0.5 and 1% and fumed silica at 7, 8, 9 and 10%. These formulations have been characterized for the sliding (flow) properties using a modified Emmamreddy and Gosh method [20]. Even if it is a relatively rough method, the values obtained are useful for the comparison of the different formulations. The results are reported in **Figure 9.**

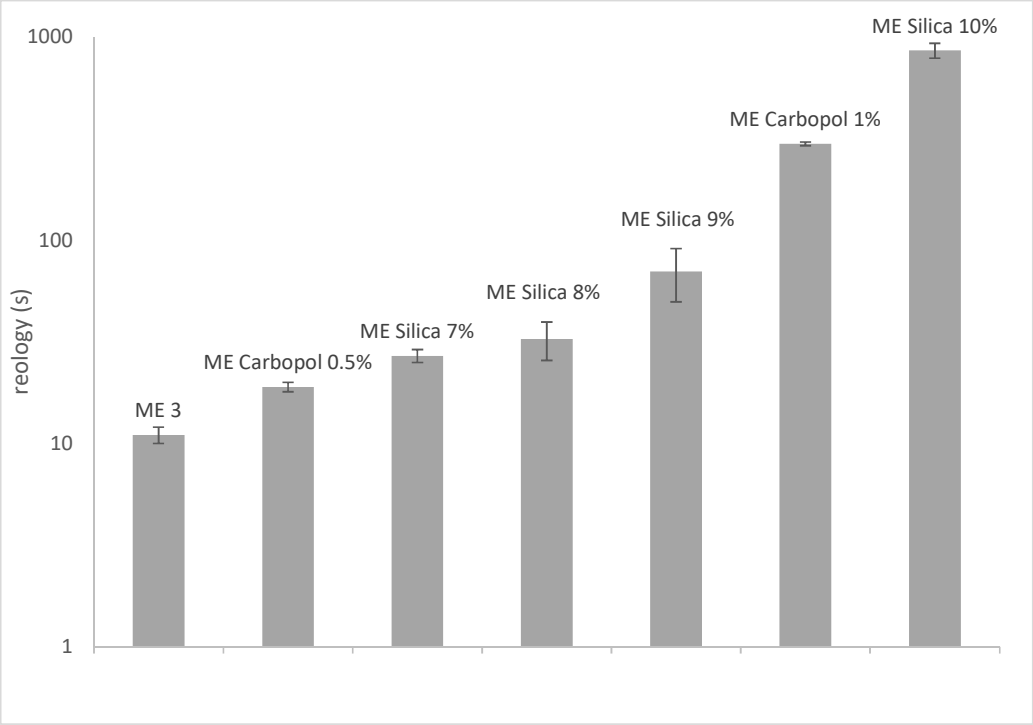

Figure 9. Reology results expressed as time (s) necessary to reach equilibrium state (log scale y axis).

When Carbopol is used at 0.5%, the viscosity increase is modest, while a significant increase is recorded at 1% in agreement with its mechanism of gelation. Carbopol 940 is a cross-linked homopolymer composed of polyacrilatunits and it is known to induce the gelation of both aqueous and polar organic solvents. In particular, there are evidences in literature [41] of its capability to form H bond not only with water but also with Tween 80.

In the case of fumed silica the viscosity increases slowly from 7 to 9% while a dramatic increase (see log scale on y axis) is recorded at 10%. Literature data [42] show that there is an inverse correlation between the capability of the silica to interact with the system and the gelation power; in fact, the higher the affinity for the system (with hydrogen bonding formation) the lower the possibility of silica-silica interaction, necessary for the network formation and the increase in system viscosity. In the present case we could hypothesize that for silica

concentration up to 9 % the interaction with the polar components of ME is predominant, when the concentration reaches to 10 % the dramatic increment of viscosity could be the reflection of the interaction between silica particles.

4.3.2.2 Thicknening of IMQ-loaded ME

The gelation tests have been initially performed on the formulation without IMQ. After the selection of the better thickeners (carbopol and silica), gels have been prepared starting from IMQ-saturated microemulsion. Interestingly, a different behaviour was found: when silica was added, at any concentration, gelation occured without any change in terms of transparency and no drug precipitation took place. In the case of carbopol (both at 0.5 and 1%), the addition of the polymer induced IMQ precipitation, suggesting a different mechanism of interaction between the two thickener and the ME components.

We tried to avoid IMQ precipitation by reducing drug concentration in the microemulsion: carbopol was added to microemulsion with lower saturation degrees (α), namely 0.7, 0.5 or 0.25. When α was 0.7, IMQ precipitation still occured, while at α = 0.25 the obtained gel was perfectly transparent; when α = 0.5 only a slight opalescence was present. We decided to use α=0.5, in order to be, after carbopol addition, at saturation conditions, i.e. in a situation comparable with the other formulations.

4.3.2.3 Evaluation of ME-based gels

Four different IMQ-loaded gelled microemulsions have been tested on the skin and their performance is reported in **Figure 10** compared to ME 3.

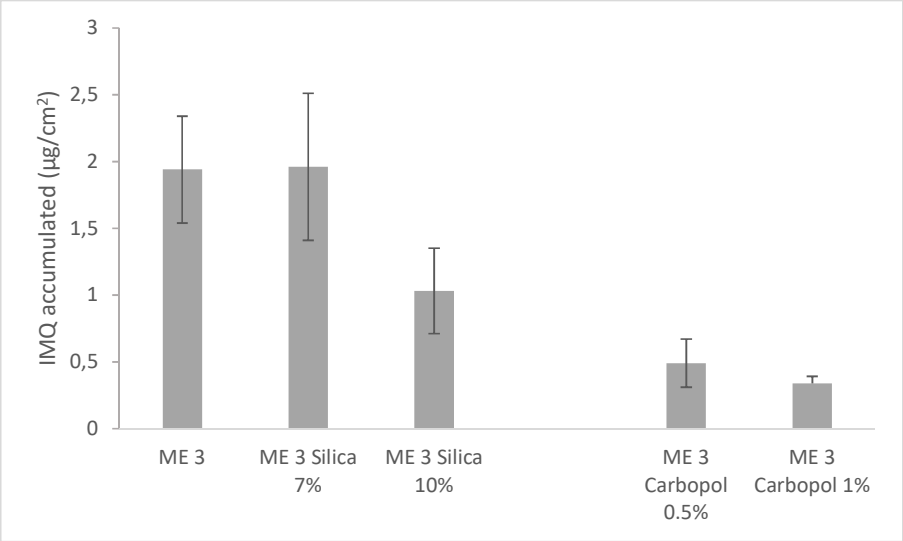

Figure 10. Total amount of IMQ accumulated in the skin (µg/cm^2) form ME 3 as such and after gelification.

In the case of carbopol-thickened MEs, the performance was definitely lower than ME3 and no statical difference was observed between 0.5 and 1%; the lower level of IMQ accumulation could be due to:

1- The lower IMQ concentration (approximately 4 mg/ml for ME 3 and 2 mg/ml for the gel). However, this factor should have a limited influence, since both formulations are close to saturation (i.e. have the same thermodynamic activity).

2- The higher viscosity. This could slow down both drug diffusion and solvent uptake, one of the important mechanism of IMQ penetration [43]. However, the viscosity increase is modest and apparently irrelevant since

silica-gel at 7% has a comparable value of viscosity (**Figure 9**) but a performance more than 4 times higher (**Figure 10**).

3- The interaction of carbopol with the microemulsion components that changes the internal structure with a negative effect on IMQ skin delivery.

When 7% silica gel is applied, no difference can be seen in IMQ accumulation in comparison to ME 3; when silica concentration increase to 10% a slight ($p > 0.05$) decrease occurs. However, as previously underlined, in this case the difference in terms of viscosity is dramatic and the gel structure - by definition a network which entraps the liquid phase - reduce solvent mobility and consequently solvent and drug uptake [43]. Additionally a modification of ME internal structure cannot be excluded.

It is possible to conclude that the addition of an external gelling agent to the microemulsion in order to obtain a usable gel, can critically affect drug bioavailability. This can be due to a decrese of drug solubility in the formulation, to a reduction of solvent mobility and, consequently, of solvent and drug uptake and/or to a change of the microemulsion internal structure. However, we are not able to say how and in what measure the internal structure of ME 3 could change. Dynamic Light Scattering (DLS) analysis on Me 3 did not shown measurable droplets of dispersed phase, suggesting a bicontinuous structure. This result is not surprising considering the high concentration of surfactants, i.e. Tween 80 and Transcutol.

4.3.3 Self-gelling microemulsions

Given the previously reported results, it is clear that the introduction of an external gelling agent into a microemulsion system could affect its structure and performance.

TPGS is a water soluble source of vitamin E, developed by Eastman in 1950. It is prepared by esterification of the acid group of crystalline d-α-tocopheryl acid succinate by polyethylene glycol 1000. This molecule has found wide utility in pharmaceutical formulations: it is, in fact, able to improve drug bioavailability by enhancing drug solubility of poorly soluble drugs, by stabilizing amorphous drug form and enhancing drug permeability by P-glycoprotein efflux inhibition. It is also used as functional ingredient in self-emulsifying formulations, as thermal binder in hot melt extrusion processing and as carrier for wound care and treatment. For the development of dermal formulation it demonstrated its utility as bioadhesive excipient for the production of films and as penetration enhancer[44, 45].

4.3.3.1 Pseudo ternary phase diagrams

In order to understand if in combination with the other excipients (oleic acid, transcutol and water) TPGS was able to form a microemulsion, we started by replacing Tween 80 in ME 3 (composition of ME 3 in **Table 5**), considering that the HLB values are relatively similar (13 TPGS vs 15 Tween 80). We obtained a transparent system (ME T 20) with a viscosity that was slightly higher than ME 3 (16 s vs 11 s) and able to solubilize at saturation a comparable amount of IMQ.

We decided then to proceed with a more systematic approach, building a pseudo ternary phase diagram. This diagram can be useful for the identification of both the region of existence of microemulsions, and the region of existence of gel systems. We built three diagrams (**Figure 12**), using two different oil phases (oleic acid and isostearic acid (**Figure 11**)), two different surfactants (TPGS and Cremophor (**Figure 11**)) and transcutol as co-surfactant (**Figure 12**).

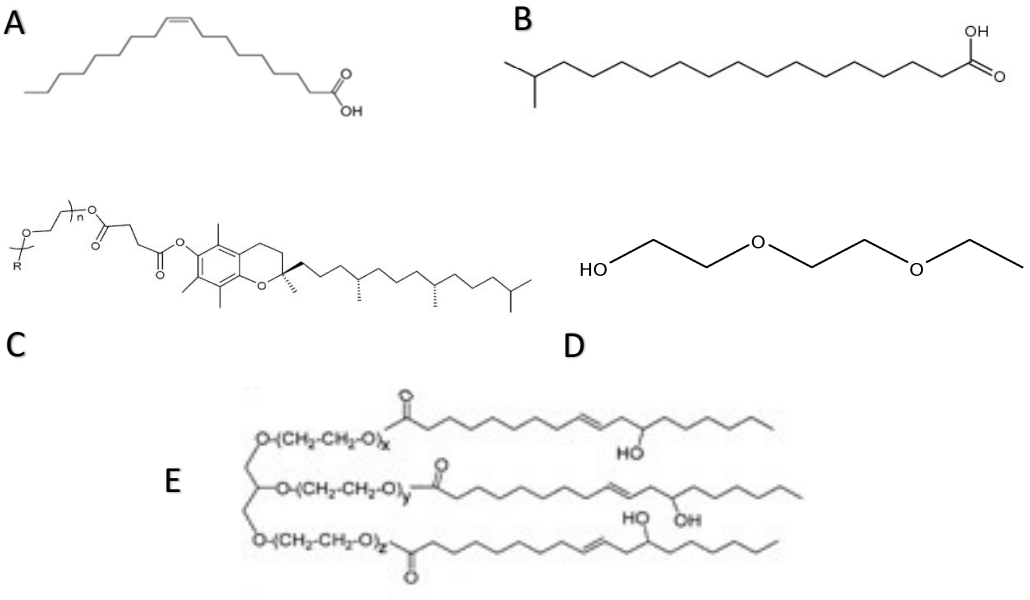

Figure 11. Chemical structures of the component investigated: A) oleic acid, B) isostearic acid, C) Vitamin E TPGS, D) Transcutol, E) Cremophor ELP.

DIAGRAM	OIL	SURFACTANT	CO-SURFACTANT	WATER
A	Oleic acid	TPGS	Transcutol	Water
B	Oleic acid	Cremophor	Transcutol	Water
C	Isostearic acid	TPGS	Transcutol	Water

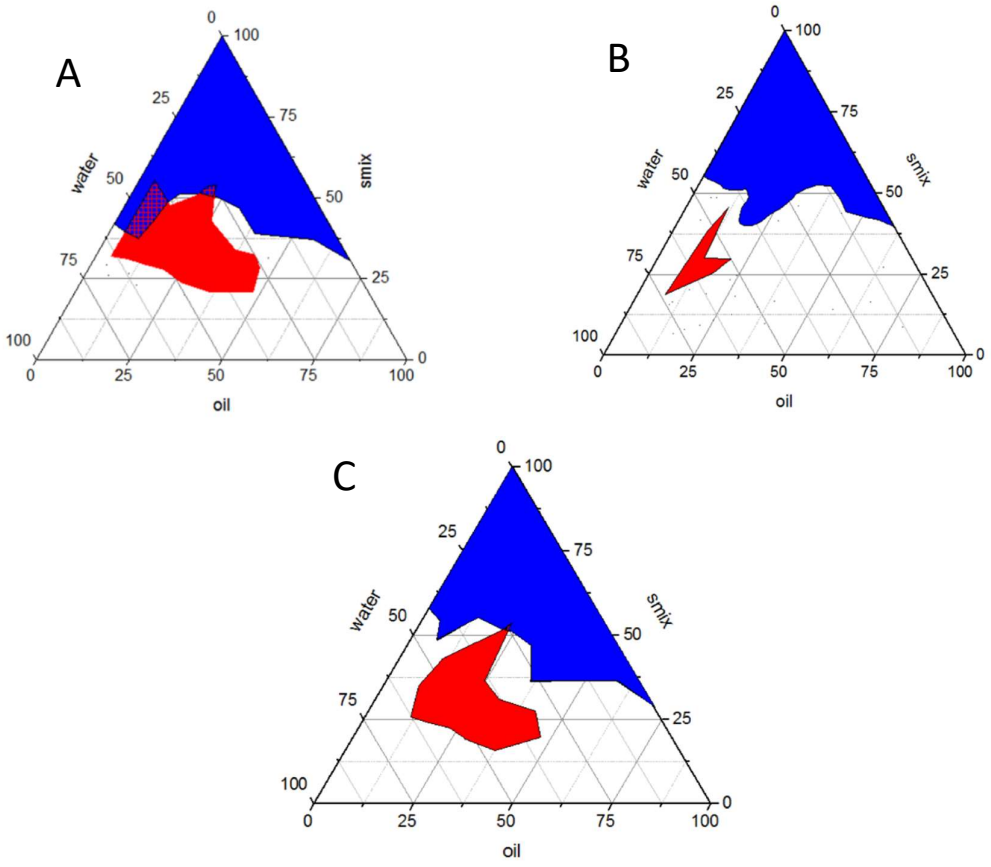

Figure 12. Pseudo ternary phase diagrams. A: oleic acid/TPGS/transcutol/water, B: oleic acid/cremophor/transcutol/water, C: isostearic acid/TPGS/transcutol/water.

In the diagrams (**Figure 12**) two different regions are highlighted: in red the region of existence of a viscous formulation, in blue the region of existence of microemulsion (transparent formulation). The overlapping of the two regions allow us to individuate the components concentration-range where completely clear gels exist. In this region –in principle- it would be possible to take advantage of both the enhancing properties of ME and the rheological properties of gels.
It is possible to see (**Figure 12** B) that using cremophor it is not possible to prepare self-gelifying microemulsions. On the contrary, TPGS permits the formation of microgel using either oleic acid and isostearic acid –even if, in this last case, in a much smaller region.

4.3.3.2 Oleic acid

In diagram A (**Figure 12**) (oleic acid/TPGS/transcutol/water) it is possible to highlight two different microgel regions:

1. oil phase/smix ratio 1/9, 1.25/8.75, 2/8, with a water content from 40% to 60%. Formulation with a water content between 55 and 60 % have, however, shown a slight opalescence which is index of a dispersed phase size higher than 150 nm. It is interesting to note that when the ratio oil/smix is 0.5/9.5, no increase of viscosity has been observed; indicating that the formation of a gel-like structure is not only ascribable to the known properties of TPGS [46], but that also a critical % of oily phase is required.
2. oil phase/smix 3/7 in a more restricted range of water (25-30%).

The oil/smix ratio 1.25/8.75 was further investigated by preparing and characterising microemulsions with a water content from 20 to 65%.
Figure 13 illustrates the appearance of the formulations, while in **Figure 14** the flow (sliding) properties are reported.

Figure 13. Appearance of the TPGS based MEs with increasing water content (from 20% to 65%).

The viscosity of the formulation increased as the water content increased up to a value of 55%. In this case, a slight opalescence can be seen, indicating the presence of a dispersed phase with a size higher than 150 nm. Formulation with a water content of 60% is more opalescent while 65% formulation has a milky appearance, but these MEs are less viscous then 55%.

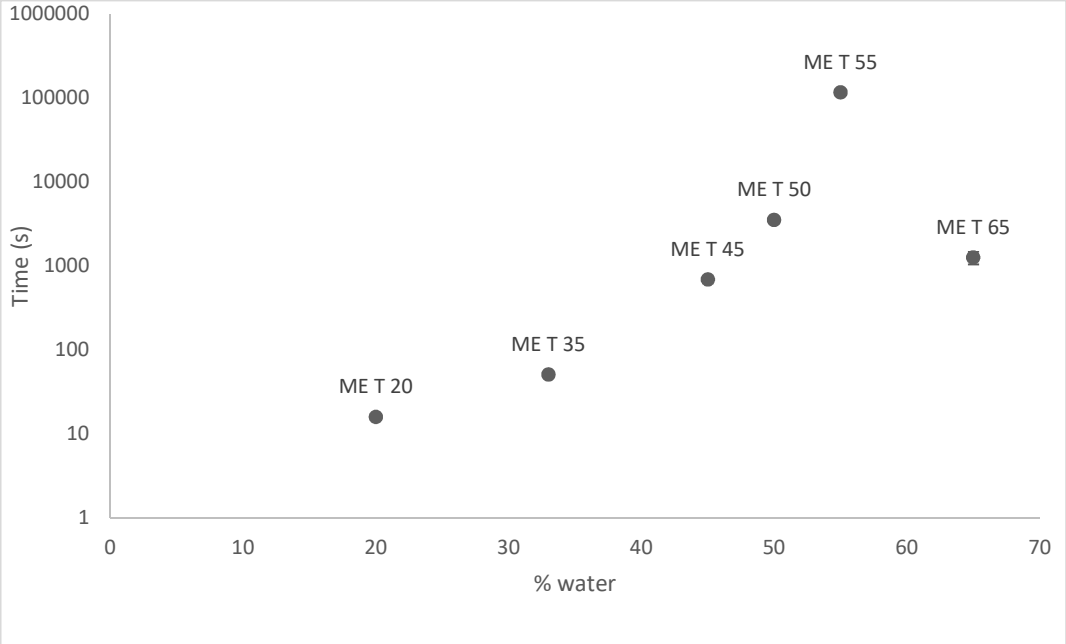

Figure 14. Time (s) necessary for reach the equilibrium state of ME T 20-35-40-45-50-55-65 as a function of water content %.

The formulations were then analysed using a polarized light optical microscope. In case of a water content of 20, 35 and 65%, no images are visible. On the contrary, ME 45, 50 and 55% once spread with a spatula on the carrying slide, show peculiar patterns (**Figure 15**) indicating the formation of birifrangent ordered structures. It was not possible to see structure differences between 45, 50 and 55% since the pattern is higly variable and depends upon the thickness of the gel layer, the temperature (that increases during the analysis due to the heat of the analysing light), the presence/absence of the covering slide, the way of spreading the formulation onto the slide and the time between formulation spreading and microscopy analysis. This suggest that the ME structure changes not only as a function of water evaporation (presence/absence of covering slide) but also as a function of the temperature and the force applied during the spreading.

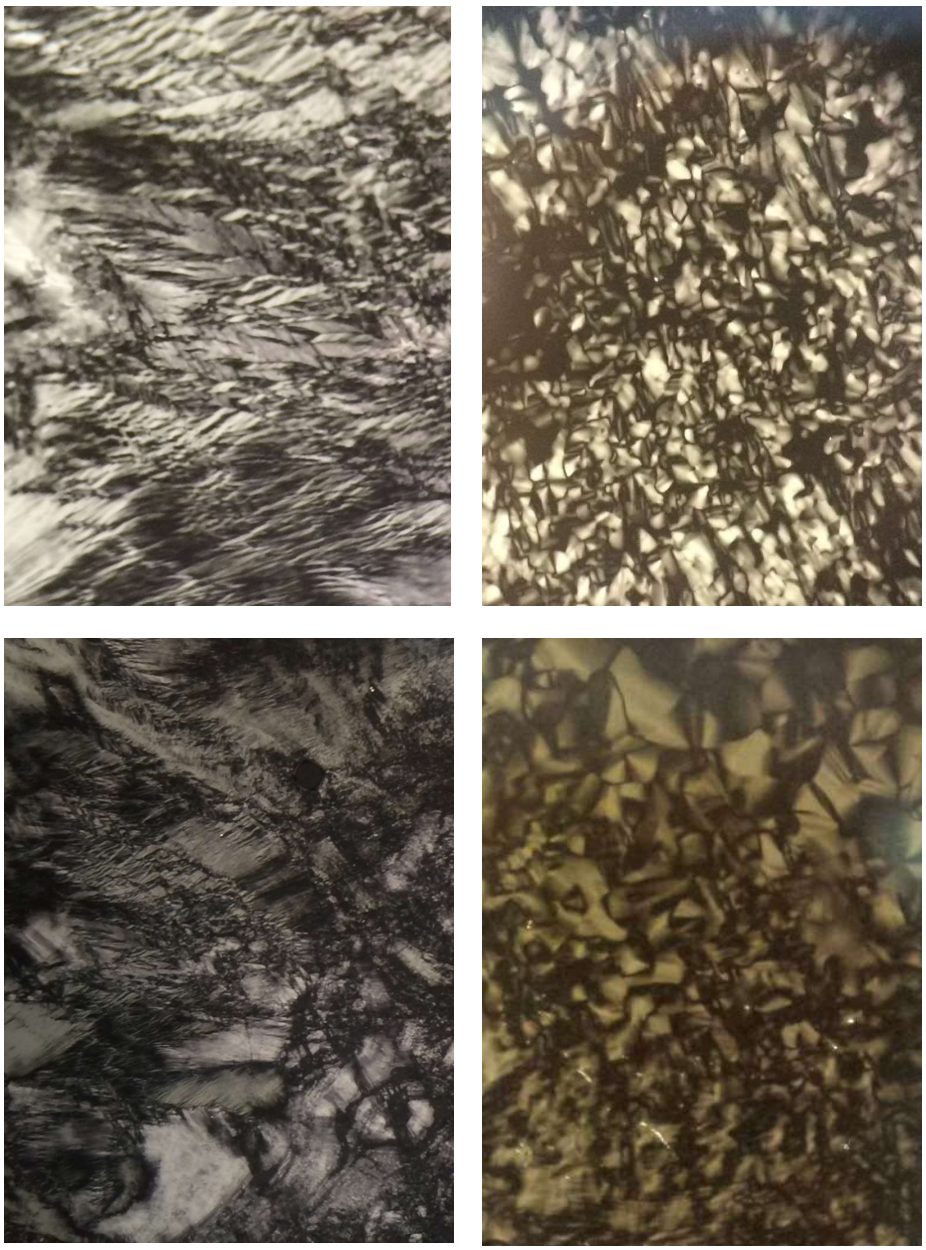

Figure 15. Polarized light microscopy images of oleic acid/TPGS:transcutol (1.25:8.75) microemulsions, with a water content between 40 and 55%.

In order to figure out the role of microemulsion composition and structure on delivery efficiency, oleic acid/TPGS:transcutol (1.25:8.75) MEs with a water content of about 20, 35, 45, 50 % (i.e. perfectly limpid and with increasing viscosities) were loaded with IMQ (see chapter 2.1.7) and evaluated for IMQ skin accumulation. The composition of the formulations is reported in **Table 7**.

	Excipients (%w/w)	ME T 20	ME T 35	ME T 45	ME T 50	ME T 25 ISO
COMPOSITION	OLEIC ACID	9.1	7.5	6.2	5.7	-
	ISOSTEARIC ACID	-	-	-	-	20.6
	VITAMIN E TPGS	35.5	29.6	24.3	22.1	27.0
	TRANSCUTOL	35.1	29.2	24.0	21.8	26.7
	WATER	20.3	33.7	45.4	50.4	25.7
IMQ SKIN RETENTION	TOT (µg/cm^2)	1	1.42	1.74 1.87	1.93 1.36	3.36
	SD	0.43	0.09	0.26 0.88	0.23 0.77	1.14

Table 7. Compositions of the selected microemulsions expressed as %w/w and amount of IMQ accumulated in the skin (epidermis + dermis) after 6 h of application at infinite (black) or finite (green) dose (average ± sd).

ME T 20 was saturated with IMQ (concentration about 4 mg/ml) added with increasing amounts of water to obtain ME T 35, 45 and 50. The resulting IMQ concentrations were 83, 75 and 69 % of the saturated ME T 20 concentration (considered as 100%). During water adddition, no precipitation occurred despite the extremely low IMQ water solubility. We can hypothesize that all the MEs are at saturation.

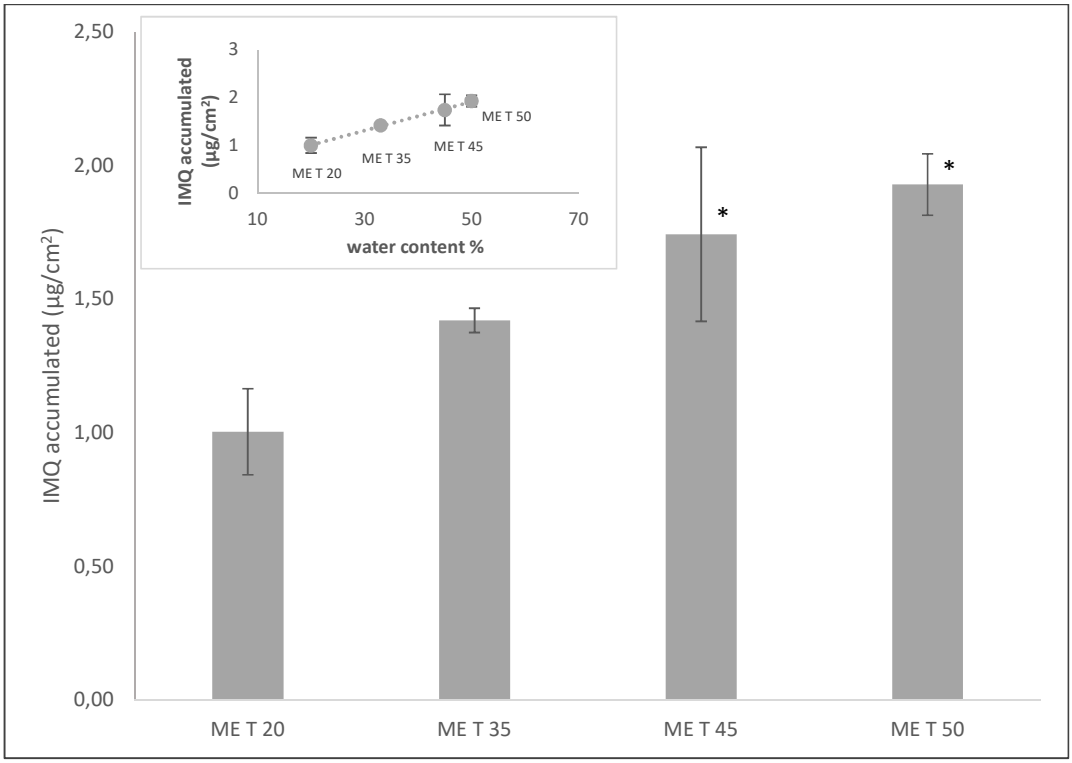

Figure 16. IMQ skin retention (µg/cm^2) from TPGS based microemulsions. The data are represented as mean ± sem. * significantly different from ME T 20 ($p < 0.05$).
The insert illustrates the accumulation as a function of water content

As can be seen in **Figure 16**, IMQ skin accumulation increased as a function of ME water content. The result is particularly relevant since the progressive improvement of performance is associated to a progressive decrease of drug concentration in the formulation and increase of formulation viscosity, both factors that could, in principle, reduce drug delivery to the skin.

Literature evidences [35] have underlined that an increase of ME water content could positively affect the accumulation efficiency on the basis of three different mechanisms:

1. Higher hydration of the stratum corneum
2. Increase of the thermodynamic activity of poorly water soluble drugs
3. Modifications of microemulsion structure

The first two mechanism are probably not important in this case, since 1) our previous study on the mechanisms of IMQ skin delivery have highlighted that the SC is not a relevant barrier for IMQ skin accumulation (see **Figure 4** and **8**) 2) all the microemulsions tested are at the saturation. However, it is possible that the higher water content increased the thermodynamic activity of the different excipients, favouring their SC uptake and, consequently, increasing the "drag effect", as described earlier (see paragraph 4.2.3).

The third hypothesis, i.e. the change of the ME internal structure, can represent another important factor. In fact, if the ME internal structure changes, drug localization inside the microemulsion can change as well, moving for instance from an internal phase of a o/w microemulsion to the interface of a bicontinuous structure. Drug localization can affect its mobility.

Up to now, the microemulsions have been tested at infinite dose, to try to elucidate the accumulation mechanisms, however, it is also important to verify the performance of the formulation in the actual "in use" conditions. In this case 10 mg/cm^2 of ME T 45 and 50 were applied to the skin. As it is possible to see from **Figure 17** the accumulation is comparable to infinite dose ($p>0.05$): this allows to conclude that the viscositity is able to preserve ME structure, reduce the water loss and guarantee a sustained release during the time of application.

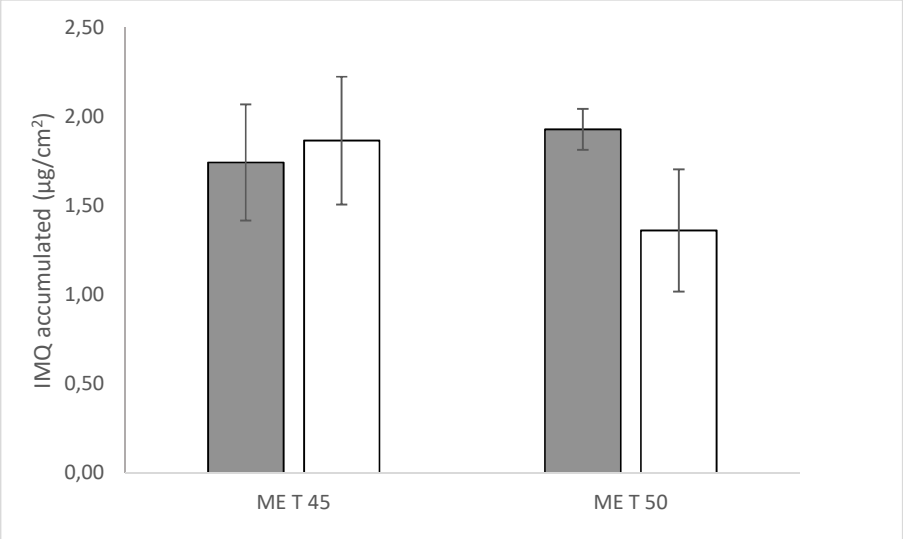

Figure 17. IMQ skin retention (µg/cm^2 ; average ± sem) from TPGS based microemulsions applied at infinite (grey bar) or finite (white bar) dose.

4.3.3.3 Isostearic acid

Recent studies have demonstrated that the therapeutic effect of Aldara, which is one of the commercially available IMQ cream formulations, is due not only to IMQ, but also to the presence of isostearic acid, used to solubilize the drug. In that paper [47], the authors reported that when the blank cream was applied to naïve murine skin, keratinocytes death and IL-1 release still occur. When tested in vitro on primary human keratinocytes isostearic acid demonstrated to induce apoptosis, inflammation and IL-1 release. Further in vivo studies demonstrated the capability of isostearic acid to induce expression of cytokines, concluding that IMQ is not the only active component of Aldara.

Starting from this evidence, we evaluated the possibility to formulate a microemulsion using isostearic acid instead of oleic acid as oily phase. As reported in **Figure 12**, it is possible to obtain a microemulsion for different oil/smix ratio, but the region of existence of the microgel is definitively small: the gelation of microemulsion occurs only in the range of water content 25-30%, when the ratio

oil/smix is 3/7. The absence of microgel region for 1/9 to 2/8 oil/smix ratio can be attributed to the different molecular structure of isostearic acid (**Figure 11** B) and in particular to the absence of insaturation and/or to the presence of a terminal ramification, causing an higher steric hindrance that could prevent gelation [48]. In order to further characterize this formulation, images at polarized optical microscope were taken and a different pattern was observed in comparison with the oleic acid based microemulsions (**Figure 15** and **Figure 18 A**). The difference was in a first moment ascribed to the different composition of the oil phase. However, since the concentration of oil, in ME T 25 ISO was higher than ME T series with oleic acid (oil/smix ratio 3/7 instead of 1/9), we decided to analyse oleic acid based microemulsions with the same oil/smix ratio, in order to have comparable oil concentration. Images are presented in **Figure 18 B**.

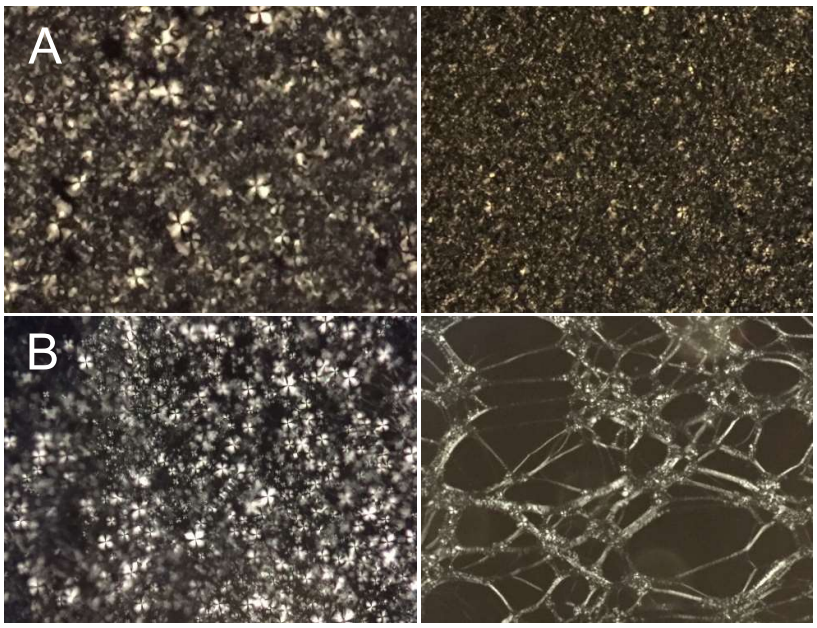

Figure 18. Polarized light microscopy images of A) isostearic acid/TPGS:transcutol and B) oleic acid /TPGS:transcutol microemulsions (oil/smix ratio 3/7).

As it is possible to appreciate, when the oil/smix ratio is the same, no significant differences can be appreciated and the different pattern found is due mainly to the higher oil concentration.

The isostearic-based microgel (ME T 25 ISO-**Table 7**) was loaded with IMQ at saturation (see chapter 3.7.1): IMQ content resulted higher (about 13 mg/ml) than in the oleic acid-based microemulsion, because of the higher % of oil phase (20.6%), in which IMQ is highly soluble [21].

Accumulation results are illustrated in **Figure 19**, compared with the one obtained with the commercial formulation.

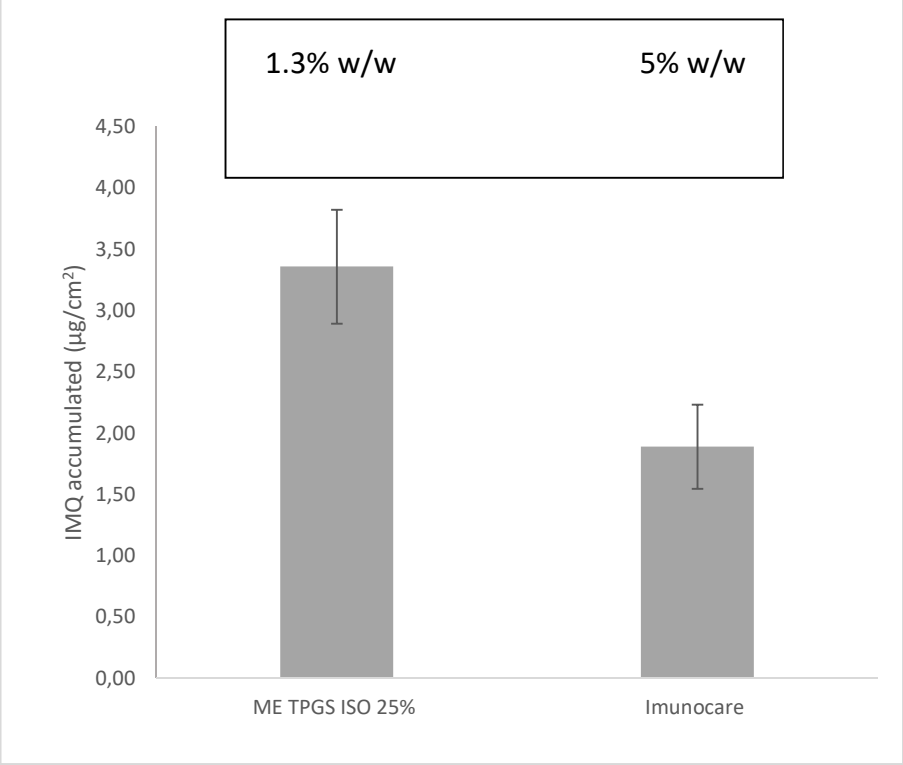

Figure 19. IMQ skin retention (µg/cm^2; average ± sem) from Imunocare® and TPGS based microemulsion.

As it is possible to appreciate, in this case the performance is definitely better ($p<0.05$) than the commercial cream, despite the about 4 times lower concentration.

5 Conclusion

The results obtained in the present work underline:

- the fundamental role of solvent in imiquimod skin penetration and retention. It was found that drug skin uptake is strictly related to solvent uptake, suggesting the relevance of solvent drag effect in imiquimod skin delivery. Concerning the localization of the barrier for transport, the stratum corneum represents a barrier towards the solvent more than towards the drug. In fact, when the stratum corneum was removed by tape stripping, imiquimod retention increased from a very lipophilic vehicle, such as oleic acid, but not from hydrophilic vehicle. The work on imiquimod skin retention from pure solvents represented the starting point for the preparation of microemulsions;
- the results obtained from MEs show that these systems are effective for IMQ skin delivery. In particular, the ME composed of 10% oleic acid, 35% Transcutol, 35% Tween 80 and 20% water is able to accumulate the same amount of drug as the commercial formulation but with far more efficiency, since its concentration is 12 times lower. These results are a valuable base to optimize imiquimod-loaded microemulsions for both the treatment of neoplastic skin diseases and as adjuvant for vaccination;
- the gelation of ME seems to negatively affect the accumulation in case of Carbopol, while, when silica is added, comparable performance are observed. This difference could be ascribed to the different mechanism of gelation, so a different interaction with ME structure/components;
- vitamin E TPGS based microemulsions are interesting systems for the local drug delivery. When oleic acid is used, an increase of viscosity as a function of water content has been found. Interestingly, the increase of

viscosity and water content is in this case associated with an increase in IMQ skin retention, despite the lower IMQ concentration. Moreover, the viscosity of these systems make them suitable for skin application.

- The possibility to formulate self gellying microemulsions with isostearic acid give the possibility to have both the beneficial effect of IMQ and isostearic acid. The accumulation results are promising since a higher accumulation was observed in comparison with the commercial formulation, despite the lower IMQ concentration.

6 Supplementary informations

Formulation	Dose	Epidermis (μg/cm^2)	SD	Dermis (μg/cm^2)	SD	Total (μg/cm^2)	SD
TWEEN 80	infinite	0.26	0.08	0.06	0.03	0.33	0.06
2-pyrrolidone	infinite	0.48	0.27	0.22	0.22	0.69	0.48
PG Transcutol	infinite	0.14	0.05	0.05	0.01	0.19	0.06
Transcutol	infinite	0.32	0.14	0.06	0.04	0.38	0.17
PEG 200	infinite	0.12	0.12	0.06	0.07	0.17	0.19
PEG 400	infinite	0.2	0.05	0.23	0.13	0.43	0.16
PEG 600	infinite	0.79	0.63	0.36	0.2	1.14	0.81
	infinite- tape stripped	1.39	1.05	0.4	0.12	1.79	0.98
	infinite- isolated dermis	-	-	61.9	20.79	61.9	20.79
Oleic Acid	infinite	1.02	0.38	0.61	0.1	1.62	0.4
	infinite-tape stripped	3.16	0.93	1.05	0.74	4.03	1.2
	infinite- isolated dermis	-	-	2.8	2.44	2.8	2.44
DMSO	infinite	0.61	0.08	0.41	0.33	1.02	0.27
ME 1	infinite	1.85	0.67	1.25	0.51	3.09	0.38
ME 2	infinite	2.41	0.72	1.77	0.23	4.18	0.9
ME 3	infinite	1.17	0.54	0.76	0.59	1.94	1.07
ME 3 carbopol 0,5%	infinite	0.37	0.24	0.12	0.08	0.49	0.31
ME 3 carbopol 1%	infinite	0.22	0.07	0.12	0.1	0.34	0.09
ME 3 silica 7%	infinite	1.38	0.71	0.58	0.43	1.96	0.96
ME 3 silica 10%	infinite	0.73	0.42	0.29	0.18	1.03	0.56
ME T 20	infinite	0.74	0.37	0.27	0.14	1	0.43
ME T 35	infinite	1.13	0.09	0.39	0.04	1.42	0.09
ME T 45	finite	1.54	0.88	0.33	0.16	1.87	0.88
	infinite	1.32	0.58	0.43	0.18	1.74	0.26
ME T 50	finite	0.97	0.58	0.4	0.33	1.36	0.77
	infinite	1.4	0.26	0.53	0.07	1.93	0.23
ME T 25 Iso	infinite	2.51	1.02	0.85	0.44	3.36	1.14
Imunocare	finite	1.67	0.14	1.04	0.43	2.71	0.42
Imunocare	finite- stripped skin	1.16	0.48	0.73	0.16	1.89	0.61
Imunocare	infinite	1.27	0.6	0.62	0.3	1.89	0.77

Table 8. Amount of IMQ accumulated from different formulations applied 6 h at infinite or finite dose conditions (average ± SD).

Formulation	pH	Reology (s)	SD
ME 3	6.2	11	1
ME 3 carbopol 0,5%	5.7	19	1
ME 3 carbopol 1%	5.5	298	6
ME 3 silica 7%	6.1	27	2
ME 3 silica 10%	6.1	860	72
ME T 20	6.5	16	1
ME T 35	6.1	51	2
ME T 45	5.7	690	46
ME T 50	5.7	3540	227
ME T 25 Iso	5.8	-	-

Table 9. Characterization of gelled and self-gelled microemulsions

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PART 2

Local delivery of corticosteroids:

Triamcinolone acetonide and dexamethasone palmitate

PART 2

In **PART 2**, the formulation of corticosteroids for local delivery is presented.
In particular, we have developed:

- microemulsions loaded with triamcinolone acetonide (TA) for the buccal delivery

- dexamethasone palmitate (DXP) nanoparticles based on hyaluronic acid conjugate. This second part of the project was conducted at Institut Galien Paris-Sud, UMR CNRS 8612, University of Paris-Sud.

DEVELOPMENT OF MICROEMULSIONS FOR BUCCAL DELIVERY OF TRIAMCINOLONE ACETONIDE: A PRELIMINARY STUDY

Table of contents

1 Introduction 101

2 Aim 103

3 Materials and methods 104

3.1 Materials 104

3.2 Methods 104

3.2.1 Solubility studies 104

3.2.2 Construction of pseudoternary phase diagram 105

3.2.3 Thermodynamic stability studies 105

3.2.4 Microemulsions characterization 105

3.2.5 In vitro permeation studies 106

3.2.6 HPLC analysis 107

3.2.7 Data analysis 108

4 Results and discussion 109

4.1 Microemulsions development 109

4.1.1 Solubility studies 109

4.1.2 Construction of pseudoternary phase diagrams 116

4.2 Microemulsions characterization 120

4.3 In vitro permeation experiments 122

5 Conclusions 128

6 Bibliography 129

1 Introduction

In the last decades microemulsions have been successfully studied for dermal application. These systems are easy to prepare and characterized by high thermodynamic stability; they can, in principle, solubilize both hydrophilic and lipophilic drugs and have been demonstrated to be able to enhance skin uptake. While microemulsions have been widely investigated for skin application, only a few studies can be found in the literature for buccal drug delivery. Ceschel et al. tested microemulsions for the buccal delivery of Salvia desoleana essential oil [1], while Chukaewrungroj studied the use of microemulsions for the delivery of fluocinolone acetonide [2]. In 2004, the efficacy of a microemulsion loaded with mometasone furoate has been demonstrated in vivo for the treatment of oral lichen planus [3]. Recently, promising results have been found from the use of microemulsions, loaded into laminated sponges, for the transbuccal delivery of carvedilol [4].

Despite the few studies, the properties of ME can be very interesting for buccal application since the presence of a specific combination of excipients could enhance and accelerate drug uptake, issue particularly relevant given the good barrier properties of the buccal epithelium and the short residence time that characterizes buccal administration. Microemulsions could be used in two different forms:

1) as mouthwashes; the liquid dosage form allows the drug to reach all the areas of the oral cavity, thus to treat diffuse diseases that affect different parts of the oral mucosa [5]. The application of a liquid vehicle can also be useful in the case of painful diseases, since –if formulated with non-irritant excipients- liquid formulation are better accepted by patients compared to the application of a solid dosage form.

2) as gels; as discussed in the previous chapter, it is possible to thicken MEs in order to obtain mucoadhesive semisolid formulations, more suitable for treating easily accessible and localised mucosal diseases.

2 Aim

The aim of the present work was to investigate the potential of microemulsions for buccal application, using triamcinolone acetonide (TA), a corticosteroid currently employed for some diseases of the oral cavity such as *r*ecurrent apthous stomatitis (RAS) and lichen planus [6, 7].

Microemulsion development was performed following a simple protocol based on a) drug solubility studies and excipients selection; b) construction of pseudo ternary phase diagrams by using the aqueous titration method; c) accelerated stability tests. Then, microemulsions were tested on buccal mucosa: permeation studies were performed in order to investigate TA transport across the mucosa and identify the best ME composition to obtain a fast and substantial drug uptake.

3 Materials and methods

3.1 Materials

Triamcinolone acetonide was purchased from Metapharmaceutical (Barcelona, Spain) while, Transcutol HP, Labrasol, Peceol, Labraphac lipophile, Lauroglycol, Plurol oleique, Maisine 35-1, Capryol 90 were a gift from Gattefossé (Saint-Priest Cedex, France). Brij78 and Tween 20 were purchase from Sigma Aldrich (St. Louis, MO, USA). PEG 200, 400, 600, isopropyl miristate and propylene glycol were obtained from A.C.E.F. (Fiorenzuola, Italy). Sorbitan monoleate 80 (Span80) was a gift from Croda Ibérica SA, Spain. Lutrol L44, Pluronic 3500, Pluronic 6200, Cremophor ELP and Vitamin E TPGS were obtained from BASF (Ludwigshafen, Germany). For HPLC analysis, bidistilled water was used. Acetonitrile and methanol were of HPLC grade; all other reagents were of analytical grade.

3.2 Methods

3.2.1 Solubility studies

The selection of microemulsion (ME) excipients was based on TA solubility. In order to measure the solubility, an excess of TA was added to 2 ml of vehicle. Suspensions were left under magnetic stirring for 24 h at room temperature, then centrifuged for 10 minutes at 13000 rpm. The concentration was determined by HPLC analysis after dilution. This method was not suitable for all the vehicles tested so for some of them an estimation of solubility was done. Aliquots of TA (1 mg each) were added subsequently to 1 ml of vehicle until the vehicle was not able to dissolve it anymore.

3.2.2 Construction of pseudoternary phase diagram

In order to identify the region of existence of microemulsions, pseudo ternary diagrams phase were built. Since microemulsions are made of 4 components, on one axis is represented the mixture of surfactant/co-surfactant (smix). For each smix ratio tested, a pseudo ternary diagram was built. Different ratio of smix were prepared (1/1, 1/2, 2/1) v/v. Different ratios of oil/smix were tested (1/9, 1.2/8.8, 1.25/8.75, 1.5/8.5, 1.7/8.3, 2/8, 2.3/7.7, 2.5/7.5, 3/7, 3.4/6.6, 4.5/5.5, 5/5, 6/4, 7/3, 8/2, 9/1,) v/v were added of known volumes of water, in order to obtain concentrations between 5 % and 95 %; after each addition of water, visual inspections were done and recorded. The visual observations measured the transparency, opalescence, fluidity, and phase separation.

3.2.3 Thermodynamic stability studies

For each pseudo ternary diagram, five MEs were selected from the region of existence. The selected MEs were added of TA at 0.1 % w/v. Accelerated stability tests were then performed: formulations were first centrifuged for 30 min at 3500 rpm, then submitted to 6 cycles of heating and cooling of 48 hours each (40 °C, 4 °C) and finally to 3 cycles of freezing and thawing of 24 hours (-20 °C, 25 °C). MEs that failed a step were not included in the following phase.

3.2.4 Microemulsions characterization

3.2.4.1 Measurement of dispersed phase size

ME drops dimensions were measured using the dynamic light scattering technique (DLS), at 25 °C with an incidence angle of 90 °. Measures were performed using a Litesizer 500 (Antoon Paar, AU).

3.2.4.2 pH determination

The pH was measured using an Orion 4 Star pH meter (Thermo Scientific, Waltham, Massachusetts, United States), at room temperature.

3.2.4.3 Determination of TA content

In order to confirm the concentration of TA in MEs, samples were directly injected in HPLC. See below for the HPLC method.

3.2.4.4 Polarized light microscopy

In order to assess the isotropy, MEs were observed at cross-polarized light microscope (Nykon, Shinjuku, Japan). Samples were deposited on a slide with a spatula, then covered with a covering slide in order to prevent the water evaporation.

3.2.5 In vitro permeation studies

Permeation experiments were performed using Franz type diffusion cells with a permeation area of 0.6 cm^2 (DISA, Milan, Italy). The receptor compartment was filled with about 4 ml of a NaCl 0.9 % solution, previously degassed in order to avoid the formation of bubbles at the skin interface. This solution was kept at 37°C under magnetic stirring. Experiments were performed on oesophageal epithelium (description of tissue preparation is reported in the next paragraph). Experiments were performed at infinite dose (1.2 ml/cm^2 of formulation) for 7 hours at 37 °C.

Samples of receptor solution were taken with a syringe at time 0 and after each hour, replaced with fresh solution, and analysed by HPLC.

3.2.5.1 Tissue preparation

All tissues were obtained from a local slaughterhouse (Macello Annoni, Madonna de' Prati, Busseto, Parma), and transported to the lab in isotonic phosphate buffer at pH 7.4.

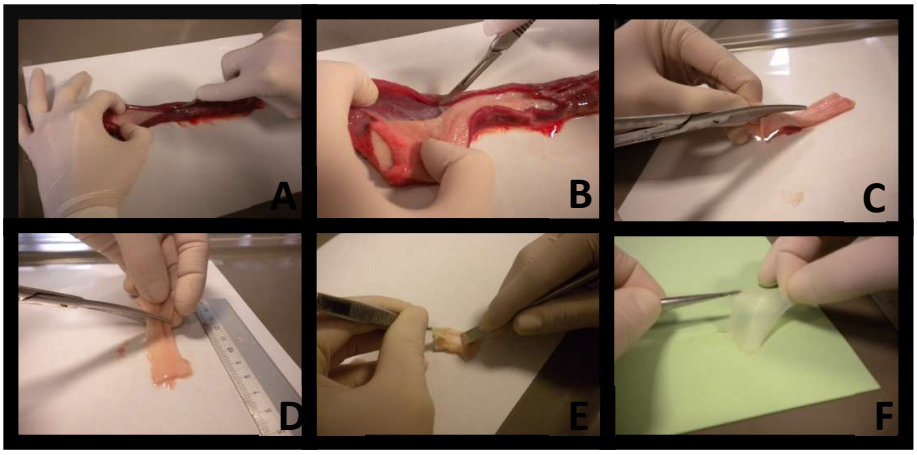

Figure 1. Preparation of porcine oesophageal epithelium

Oesophagus was separated from the muscular underlying tissue using a scalpel (1 A-B), washed with saline solution and cut longitudinally (1 C). Pieces of about 1 cm^2 were cut (1 D) and immersed for 2 minutes in distilled water at 60 °C. Epithelium was then detached from the connective tissue using a spatula (1 E), then placed on filter paper previously soaked in saline solution (1 F). Samples were then frozen at – 20 °C for a maximum period of 3 months.

When needed, the tissue was thawed for 30 minutes in saline before mounting them on the diffusion cells

3.2.6 HPLC analysis

For the quantitative determination of TA, an HPLC analysis was performed using an Agilent 1260 Infinity, equipped with a quaternary pump and automated autosampler. The column was a Bondclone 10 µm C18 300 x 3.9 mm (Phenomenex Columbus, USA). The mobile phase was water/acetonitrile (60/40,

v/v) eluted at 1 ml/min. Injection volume was 100 µl and the UV detection was performed at 240 nm. Using these conditions, the retention time was about 9 min.

3.2.7 Data analysis

In permeation experiments, the cumulative amount of drug permeated was plotted as a function of time. TA steady state flux (J, µg/cm^2 h) was calculated as the slope of the regression line in the interval 4-7 h. Time lag (h) was calculated as the intercept of the regression line on the time axis.

4 Results and discussion

4.1 Microemulsions development

4.1.1 Solubility studies

For the ME development, a simple procedure, largely described in literature, was followed [8]. The first step was the evaluation of TA solubility in different excipients. TA (**Figure 2**) is a corticosteroid anti-inflammatory drug, with a molecular weight of 434.49 g/mol, sparingly soluble in water (21 µg/ml at 28 °C [9]) and in ethanol, partially soluble in vegetable oils and insoluble in mineral oils.

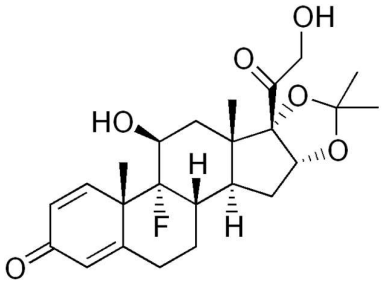

Figure 2. Triamcinolone acetonide

The different vehicles tested were chosen among oil phases, surfactants, co-surfactants and aqueous co-solvents. The values of solubility (measured or estimated) are reported in **Table 1** where the vehicles are classified as a function of their typical use in MEs.

VEHICLE	TA SOLUBILITY (mg/ml)
OILS	
TRIACETIN *	4 < X < 5
OLEIC ACID	< 1
ISOPROPYL MYRISTATE	< 1
MYRISTIL MYRISTATE	<1
LABRAFAC LIPOPHILE (Caprylic/Capric triglyceride)	< 1
LABRAFAC PG (Propylene glycol dicaprylocaprate)	< 1
MAISINE 35-1 (Glycerol monolinoleate)	< 1
PECEOL- GMO (Glyceryl monoleate)	1 < X < 2
AQUEOUS CO-SOLVENTS	
PROPYLENE GLYCOL	< 1
PEG 200	17.6 ± 1.5
PEG 400	14.8 ± 5.2
PEG 600	8.4 ± 0.9
SURFACTANTS	
CAPRYOL 90 (Propylene glycol monocaprylate)	6 < X < 7
SPAN 80 (Sorbitane monooleate)	1 < X < 2
LAUROGLYCOL 90 (Propylene glycol monolaurate)	1 < X < 2
BRIJ 78 (5% w/v) (Polyethylene glycol octadecyl ether)	< 1
PLURONIC 3500	4.1 ± 1.2
PLURONIC 6200	1.9 ± 0.6
TWEEN 20	< 1
TWEEN 80	5.9 ± 1.5
CREMOPHOR ELP (Polyoxyl castor oil)	4 < X < 5
LUTROL L44	3 < X < 4
LABRASOL (PEG-8 Caprylic/Capric Glycerides)	9 < X < 10
PLUROL OLEIQUE (Polyglyceryl-3 dioleate)	1 < X < 2
TPGS 50 mg/ml	< 1
CO-SURFACTANTS	
Transcutol	25.9 ± 3.3

Table 1. Estimated and measured TA solubility in different vehicles. When the solubility was measured by HPLC analysis, n=3.

4.1.1.1 Mechanisms involved in TA solubilization

TA has a log P value of 2.53, this is generally considered lipophilic. Despite this, the solubility of TA was lower than 1 mg/ml in almost all the lipophilic phases tested. To try to understand the solubility data, initial efforts were directed towards the analysis of the molecular structures of the vehicles tested, starting from fatty acids and their esters (**Table 1**, oils and surfactants sections). TA solubility is not related to hydrophobic interactions with long alkyl chain but, instead, to hydrogen bond formation with the polar moiety. In fact, the solubility was lower than 1 mg/ml in oleic acid and IPM (**Figure 3** A-B) while a slight increase (between 1 and 2 mg/ml) was found increasing the MW of the hydrophilic part as in case of GMO, Span 80 (**Figure 3** C-D) and lauroglycol. Additionally, a further solubility increase can be seen when the alkyl chain length is reduced to C8 (Capryol 90 between 6 and 7 mg/ml).

Then, we extended the analysis to vehicles with a significant number of H bond acceptor or donor sites, such as polyoxyethylene based excipients (PEGs, Pluronics, Transcutol and Tween 80). As it is possible to see from **Figure 3** E-F-G-H, these molecules are characterised by the presence of, at least, 2 ethylene oxide moieties, which are good H bond acceptors, and one or more –OH terminal group, which could act as H bond donors.

Additionally, higher solubility is measured in PEGs and Transcutol, without lipophilic chains, while lower values are measured for Pluronics and Tween 80, which present a polypropylene glycol block and an ester group with oleic acid, respectively.

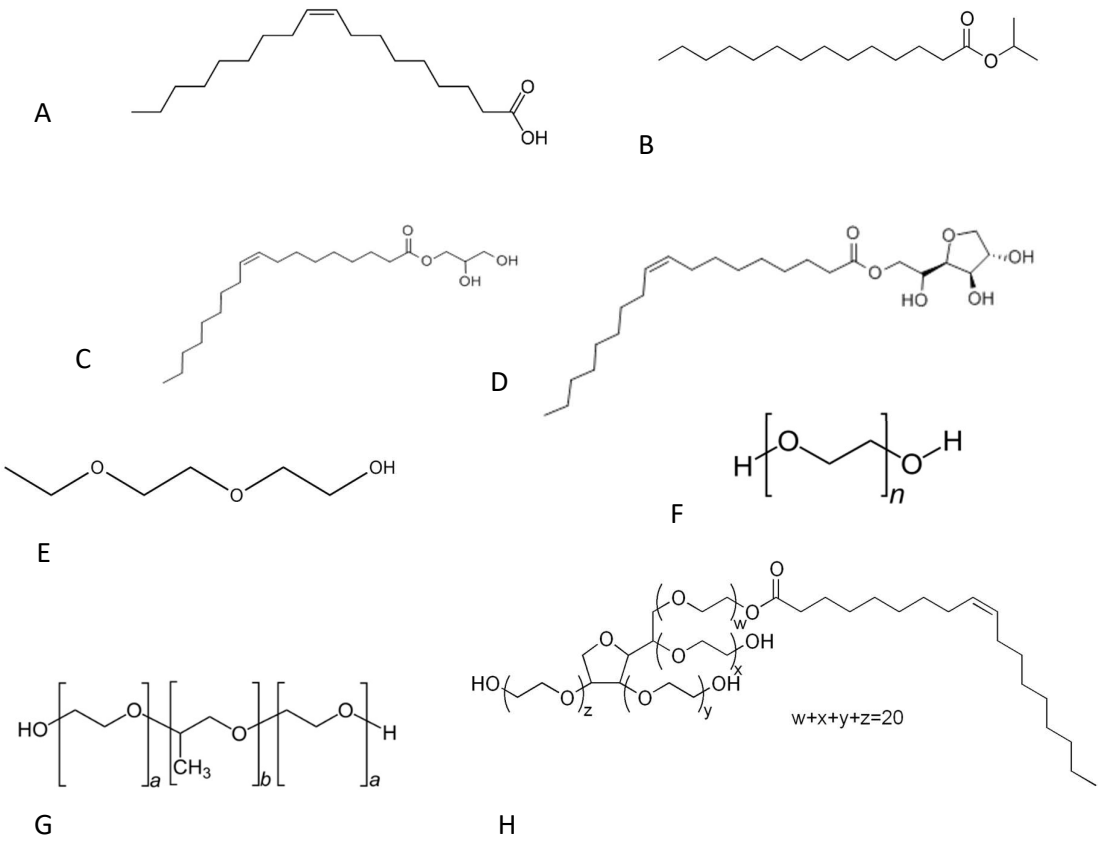

Figure 3. Molecular structure of A) Oleic acid B) Isopropyl myristate C) GMO D) Span 80 E) Transcutol F) PEGs G) Pluronics H) Tween 80

Moreover, another observation can be done: the solubility in surfactants is inversely related to the molecular weight of the vehicle and this can be clearly observed from **Figure 4**.

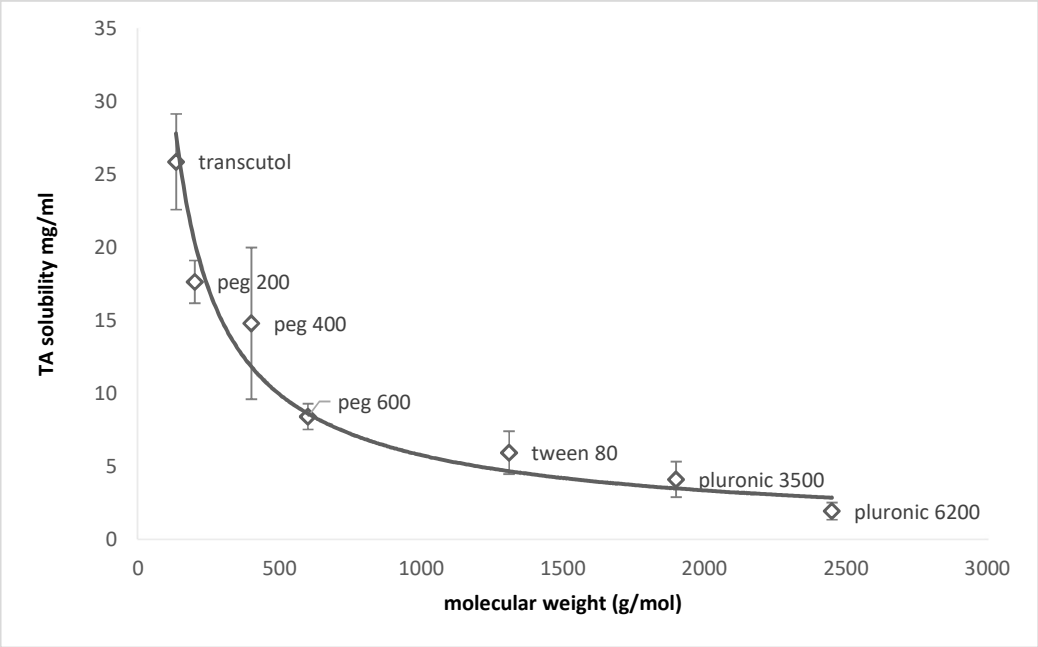

Figure 4. Correlation between solubility (mg/ml) and molecular weight (g/mol) of the vehicle

The inverse relation suggests that as the molecular weight increase, there is a decrease in the interaction capability with TA. The same relation is valid also in the case of other polyoxyethylene derivatives, such as Cremophor EL (MW ≈ 3000 g/mol). However, since the solubility value was only estimated, it was not included in the elaboration.

Further considerations can be made if we report TA solubility (moles/ml) as a function of the calculated moles of vehicles contained in 1 ml (**Figure 5**).

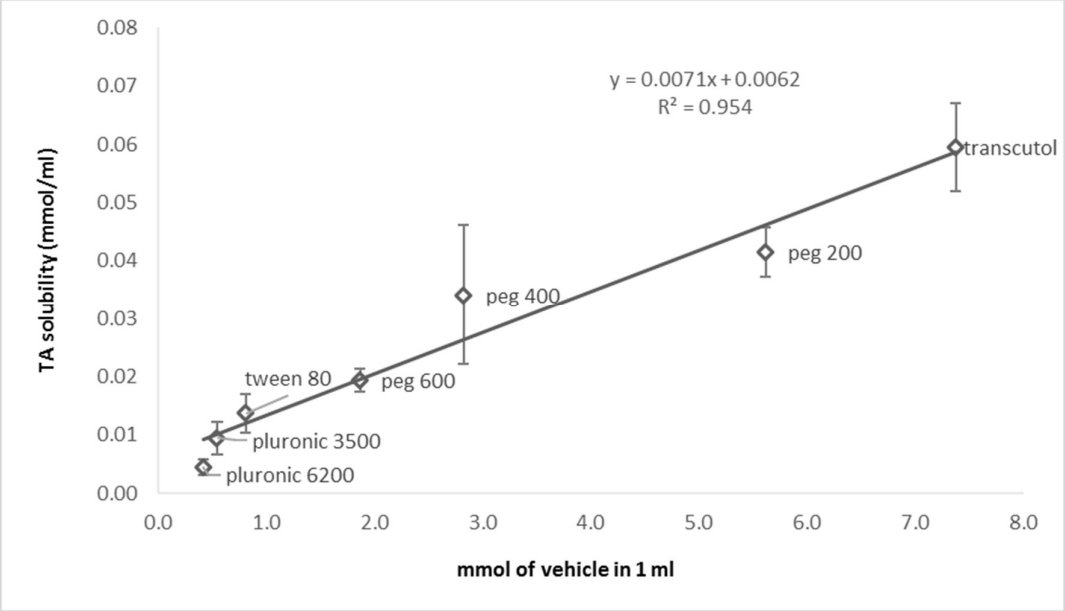

Figure 5. Correlation between moles of solubilized TA and relatively moles of vehicles.

This graph shows a linear correlation, indicating that when these relatively homologous structures are considered, a constant number of molecules of vehicle is necessary for the solubilisation of one molecule of TA. This value can be estimated by the slope of the correlation line.

Labrasol was not included in the previous elaboration because its solubility was only estimated (approx. 9 mg/ml) and because it is difficult to estimate its MW since it is a mixture of monoesters, diesters, and triesters of glycerol and monoesters and diesters of polyethylene glycols with a mean relative molecular weight between 200 and 400 [10]. According to the HLB value, approx. 12, it is possible to hypothesize that also in this case, the good solubility is provided by the predominant hydrophilic domain, represented by polyethylene glycols.

4.1.1.2 Excipients selection

On the base of the solubility results obtained, the following excipients were selected:

- Glyceryl monooleate (GMO) is a common pharmaceutical and nutraceutical excipient. It is recommended for use in formulations administered by oral, topical and in rectal/vaginal routes. For oral drug delivery in particular, it is used as an oily carrier, a solubility and bioavailability enhancer. (TA solubility 1 - 2 mg/ml).
- Labrasol, commercial name for caprylocaproyl polyoxyl-8 glycerides, employed both for oral and topical drug delivery. It is a water dispersible surfactant, used for self-emulsifying lipidic formulations, as solubility and bioavailability enhancer and stabilizer for emulsions (in particular for o/w, HLB 12). (TA solubility 9 - 10 mg/ml)
- Transcutol, diethylene glycol monoethyl ether, is widely used in dermal drug delivery as absorption enhancer, because of its ability to increase the solubility of drugs into the stratum corneum [11]. Moreover, the enhancer properties on the buccal mucosa have been suggested also by Ceschel et al. [1]. In general, it is used as a solubilizer, both for topical and oral administration. (TA solubility ≈25 mg/ml)

However, in the choice of excipients for buccal application careful attention should be dedicated also to other aspects, such as the irritation potential, the mucoadhesive properties and the organoleptic characteristics. GMO in particular, is interesting since it has shown mucoadhesive properties: Lee at al., studied the mucoadhesive properties of the liquid crystalline phase of GMO, demonstrating that are dependent of the water uptake into a water rich environment [12]. For this reason, it is a particularly interesting excipient for buccal administration.

4.1.2 Construction of pseudoternary phase diagrams

Pseudo ternary phase diagrams are a predictive tool, useful to identify the region of existence of microemulsions. It is important to underline that this kind of diagrams can be used also to identify the region of existence of different phases, like gels or coarse emulsions.

If the microemulsion consists in 4 components, usually a fixed ratio of two components is chosen: in general, a fixed surfactant and co-surfactant ratio is used. The percentage of this fixed mixture (smix) is represented on one axis, while on the others are represented the percentage of water and oil. The construction of these phase diagrams is performed by simple visual inspection.

Once fixed the smix, the oil and the smix are blended at pre-determined ratios and slowly titrated with the aqueous phase. After each addition (usually water is added to change the percentage from 5 to 95 %), the system is inspected for clearness and viscosity, and the proportion of each component is calculated and noted. The various proportions at which microemulsions are formed are then plotted to generate a pseudo-ternary phase diagram. Three different smix ratios were chosen (1/1, 1/2, 2/1), using labrasol and transcutol. The results are shown in **Figure 6**.

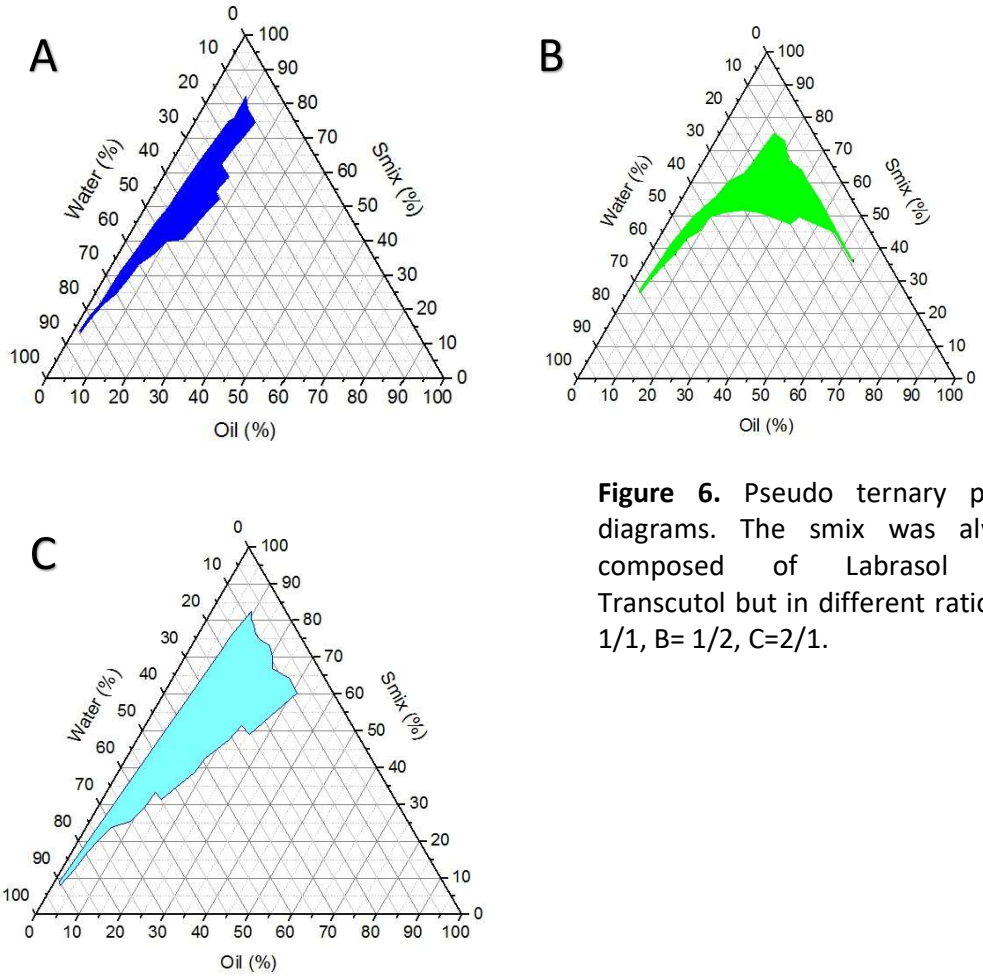

Figure 6. Pseudo ternary phase diagrams. The smix was always composed of Labrasol and Transcutol but in different ratio. A= 1/1, B= 1/2, C=2/1.

The surfactant chosen must be able to reduce the interfacial tension to suitable values. According to the literature, suitable values are around $10^{-2}/10^{-3}$ mN/m [13]. Usually it is not possible to reach these values with the presence of only the surfactant but it is possible with the addition of a co-surfactant, in this case Transcutol. The combination helps the dispersion process, providing the formation of a flexible film around the droplets.

In this particular case, when the ratio of smix is 1/1 (**Figure 6** A), 5 % of oil could be solubilized by about 25 % of smix. Increasing the concentration of smix up to 50 %, it is possible to solubilize a maximum of 15 % of oil.

Interestingly, increasing the percentage of transcutol (smix 1/2) (**Figure 6** B), the area of existence of ME changes completely and, with the same percentage of smix, up to 40 % of oil is solubilized. When the smix contains a higher percentage of Labrasol (smix 2/1) (**Figure 6** C) the region of existence increase, but a lower % of oil can be included in the ME: it is possible to reach an oil percentage of 30 % using 60 % of smix. This confirms that the use of the co-surfactant is crucial.

From each diagram it is possible to prepare hundreds of formulations. Five microemulsions were selected from each diagram (**Table 2**). As a criterion we decided to select a maximum concentration of smix of 50 % since it is well reported that large amounts of surfactants (even if non-ionic, thus better tolerated) can cause irritation.

CODE	Peceol-GMO %	Water %	Labrasol/transcutol 1/1 %	Labrasol/transcutol 1/2 %	Labrasol/transcutol 2/1 %
ME 1	10	40	50	-	-
ME 2	40	10	-	50	-
ME 3	15	40	-	-	45
ME 4	10	45	45	-	-
ME 5	5	55	40	-	-
ME 6	5	60	35	-	-
ME 7	5	65	30	-	-
ME 8	30	20	-	50	-
ME 9	5	60	-	35	-
ME 10	5	55	-	40	-
ME 11	7	55	-	38	-
ME 12	10	50	-	-	40
ME 13	10	60	-	-	30
ME 14	5	60	-	-	35
ME 15	5	70	-	-	25

Table 2. Selected formulations for accelerated stability tests

These fifteen formulations were added of TA at 0.1 % w/v and then submitted to accelerated stability tests, such as heating cooling cycles and centrifugation, in order to assess the stability.

The addition of TA did not affect the microemulsion structure since all the formulations remained clear and transparent. The accelerated stability tests were successfully passed for all the microemulsions prepared from a smix ratio of 1/1 (ME 1, ME 4, ME 5, ME 6, ME 7). When smix is 1/2, meaning higher percentage of co-surfactant, only the formulation 40 % oil, 50 % smix and 10 % water (ME 2) was stable in all the conditions; it is interesting to underline that this is the formulation with the lower water percentage, being only 10 %, indicating that Transcutol favours the formation and stability of more hydrophobic systems. When 2/1 smix was used, the stability was possible only in the smix range of 35 - 45 % and water range of 40 – 60 % (ME 3, ME 13, ME 14). The results are not surprising since using a surfactant with HLB of 12, the formation of an o/w system is favoured.

Three formulations, ME 1, ME 2 and ME 3 were selected for further investigations.

4.2 Microemulsions characterization

The pH was in all cases about 5, similar to the pH of the oral environment [14].

The formulations were investigated under polarized light microscopy and no images were visualized, meaning there is no liquid crystals or other birefringent structures in the material, confirming the microemulsion character of these formulations. ME can be o/w, w/o or bicontinuous. In the case of dispersed ME, another important parameter is the size of the dispersed phase. It is generally accepted that to have what is called a microemulsion, a system visually clear and transparent, the size of the dispersed phase has to be lower than 150 nm [13].

DLS analyses of ME 1 and ME 3, showed a dispersed droplets size of approx. 100 nm (**Table 3**). Given the low amount of GMO (**Table 2**), we can hypothesize an o/w ME. In the case of ME 2 no result was obtained, due to either the presence of a w/o Me (having an unknown refractive index) or to the presence of a bicontinuous structure.

Sample	Size (nm)	Polidispersity index (%)
ME1	90.3	0.22
ME2	ND	ND
ME3	105.7	0.21

Table 3. Size and polydispersity index of selected MEs.

It is interesting to note that in the case of ME 1, two different populations of particles can be visualized (**Figure 7**).

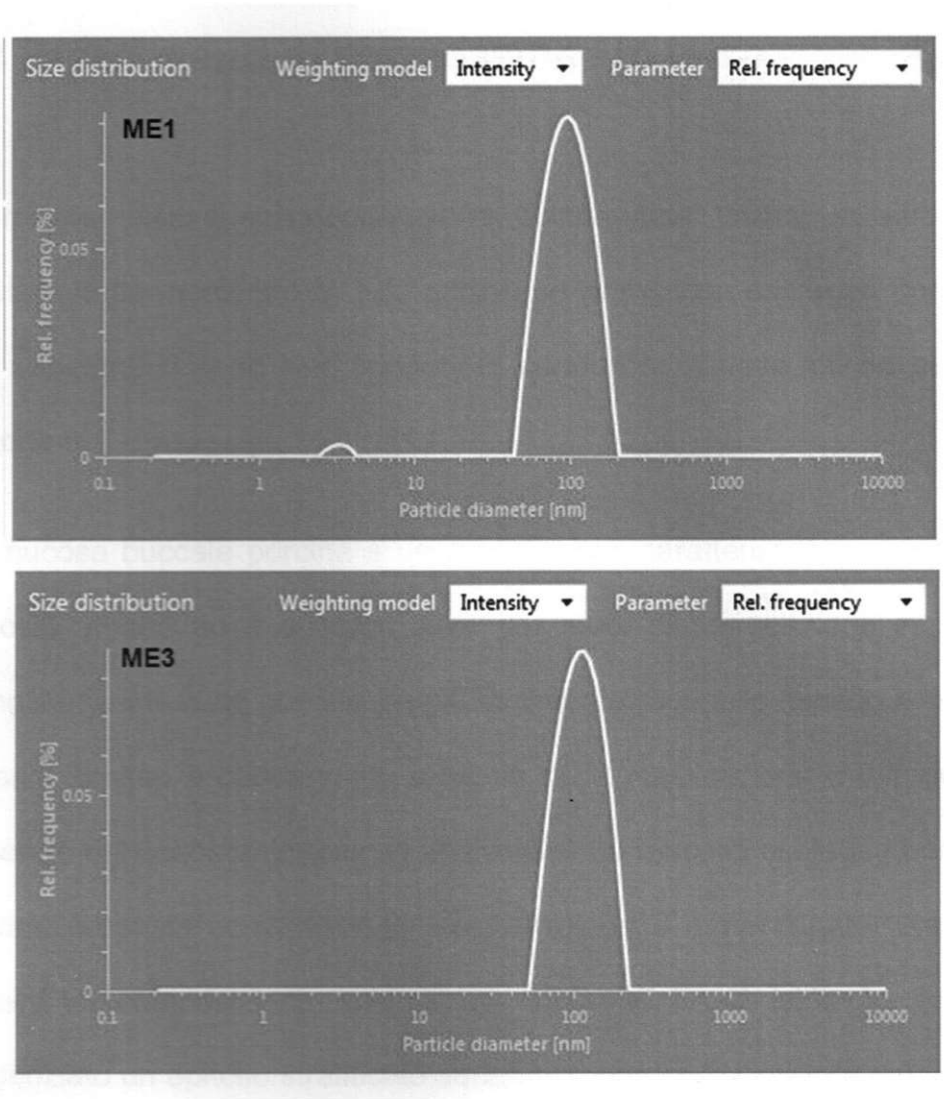

Figure 7. Size distribution (intensity) for ME 1 and ME 3.

Probably, due to the high concentration of surfactant present in the system, the formation of a population of micelles is possible. This hypothesis could eventually be confirmed by Cryo-TEM analysis.

4.3 In vitro permeation experiments

In vitro evaluation of TA permeation through porcine oesophageal epithelium was performed. The buccal porcine mucosa is a well characterized model for buccal human mucosa and it is widely used for accumulation and permeation experiments. However, even if porcine tissues are more easily available than human tissue, they are frequently damaged by mastication. Moreover, the separation of the mucosa from the underlying muscular tissue is not an easy task. For these reasons, Diaz del Consuelo at al., proposed in 2005 the oesophageal porcine mucosa as an alternative to buccal porcine mucosa because it is easier to prepare and less damaged. The lipid characterization and the permeation studies showed that the oesophageal mucosa is a suitable model for buccal human mucosa. [15].

We used this model to investigate ME performance, using TA as model corticosteroid. Even if the final use of these systems is intended for a local delivery, we studied drug permeation across the mucosa in order to understand the mechanisms underlying the penetration and permeation across the tissue.

In **Figure 8** the permeation profiles of the selected MEs, applied on the mucosa at infinite dose, are reported. Data are shown as amount of drug permeated per unit area ($\mu g/cm^2$) as a function of time.

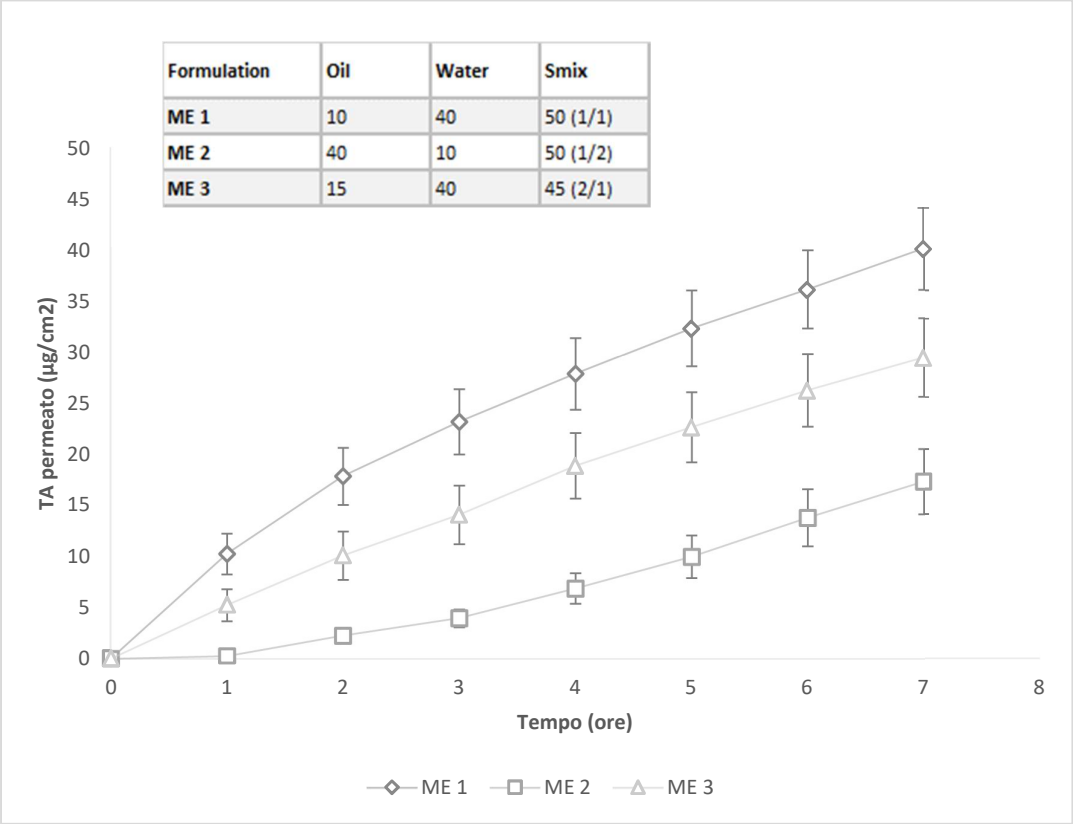

Figure 8. TA permeation profiles, starting from three different ME containing 0.1 % of TA. (average ± sem)

The three microemulsions show three different profiles:

- ME 2 shows the typical diffusion profile across a membrane, with a lag time value of approx. 2h.
- ME 1 is characterized by a burst effect
- ME 3 shows an almost linear profile

Despite these differences, all the profiles are characterized by the same flux, calculated at the steady state i.e. between 4 and 7 h (**Table 4**).

	Flux (μg cm^{-2} h^{-1})	SD
ME 1	4.05	0.80
ME 2	3.51	1.46
ME 3	3.54	0.76

Table 4. TA steady state flux, was calculated as the slope of the regression line in the interval 4-7 h

In case of ME 1 and ME 3 it is not possible to calculate the time lag.

Apparently, the second part of the permeation profile describes TA diffusion across the buccal epithelium (the same for all the MEs), while the first part of the profile describes the release of TA from the formulation and its partition into the tissue. In the case of ME 2, with low water content, its partition into the tissue is unfavourable. On the contrary, in the case of ME 1 and ME 3, the higher water content ensures a faster tissue uptake.

This behaviour can be explained considering that the solubility in the excipients used has the following trend: Transcutol > Labrasol > Peceol > water. Probably, TA is localized mainly at the interface, but it is distributed also in the oil and water phases in a percentage that is a function of the solubility. When water is the external phase TA partition into the tissue is favoured because of its low solubility in water.

For ME 1 it is also to highlight a burst effect: in fact, it is characterized by an initial and rapid permeation, which tends to level out over time. From the measurement performed with dynamic light scattering, it has been shown that ME 1 contains two different populations (**Figure 7**): the big can be attributed to the droplets of the dispersed phase, while the small is probably due to micelles formation. We can hypothesize that the micelles formation increases TA concentration in the water phase, enhancing the uptake of drug into the epithelium.

In order to understand the role of microemulsion structure in TA permeation, simple solutions of TA in mixtures of oil/smix or w/smix were tested (**Table 5**). The permeation profiles are represented in **Figure 9**, **10** and **11**.

Formulation	Peceol %	Water %	Labrasol/transcutol 1/1%	Labrasol/transcutol 1/2%	Labrasol/transcutol 2/1%
ME 1 no w	17	-	83	-	-
ME 2 no w	44	-	-	56	-
ME 3 no w	25	-	-	-	75
ME 1 no oil	-	44	56	-	-
ME 2 no oil	-	17	-	83	-
ME 3 no oil	-	47	-	-	53

Table 5. Composition of oil/smix or water/smix mixtures tested.

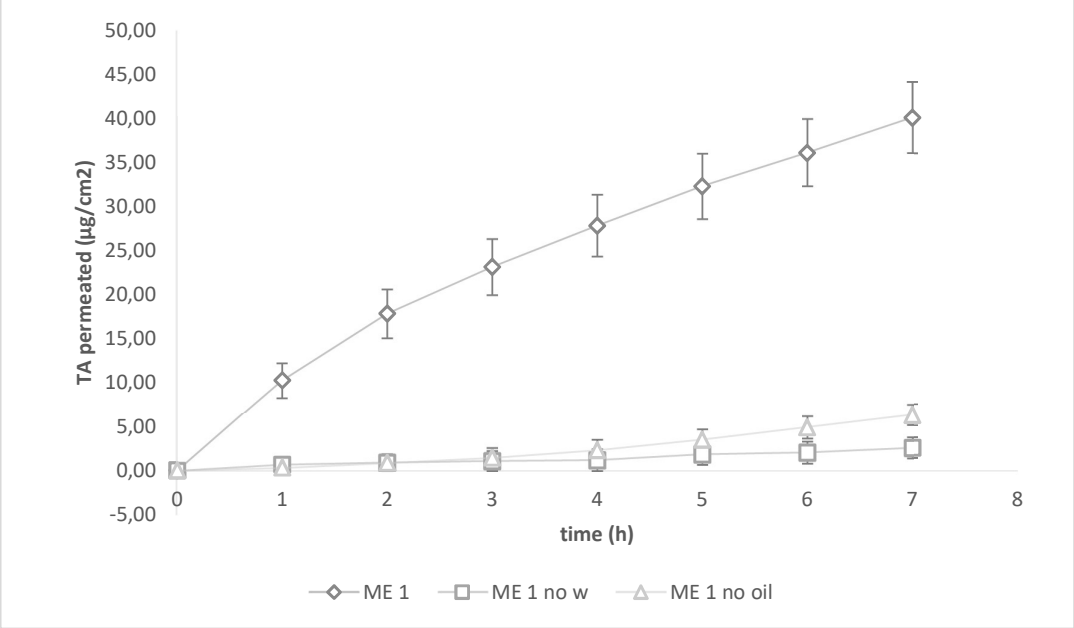

Figure 9. TA permeation profiles from ME1 and its respective mixtures water/smix (ME1 no oil) and oil/smix (ME1 no w), expressed as average ± sem.

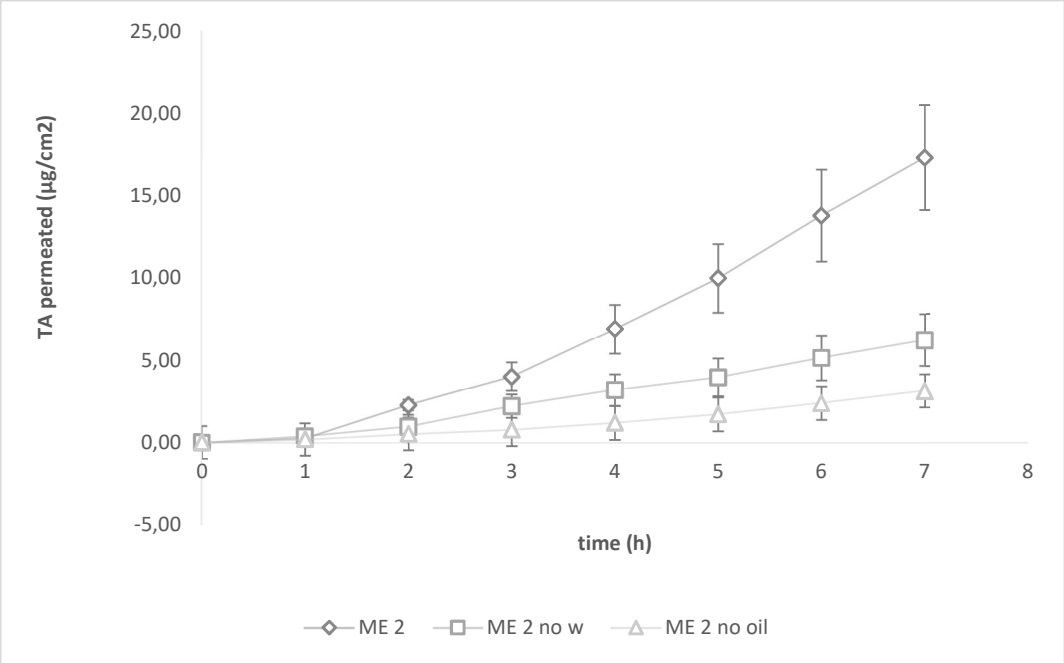

Figure 10. TA permeation profiles from ME2 and its respective mixtures water/smix (ME2 no oil) and oil/smix (ME2 no w), expressed as average ± sem.

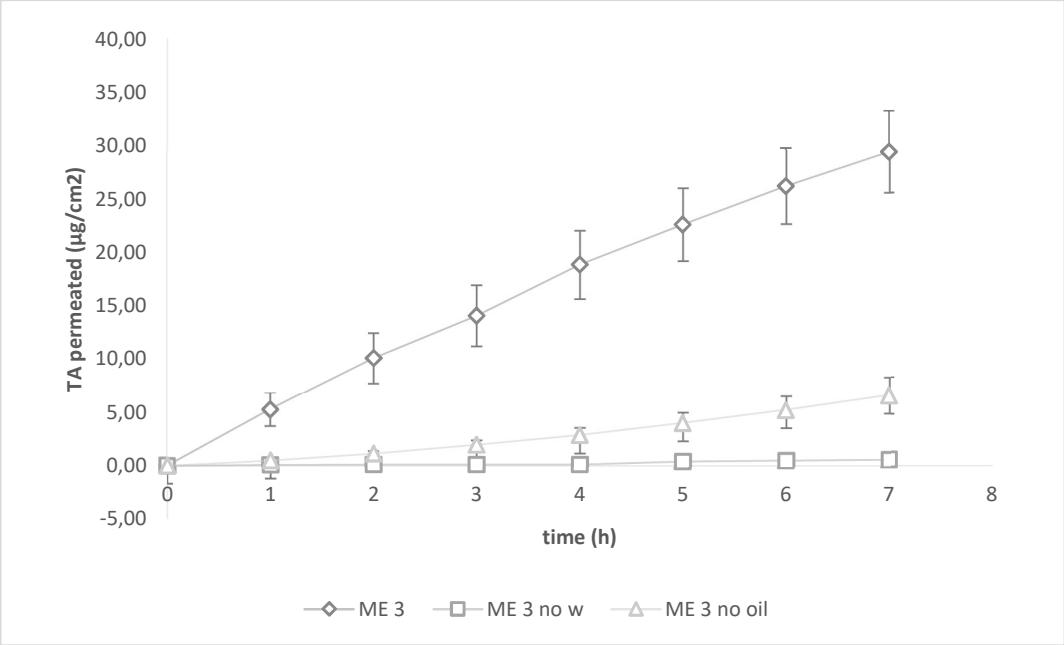

Figure 11. TA permeation profiles from ME3 and its respective mixtures water/smix (ME3 no oil) and oil/smix (ME3 no w), expressed as average ± sem.

Overall conclusions can be drawn: the lack of either water or oil phase leads to a decrease of permeation, meaning that apparently, the microemulsion formation is essential to have a permeation enhancement. The presence of high percentages of surfactant is not enough to guarantee high permeation profiles.

However, some differences between the different MEs can be highlighted: in the case of o/w ME (ME 1 and ME 3), the decrease in permeation is dramatic, while it is less important for ME 2. If it is true that the permeation is sustained by TA release from oil to water, the lack of one of the two is critical only when water is the external phase in contact with the tissue and oil the internal matrix. In the case of ME 2, the loss of structure is probably less incident since the permeation was not, probably, sustained from the internal phase.

5 Conclusions

The results obtained in the present work underline that:

- MEs are valuable formulations for buccal delivery. By modifying the composition, it is possible to modify the absorption rate across porcine oesophageal epithelium. The results obtained from oil/smix or water/smix mixtures confirm that the ME structure is essential for drug delivery, so the enhancement is not only due to the high surfactant and co-surfactant content.

- Additionally, solubility studies demonstrate that TA solubility is linked- at least to a certain extent- to the vehicle capability to form H bond instead of hydrophobic interactions. This could be potentially useful in order to predict TA solubility; however further investigations, extending the analysis to a larger number of vehicles, have to be done.

- Despite the promising results, further studies should be performed, for example performing experiments on short times in order to better simulate the in vivo conditions, evaluating drug concentration into epithelium. Finally, tolerability, mucoadhesion and organoleptic characteristics should be evaluated as well.

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DEVELOPMENT OF HYALURONIC ACID CONJUGATE NANOPARTICLES FOR LOCAL DELIVERY OF DEXAMETHASONE PALMITATE.

Table of contents

1 Introduction 133

2 Aim 134

3 Materials and methods 135

3.1 Materials 135

3.2 Methods 135

3.2.1 Synthesis conjugate hyaluronic acid-DPPE 135

3.2.2 Characterization of HADPPE conjugate 136

3.2.3 Nanoparticles preparation 137

3.2.4 Nanoparticles characterization 137

4 Results and discussion 140

4.1 Conjugation of HA with phospholipid (DPPE) 140

4.1.1 Characterization of the conjugate (HADPPE) 141

4.2 Nanoparticles preparation 143

4.2.1 Nanoprecipitation 143

4.2.2 Emulsion/evaporation 145

4.3 Nanoparticles characterization 148

4.3.1 Stability 148

4.3.2 TEM Images 149

4.3.3 Quantification of HADPPE 150

5 Conclusion 152

6 Bibliography 153

1 Introduction

The use of topical glucorticoids (GCs) has been introduced in 1952, when hydrocortisone was successfully employed for the treatment of dermatoses. Since then, a large number of formulations, usually semisolid conventional formulations like cream, ointment or gels, loaded with different GCs, have been commercialized. In 1958 some studies demonstrated the great potential of dexamethasone that, since then, has been widely employed both as such and as prodrug. Dexamethasone prodrugs are obtained by esterification with phosphate (to increase water solubility) or with lipophilic molecules like valeric or acetic acid, in order to increase the lipophilicity and, as a consequence, the permeability across biological barriers. In 2001, dexamethasone as ester with palmitic acid (**Figure 1**) has been evaluated for the treatment of osteoarthritis [1]. This compound is presently commercialised in Germany as an injectable formulation. However, this prodrug could be particularly interesting also for local application. In principle, its very high lipophilicity (log P of about 9 [2]) could potentially lead to high accumulation into the SC or epithelia. The presence, widely reported, of esterases into the SC and viable tissues could then ensure the slow activation of palmitate into dexamethasone and its diffusion into the deeper tissues. Being highly hydrophobic, it is important to develop a formulation which is able to both solubilize ad release DXP, in order to favour the partition into epithelia.

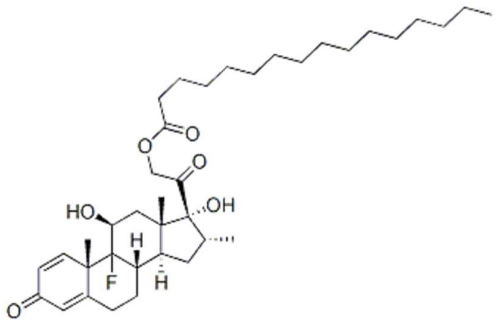

Figure 1. molecular structure of dexamethasone palmitate

2 Aim

We decided to prepare and characterize nanoparticles loaded with DXP, suitable for local application, mainly because: 1) nanoparticles allow to formulate DXP into an aqueous vehicle and 2) different authors demonstrated that this kind of system could improve, for example, skin retention while limiting the systemic absorption [3, 4].

We used high molecular weight hyaluronic acid (HMWHA) as polymer because it is safe and biocompatible, can provide good hydration of the tissue and can eventually contribute to the anti-inflammatory activity [5].

In order to have an interaction between DXP and HA strong enough to obtain stable nanoparticles but, at same time, sufficiently weak to guarantee the release of DXP, HA was conjugated with a lipid molecule containing two palmitate chains, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE).

This preliminary work consisted in the synthesis of the conjugate, nanoparticles preparation and characterization.

3 Materials and methods

3.1 Materials

High molecular weight hyaluronic acid (HA) (sodium salt, 1500 kDa, purity of 95%) was purchased from Acros organics (Geel, Belgium) while HA 1000 kDa was a gift of IBSA Farmaceutici (Roma, Italy). 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) was provided by Avanti Polar Lipids (USA), while 1-ethyl-3-[3-dimethyl)aminopropyl]carbodiimide (EDC) and perchloric acid 70% were purchased from Sigma Aldrich (St. Louis, MO, USA). Dexamethasone palmitate (MW. 630.87 g/mol, pKa 12.14) was provided by Interchim (Montluçon, San Diego, United States). For HPLC analysis, bidistilled water was used. Acetonitrile and methanol were of HPLC grade; all other reagents were of analytical grade.

3.2 Methods

3.2.1 Synthesis conjugate hyaluronic acid-DPPE

The synthesis of HA-DPPE conjugate was performed according to Surace and al [6].

14 mg of HA (1000 and 1500 kDa) were dissolved in 5 ml of water and the pH adjusted to 4 using HCl 0.1N. In order to activate the carboxylic groups, 6 mg of EDAC were added. The reaction was thermostated at 37°C. After 2 h the activated HA was transferred onto a DPPE film previously prepared in a ballon, starting from a $CHCl_3$ solution, by complete solvent evaporation. For 14 mg of HA, 360 µg of DPPE were used. The solution was submitted to sonication for 30 minutes, then the pH was adjusted to 8.6 using a 0.1 M borate buffer pH 9.4. The reaction was monitored up to 24 h, by thin layer chromatography (TLC) using F254 silica gel precoated sheets (Saint-Quentin-Fallavier, France). After migration of the mobile phase ($CHCl_3$/CH_3OH 2:1 v/v), the sheets were exposed to ninhydrin (2,2-

dihydroxyindene-1,3-dione) solution 1% w/v in ethanol and heated with a hair drier until spot formation.

Purification of the product, meaning removal of undissolved and unreacted DPPE, was performed by centrifugation, followed by dialysis against milliQ water (Spectra/Por regenerated cellulose membrane with a molecular weight cutoff of 50000) for 48h. After the complete removal of DPPE the solution containing the conjugate was frozen at -20°C, then freeze-dried.

3.2.2 Characterization of HADPPE conjugate

3.2.2.1 Bartlett Assay

In this method phospholipids phosphorous is acid-hydrolysed to inorganic phosphate. This is converted to phosphomolybdic acid by the addition of an ammonium molybdate solution, then the phosphomolybdic acid is quantitatively reduced to a blue coloured compound using ascorbic acid. The intensity of the colour is measured spectrophotometrically.

The majority of phospholipids contain exactly one mole of phosphorous per mole of phospholipid, hence the concentration of phospholipid could be derived directly from the measurement of the phosphorous content in the sample.

A known volume (from 50 to 250 µl) of sample was introduced in glass tubes and the solvent evaporated by heating at 110°C using a sand bath. After complete evaporation, tubes were heated at 200°C and 300 µl of perchloric acid 70% were added. In this phase the phosphorous was mineralized. After 30 minutes, the tubes were removed from the bath and left to room temperature. Then 1 ml of water and 400 µl of ammonium molybdate 1.25%w/v and 400 µl of ascorbic acid 5% w/v were added. After each addition, samples were vortexed for 1 minute. In order to help the coloured complex formation, tubes were heated for 5 minutes in boiling water.

Linearity was assessed using a standard solution of NaH_2PO_4 in the concentration interval 10-40 µM. The absorbance is measured at a wavelength of 820 nm.

3.2.3 Nanoparticles preparation

3.2.3.1 Nanoprecipitation

DXP was solubilized in 1ml of acetone while HADPPE was dissolved in 10 ml of water at 60°C. DXP solution was added to the water solution and the mixture was stirred for 5 minutes. Acetone was then evaporated using a rotavapor.

3.2.3.2 Emulsion/evaporation

DXP was solubilized in 1 ml of chloroform, HADPPE in 10 of water at 4°C. DXP solution was added to the water solution then the mixture was vortexed for 30 seconds and sonicated for 2 minutes at 40% of amplitude. Chloroform was evaporated using a rotavapor.

3.2.4 Nanoparticles characterization

3.2.4.1 Size and Zeta Potential

Samples for size measurements were diluted usually 1:10 in MilliQ water. For each measure 3 measure of 60 seconds were taken.

Samples for Zeta Potential measurement were usually diluted 1:10 with NaCl 1mM. Measurements were performed with Malvern zetasize nano ZS (Malvern, UK).

3.2.4.2 Quantification of DXP and HADPPE in the formulation

A known volume (200-500 µl) of nanoparticles suspension was inserted in a tube. And diluted with MilliQ water up to 3 ml. The suspension was centrifuged at 4°C and 40000 rpm: different times have been evaluated (1h, 2 h, 4h, 10h).

After centrifugation the supernatant was carefully removed using a pipette. The supernatant was analysed by DLS and for HADPPE and DXP content.

3.2.4.3 HPLC condition for DXP detection

DXP quantification was performed by HPLC using a Flexar instrument (Perkin Elmer, Waltham, MA, USA) and a C18 column (Symmetry Shield RP18/5 µm/4,6x250mm, Waters, Massachusetts, CA, USA) thermostated at 40°C. Mobile phase was a mixture of CH_3CN/H_2O 85/15 v/v, the flux was 1.2 ml/min and the injection volume of 50 µl. Samples were analysed by UV absorbance (λ 240 nm). Stock solution was prepared by dissolving approx. 2 mg of DXP in 20 ml of CH_3CN. Calibration curves were prepared in the interval 10-150 µg/ml.

3.2.4.4 Colorimetric assay for HADPPE detection (HA detection)

In order to quantify the conjugate not associated to nanoparticles, a colorimetric assay was performed. Two different methods have been used:

METHOD A

100 µl of sample is diluted to 1 ml with water then 25 µl of phenol solution 80% p/p at and 2.5 ml of sulphuric acid 95% v/v are quickly added.

The tubes are well-shaken 30 s then rest for 10 minutes before heating to 40°C for 90 minutes. Absorbance of the sample was recorded in 600-400 nm range and the maximum value was used for quantification.

METHOD B

REAGENTS:

100 µl of sample is diluted to 250 ul with water then 1.25 ml of sodium tetraborate solution 0.025 M are added to each sample and shaken for 30 seconds. In order to help the reaction, samples are heated at 100°C for 10 minute After the complete cooling 50 µl of carbazole 0.125%w/v in EtOH are added, shaken for 30 seconds and heated for 15 minutes at 100°C. Absorbance of the sample was recorded in 600-500 nm range and the maximum value was used for quantification.

3.2.4.5 TEM

Images at electronic transmission microscopy have been taken at IMAGIF (CNRS, Gif-sur-Yvette, France). Samples were treated as following: 5 µl of nanoparticles suspension were placed for 1 minute on a copper grid covered with formwar. A negative coloration was performed by adding a drop of uranyl acetate at 2%w/w for 30 seconds. The excess of solution was carefully wiped using filter paper, then the grids was left at air until observation. The images were taken using a microscope JEOL at 80 kV of acceleration voltage.

4 Results and discussion

4.1 Conjugation of HA with phospholipid (DPPE)

The aim of the reaction was to link the DPPE to HA through the formation of an amidic bond between the –COOH group of HA and the $–NH_2$ of DPPE (**Figure 2**).

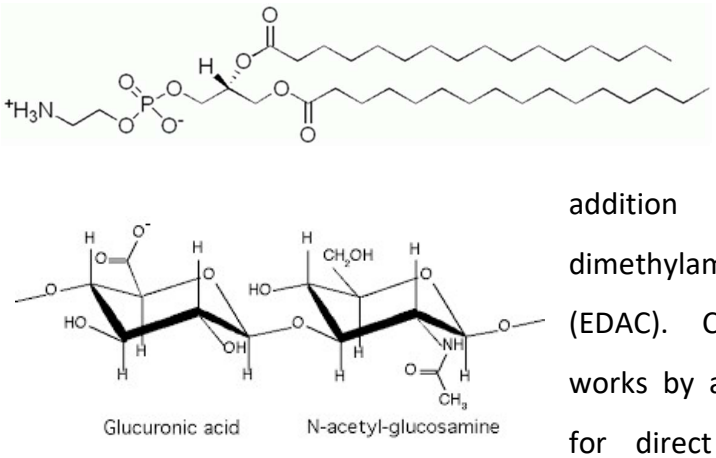

Figure 2. Molecular structures of DPPE and hyaluronic acid

In order to favour this reaction, the carboxylic group is activated by the addition of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC). Carbodiimide conjugation works by activating carboxyl groups for direct reaction with primary amines via amide bond formation. EDAC crosslinking is more efficient in acidic conditions and must be performed in buffer devoided of extraneous carboxyls and amines. Neutral pH is also compatible with the activation but reduce the efficiency. EDAC acts on carboxylic group giving and intermediate, o-acylsourea that is easily displaced by the primary amino group of DPPE. The o-acylsourea intermediate is unstable in aqueous solution. In order to help the reaction, when DPPE is added the pH conditions are adjusted to 8.6 with a borate buffer 0.1 M. Description of the mechanism in **Figure 3**.

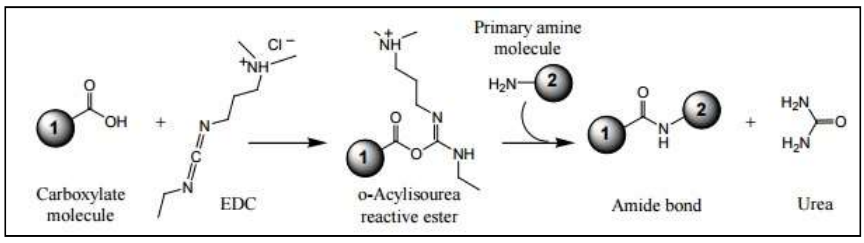

Figure 3. Mechanism of reaction.1= Hyaluronic acid 2= DPPE

The synthesis was followed using TLC. Different system of revelation can be used but the most efficient is ninhydrine.

Ninhydrine revelation is a common method used for the detection of primary amines. When applied on TLC plate in presence of primary amines a red spot is developed. This method could be, at least in principle, useful for the detection of the unreacted DPPE in the reaction mixture. However, since the DPPE solubility in aqueous phase was very low, this procedure is not suitable to follow the reaction. Instead, it is possible to detect the formation of the conjugate: in fact, even if the conjugate does not present any amino group, is possible to break the amide bond by heating and release of the amine group. Since the conjugate has a low mobility, the spot will be detected at the baseline (Rf=0).

4.1.1 Characterization of the conjugate (HADPPE)

4.1.1.1 Bartlett assay

The reaction yield has been determined using the Bartlett assay. This colorimetric method allows to quantify the amount linked in the conjugate through the mineralization of the organic phosphorus contained in the DPPE residues and. The method is however very sensitive to the presence of very low amount of P that could contaminate the samples. For this reason, it was not possible to quantify the amount of DPPE linked to HA 1500 kDa because of the presence of a contamination. In case of the conjugate prepared with HA 1000 kDa, the reaction yeld, expressed as percentage of DPPE linked in comparison with the initial amount added to HA-activated solution, was about 60%.

4.1.1.2 DLS measurements

The solubilisation of HA in water (at 0.5% w/v) give viscous solutions. When the conjugate is solubilized in water, at comparable concentrations, no increase of viscosity is observed, for both the MW tested. This allows to affirm that the presence of the lipid prevents the formation of a gel-like structure, typical of HMWHA.

Since the conjugate is an amphiphilic molecule, it is possible to hypothesize that it is able to form aggregates when dissolved in water. For this reason, we decided to performed analysis of HA and HADPPE solutions in water.

Measurements of particle size was performed on HA 1000 kDa and HADPPE 1000 kDa and 1500 kDa solutions. Results are reported in **Table 1**

Sample	Size (nm)	SD	PDI	SD	Z Pot	SD
HA 1000 kDa 0.25 mg/ml	1223,9	140,5	0,472	0,034	-	-
HADPPE 1000 kDa 0.5 mg/ml	535	6,8	0,301	0,008	-25.2	1.8
HADPPE 1500 kDa 0.5 mg/ml	372,6	5,3	0,281	0,006	-28.3	0.6

Table 1. Measurements of size and Zeta Potential for HA and HADPPE solutions

Some general considerations can be made:

1. The different size and PDI measured for HADPPE in comparison with HA confirms the presence of coherent aggregates.
2. Smaller size was measured for HADPPE 1500 kDa.
3. The measures of Zeta Potential show negative values for both conjugates. The net negative charge measured is ascribable to the presence of free carboxyl groups and, in case, of the negatives phosphate groups of DPPE.

4.2 Nanoparticles preparation

4.2.1 Nanoprecipitation

Nanoprecipitation is a widely used method for polymeric nanoparticles production. It is a flexible method that can be performed with a wide range of polymers and different non-toxic organic solvents miscible with water. Developed by Fessi and co-workers in 1989 [7] it is used when both polymer and drug were soluble in the organic phase (and not in water). Since the organic and water phases are miscible, nanoprecipitation occurs by a rapid desolvation of the polymer when the polymer solution is added to the non-solvent.

In our case this method was modified given that, despite the conjugation, the polymer is still highly hydrophilic, and has to be dissolved in the water phase.

Different weight ratio DXP/HADPPE were tested, for all the formulations size and zeta potential were measured. Results are reported in **Table 2**

NAME	DXP (mg/ml)	HA-DPPE (mg/ml)	DXP precipitate	Size		PDI		Zeta Potential	
				mean	SD	mean	SD	mean	SD
F1	2.5	0.25	Y	225.8	6.3	0.28	0.04	-61.4	2.6
F2	1	1	N	340.1	39.8	0.38	0.04	-51	1.73
F3	1	0.5	Y	194.4	4.3	0.19	0.06	-63.4	3.7
F4	0.5	0.5	N	194.5	4.5	0.06	0.02	-40.9	0.2
F4 B	0.5	0.5	N	287	1.6	0.14	0.01	-57	1.6

Table 2. Size and Zeta Potential of formulations prepared with nanoprecipitation method. F1-F2-F3-F4 are prepared with HADPPE 1500 kDa, F4 B is prepared with HADPPE 1000 kDa.

Some general considerations can be done:

1- all the formulations show a negative value of Zeta Potential, lower than -30 mV, which is widely accepted as minimum value in order to have a stable formulation. In fact, when the value is close to neutrality, nanoparticles tend to aggregate.

   The superficial negative charge is likely due to free-carboxyl groups of HADPPE conjugate even if also the phosphate group of DPPE can contribute.

2- When the weight ratio is ≥2 (F1 and F3) DXP precipitation occurs and the Zeta Potential is highly negative. It is possible to hypothesize that the higher Z potential (compared to F2 and F4) is due to the complete association of HADPPE to nanoparticles.

3- On the contrary, when the weight ratio is 1, so for formulations F2 and F4, no precipitate is detectable. Since DXP solubility in water is lower than 0.1 µg/ml (measured value), we can hypothesize that in these formulations DXP is completely associated to nanoparticles (or maybe partially solubilised by micelles). The values obtained for zeta potential are lower in absolute terms. Starting from our previous assumptions it is possible to hypothesis that in this case the amount of HADPPE associated is lower and a part of the total amount is solubilized in water along with the suspension of nanoparticles.

4- From the comparison of F4 and F4 B, the MW of HA affects the size and Zeta Potential of the nanoparticles. However, the two measurements were done with different instruments so we have to be careful in the comparison.

Comparing the results obtained from DLS analysis of HADPPE 1000 kDa (Table 1) and the respective formulation, F4 B (Table 2) analysed with the same

instrument, it is possible to see, in case of F4 B, a lower size (287 vs 535 nm) and z potential (-25.2 vs -57 mV). This could be due to a stronger interaction with palmitate chains of DXP and to a different conformation that exposes more free –COOH groups.

4.2.2 Emulsion/evaporation

Other formulations were prepared using the emulsion/evaporation method. Also in this process, the polymer is usually dissolved in the organic phase along with the lipophilic drug. Normally the organic phase, which is by definition not miscible, is mixed with a water phase usually added with a stabilizer. Sonication is performed to facilitate the formation of small dispersed droplets, then the organic solvent is evaporated. However, as already mentioned, HADPPE is not soluble in organic phase and solubilized in water. Being an amphiphilic molecule, it is probable that HADPPE acts as a stabilizer and that the amount linked to nanoparticles is governed by the degree of substitution with the lipid.

Starting from the evidence that when the weight ratio is equal to 1 no precipitation of DXP occurred (see 3.2.1), formulations with the same ratio of DXP and HADPPE were prepared. The formulations compositions along with size and zeta potential are reported in **Table 3**

Name	Volume CH_3Cl (ml)	Sonication time (min)	DXP (mg/ml)	Ha-DPPE (mg/ml)	Size		PDI		Zeta potential	
					mean	SD	mean	SD	mean	SD
F5	1	2	1	1	316,8	3,8	0,2	0,00	-31,8	0,2
F6	2	2	1	1	367,7	5,9	0,2	0,04	-32,4	0,9
F7	1	1	1	1	355,8	2,8	0,3	0,01	-35,2	0,9
F8	1	2	0.5	0.5	337,5	2,8	0,2	0,01	-33,3	0,8

Table 3. Size and Zeta Potential of formulations prepared with emulsion/evaporation method.

Also for the emulsion/evaporation method, all the formulations were characterized by a net negative charge. Comparing these formulations with the ones obtained with the nanoprecipitation method, it is possible to highlight a lower superficial charge and a larger size. This could be ascribed to a lower amount of HADPPE linked.

Modifications in the preparation procedure have been tested, mainly in order to reduce the size. Neither the increase of the organic phase volume nor the reduction of the sonication time were able to modify significantly the size, nor the superficial charge.

Interestingly, the reduction of sonication time affects the stability: the measurements of size taken after 24 h for F7 show an increase of both size and polydispersity index (**Table 4**, **Figure 4**), while the other formulations were stable.

	F7					
Time	Size (nm)		PdI		Z Potential (mV)	
	mean	sd	mean	sd	mean	sd
0	355,80	2,84	0,26	0,01	-35,23	0,91
24	724,60	85,36	0,59	0,06	-34,97	0,21

Table 4. Measurements of size and Zeta Potential at time 0 and after 24 h for F7.

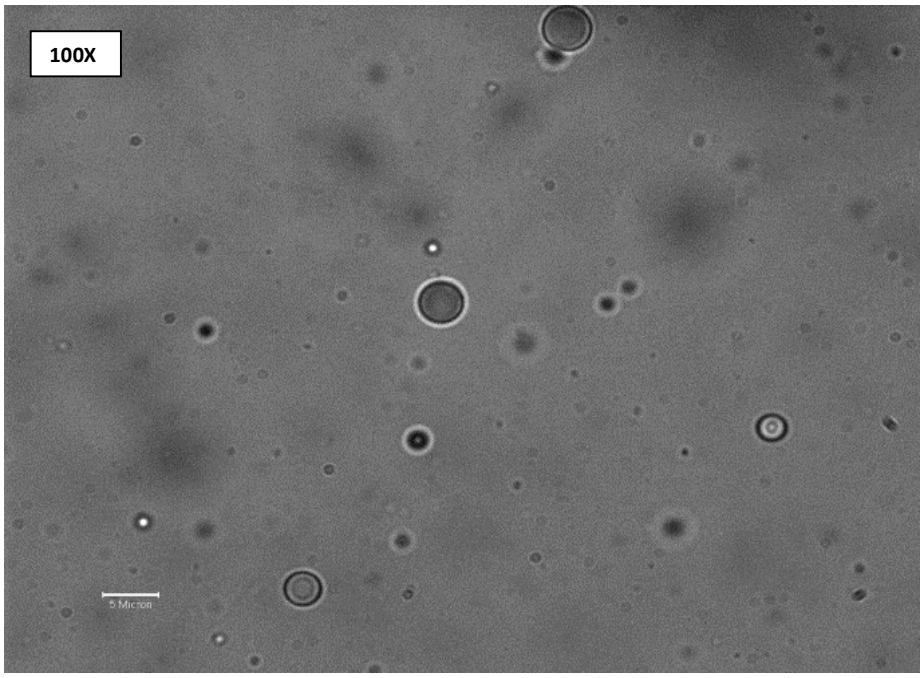

Figure 4. Optical microscope images of F7 after 24 h. Bar= 5µm.

This phenomenon is interesting since it is not ascribable to a low value of zeta potential because it is lower than -30 mV and it is the same for all the formulations.

4.3 Nanoparticles characterization

4.3.1 Stability

Size and zeta potential have been followed for 1 month in order to assess the stability of the selected formulation, when kept at 4°C. The results are reported in **Table 5.**

FORMULATION		T 0			T 15			T 30		
		size	PdI	Z potential	size	PdI	Z potential	size	PdI	Z potential
F2	mean	340.1	0.4	-51	260.5	0.2	-43.8	267.5	0.1	-42.2
	SD	39.8	0.04	1.7	4.2	0.03	1.2	3.7	0.01	0.8
F4	mean	194.5	0.1	-40.9	197.3	0.1	-45.5	198.8	0.1	-39.5
	SD	4.5	0.02	0.2	0.2	0.03	0.4	2.1	0.01	0.9
F5	mean	316.8	0.2	-31.8	324.8	0.2	-30.7	326.8	0.1	-29.8
	SD	3.8	0.01	0.2	5.1	0.03	0.4	1.7	0.01	0.5
F8	mean	337.5	0.2	-33.3	325.6	0.2	-29.9	334.3	0.2	-29.6
	SD	2.8	0.01	0.8	2.3	0.01	0.5	1.1	0.01	0.3

Table 5. Measurement of selected formulations at time 0, after 15 and 30 days.

In general, all the formulations show, at least during 1 month, a satisfying stability.

The formulation F2, prepared by the nanoprecipitation method, show a change in terms of both size and zeta potential. The decrease of both these values allows to hypothesize that there is a detachment of HADPPE. This does not, however, affect the stability since not aggregates were detected.

4.3.2 TEM Images

In order to further characterize the nanoparticles, TEM images were taken.

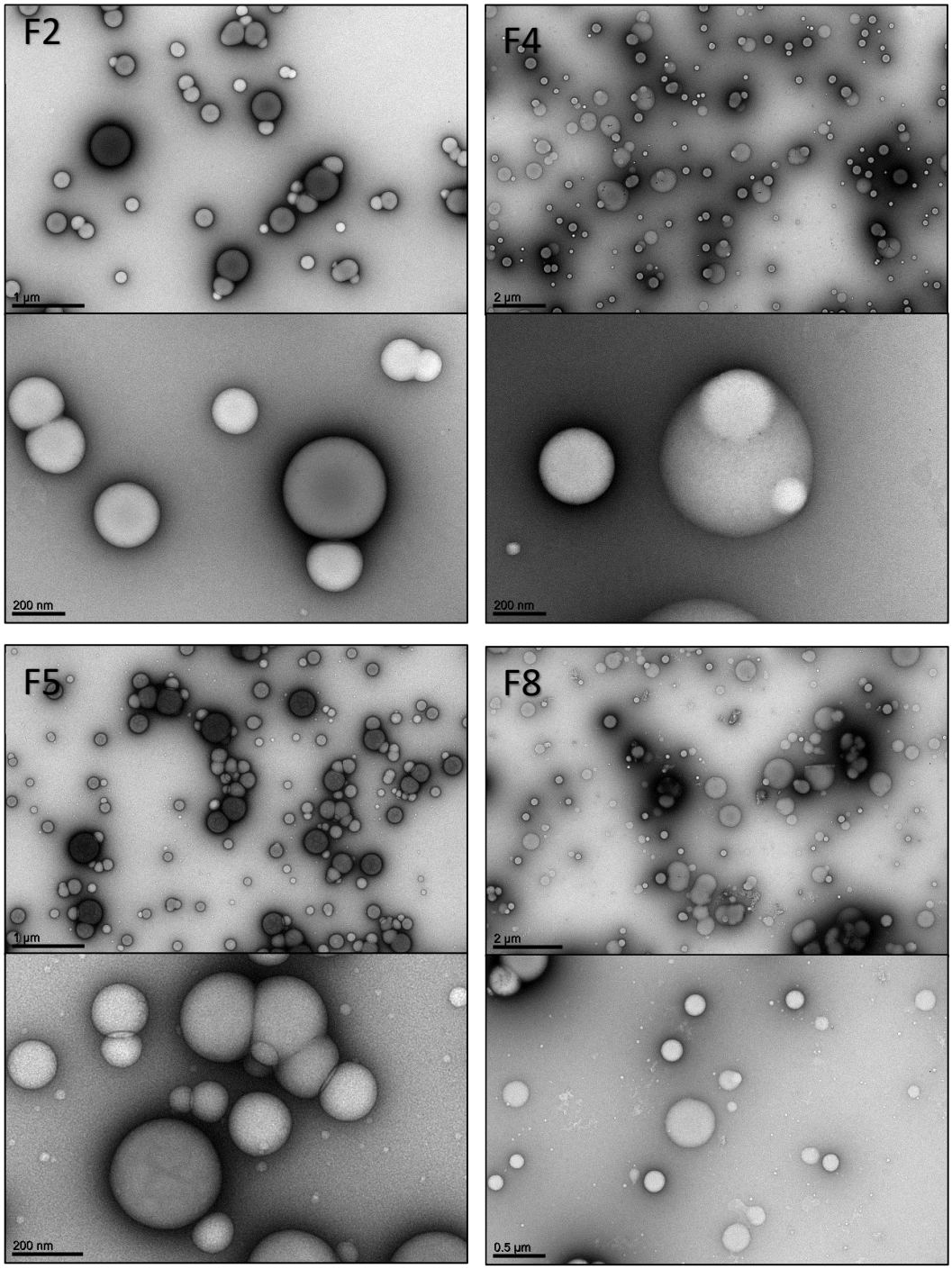

Figure 5. TEM images of F2-F4-F5-F8

The images confirm nanoparticles formation. As it is possible to see, all the formulations present particles characterized by smooth surface and spherical shape, with a diameter consistent to DLS results. No significant differences could be detected when nanoprecipitation method (F2 and F4) was used in comparison with emulsion/evaporation (F6 and F8). Interestingly, the degree of dispersion seems to be the same in all formulations: despite the difference in terms of mean size, the two procedures gave comparable results.

4.3.3 Quantification of HADPPE

In order to well characterize the selected formulations, efforts were directed towards the quantification of the amount of HADPPE in the nanoparticles.

To perform the separation of nanoparticles from the aqueous phase different techniques could be used. Since filtration cannot be used, ultracentrifugation has been tested.

Different attempts were done in order to obtained nanoparticle precipitation, modifying the centrifugation time (from 2 to 10 h) and the volume of suspension (0.2-2 ml). After each trial, the presence of a dense precipitate, well separated was always detected. The supernatant was always optically clear.

The supernatant was analysed for HADPPE using two different colorimetric assays based on coloured complex formation with HA. The results, calculated as the percentage of HADPPE in the supernatant in comparison with the theoretical concentration, were about 100% with method A and about 90% with method B.

Apparently, all the conjugate is in the supernatant after ultracentrifugation. In order to check if the results could be ascribed to an analytical interference given by other components of formulation, a saturated solution of DXP in water was treated as well. No interference was found for method B and the small interference found in case of method A is not sufficient to justify the results obtained. It is possible that the hydrophobic interaction, present between the

palmitate chain of DXP and the HADPPE moiety of the conjugate, is too weak and that HADPPE is detached during ultracentrifugation.

The supernatant was also analysed at DLS and the presence of a coherent population was always detected for any condition tested (time and volume centrifuged).

Samples of supernatant were also analysed by HPLC in order to detect the possible presence of DXP. Small amounts of DXP (about 10% in comparison with the initial amount) were found, indicating the solubilisation properties of HADPPE aggregates.

5 Conclusion

This preliminary work indicates that:

- Using HA-DPPE conjugate, it is possible to obtain nanoparticles with negative superficial charge and a mean size included between 200 and 350 nm.

- All the selected nanoparticles showed satisfactory stability, at least for 1 month.

- Nanoparticles characterization is a challenging step because of the relatively weak hydrophobic interactions between DXP and conjugate. Further investigations, using different techniques (like electrophoresis) will be evaluated. Additionally, in vitro experiments will be performed to assess the capability of these systems to deliver DXP to different tissues.

6 Bibliography

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