UNIVERSIDADE DE LISBOA

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Pharmaceutical topical dosage forms as carriers for glucocorticoids

Sara Sofia Caliço Raposo

Doutoramento em Farmácia

(Tecnologia Farmacêutica)

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Aos meus pais e avós

Ao Ludo

Pelo amor e perseverança

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Resumo

Emulsões e dispersões semi sólidas e fluidas são largamente usadas em produtos farmacêuticos para a veiculação tópica de fármacos.

Para terem atividade terapêutica, os fármacos têm que penetrar na pele, o que consiste no transporte de uma substância para uma determinada camada da pele. A maior parte dos fármacos não consegue permear a pele, pelo que é necessário um veículo para os transportar ou para aumentar a libertação no local de ação.

Os glucocorticóides tópicos (GT) são os fármacos mais frequentemente prescritos por dermatologistas. A sua eficácia clínica no tratamento da psoríase e de dermatites está relacionada com os seus efeitos vasoconstrictores, anti-inflamatórios, imuno supressores e antiprofliferativos. As células alvo para os GT são principalmente os queratinócitos (maioritariamente localizados na epiderme) e os fibroblastos (maioritarimanete localizados na derme). Os efeitos adversos observados para os GT são devidos à ação destes nos fibroblastos. Assim, se a permeação for direcionada para as camadas mais superficiais da pele em detrimento das camadas mais profundas, os efeitos colaterais podem ser diminuídos. O furoato de mometasona é um corticóide sintético, lipofílico e classificado como "potente". Estudos i*n vitro* demostraram que o furoato de mometasona está entre os mais potentes GT na inibição da produçaõ de citoquinas, libertação de histamina e de eosinófilos. Por outro lado a biodisponibilidade deste farmaco é muito baixa havendo assim uma minimização dos efeitos adversos.

Ao longo dos últimos anos vários grupos científicos têm tentado optimizar a potência dos corticóides e minimizar os seus efeitos secundários. Várias tentativas têm sido feitas de forma a aumentar a segurança e eficácia dos GT, nomeadamente na aplicação de novos regimes de aplicação, o desenvolvimento de veículos baseados em nanotecnologias e na síntese de novos fármacos.

Nesta dissertação foram revistas e avaliadas as estratégias mais recentes para a veiculação de GT aumentando a sua permeação e acumulação. Os veículos mais recentes incluem, partículas lipídicas, lipossomas, transferosomas, partículas poliméricas entre outros. Estas formulações são relativamente recentes e a indústria farmacêutica ainda não possui as metodologias adequadas para a sua produção. Para além disto, diversos desafios como armazenamento, manipulação e fabrico emergem aquando da avaliação da estabilidade, compatibilidade e transposição de escala destas novas tecnologias.

A interface entre a ciência da formulação e a engenharia continuará a ser uma fronteira no desenvolvimento de novos produtos pois o ratio custo / benefício destas novas tecnologias é difícil de antecipar.

No nosso entender, um desenvolvimento farmacêutico racional, que integre formulações simples e facilmente traspostas de escala para a indústria farmacêutica, irá ajudar no desenvolvimento de veículos adequados.

As emulsões convencionais são preparadas aquecendo as fases aquosa e oleosa à mesma temperatura. Posteriormente estas fases são misturadas e homogeneizadas até atingirem a temperatura ambiente. Actualmente, os pocessos de fabrico têm ser optimizados e os custos reduzidos. O método a quente apresenta a desvantagem, quando comparado com o processo a frio, de apresentar mais custos. As vantagens da emulsificação a frio não estão limitadas à diminuição dos custos de produção, mas também são mais fáceis de processar, diminuindo o tempo de produção e consequentemente aumentando a capacidade de produção. Não é necessário tempo de aquecimento e consequentemente não é necessário o passo de arrefecimento pelo que a estrutura das emulsões é mais facilmente previsível. No entanto, as emulsões preparadas pelo processo a frio são mais difíceis de estabilizar e de obter cremes com uma cosmeticidade adequada, principalmente devido a limitação dos excipientes que podem ser usados (apenas excipientes líquidos ou solúveis numa das fases). Estes problemas aumentam quando fármacos como os GT são incorporados, maioritariamente devido a problemas de solubilidade. Os GT são insolúveis em água e apresentam uma baixa solubilidade nos solventes mais utilizados em preparações tópicas.

Este projeto teve como principais pressupostos o desenvolvimento e caracterização de emulsões para uso dermatológico, de valor acrescentado para a empresa financiadora.

Os principais objetivos desta dissertação foram o desenvolvimento de formulações tópicas, contendo furoato de mometasona (0.1 % m/m) estáveis a pH ácido, contendo o menor número possível de excipientes e usando a menor energia possível durante o seu fabrico. Após desenvolvidas e caracterizadas, o objetivo foi estudar os perfis de libertação e permeação através de membranas sintéticas e pele humana, e realizar estudos *in vivo* da ação anti inflamatória comparando os resultados obtidos com a formulação de referência no mercado.

Seguidamente, a estabilidade física, química e microbiológica da formulação final, de acordo com as guidelines farmacêuticas, foi avaliada. Finalmente, os efeitos biológicos

e a avaliação de segurança e a transposição de escala foram conduzidos para o placebo (emulsão sem fármaco) de acordo com a legislação europeia de cosméticos em vigor.

Várias formulações foram desenvolvidas e analisadas, quatro emulsões foram seleccionadas por apresentarem uma estabilidade química e física adequada. Estas quatro emulsões diferiram no co tensioactivo (*PEG-20 glyceryl laurate* ou *polyglyceryl-4-isostearate*) utilizado e no glicol (*2- methyl-2,-4 pentanediol* ou *etoxydiglycol*).

A utilização de métodos reológicos, calorimetria de varrimento diferencial, análise microscópica e determinação do tamanho da gotícula permitiram seleccionar as emulsões mais estruturadas, ou seja com uma estabilidade física mais promissora (emulsões contendo *PEG-20 glyceryl laurate*).

Estudos de libertação e permeação *in vitro* demostraram que o glicol utilizado tem pouca influencia na permeação do furoato de mometasona. Estes resultados estão de acordo com os resultados de solubilidade do fármaco em ambos os glicóis. Foi demostrado que o co - tensioactivo influenciou a permeação do furoato de mometasona, sendo a permeação mais elevada para o co - tensioactivo mais hidrófilico devido a uma menor afinidade do fármaco para este. Foi demostrado um aumento do coeficiente de permeabilidade entre 2.7 a 7.8 vezes, comparando o valor experimental com o valor teórico esperado para este fármaco. Este aumento foi atribuído aos excipientes presentes na emulsão que funcionaram como promotores cutâneos.

Os resultados obtidos no ensaio de *tape stripping* em pele humana, demostraram que a quantidade de furoato de mometasona que atingiu as camadas viáveis da pele é baixa (1.99 %), ficando parte do fármaco retido no estrato córneo (10.61 %). Apesar disto, os estudos *in vivo* demostraram que as formulações desenvolvidas diminuíram o edema e o eritema na orelha do rato em mais de 90 %. Adicionalmente, foi demostrado que a eficácia das formulações é semelhante à da formulação comercial relativamente aos estudos anti inflamatórios.

A formulação selecionada tendo em conta a análise estrutural e os ensaios *in vitro* e *in vivo* foi a emulsão contendo *PEG-20 glyceryl laurate* e *2- methyl-2,-4 pentanediol*. Os estudos de estabilidade física, química e microbiológica demostraram que esta formulação é estável, pelo menos, durante 12 meses nas presentes condições experimentais.

Relativamente aos estudos dos efeitos biológicos (perda de água trans epidérmica, corneometria e sebometria) do placebo, foi possível observar que este contribuiu para

restaurar a barreira cutânea devido a um aumento significativo dos lípidos à superfície da pele. Adicionalmente a avaliação de segurança concluiu que este veículo é seguro para aplicação tópica nas condições previstas.
Finalmente, os estudos preliminares de transposição de escala para o placebo, demostraram que o perfil reológico não sofreu alterações significativas e a distribuição do tamanho das gotículas foi monomodal, no placebo produzido na escala piloto industrial e, bimodal nas escalas laboratoriais, indicando um possível aumento da estabilidade física para o primeiro. Conclui-se que os riscos associados à transposição de escala não foram elevados podendo-se assim prosseguir para a validação do processo à escala industrial. Foi ainda observado que o processo a frio utilizado diminuiu os custos totais de produção em mais de 17 % quando comparado com um processo a quente.

De uma forma geral pode concluir-se que o desenvolvimento de uma emulsão produzida a frio foi conseguido com um perfil de estabilidade adequado à sua comercialização. Os resultados obtidos indicam que o ratio benefício risco poderá ser melhorado, e que a emulsão desenvolvida apresenta a mesma eficácia quando comparada com o produto de referência em termos de atividade anti inflamatória.
A produção industrial desta emulsão irá reduzir substancialmente os custos associados à produção.

Palavras-Chave: emulsificação a frio, furoato de mometasona, mico estrutura, custos de produção.

Abstract

With rapid developments in materials science, pharmaceutics and biotechnology, new systems have emerged for topical glucocorticoids delivery. Despite being a mature class of drugs, they are still the most frequently prescribed drugs by dermatologists, explaining the interest on this field.

Over the years, research has focused on strategies to optimize the potency of steroids while minimizing adverse effects. Mometasone furoate (MF) is a synthetic, lipophilic, 16 alpha methyl analogue of beclomethasone, classified as class III (European Classification).

The development of simple formulations for MF delivery, easily scaled-up to industry, produced by methods that can allow the decrease of production costs, will be the rational beyond this project.

Emulsions suitable for cold process emulsification were developed and optimized. Four formulations were created differing on the co-emulsifier used (PEG-20 glyceryl laurate and polyglyceryl-4-isostearate) and the glycol (2-methyl-2,4-pentanediol and ethoxydiglycol). Formulation design coupled with structure analysis allowed the selection of the most stable emulsions, emulsions containing PEG-20 glyceryl laurate.

In vitro permeation studies demonstrated that these emulsions, containing MF (0.1 % w/w), were responsible for a increased on the permeability coefficients of MF. The *in vivo* studies showed that, the topical application of the formulation would assure, at least, the same efficacy compared with the commercial cream.

Additionally, it was demonstrated that the selected emulsion (PEG-20 glyceryl laurate with 2-methyl-2,4-pentanediol) is physical, chemical and microbiological stable during 12 months.

In vitro and *in vivo* studies showed that the placebo (emulsion without MF) was not skin-irritant and it was demonstrated to contribute to restore the skin barrier by increasing the amount of lipids within the skin.

Finally, the cold process allowed a total production savings of more than 17% when compared to the traditional hot process and preliminary scale-up studies suggest that the risk associated to the scale-up is minor.

Keywords: cold process emulsion; mometasone furoate; microstructure; production costs.

Résumé

En raison d'évolution rapide en science des matériaux, pharmaceutique et biotechnologie, des nouveaux systèmes ont émergé pour l'administration de glucocorticoïdes topiques.

Bien qu'étant une classe de médicaments matures, ce sont toujours les médicaments les plus prescrits par les dermatologistes, ce qui explique l'intérêt porté à ce domaine.

Pendant des années, les recherches se sont concentrées sur les stratégies visant à optimiser la puissance des stéroïdes en minimisant les effets indésirables. Le furoate de mometasone (FM) est un 16 alpha méthyle analogue du beclomethasone, synthétique et lipophile, classe class III (Classification européenne).

Le développement de formules simple pour l'administration du MF, facilement applicable à un accroissement d'échelle pour l'industrie et produit par des méthodes qui peuvent permettre la réduction des coûts de production, sera le rationnel au-delà de ce projet.

Des émulsions, adaptées aux émulsions à processus à froid, ont été développées et optimisées.

Quatre formulations ont été créées, se différenciant par le co-tensioactif utilisé (PEG-20 glyceryl laurate et polyglyceryl-4-isostearate) et le glycol (2-methyl-2,4-pentanediol et ethoxydiglycol).

La conception de la formulation associée à l'analyse de la structure, à permis la sélection des émulsions les plus stables, émulsions contenant du PEG-20 glyceryl laurate.

Des études *in vitro* de permeation ont montre que ces émulsions, contenant du FM (0,1% m/m), sont responsables d'une augmentation des coefficients de perméabilité du FM.

Les études *in vivo* ont montré que l'application topique de la formulation assurerait, au minimum, la même efficacité par rapport à la crème commerciale.

De plus, il a été démontré que l'émulsion sélectionnée (PEG-20 glyceryl laurate avec du 2-methyl-2,4-pentanediol) est stable physiquement, chimiquement et microbiologiquement pendant 12 mois.

Les études *in vitro* et *in vivo* ont montré que le placebo (émulsion sans médicament), n'irritait pas la peau et contribue à restaurer l'épiderme, en augmentant la quantité de lipides dans la peau.

Enfin, le processus à froid a permis une économie de production totale de plus de 17% compare aux processus chaud traditionnel. Les études préliminaires d'accroissement d'échelle suggèrent que le risque associé à l'accroissement d'échelle est mineur.

Mots clés: émulsion processus à froid ; furoate de mometasone, microstructure, coût production.

List of Publications

Patents

Portuguese Patent nº105982 M: submitted at 3rd November 2011 – Cold Process Emulsion as Vehicle for Anti-Inflammatory Drugs: Composition and Preparation Method. Helena Margarida Ribeiro and Sara Raposo; Faculty of Pharmacy University of Lisbon and Laboratório Edol Produtos Farmacêuticos S.A.

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Abbreviations

ANOVA - Analysis of variance

ATR - Attenuated total reflectance infrared

BD - Betamethasone dipropionate

BMV - Betamethasone-17-valerate

COX-2 - Cyclooxygenase-2

CP - Clobetasol-17-propionate

DMSO - Dimethyl sulfoxide

DSC - Differential scanning calorimetry

EMEA - European Agency for the Evaluation of Medicinal Products

*f*2 - similarity factor

FDA - Food and Drug Administration

FTIR - Fourier transform infrared spectroscopy

G' - Storage modulus

G´´ - Loss modulus

HA - Hydrocortisone acetate

HC - Hydrocortisone

H&E - Hematoxylin and eosin

HLB - Hydrophilic lipophilic balance

HPA - Hypothalamic pituitary adrenal

HPLC - High-performance liquid chromatography

HPMC - Hydroxypropylmethylcellulose

HRIPT - Human repeated insult patch test

IC_{50} - Half maximal inhibitory concentration

ICH - International conference on Harmonization

IL - Interleucine

IPM - Isopropyl myristate

J - Flux

Kp - Permeability coefficient

Log P - Partition coefficient

MC - Methylcellulose

ME - Microemulsions

MF - Mometasone furoate

MIA - Market introduction authorization

MoS - Margin of safety

MTT - 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide

Na-DOC - Sodium-deoxycholate

NLC - Nanostructured lipid carriers

NMR - Nuclear magnetic resonance

NOAEL - No observed (adverse) effect level

PBS - Phosphate buffer saline

PC - Prednicarbate

PEG - Polyethylene glycol

PGA- Poly glutamic acid

PLA - Poly lactic acid

PLGA - Poly (lactide-co-glycolide) acid

PLP - Prednisolone phosphate

PPG - Polypropylene glycol

PS - Parelectric spectroscopy

PVM/MA - Methyl vinyl ether/maleic anhydride copolymer crosslinked with decadiene

PVP - Polyvinyl pyrrolidone

RH - Relative humidity

RP - Reversed phase

RT - Room temperature

SC - S*tratum corneum*

SDS - Sodium dodecyl sulfate

SED - systemic exposure dose

SLN - Solid lipid nanoparticles

SLS - Sodium lauryl sulfate

TCA - Triamcinolone acetonide

TEM - Transmission electron microscopy

TEWL - Trans epidermal water loss

TG - Topical glucocorticoids

tanδ = G''/G' - Loss factor

η’ - Dynamic viscosity

γc – Critical strain

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1. Introduction

All the aims, objectives, theoretical background, materials, methodologies, findings, discussions and conclusions of my PhD research project are presented in this thesis. The research project, which began 1st May 2009, integrated the development of innovative topical systems for the delivery of corticoids and was especially oriented to meet the industrial needs of a pharmaceutical Portuguese company, yet it has had a science based approach.

All the research and scientific work, results from a joint partnership between a Portuguese pharmaceutical company – Laboratório Edol, Produtos Farmacêuticos S.A (www.edol.pt) – and the Faculty of Pharmacy of the University of Lisbon, Portugal (www.ff.ul.pt).

The financial support of the entire research project, including the PhD grant, was equally shared by Laboratório Edol, Produtos Farmacêuticos S.A and by Portuguese Foundation for Science and Technology (grant number: SFRH/BDE/33550/2009), between May 2009 and May 2013.

The experimental work that supports this thesis was performed at the Departamento de Farmácia Galénica e Tecnologia Farmacêutica of the Faculty of Pharmacy of the University of Lisbon, with the exception of the structural analysis of the emulsions that was performed at the University of Strathclyde in Glasgow (United Kingdom), and both the validation of the high-performance liquid chromatography (HPLC) methods and the development of the scale-up process, which were respectively conducted at the Department of Quality Control and the Department of Production of Laboratório Edol Produtos Farmacêuticos S.A.

2. Motivation

Considering the increase of the complexity and competitiveness of the pharmaceutical market, it is of high importance for all pharmaceutical companies to pursuit the development of innovative pharmaceutical forms and products, in order to guarantee the quality of the products and, consequently, to strengthen the position of the companies in the market. In this sense, Laboratório Edol Produtos Farmacêuticos S.A needs to be constantly watchful to the feedback from consumers, as well as to the market

developments in order to detect gaps, new opportunities and/or possible ways of improving their products. Thus, it is very important for Laboratório Edol Produtos Farmacêuticos S.A to improve their products, as well as to develop new pharmaceutical dosage forms or products.

To better address this need, the know-how transfer from the university to the company is crucial to improve the performance of the company. In the last years, the marketing of new products was achieved by purchasing technical dossiers that include a complete set of data and supportive information, which allowed a quick introduction of new products on the market. However, this easy solution represents also a drawback to the development of the company.

The cooperation activities between the Departamento de Farmácia Galénica e Tecnologia Farmacêutica of the Faculty of Pharmacy of the University of Lisbon and Laboratório Edol Produtos Farmacêuticos S.A., for the development of new products and for the improvement of the quality and performance of the existing ones, for dermatologic and cosmetic purposes, started in 2005. This research project follows on the established cooperation partnership and aims to respond more effectively to the increasing demands of Laboratório Edol Produtos Farmacêuticos S.A.

Laboratório Edol Produtos Farmacêuticos S.A decided to develop a new area: unlike in the USA or in the rest of Europe, the cream and lotion compositions for the topical application of mometasone furoate (MF) marketed in Portugal are not protected. MF had its first market introduction authorization in Portugal on 22/08/91 as an ointment and cutaneous solution, having been protected by a Portuguese patent (PT 74357) that only protected the "process for preparing aromatic heterocyclic esters of steroids", which has lapsed on 29/01/2002. Nowadays, MF is marketed as powder and suspension for inhalation, cutaneous solution, cream and ointment.

Following this opportunity, our research project also aims to explore and develop possibilities of using drugs with unprotected patents and/or market introduction authorizations expired, but with an improved therapeutic activity.

3. Aims of the research project

The emphasis of this project relayed on the development and evaluation of emulsions for dermatologic applications. Also, the project activities were directed for achieving a main and final goal of creating added value for the company.

The aim of the present work was to develop pharmaceutical emulsion(s) for dermatologic use that are physically stable at acidic pH and including the minimum number of excipients, and requiring as little energy as possible during their preparation, i.e. by using a new and more economical method of emulsification: a cold process. During the formulation development, the aim was also to investigate the influence of the type of co-emulsifier and the type of glycol on the microstructure of the emulsions by rheological, thermal and microscopic techniques.

For this research thesis, we elected the dermatologic delivery of topical glucocorticoids, such as MF, as the main therapeutic application. The delivery of such drugs is challenging due to their chemical characteristics, namely their poor solubility in water and their stability in acidic conditions, which is often poorly compatible with emulsion stability.

Secondly, the final emulsions were studied using *in vitro* release and permeation tests and also *in vivo* studies, comparing the results to the performances of other benchmark products.

Finally, one of the studied emulsions was selected and a complete physical, chemical and microbiological stability assessment was conducted, according to the international pharmaceutical guidelines and standards. Additionally, the safety and biological effects of the placebo (product without drug) was assessed by using both *in vitro* and *in vivo* studies, as an adequate equilibrium between the safety and efficacy effects is of high importance.

4. Structure of the thesis

The thesis is divided in nine chapters:

- Chapter II consists on a literature review about the main functions of the skin, a detailed description about the physiology and anatomy of the main barrier for the percutaneous absorption – the *stratum corneum* (SC), as well as the main diffusion routes through the skin. This chapter also includes a literature review on the topical vehicles for dermal delivery of corticoids.
- Chapter III describes the pharmaceutical development according to the guideline ICH Q8 (R2). Throughout the formulation pharmaceutical development, four emulsions were selected.
- Chapter IV describes the structural analysis of these four emulsions, comprising all the data and information that allowed the selection of the best two of them.
- Chapter V presents the *in vitro* release and permeation studies as well as the *in vitro* cytotoxicity and the *in vivo* anti-inflammatory studies. At this stage, only one of the tested emulsions was selected to the complete physical, chemical and microbiological stability studies.
- Chapter VI describes the physical, chemical and microbiological stability studies according to the guideline ICH Q1A (R2).
- Chapter VII describes the safety assessment of the placebo (emulsion without drug) as well as its biological effects on human volunteers as the emulsion has also potential for being marketed as a cosmetic product.
- Chapter VIII addresses the first scale-up studies of the placebo.
- Chapter IX summarizes the highlights of the thesis regarding the experimental results and the impact of the work in the industrial field.

Finally, the annex I describes the validation procedures of the high-performance liquid chromatography (HPLC) method for the assay of MF in the final emulsion.

1. The Epidermal barrier

The main barrier to the percutaneous absorption of topically applied drugs is the SC. The structure of the SC can be described by a multilayer matrix of hydrophobic and hydrophilic components [1], which form a barrier to penetration of irritants, allergens and pathogenic microorganisms through skin.

The structural integrity of the SC is maintained by the presence of modified desmosomes, called corneodesmosomes, which lock the corneocytes together and provide tensile strength for the SC to resist to shearing forces [2]. Elias [3] visualized the SC as being similar to a brick wall, with the corneocytes analogous to bricks, and the lipid lamellae acting as mortar.

The barrier nature of the SC depends critically on its unique constituents; unlike the typical biological membranes mainly composed by phospholipids, the hydrophobic lipids present in the intercellular spaces of the SC are ceramides (45–50% consist of a sphingosine or a phytosphingosine base to which a non-hydroxy fatty acid or an alpha-hydroxy fatty acid is chemically linked), cholesterol (25%), long-chain free fatty acids mostly with chain lengths C22 and C24 (15%, highly enriched in linoleic acid), and 5% other lipids, the most important being cholesterol sulfate, cholesterol esters, and glucosylceramides [4]. These lipids, which are organized in multilamellar bilayers, regulate the passive flux of water through the SC and are considered to be very important for skin barrier function [5].

Due to the barrier nature of the SC, topically applied compounds may accumulate, that is. the SC may serve as a reservoir from which substances can be subsequently absorbed over long periods of time [1]. The reservoir function of SC was first reported by Vickers in 1963 [6], who demonstrated that topically applied corticosteroid forced into SC by occlusion remained there for 7-14 days, as observed by the development of a physiological marker, the vasoconstriction.

Almost since the introduction of the modern scientific study of percutaneous absorption, authors have debated the relative importance of three potential routes of entry from the surface of the skin into the sub-epidermal tissue (Fig. 2.1). Hence, the absorption of drugs through the skin can occur through intact epidermis – transepidermal route and/or skin appendages – transappendageal route [8]. Since skin appendages occupy less than 0.1% of the total human skin surface, the transappendageal route has generally been

considered to contribute minimally to the overall permeation [9]. However, these calculations did not take into the account that the hair follicles represent invaginations, which extend deep into the dermis with a significant increase in the actual surface area available for penetration.

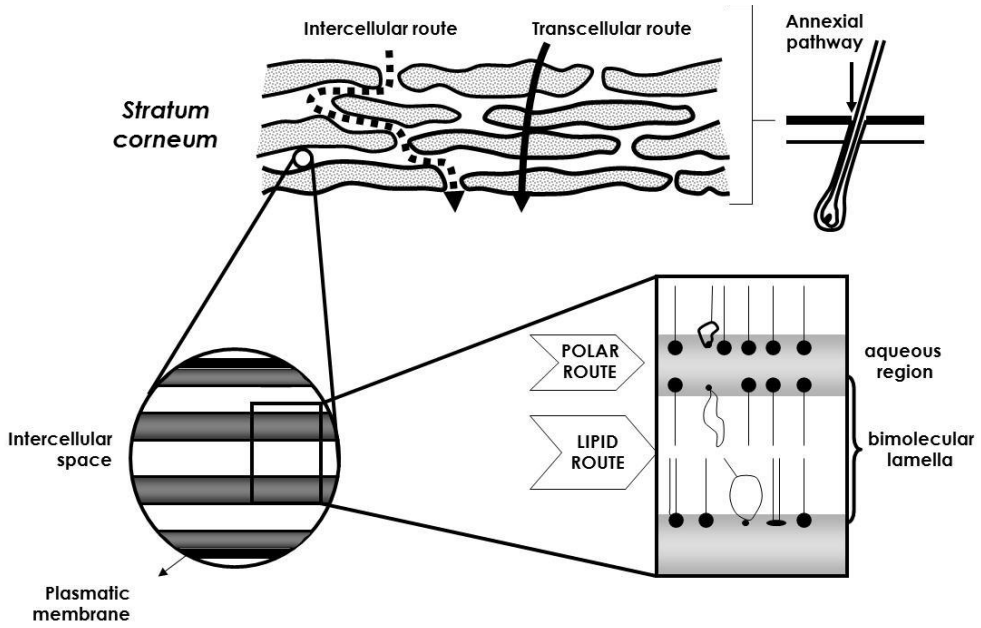

Fig. 2.1. SC model scheme ("brick-and-mortar") with the lipidic intercellular matrix and the possible penetration pathways (intercellular: dashed arrow; transcellular: black arrow); annexial route is also represented. Substances permeate mainly along the tortuous pathway in the intercellular lamellar regions which are oriented parallel to the corneocyte surface. Hydrophobic permeants diffuse through the SC intercellular bilayers – lipid route; Polar route could be a domain for hydrophilic molecules to penetrate the SC (adapted from [7]).

Transport across the SC is largely due to passive diffusion [10] and depends on a number of physicochemical properties of the vehicle, the skin and the permeant. Four physicochemical parameters pertaining to the drug were identified: the molar mass which determines the diffusion coefficient, the number of hydrogen-bond donors and the number of hydrogen-bond acceptors that control the interactions with the surface of corneocytes, and the octanol–water partition coefficient (logP) that represents the SC–water partition [11, 12].

On the other hand, recent advances in this area have demonstrated the important role of hair follicles as penetration pathways and reservoir structures for topically applied

compounds. Furthermore, it has been demonstrated that the penetration depth of the particles can be influenced by their size resulting in the possibility of a differentiated targeting of specific follicular structures. Thus, the selective delivery of topically applied substances to the contemplated target sites offers a diversity of therapeutic options [13-15].

There has been much debate over the past decades on the route of penetration but experimental evidence suggests that, under normal circumstances, the predominant route is through the intercellular spaces (transepidermal route). The diffusion path length is therefore much longer than the simple thickness of the SC (20 µm) and has been estimated as long as 500 µm. Importantly, the intercellular spaces contain structured lipids and a diffusing molecule has to cross a variety of lipophilic and hydrophilic domains before it reaches the junction between the SC and the viable epidermis. The nature of the barrier is thus very heterogeneous and it is perhaps surprising that diffusion through the skin can be described by simple solutions to Fick's laws of diffusion [16, 17].

Many experimental methods for assessing percutaneous absorption are available now; this has largely been brought about by the development of sophisticated biophysical techniques and increased computing powers. The advanced technology has clearly provided indications, at a molecular level, about routes of permeation and how the barrier function can be modulated by excipients with which actives are formulated. Techniques such as attenuated total reflectance infrared (ATR) spectroscopy and fourier transform infrared spectroscopy (FTIR) [18, 19], nuclear magnetic resonance (NMR) [20] and transmission electron microscopy (TEM) [21] have been crucial to understand the routes of topical permeation. Barry *et al*. [22] explored a novel technique employing two human skin membranes to differentiate shunt route delivery from bulk transepidermal input. The method monitors penetration through epidermal membranes and compares it with delivery through a sandwich of SC and epidermis, with the SC forming a top membrane. The approach was particularly valuable for shunt route analysis, being also useful for passive diffusion and iontophoretic drug delivery.

2. Topic corticosteroid classification and relevance

The development of topical products for dermatological diseases represents an untapped opportunity for clinical pharmacology since they represent the most widely used preparations in dermatology [5].

The introduction of topical hydrocortisone (HC) by Sulzberger and Witten in 1952 [23] provided a major pharmacologic breakthrough for dermato-therapy, since the "compound F", as they described HC, was for the first time, topically effective.

Chemical substitution at certain key positions is able to modify the potency of corticosteroids. For example, halogenation at the 9-α position enhances the potency by improving activity within the target cell and decreasing breakdown into inactive metabolites. Along the same lines, masking or removing the hydrophilic 17-dihydroxyacetone side-chain or the 16-α-hydroxy group will increase the molecule's lipophilicity [24], thus enhancing penetration through the SC. The most important group's modifications are described in Table 2.1.

Table 2.1. Beneficial effects of the most important functional groups of topical corticoid molecules (adapted from Katz and Gans [24]).

C – 11 β	Converting = O to –βOH provided topical activity
C - 9	Fluorine increased potency
C - 6	Fluorine increased potency
C - 9 and C - 6	Fluorines at both positions further increased potency as compared to only one fluorine.
C – 16, 17	The acetonide group provided increased penetrability and enhanced percutaneous absorption (e.g., acetonide was 10 times more active than parent topically but equal systemically).
C – 1, 2	The formation of a double bond between carbons 1 and 2 increased activity.
C - 21	Esterification with an acetate resulted in increased resistance to metabolic breakdown. This enhancement of lipophilicity resulted in optimization of percutaneous absorption.

Although many topical corticoids have been used for numerous skin disorders, their only "approved usage" remains for atopic dermatitis and psoriasis.

The acute and chonic dermatoses in which corticosteroids are the most effective treatment are seborrheic dermatitis, atopic dermatitis, localized neurodermatitis, anogenital pruritus, psoriasis (particularly of the face and between skinfolds), inflammatory phase of xerosis, and late phase of allergic contact dermatitis or irritant dermatitis.

Early in the era of topical corticoid, the U.S. Food and Drug Administration (FDA) developed a regulatory appeal system now almost universally accepted, whereby sponsors need only to demonstrate activity via parallel placebo-comparison studies in atopic dermatitis and psoriasis to obtain "class" labeling [25]. In fact, the only acceptable methods to assess bioavailability and bioequivalence of topically applied drug formulations are clinical trials between generic and original products and pharmacodynamic response studies, measured by the vasoconstrictor assay. A search in the *clinicaltrials.gov* [26] database showed that the main clinical trials in this area are focused on application regimens, dosage schedule, combine therapies, efficacy and safety assessment, or other therapeutic uses such as sun protection. Furthermore, all of these studies are being carried out with conventional formulations, i.e., creams, ointments and sprays, with no evidence for the use of advanced systems, such as nanoparticulate carriers, in glucocorticoid topical delivery.

Although clinical trials are considered the 'gold standard', these studies are relatively insensitive, costly, time-consuming and require large numbers of subjects. In contrast, pharmacodynamic response studies are relatively easy to perform and allow obtaining relevant information. Montenegro *et al*. [27] studied the effect of application time on skin blanching response and SC concentration after topical application of 0.1% betamethasone-17-valerate (BMV) cream on healthy volunteers.

The importance of corticoid therapy in skin diseases is associated to their long history of safety and effectiveness for certain conditions. This approach remains one of the most useful and widely prescribed treatments in day-to-day dermatologic practice, nearly 1.7 million prescriptions are dispensed each year for treatment of dermatological conditions [28]. It is, thus, not surprising that the global corticosteroid market grew by 9.7% over the period 2006–2007, accounting for $1.4bn in sales. Topical dermatological products with one or more corticosteroids and no other active

ingredients form the major share of the market at 75.5%. This class of corticosteroids also grew at a higher rate of 11%, compared to the second class of corticosteroids, which has an anti-infective agent in combination. The latter class recorded a growth of 6.1% over its 2006 sales of $336m. Increased safety concerns will restrict future growth for topical drugs that could be a suitable alternative to topical corticoids. As immunosuppressants these products have a declining market presence due to the FDA's black box label warning in 2005 for potential cancer risk, which emphasises the important role of corticoids in the present and future economic trends [29].

3. Mechanism of action of topical glucocorticoids

The target cells for TG are, not only the keratinocytes and fibroblasts, but also immune cells (Tcells, monocytes, macrophages, langerhans cells), within the viable epidermis and dermis, where the glucocorticoid receptors are located [5, 30]. The transport across the cell membrane of TG is a non-mediated, passive diffusion process related to drug lipophilicity. Within the cytoplasm, the steroid molecule binds to the glucocorticoid receptor, forming a complex that is rapidly transported to the nucleus [5, 31]. Briefly, TG-receptor binding causes a conformational change of the receptor with consequent shedding of the DNA-binding domain capping protein, hsp90. Exposure of the DNA-binding site allows binding of the glucocorticoid-receptor complex to the glucocorticoid responsive element.

This interaction stimulates alterations in transcription, either positively or negatively, and thereby translation of proteins [32].

In addition to this direct regulatory effect on gene transcription, TG are also able to indirectly regulate transcription by blocking the effects of other transcription factors as nuclear factor-κB. TG may inhibit the transcription of proinflammatory cytokine genes (including the interleukins IL-1, IL-2, IL-6, interferon γ, and tumour necrosis factor-α genes), T-cell proliferation and T-cell dependent immunity. In fibroblasts, IL-1α is responsible for proliferation, collagenase induction, and IL-6 synthesis, which control skin thickness. The inhibition of IL-1α in keratinocytes has anti-inflammatory effects, whereas the same inhibition in fibroblasts has anti-proliferative and atrophogenic effects [5, 32, 33].

4. Topical delivery systems

Paradoxically, the skin, the most exposed organ, still holds many secrets concerning the mechanisms of cutaneous permeation [34]. Once applied on the intact skin, low-molecular-weight molecules generate a transcutaneous concentration gradient capable of pushing molecules into the skin. The idea is to simplify the enormous complexity of the skin and to consider diffusion through the skin governed by Fick's law of diffusion [35]. A critical parameter that controls the skin permeation is the drug solubility in the vehicle because its solubility influences both the drug concentration gradient in the solution and partition coefficient between the vehicle and the skin [36]. It has been demonstrated that at a given concentration, release is ordinarily faster from the vehicle in which the drug is completely solubilized [37]. A full study on the drug's solubility for each vehicle composition is necessary to avoid supersaturation, that is, when the drug concentration is increased above its equilibrium solubility, the system becomes thermodynamically unstable and crystallization and precipitation of excess drug occur over time [36]. As corticoids are usually insoluble or have a very poor solubility in water [38], solvents and co-solvent systems are widely used to improve both, the amount and range that can be administered at therapeutic levels through the skin.

To overcome the excellent barrier properties of the human skin, penetration enhancement techniques based on delivery systems have been considerably exploited [39]. Penetration enhancers promote skin permeability by altering the skin as a barrier to the flux of a desired penetrant. Chemical penetration enhancers are incorporated into a formulation to improve the diffusivity and solubility of drugs through the skin that would reversibly reduce the barrier resistance of the skin [40]. To enhance skin permeation, chemical enhancer targeting the SC lipid domain requires the enhancer to orient itself within that microenvironment to perturb and alter the SC lipid lamellae structure [41].

Other penetration enhancement techniques have been largely studied such as iontophoresis, electroporation, eutectic mixtures [42], supersaturated systems [43, 44] and nanoparticles including deformable vesicles [45]. However, enhancement strategy may differ for each drug, and requires optimization. It is well known that the vehicle used in a topical formulation greatly influences the rate and extent of drug permeation, thus modifying the potency of the drug in the formulation [46].

4.1 Conventional delivery systems for TG delivery

Over the past few decades there have been many advances in our understanding of the physicochemical properties of both formulation systems and their ingredients. Dosage forms for dermatological drug therapy are intended to produce desired therapeutic action at specific sites in the epidermal tissue. The drug's ability to penetrate the epidermis, dermis, and subcutaneous fat layers depends on the physicochemical properties of the drug, the carrier base and skin condition.

In modern-day pharmaceutical practice, semisolid formulations are the preferred vehicles for dermatological therapy because they remain *in situ* and deliver the drug over extended time periods. To be effective, topically applied agents, such as corticoids, must gain entry to the skin and pass from one layer of tissue to the next. Most of the drugs cannot achieve this if administrated alone, but only if a part of a formulation, that is, as a solute in a vehicle or solvent that carriers the active agent or at least enhances its delivery [47].

Topical formulations of corticosteroids are usually administered in the pharmaceutical form as ointments, creams, lotions, gels, aerosol sprays, powders and foams [48]. Concerning TG, most research papers refer gels and emulsions as vehicles (Table 2.2).

Table 2.2. Conventional delivery systems for glucocorticoid delivery.

Drug	Vehicle	Physicochemical parameters	Chemical stability	Permeation	Antiinflammatory activity	Ref
MF 0.1%	HPMC gel	pH – 4.35-4.69 (over 365 days at RT). pH – 4.34-4.96 (over 365 days at 40 ºC).	109.6 ± 3.7% (accelerate conditions during 180 days) and within the limits at RT during 365 days.	**Human skin**: 0.11% ± 0.07 (after 8h) 0.36 % ± 0.06 (after 24h)	n.a.	50
MF 0.1%	Carbopol® 940/ethanol/propyleneglycol 0.75%/20%/15 % gel	pH: 7.1 apparent viscosity: 38600 ± 320 CPS spreadability: 26.31 ± 1.38 g.cm/s	100.8 ± 0.47% (T0)	**Rat skin**: 63.12 ± 0.27 % after 8h	n.a.	51
MF 0.1%	Carbopol® 940/ethanol/propyleneglycol 1.25%/20%/15 % gel	pH: 6.9 apparent viscosity: 43500 ± 250 CPS spreadability: 20.83 ± 0.86 g.cm/s	99.05 ± 0.56% (T0)	**Rat skin**: 51.80 ± 0.14 % after 8h	n.a.	51
MF 0.1%	Carbopol® 940/ethanol/propyleneglycol 1%/20%/10% gel	pH: 6.9 apparent viscosity: 45100 ± 210 CPS spreadability: 20 ± 0.8 g.cm/s	99.85 ± 0.52% (T0)	**Rat skin**: 60.01 ± 0.15 % after 8h	n.a.	51
MF 0.1%	Carbopol® 940/ethanol/propyleneglycol 1%/30%/15% gel	pH: 7.1 apparent viscosity: 39200 ± 290 CPS spreadability: 25 ± 1.25 g.cm/s	99.7 ± 0.37% (T0)	**Rat skin**: 81.14 ± 0.20 % after 8h	n.a.	51
CP 0.05%	Na-DOC gel	n.a.	n.a.	**Pig ear skin**: Accumulation enhancement ratio on epidermis and dermis compared with commercial cream: 22.33 and 2.73	n.a.	53
CP 0.05%	Chitosan gel	n.a.	n.a.	**Pig ear skin**: Accumulation enhancement ratio on epidermis and dermis compared with commercial cream: 2.33 and 0.90	n.a.	53
MF 0.1%	Na-DOC gel	n.a.	n.a.	**Pig ear skin**: Accumulation enhancement ratio on epidermis and dermis compared with commercial cream:	n.a.	53

				1.78 and 0.65		
MF 0.1%	Chitosan gel	n.a.	n.a.	**Pig ear skin**: Accumulation enhancement ratio on epidermis and dermis compared with commercial cream: 1.32 and 0.47.	n.a.	53
BMV 0.1%	Chitosan gel	n.a.	n.a.	**Rat abdominal skin**: 7.42 ± 1.1 µg/cm^2	n.a.	46
BMV 0.05% and 0.1%	Na-DOC gel	pH - 6.73 ± 0.08 for 0.05% and 6.7 ± 0.06 for 0.1% during 3 months Viscosity at 1420 s^{-1}: 6.864 mPa.s for 0.05% and 10.680 mPa.s for 0.1%	99.46 ± 0.29 and 99.39 ± 0.16 for 0.05% and 0.1%, respectively during 3 months	**Rat abdominal skin**: 11.01 ± 1.3 µg/cm^2 and 36.09 ± 0.6 µg/cm^2 for 0.05 and 0.1%, respectively.	**Rat** (acute edema model): both formulations produced higher edema inhibition compared to commercial cream	46
BD 0.1%	Ointment with FO	n.a.	n.a.	**Tape stripping in porcine ear**: two-fold increase in the BD amount recovered in lower and basal layer of the skin with FO formulation	**COX-2 expression**: FO alone reduced the levels of COX-2. FO with BD: the reduction in COX-2 was more apparent, denoting synergistic or additive potentiation.	60
HC 1%	O/W emulsion	pH: 4.5-5.5 viscosity: 2000 mPas	n.a.	**Silicone membrane**: amount transferred over time per unit area was 4.1% after 48h.	**Rat** (croton oil-induced ear inflammation model): no statistically significant differences between the anti-inflammatory actions compared with commercial emulsion.	63
HC 1%	O/W emulsion with 5% ceramide	pH: 4.5-5.5 viscosity:5000 mPas	92 ± 3.7% (accelerate conditions during 180 days).	**Silicone membrane**: amount transferred over time per unit area was 4.9% after 48h.	**Rat** (croton oil-induced ear inflammation model): no statistically significant differences between the anti-inflammatory actions compared with commercial emulsion.	63
HC 1%	O/W emulsion with alkylpoly Glucosides and Miglyol®812 (MG)	pH: 6.21 Conductivity: 13.53 µS/cm G': 1598.7 Pa	n.a.	**Artificial skin constructs:** Flux (x10^{-9} g/cm^{2}s): 5.26, 3.31, 5.54 for MG; IPM and liquid paraffin (LP), respectively.	**Human volunteers** Skin blanching effect: LP>IPM>MG. Hydration: LP increased hydration TWEL: was increased with IPM and LP and decreased	
HC 1%	O/W emulsion with alkylpoly Glucosides and	pH: 5.70 Conductivity: 15.24 µS/cm	n.a.			65

	isopropyl myristate (IPM)	G': 1395.2 Pa			with MG.	
HC 1%	O/W emulsion with alkylpoly Glucosidesand LP	pH: 5.81 Conductivity: 11.28 µS/cm G': 2411.7 Pa	n.a.			
CP 0.05%	Microemulsion: 3% isopropyl miristate, 15 % Cremophor EL®, 30% of isopropyl alcohol and 50% of water	Globule size: 18.26 nm Solubility: 36.42 mg/ml	n.a.	**Albino Wistar rats**: cumulative amount of CP permeated after 8h was 53.6 ± 2.18 and 37.73 ± 0.77 µg cm^{-2} for market formulation.	n.a.	68
CP 0.05%	Microemulsion based gel (Carbopol® 934 P): 3% isopropyl miristate, 15 % Cremophor EL®, 30% of isopropyl alcohol and 50% of water	n.a.	n.a.	**Albino Wistar rats**: cumulative amount of CP permeated after 8h was 28.43 ± 0.67 and 37.73 ± 0.77 µg cm^{-2} for market formulation.	n.a.	68
HC	Microemulsion: isopropyl myristate; sucrose laurate L 595 and L1695	pH: 6.4 Viscosity: 26 mPa Surface tension: 26.49 mN/m;	n.a.	n.a.	**Human volunteers** Increase on skin redness and increase in blanching effect for both micro emulsions compared with a commercial cream.	
HC	Microemulsion: isopropyl myristate; polyoxyethylene glycerol monostearate and polyglyceryl-6-dioleate	pH: 7.75 Viscosity:141mPa Surface tension: 27.78 mN/m	n.a.	n.a.		71

4.1.1 Gels

The common characteristic of gels is that they contain continuous structures that provide solid-like properties. Depending on their constituents, gels may be clear or opaque and be polar, hydroalcoholic, or nonpolar. The simplest gels are hydrogels comprising water thickened with natural gums (e.g., tragacanth, guar, or xanthan), semisynthetic polymers (e.g., methylcellulose (MC), carboxymethylcellulose, or hydroxyethylcellulose), synthetic polymers (e.g., carbomer – Carbopol® or carboxyvinyl polymer), or clays (e.g., silicates or hectorite) [49].

Polymers have been used as delivery systems for topical corticoids; they are suitable systems for topical skin application due to their rheological properties, which can be considered as an additional advantage for an easier application to large skin areas. For example, Salgado *et al*. [50] developed a gel containing MF as an alternative to lotions for the application on anatomical regions with hair. The authors refer a hydroxypropyl methylcellulose (HPMC) gel with isopropyl alcohol and propyleneglycol as a delivery system to MF (0.1%). The vehicle presented suitable organoleptic characteristics as well as chemical, physical and microbiological stability for 1 year when stored at room temperature (RT). Patel and Kamani [51] also developed a gel formulation for the delivery of MF containing Carbopol® 940 as polymer and propylene glycol and ethanol as solvent/co-solvent system. They found that the concentrations of polymer, solvent and co-solvent significantly affected the *in vitro* permeation and flux through the rat's epidermal membrane. However, as Carbopol® 940 must be neutralized in order to achieve the desired viscosity, the pH values of the formulations were between 6.8 and 7.2, which according to Teng *et al*. [52] is not appropriate for MF stability. In aqueous solutions stability of MF was found to decrease with an increase in pH with the maximum stability below pH 4. Thus, it seems that the above described Carbopol® 940 gel is not suitable for the delivery of the included glucocorticoid.

Some polymers have been described as penetration enhancers like sodium-deoxycholate (Na-DOC) gel. Senyiğit *et al*. [53] studied the influence of the vehicle (chitosan and Na-DOC gels), the effect of penetration enhancers (terpenes and diethylene glycol monoethyl ether - Transcutol® P), and the effect of iontophoresis on the skin accumulation of clobetasol-17-propionate (CP) and MF. The results showed that Na-DOC gel increased the amount of CP retained in both epidermis and dermis in 20-fold

and 2-fold, respectively, when compared with a commercial cream. The MF data followed the same trend but less significantly. Concerning the effect of penetration enhancers and iontophoresis, terpenes showed the best results in accumulation data and iontophoresis did not significantly produced further enhancement in CP skin retention, but increased MF skin concentration.

Na-DOC gels were used to deliver BMV at two different strengths, 0.05 and 0.1%. *In vitro* permeation studies showed that the flux was 2.5 (0.05% gel) and 8.5 times (0.1% gel) higher compared to the commercial cream (0.1%) [46]. The pharmacodynamic responses after *in vivo* topical application in rats showed a significant agreement between anti-inflammatory activity and *in vitro* permeation of BMV.

These results confirm that vehicles have a major influence on the permeation of drugs through the skin. The development of suitable vehicles can therefore reduce the systemic adverse effects of TG, enhancing their permeability thus reducing the amount of topically applied drug.

Polymers seem also to play an important role on the control of drug crystallization in some vehicles. The control of drug crystallization is of particular interest for the efficiency and quality of topical formulations since drug crystallization within a matrix may cause a reduction in skin permeation [54], which assumes a major importance in the case of supersaturated systems.

Supersaturation technique has been extensively used to enhance the permeation of drugs through human skin [43, 44]. However, these systems tend to crystallize by spontaneous nucleation. Raghavan *et al*. [55] described the effects of HPMC, MC, polyvinyl pyrrolidone (PVP) and polyethylene glycol (PEG 400) on the crystallization of hydrocortisone acetate (HA). It was observed that nucleation was delayed in the presence of polymers, with a more pronounced delay in the presence of cellulose polymers. The mechanism of nucleation retardation by the polymers can be explained in terms of association of the drug with the polymer through hydrogen bonding. Moreover, HPMC is an antinucleant polymer that is adsorbed on the hydrophobic surface of crystals, thus stabilizing the precipitates and increasing the thermodynamic activity of the drug.

4.1.2 Emulsions and microemulsions

An emulsion is a heterogeneous preparation composed of two immiscible liquids, one of which is dispersed as fine droplets uniformly throughout the other. These systems are thermodynamically unstable [56] and are rarely simple two-phase oil-and-water systems. Their study and development is one of the most difficult and complex subjects in the pharmaceutical field [57]. Both oil-in-water and water-in-oil emulsions are extensively used for their therapeutic properties and/or as vehicles to deliver drugs and cosmetic agents to the skin. The emulsion facilitates drug permeation into and through the skin by its occlusive effects and/or by the incorporation of penetration-enhancing components.

Another important parameter on the dermal delivery is the dissolved fraction of a drug in a vehicle, making solubility properties one of the initial objectives for a novel pharmaceutical formulation. Furthermore, as the cutaneous drug delivery rate of formulations is generally related to the concentration (activity) gradient of the drug toward the skin, the solubility potential of microemulsions (MEs) may be an important factor in increasing skin absorption of drugs [58]. The term ME, which implies a close relationship to ordinary emulsions, is misleading because MEs are readily distinguished from normal emulsions by their transparency, low viscosity and more fundamentally their thermodynamic stability and ability to form spontaneously [56].

4.1.2.1 Emulsions

Many studies have been performed to investigate the effect of emulsions on dermal and transdermal drug delivery. Emulsions have been compared with ointments, MEs, aqueous suspensions and gels. From these studies, it is difficult to draw general conclusions because the various systems differed in their composition as well as physicochemical properties.

For instance, Borgia *et al*. [59] evaluated the influence of different commercial prednicarbate (PC) preparations in the topical permeation of the drug through reconstructed human epidermis. They observed that the release ranks were in the order cream (oil-in-water) < fatty ointment (water-free) < ointment (water-in-oil), suggesting that the increased steroid permeation should be due to the composition and inner structure of preparations together with the high lipophilicity of PC. The drug that is

solubilised in the fatty ointment and in the external phase of ointments can freely diffuse through the membrane at the interface with the preparation. The enhancing effect in ointment seems also to be linked to the increased thermodynamic activity of the drug in the ointments containing water in significant amounts.

Despite belonging to different categories, vehicles are often structurally similar and affect the diffusion of the active agent through the matrix. Over the years, research has focused on strategies to optimize the potency of steroids, while minimizing adverse effects. One of the possibilities to reduce the systemic adverse effects of TG is to improve their retention into the skin without augmenting the amount permeated, so as to reduce the applied dose [46]. The penetration enhancers most widely used are polyalcohols that are commonly used as emollients and solvents in emulsions. The use of natural oils, like omega-3 fatty acids, with both enhancement activity and improved corticoid anti-inflammatory activity has been also studied. Zulfakar *et al*. [60] investigated the influence of fish oil (FO) on the topical delivery and anti-inflammatory properties of betamethasone dipropionate (BD). The authors evaluated the drug penetration into porcine ear skin by tape stripping and concluded that the deposition of the active in the lower layers of the skin was increased in the presence of FO. To gauge anti-inflammatory activity, they used the expression of cyclooxygenase-2 (COX-2) that was indicated by a brownish-red staining. At 0 h the presence of staining proved that a high level of COX-2 was expressed and after 6 h a reduction in the intensity of staining in the skin treated with BD and with FO alone was observed. The reduction was even more evident when the two compounds were combined, denoting synergistic or additive potentiation, which could allow the reduction of corticosteroid doses administered.

It is well known that the vehicle used can substantially affect the individual agent's clinical action, potency, and acceptability to the patient. In addition of being a carrier for the active drug, the vehicle may also have the functions of hydrating the skin and increasing drug penetration. The key factors in the management of inflammatory skin diseases like AD are i) skin hydration and barrier repair; ii) use of effective topical anti-inflammatory agents, such as corticosteroids and iii) avoidance of allergenic triggers [61]. Concerning the first point, physiologic lipids such as ceramides, cholesterol, and fatty acids reduce trans epidermal water loss (TEWL) of the skin through the different mechanisms of action from non-physiologic lipids. Specifically, these lipids penetrate into the skin and modify endogenous epidermal lipids and the rate of barrier recovery.

The supplementation of lipid mixtures can contribute to the improvement of the defective barrier function of the skin, decreasing the amount of the corticoid needed [62]. In fact, oil-in-water emulsions containing ceramides have demonstrated a decrease in the TEWL levels in human volunteers and therefore highest skin repair. The same formulation was also studied concerning anti-inflammatory effect in the presence of 1% HC, and *in vivo* tests showed that the local inflammation model was similar to that obtained for a control HC formulation [63].

According to these studies, formulations containing physiologic lipid mixtures seem to be suitable therapeutic approaches to minimize side effects, mostly happening with steroid formulations.

Surfactants can also interact with skin surface lipids as they have the potential for solubilizing the SC lipids due to their capacity to interact with keratin. As a disruption of the order of corneocytes occurs [64], the improvement on drug penetration is frequently accompanied by cutaneous adverse reactions, making the selection of excipients an issue of major importance.

Nowadays we assist to an increased interest in the field of natural surfactants, as they can avoid the adverse reactions caused by synthetic surfactants, and especially due to the presence of large number of hydroxyl groups in their chemical structure able to increase skin hydration, increasing the permeability of the SC. These natural surfactants include a group of alkylpolyglucosides; Savić *et al*. [65] developed a vehicle based on cetearyl glucoside and cetearyl alcohol to deliver HC. They found that the formulation presented a favorable safety profile indicated by the lack of adverse effects during the *in vivo* study.

4.1.2.2 Microemulsions

Generally, MEs have favorable solvent properties due to the potential incorporation of large fraction of lipophilic and/or hydrophilic phases. Furthermore, investigations have indicated that the unique structural organization of the phases in MEs may contribute to additional solubility regions, increasing the loading capacity of MEs, compared to nonstructured solutions containing the same fraction of the constituents [58]. Studies with hydrophilic and hydrophobic MEs containing Transcutol® showed a great potential in increasing solubility and flux of HA, when compared to a gel and an ointment; it

seems that the high solubility provides the necessary concentration gradient for high permeation [48].

The potential of MEs in the improvement of drug penetration throught the skin was extensively reported [66, 67]. Recent studies [68] showed that a ME containing 3% isopropyl miristate, 15% polyoxyethylene castor oil (Cremophor EL®), 30% isopropyl alcohol and 50% water, after gelification with Carbopol® 934P, was suitable for the retention of CP in the skin. The e*x-vivo* permeation studies were performed using healthy male albino Wistar rats and this model was found to have a higher permeability character than human skin, particularly for lipophilic penetrants [69, 70]; thus, the permeation results through rat skin are usually over-expressed. The results also showed a lower irritation potential compared to a market formulation using Draize primary skin irritation test on albino rabbits [68]. Other studies also confirm no *in vitro* irritability in the hen's egg test on chorioallantoic membranes of HC formulated in MEs (both high-water-content and low-water-content); however, *in vivo* studies showed an increase in redness and irritation in the skin produced by MEs containing HC when compared with a commercial cream as well as an increase on the blanching effects [71]. The authors concluded that the use of MEs might be more useful with drugs used in conditions where irritation is negligible and in transdermal therapeutic systems.

4.1.3 Foams as delivery systems for TG

More recently, pharmaceutical foams appeared as the solution for skin drug deposition [72]. To overcome the poor drug release from the vehicles, a study investigated the capability of a thermolabile, triphasic foam delivery BMV and CP through the skin [47]. A series of *in vitro* studies have demonstrated that the new foam has the ability to deliver the active drug at an increased rate compared with other vehicles. These findings suggest that the new foam utilizes a nontraditional ‘‘rapid-permeation’’ pathway for the delivery of drugs. It is likely that components within the foam (probably the alcohols) act as penetration enhancers, and reversibly alter the barrier properties of the outer SC, thus driving the delivered drug across the skin membrane via the intracellular route. Moreover, clinical trials have demonstrated that BMV foam [73-76] and CP foam [73, 77] are safe and effective treatments for psoriasis, affecting not only scalp but also nonscalp regions of the body. The BMV and CP foams demonstrated better or

equivalent results concerning effectiveness when compared with BMV lotion and CP solution, respectively, and increased absorption speeds when compared with the same formulations above described.

The proportion of patients reporting adverse events or the incidence of treatment-related adverse events typically does not significantly differ between the foam formulations and placebo or other standard topical medications used to treat psoriasis.

Hypothalamic-pituitary-adrenal (HPA) axis studies demonstrated that BMV foam does not cause HPA suppression [73] despite two fold increase on penetration into the skin compared to BMV lotion [74] and CP foam causes no greater suppression than CP ointment [73].

Another study [78] showed that BMV foam is effective for scalp psoriasis with both once-a-day and twice-a-day uses. This feature of the BMV foam is encouraging for expected improvement in clinical use.

4.2 Nanoparticulate delivery systems

Novel drug delivery systems have been introduced in the topical delivery of drugs with special incidence on particulate carriers, which are also known as colloidal carrier systems. Much has been written about the ability of lipid-based particles and other vesicular colloidal carriers to penetrate the SC. The possibility of using such particles for topical drug delivery has been widely discussed [79-81], including glucocorticoid nanoparticulate formulations for the treatment of inflammatory skin diseases such as atopic dermatitis and psoriasis. To overcome low uptake rates, current research in the field of pharmaceutical technology investigates micro and nanoparticulate systems which do not only enhance percutaneous absorption but may even allow for drug targeting to the skin or even to its substructure. Thus, they might have the potential for an improved benefit/risk ratio of topical drug therapy [82, 83].

Müller *et al*. [84] proposed a model of film formation on the skin dependent on the particle size to explain the occlusion effect after application of solid lipid nanoparticles (SLN) onto the skin. Comparing two populations of particles, one with 2 µm and the second with 200 nm, they observed that in hexagonal packing, the uncovered surface is identical for both populations; however, the empty spaces between the microparticles are relatively large and favour the evaporation of water hydrodynamically. In contrast,

only tiny nanosized spaces exist in the nanoparticle monolayer avoiding water evaporation and exerting an occlusive effect that will promote the permeation of topically applied drugs, such as TG.

4.2.1 SLN and nanostructured lipid carriers for TG delivery

The SLN have been proposed as a promising and versatile colloidal drug carrier system intended for several administration routes and are currently extensively studied for topical application [85, 86]. The system consists of 0.1-30% (w/w) solid lipid particles in the nanometer range, which is dispersed in water and stabilized with 0.5-5% (w/w) surfactant [86]. The solid core consists of a high melting lipid matrix that contains the drug dissolved or dispersed. In general, solid lipids can be such as mono-, di- and triacyl-glycerols (e.g., glyceryl behenate; tristearin; tripalmitin), fatty acids (e.g., stearic acid), steroids (e.g., cholesterol) and waxes (e.g., cetyl palmitate) [80]. All classes of emulsifiers have been used to stabilize the lipid dispersion. It has been found that the combination of emulsifiers might prevent particle agglomeration more efficiently [81, 87].

Several studies have demonstrated the great potential of lipid nanoparticles to improve corticoid absorption by the skin (Table 2.3). Maia *et al*. [88] developed PC-loaded SLN to induce glucocorticoid targeting to upper skin strata. Three formulations were developed; the keratinocyte and fibroblast viability were used to the selection of the best formulation. The formulation containing glyceryl dibehenate (Compritol® ATO 888) and poloxamer 188 was compared with the PC cream. The PC penetration into human skin increased by 30% as compared to the cream formulation and the permeation through reconstructed epidermis increased three-fold. However, the amount of PC reaching the dermis increased even more, failing the objective of decreasing the antiproliferative action occurring mainly on dermis. The same authors demonstrated that the same lipid dispersion containing PC, when incorporated in a cream base (10:90), accelerated the immediate uptake by the skin [82]. This mechanism was reported as the basis for the epidermal targeting effect, which should be due to a polymorphic transition of the lipid structures of the nanoparticles due to water evaporation. Although an increase of PC levels in epidermis has been demonstrated, it is not clear what happens

on dermis. Apparently the levels on dermis increased even more, which is not suitable for decreasing the adverse effects of TG [88].

An important parameter to control the distribution of the drug within the skin seems to be the location of the active compound within the lipid matrix. Several methods have been employed to study the active location, such as NMR spectroscopy, electron spin resonance spectroscopy, atomic force microscopy and TEM [89]. As these methods display some disadvantages and limitations, parelectric spectroscopy (PS) was used to study the influence of BMV localization on the dermal uptake of the drug [90]. Formulations were prepared differing on the lipid and surfactant used, but only glyceryl palmitostearate (Precirol® ATO 5) and Compritol® -based particles with poloxamer 188 obey to the stability criteria (absence of phase separation and absence of drug crystallisation). The cutaneous uptake of BMV-SLN made of Compritol®/poloxamer was almost four-fold higher when compared to a BMV cream, but the BMV targeting to the epidermis was not obtained. Results obtained with PS showed that the mobility f_0 and the density ($\Delta\varepsilon$) of the permanent electric dipole moments, obtained by Debye's equation, in drug-free Compritol® and Precirol® dispersions changed in a concentration-dependent manner upon the addiction of BMV. Moreover, the striking deviation from straight lines excluded an incorporation of drug molecules in the particle cores or shell. In fact, the results demonstrated a poor particle attachment of BMV. The drug substance distribution is influenced by several factors such as its physicochemical properties, surfactant type and concentration, lipid type and production method. Some authors defend that the lipid polarity has a great influence on this parameter [91, 92]. For example, Jensen *et al*. [93] concluded that the solubility and the release of BMV from SLN depended mainly on the monoglyceride content of the lipid used. Precirol® ATO 5 and Compritol® 888 ATO are composed by a mixture of mono, di and triglycerides, the latter being the major components. BMV showed a poor solubility in these lipids and it was observed a tendency for BMV to be more soluble in lipids presented high monoglyceride content with the best result for monomyristate, which can also be linked to the surfactant properties of monoglycerides. The same trend was observed when the release profiles were studied, indicating that BMV was incorporated in the particle surface layer and not in the core; however, it was demonstrated that as the monoglyceride content of the lipids increased, the size of the SLN increased as well. Zhang *et al.* [94] formulated BMV-loaded SLN for prolonged and localized delivery of

active drugs into the skin, showing remarkable controlled release properties of monosterarin SLN and a significant epidermis drug reservoir, while beeswax SLN failed this aim. These results emphasize again the role of the lipid composition of SLN in the diffusions of corticosteroids into the skin.

Another publication [95] described the results of drug release and skin penetration from SLN of prednisolone, PC and BMV. The results showed that SLN influence skin penetration by a mechanism of interaction between drug carrier and skin surface. This interaction appears to be strongly influenced by the lipid nature and the nanosize of the carrier but not to be derived by testing drug release.

The follicular penetration of solid particles has also been studied, since hair follicles represent interesting target sites for topically applied substances. Once they penetrate into a hair follicle, particles can follow different routes, according to their size. Small particles can penetrate through the follicular epithelium into the living tissue where they may be taken up by the blood circulation. Patzelt *et al*. [15] demonstrated that the particles of medium size (643 nm) penetrated deeper into the porcine hair follicles than smaller or larger particles. It was concluded that by varying the particle size, different sites within the porcine hair follicle can be targeted selectively. The sebaceous glands are of particular interest for topical corticoids delivery due to the physiopathology of diseases like seborrheic dermatitis; however, so far there are no scientific reports in this topic. Nevertheless, a few research works have already proved that also for corticoids, the follicular pathway is of relevant importance. It has been found that when applied as a saturated aqueous solution, 46% of the HC that permeated entered the skin through follicular orifices [96, 97].

TG are used for the local treatment of skin disorders like AD and psoriasis involving a dysfunction on the main protective skin barrier and the results obtained with intact skin may find other expression than that obtained with diseased skin. This emphasizes the importance of studying the penetration profiles of corticoids both in healthy and in diseased skin. The later assumes even higher importance, as drugs will be applied for re-establishing the protective barrier. Using BMV-loaded SLN as delivery system, the reservoir effect in the skin, when the barrier was impaired, was studied indicating that the effect was more evident when the barrier was intact. However, SLN formulation was superior to the ointment in achieving a high amount of drug substance, both, in

intact and barrier impaired skin [98]. Nevertheless, most of these studies are based on *in vitro* experiments that are often not reproducible in *in vivo* testing. In fact, there is a lack of clinical studies in this area. An exceptional example is a clinical study on CP-loaded SLN (CP-SLN) in patients with eczema. In the investigation, the application of CP-SLN cream registered significant improvement in therapeutic response in terms of reduction in degree of inflammation and itching, against marketed cream [99].

SLN are also promising delivery systems for TG, presenting different advantages including: good tolerability (avoidance of organic solvents), simple and cost effective large scale production, stability (by surfactants or polymers), site-specific targeting, controlled drug release and protection of liable hydrophilic or hydrophobic drugs from degradation [100]. However, some disadvantages have been pointed out, for instance, particle growing, unpredictable gelation tendency, unexpected dynamics of polymorphic transitions, and inherent low incorporation rates resulting from the crystalline structure of the solid lipid [101, 102]. It is very important that the drug loaded to SLN is soluble in the lipid matrix. Concerning TG, they are poorly soluble drugs that can make the permeation through the skin difficult.

This drawback may be overcome by oil-loaded lipid nanoparticles (also described as nanostructured lipid carriers, or NLCs) [103]. Liquid lipids solubilize the drug to a much higher extent than solid lipids [89, 104]. Fluticasone propionate loaded-NLCs were developed with the aim of reducing adverse-side effects and improving safety profile [105]. According to drug solubility, the authors developed two systems with Precirol® as solid lipid, PEG-6-Caprylic/Capric triglycerides (Labrasol®) or PEG-8-Caprylic/Capric triglycerides (Softigen® 767) as liquid lipid, and polysorbate 80 (Tween® 80) and Soybean lecithin as surfactants. Formulations showed suitable physical characteristics in terms of particle size (between 316 and 408 nm) and entrapment efficacy (96 and 97%); however, for polydispersity index the values obtained were very high (> 0.4). Unfortunately no studies on epidermal targeting were performed. Studies with Nile red-loaded NLCs [103] showed the Nile red distribution and penetration into skin. This study also showed that epidermal targeting was achieved by NLC application. The use of NLCs could be, thus, a suitable approach to reduce adverse side effects of TG by epidermal targeting. Moreover, the content of medium

chain triglycerides seems to influence the degree of permeation since lower medium chain triglycerides content showed the highest intensity of fluorescence.

Finally, in an interesting work of co-encapsulation of two different drugs, the concomitant use of microneedles with a NLC formulation has been recently reported, which resulted in a suitable reservoir system for transdermal delivery with a good *in vivo/in vitro* correlation [106].

Table 2.3. Glucocorticoid drug molecules incorporated in SLN and NLC.

Components	Drug	Size (nm) and PI	Entrapment efficiency (%)	*In vitro* tests	Aim / Achievements	Ref
Compritol® ATO888 / Poloxamer®F68	PC	144 / 0.34	> 90	**% of cell viability** (MTT in keratinocyte) after 6h: 87.5 ± 4.7 **Permeation through human skin:** 30% increase comparing with PC cream	Epidermal targeting / failed	88
Precirol® / Poloxamer®F68	PC	154 / 0.54	> 90	**% of cell viability** (MTT in keratinocyte) after 6h: 87.3 ± 7.0		
Dynasan 114-Lipoid S75 / Poloxamer®F68	PC	206 / 0.17	> 90	**% of cell viability** (MTT in keratinocyte) after 6h: 35.6 ± 1.7		
Compritol® ATO888 / Poloxamer®F68 in a cream base (10:90)	PC	144 / 0.34	> 90	**Permeation through reconstructed epidermis:** 6.65± 2.86% after 6h for SLN suspension and 0.82 ± 0.29% for native PC. **Penetration into excised human skin:** 6.90 ± 0.38% at 6 h and 14.83 ± 3.76% at 24 h for PC suspension in a cream base versus 1.60 ± 1.20% at 6 h and 7.53 ± 1.64% at 24 h in a PC cream.	Epidermal targeting / achieved	82
Precirol® and Compritol® ATO888 / Poloxamer®	BMV	300 / 0.22 one week after preparation and 300 /0.47 after 3 months.	n.a.	**Cutaneous absorption through human skin:** 4 fold increased penetration into the first and second 100 µm layer compared with a cream.	**1**.Increased cutaneous uptake / achieved **2**.Epidermal targeting / failed	90
Dynasan® 114 / Tween® 80	BMV	151 / 0.21	n.a.	**Release studies with cellulose membrane:** < 5 % after 6 h and 23.7% after 24h	**1.** Influence of lipid composition on the drug solubility / solubility increases with increase in monoglyceride content	93
Rylo™ MG 14 Pharma/ Dynasan® 114/ Tween® 80	BMV	250 / 0.25	n.a.	**Release studies with cellulose membrane:** < 5 % after 6 h and 20.7% after 24h		
Dynasan® 118/ Tween® 80	BMV	200 / 0.20	n.a.	**Release studies with cellulose membrane:** < 5 % after 6 h and 12.8% after 24h.	**2**. Influence of lipid composition on the drug release /	

Precirol® ATO 5/ Tween® 80	BMV	175 / 0.22	n.a.	**Release studies with cellulose membrane:** < 5 % after 6 h and 16.0% after 24h	highest release with high monoglyceride content	
Tegin® 4100 / Tween® 80	BMV	461 / 0.48	n.a.	**Release studies with cellulose membrane:** 11.7% after 6 h and 31.9% after 24h	**3**. Influence of lipid composition on the physicochemical properties / high content of monoglyceride creates more unstable particles	
Monostearin and Lecithin	BMV	136 / < 0.30	90 – 94	**Permeation through human epidermis:** J = 0.155 ± 0.009 µg/cm² h	Prolonged and localized delivery of the BMV into the skin / achieved only for SLN containing monostearin	94
Beeswax and Lecithin	BMV	126 / < 0.30	37 – 39	**Permeation through human epidermis:** J = 0.397 ± 0.037 µg/cm² h		
Compritol® ATO888 / Poloxamer®	Prednisolone	173 / 0.14		**Release studies with polyamide membrane after 8h,** flux, µg/cm²/h$^{0.5}$: 43.44 ± 7.42 and 9.90 ± 0.80, SLN and a cream base, respectively. **Human skin uptake after 6h (µg):** 1.72 ± 0.54.	**1**. Examine the TG-particle interaction and its influence on skin penetration / SLNs influence skin penetration by an intrinsic mechanism linked to a specific interaction of the drug-carrier complex and the skin surface.	95
Compritol® ATO888 / Poloxamer®	PC	173 / 0.14		**Release studies with polyamide membrane after 8h,** flux, µg/cm²/h$^{0.5}$: 14.62 ± 1.69 and 1.16 ± 0.14 SLN and a cream base, respectively. **Human skin uptake after 6h (µg):** 2.06 ± 0.70.		
Compritol® ATO888 / Poloxamer®	BMV	173 / 0.14		**Release studies with polyamide membrane after 8h,** flux, µg/cm²/h$^{0.5}$: 7.30 ± 1.59 and 0.65 ± 0.07 SLN and a cream base, respectively. **Human skin uptake after 6h (µg):** 1.37 ± 0.29.	**2**. Epidermal targeting / achieved for prednisolone and PC loaded SLN.	
Dynasan® 116 / Tween® 80	BMV	212 / 0.16	n.a.	***In vitro* penetration through porcine skin** % of BMV after 24 h in the SC: 27 versus 19 for a BMV ointment. % of BMV after 24 h in the receptor medium: < 1 versus 8.4 for a BMV ointment. ***In vitro* penetration through barrier-impaired porcine skin** % of BMV after 24 h in the SC: 40 versus 20 for a BMV ointment. % of BMV after 24 h in the receptor medium: 17 versus 15 for a BMV ointment.		
Precirol® ATO 5 / Tween® 80	BMV	151 / 0.19	n.a.	***In vitro* penetration through porcine skin** % of BMV after 24 h in the SC: 38 versus 19 for a BMV ointment.	**1**.Increase the amount of drug kept in skin / achieved **2**.Create a reservoir	

				% of BMV after 24 h in the receptor medium: < 1 versus 8.4 for a BMV ointment. ***In vitro* penetration through barrier-impaired porcine skin** % of BMV after 24 h in the SC: 50 versus 20 for a BMV ointment. % of BMV after 24 h in the receptor medium: 14 versus 15 for a BMV ointment.	of the drug in the SC / achieved with more evidence in intact skin and dependent on lipid properties	98
Cetylpalmitate / Tween® 80	BMV	179 / 0.12	n.a.	***In vitro* penetration through porcine skin** % of BMV after 24 h in the SC: 37 versus 19 for a BMV ointment. % of BMV after 24 h in the receptor medium: < 1 versus 8.4 for a BMV ointment. ***In vitro* penetration through barrier-impaired porcine skin** % of BMV after 24 h in the SC: 55 versus 20 for a BMV ointment. % of BMV after 24 h in the receptor medium: 22 versus 15 for a BMV ointment.		
SLN in a cream base	CP	177 / n.a.	92	***In vivo* studies:** 1.9-fold inflammation; 1.2-fold itching increase in terms of percent reduction in degree of inflammation and itching against marketed cream.	Improvement in therapeutic response compared to market cream / achieved	99
Precirol® / Labrasol® / Tween® 80 and Soybean lecithin	FP	Day 2: 400 / 0.90 Day 30: 343 / 0.51	96	n.a.		
Precirol® / Softigen®767 /Tween® 80 and Soybean lecithin	FP	Day 2: 316 / n.a. Day 30: 388 / 0.37	97	n.a.	Reduction of adverse effects / not demonstrated	105

4.2.2 Polymeric nanoparticles intended for TG delivery

Nanoparticles composed of polymeric materials have been extensively investigated for their use in delivery and controlled release of low-molecular-weight drugs, peptides and nucleotides via oral, topical and parental routes [107, 108].

The most common types of polymer used for the production of nanoparticulate systems are FDA-approved hydrophobic materials such as poly-lactic acid, poly (lactide-co-glycolide), poly(ε-caprolactone), chitosan, and a combination of chitosan and poly(gamma-glutamic acid) and (gamma-PGA) [109]. These polymeric carriers can

potentially i) protect labile compounds from premature degradation, ii) provide controlled and sustained release via modification of polymer composition, iii) increase localized targeting hence reducing systemic absorption, and iv) reduce irritation [110].

Despite the apparent advantages of polymer-based nanoparticulate delivery systems, these still appear rather unexplored and only a handful have been evaluated for the delivery to superficial or deeper skin layers. Concerning TG only a few studies exist, for instance, Senyiğit *et al*. [111] developed lecithin/chitosan nanoparticles intended for topical delivery of CP. They found that the skin accumulation of CP was significantly increased with this delivery system using pig ear skin, when compared with CP chitosan gel and commercial creams. Moreover, this accumulation was more evident in epidermis, which contributes to decrease side effects of steroids.

Recently, Abdel-Mottaleb *et al*. [112] reported the behaviour of polymeric submicron particles for selective betamethasone delivery to the inflamed skin. Polymeric particles of nominal diameters from 50 to 1000 nm were administered to an experimental dithranol-induced dermatitis inflammation model in mice ears. The results revealed that smaller particles had three-fold stronger and deeper penetration tendency with a preferential accumulation in inflamed skin hair follicles and sebaceous glands.

4.2.3 Liposomes and other vesicles

Liposomes are vesicular systems composed of bimolecular phospholipid layers enclosing aqueous compartments. Classical or deformable liposomes have shown their ability to increase permeation of topically applied drugs. Since the 1980's liposomes are assumedly selective drug delivery systems for the topical route of administration of glucocorticoids. In the early 1980's Mezei *et al*. [113, 114] started the research into the use of liposomes for topical skin application. Comparisons between liposomal and conventional formulations of triamcinolone acetonide (TCA) were tested in two *in vivo* rabbit studies, and for both cases, with the application of the liposomal preparations a greater steroid concentration in the epidermis and dermis and less systemic absorption than the conventional formulations were achieved. Interestingly, almost 30 years later, TCA liposome formulations are still under investigation. Recent formulation studies deal with encapsulation parameters of multilamellar liposomes as delivery vehicle for this drug [115].

Encapsulation of various glucocorticoids in liposomes enhanced their delivery into animal skin. The formation of a large drug reservoir in the skin obtained with liposome deposition can be considered for local treatment. The incorporation of HC and fluocinolone acetonide into liposomes resulted in the increased uptake into the cornified layer of hairless mice and/or guinea pigs [116, 117]. Liposomes thus increase local and decrease systemic drug concentration [118]. Cortisol-loaded liposomes presented a very much improved concentration-time profile in the different layers of human skin when compared with conventional cortisol in the ointment [119].

Liposomal preparation of BD was tested in patients with atopic eczema and in patients with psoriasis vulgaris. In eczema, the liposome preparation tended to reduce erythema and scaling more than the conventional gel used for comparison, however, did not improve the efficacy of BD in psoriasis [120].

Glucocorticoid association to liposomes was also studied in ulcer treatment and wound healing. Dexamethasone sodium phosphate liposomes were tested *in vivo* in the treatment of oral ulcers. Liposomes increased local and decreased systemic drug concentration and localized the drug in the ulcerated area [121]. The local administration of prednisolone phosphate (PLP) liposomes was tested in rat wounds, comparative to free PLP. The results showed that a single application of liposomal PLP (not observed for free PLP) applied direct after wounding could reduce wound contraction, suggesting a potential therapeutic effect in burn wounds [122].

The use or absence of occlusive conditions for topical application of liposomal HC was evaluated and the results demonstrated that penetration depth of the drug into the SC was not affected significantly by the application conditions, however, both excessive dehydration and hydration of the liposomes should be avoided in the topical application of liposomal formulations for efficient delivery of HC to the skin for a prolonged period of time [123].

The use of liposomes may be helpful as it is known that macrophages internalize particulate carriers by endocytosis as secondary drug depot, helping in localized delivery of the drug [124]. Langerhans cells are dendritic cells located in the skin, specialized for processing antigens. Moreover, in skin inflammatory disorders, inflammatory macrophages could be attracted to this compartment. If topically applied liposomes could reach deeper skin layers intact, which is only possible with very

deformable vesicles able to pass through SC of intact skin and reach epidermis and deep dermis, liposomes uptake by macrophages in the dermis may occur [125].

HC and dexamethasone were formulated in very deformable vesicles, Transfersomes®, and their biological activity were tested in an acute cutaneous inflammation model. Additionally, the drug biodistribution data were reported. The results showed an increase in biological potency, prolonged effect and reduced therapeutic dosage, comparative to commercial HC and dexamethasone products. In this case the non-occlusive application had to be assured to promote drug transport into the skin [45]. Same authors have also published the biological activity of TCA-loaded Transfersomes® and topical use of such system was found to reduce the necessary drug dosage and prolonged biological response time was achieved, comparing to TCA in commercial lotion [126]. These deformable vesicles primarily developed for transdermal delivery have achieved very good results on controlling drug deposition into the skin [127, 128].

The biological findings using this type of modified liposome drug carriers are connected to deformable vesicle features resulting from the presence of edge-active substances, surfactants, on their composition.

The appearance of Transfersome® studies dates back to early 1990's. However, the development of liposome-like carriers introducing some modifications on their membranes in order to create elastic, deformable, or electrostatic favourable structures to promote skin delivery is still under extensive research. This is also true for the promotion of skin delivery of glucocorticoids. Archaesomes, designating lipid lamellar vesicles made from archaea polar lipids, as carriers of BD were compared to conventional phospholipid liposomes for *in vitro* skin permeation, and archaeosomes appeared as the most effective carrier for the incorporated glucocorticoid [129]. Skin-lipid liposomes, non-phospholipid-based vesicles made up of lipids commonly occurring in the lipid pool of human SC, were also proposed as possible dermal glucocorticoid delivery systems. HC, betamethasone, and TCA were incorporated in skin-lipid liposomes and the results of dermal delivery, body distribution, and biological effectiveness in guinea pigs were compared with those of phospholipid-based formulations and semi-solid dosage forms. Comparative to the other formulations, skin-lipid liposomes provided the highest drug disposition within the deeper skin layers, higher blanching effect, and a reduction in drug levels in the blood and urine [130]. It

has been outlined that skin drug penetration can be influenced by modifying the surface charge of liposomes. A recent study showed the potential of negatively charged liposomes to enhance the skin penetration of betamethasone and BD using confocal microscopy to visualise the penetration of fluorescently labeled liposomes [131]. Betamethasone was encapsulated, as well, either alone into the lipid bilayer or in the aqueous compartment of liposomes by the help of betamethasone–cyclodextrin complexes. The use of drug–cyclodextrin inclusion complexes enhanced the stability of the formulation but did not improve the penetration of betamethasone than the corresponding formulation containing betamethasone alone [132].

Niosomes, also known as non-ionic surfactant vesicles, are concentric vesicles in which an aqueous volume is entirely enclosed by a membranous bilayer mainly composed of non-ionic surfactants and cholesterol. Like liposomes, niosomes can be used to deliver both hydrophobic and hydrophilic drugs and are claimed to increase skin penetration of drugs, acting as local depot for sustained release of dermal active compounds. A gel prepared with niosomes containing CP presented a sustained and prolonged drug action compared to a CP marketed gel [133].

5. Conclusion

Since most dermatological preparations represent a mixture of several materials that are not miscible, they often form dispersed systems that are thermodynamically unstable. Several approaches have been made in order to improve stability, drug release, permeation and the benefit/risk ratio of topical corticosteroid treatment. The pharmaceutical development of such carriers represents an enormous challenge.

The vehicles for TG delivery have to obey several demands:

- presence of specific ingredients: emollients, preservatives, soft surfactants,
- presence of other active ingredients: cutaneous antiseptics, local anti-inflammatory or immunomodulating agents and/or local antibiotics,
- limitation of the transepidermal water loss, film formation properties, which influences bio-availability and local tolerance,
- avoid the degradation of the corticoid that is a real challenge due to solubility limitations and pH stability.

Also, there is a lack of comparative studies between different formulations, which makes it difficult to understand what are the critical physicochemical factors to take into account when designing delivery systems dedicated to topical application, and, more importantly, how these different factors interact with each other. Furthermore, it is difficult to interpret and correlate each delivery system because there is also a lack of quantitative percutaneous data. Considering that most of the formulations are intended for skin disease treatment, surprisingly, the available data on the performance of glucocorticoids delivery systems on inflamed skin is scarce. These limited answers related to corticoid delivery systems should be addressed in future research.

During the past 30 years, pharmaceutical scientists and technologists concentrated on the design, development, validation, and manufacture of various traditional pharmaceutical formulations. Consistent with the technological advancements during the past 10 - 15 years, numerous formulations and drug delivery concepts emerged for enhanced therapeutic applications. Examples include vehicles using nanotechnology. Because these formulations are relatively new, the industry needs to address several challenges such as storage, handling, and manufacturing while assessing their stability, compatibility, and scale-up and manufacturing issues before commercial distribution.

The interface between formulation science and engineering will continue to be at the frontier of new product development, with applications extending ever further into targeted delivery and monitoring, although the cost-benefit of such developments is difficult to anticipate. On the other hand, the blockbuster business model for drug discovery and development is unlikely to be sustainable as increased R&D costs are coupled with a disproportionately lower financial yield from new pharmaceutical products.

It is our opinion that formulators should be focused on the development of reliable dosage forms that could be easily scaled-up to industry or even the development of vehicles that could allow the decrease of production costs.

From this extended data collection in the field of TG delivery systems, one may conclude that vehicles presenting epidermal targeting and lower dermis uptake, with reliable features that can be easily industrialized, are still on demand.

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1. Introduction

According to the International Conference on Harmonization (ICH) Q8 (R2) [1], the aim of the pharmaceutical development is to design a product with quality and to define its manufacturing process to consistently deliver the intended performance of the product. The information and knowledge gained from pharmaceutical development studies and manufacturing experience provide scientific understanding to support the establishment of the design space, specifications, and manufacturing controls. In recent years, the "quality by design" concept has been introduced by the ICH Q8 guideline. This guideline has recommended the establishment of a science-based rationale in pharmaceutical development studies for both, formulation development and manufacturing process development.

The selection of ingredients and their assembly, the selection of the production method and the stability assessment of the formulations is an ambitious process. It is the responsibility of the formulator to design a drug-delivery system capable of consistently achieve the desired pharmacokinetic–pharmacodynamic profile as an outcome of formulation and manufacturing process development and optimization. To achieve a robust formulation and reproducible product manufacturing process in which the product quality specifications are consistently met is not always an easy and straightforward exercise [2].

The development of emulsions intended for dermatological purposes encompasses not only the development of a vehicle with suitable characteristics for topical application, such as the spreadability and physical stability, but also the assurance that the vehicle provides a good environment for the drug which is translatted into a suitable chemical stability.

It is well known that emulsions are thermodynamically unstable. They possess a positive interfacial free energy and, in order to reach thermodynamic equilibrium, will continually attempt to separate back into their original oil and water phases. Thus, to manufacture a consistent dermatological product with a realistic shelf-life, the formulator must attempt to delay this separation process by kinetically stabilizing the emulsion [3].

To provide kinetic stability to emulsions, several excipients may have an important role. Surfactants, co-emulsifiers, and/or polymers, when incorporated in these systems, can delay phase separation, by forming an interfacial film and/or a rheological barrier.

A high-quality product often needs a high concentration of surfactant(s), however, the intrinsic toxicity of these ingredients limit their use. The incorporation of other molecules that allow the minimization of the concentration of surfactant is an advantage. It is well established that the incorporation of another amphiphilic substance, such as co-emulsifier, enables the adjustment of the surfactant's efficiency and its concentration to form an emulsion [4]. Another approach involves the maximization of the amount of surfactant in the hydrophilic-lipophilic balance (HLB) region of 9-13, where the surfactant solubility is between the high oil solubility and high water solubility. This results in most surfactant partitioning to the interface as opposed to partitioning into the bulk phase, and allows the stabilization of a larger interfacial area [5]. Another possibility involves the co-stabilization by adding appropriate macromolecules or polymers also to form structured interfacial films, which prevent the coalescence of oil drops [6]. However, decreasing the concentration of the surfactant in an emulsion often leads to the inclusion of a large number of additional ingredients with different natures. This makes more difficult to predict interactions between excipients and thus, to understand the complex microstructure of the emulsion.

The selection of multifunctional components may help to solve these problems. For instance, cationic surfactants such as quaternary ammonium compounds have the ability to disrupt the microorganism's membranes [7]; glycols are powerful solubilizing agents used in several dosage forms, as well as penetration enhancers [8, 9], and polymers may have a double rule: improve the stability by the addition of a yield value and also prevent the crystallization of poorly soluble drugs such as the glucocorticoids.

2. Materials and methods

2.1 Materials

MF was purchased from Cristal Pharma (Spain). Ethoxydiglycol (Transcutol® CG), caprylocaproyl macrogol-8 glycerides (caprylocaproyl) (Labrasol®) and glyceryl dibehenate and tribehenin and glyceryl behenate (Compritol® 888 ATO) were a kind

gift from Gattefossé (France); 2-methyl-2,4-pentanediol (99% grade) (pentanediol), pentane-1,5-diol, 1-propanol, 1,4-butanediol (99% grade), hydroxypropylmethylcellulose (HPMC) and hydroxypropylcellulose (HPC) were obtained from Sigma Aldrich, (Germany); Bis-PEG/PPG-16/16 PEG/PPG-16/16 dimethicone and caprylic/capric triglyceride (Abil® Care 85), Bis-PEG/PPG-20/5 dimethicone; methoxy PEG/PPG-25/4 dimethicone; caprylic/capric triglyceride (Abil® Care XL), C12-15 alkyl benzoate (Tegosoft® TN), PEG-20 glyceryl laurate (Tagat® L2), PEG-12 dimethicone (BRB 526), PEG-18 glyceryl oleate/cocoate (Antil® 171), polyglyceryl-4-isostearate (Isolan® GI 34), polyethylene glycol (PEG) 400 and cetrimide BP were a kind gift from Evonik Industries AG (Germany). Methyl vinyl ether/maleic anhydride copolymer crosslinked with decadiene (PVM/MA) (Stabileze® QM) was a gift from ISP (EUA). Isopropyl myristate (IPM), isopropyl alcohol, sorbitan oleate (Span® 80) and polysorbate 80 (Tween®80) and propylene glycol were obtained from José Vaz Pereira, S.A., (Portugal). All other reagents were of pharmaceutical or HPLC grade and used as received. Deionized water was obtained by inverse osmosis (Millipore, Elix 3).

2.2 Methods

2.2.1 Manufacturing process

Several methods suitable for the production of emulsions were considered. Among them the hot and the cold processes were compared regarding industry benefits and scale-up process. The cold process method is described in section 2.2.4.

2.2.2 Pre-formulation studies

2.2.2.1 Selection of cellulose polymers

Two gels, differing in the gelling agent (HPMC or HPC; 1.5% w/w) where prepared containing MF (0.1 % w/w), water, isopropyl alcohol and propylene glycol (40:40:20). The gels were prepared by dispersing the gelling agent (HPMC or HPC) into water and, separately, the MF was dispersed into the mixture of isopropyl alcohol and propylene glycol. This second mixture was then added to the first preparation. The two gels, stores

at 22 ºC, were evaluated by optical microscopy (Olympus CX40 microscope with soft imaging system Cell D, New York, US), concerning the crystallization of the drug just after the preparation and 15 days after preparation. The permeation profiles of MF from the gels were assessed through silicone membrane and human skin (Chapter V – section 2.2.3).

2.2.2.2 Selection of glycols

2.2.2.2.1 Solubility studies

- MF was added to several solvents or mixtures of solvents: pentanediol/caprylocaproyl (7:3 w/w); ethoxydiglycol; PEG 400; pentanediol; propylene glycol; 1-propanol; 1,4-butanediol; pentane-1,5-diol; PEG-20 glyceryl laurate; PEG-12 dimethicone and PEG-18 glyceryl oleate/cocoate until saturation. Saturation was achieved when excess solid persisted for more than 12 h with a constant shaking at 22ºC.
- MF was added to a series of ethoxydiglycol – water; pentanediol/caprylocaproyl (7:3 w/w) – water and pentanediol – water mixtures varying from 100% of water to 100% of the glycol and stirred at 22ºC during 48h.

After ensuring that the solute-solvent equilibrium had been reached, the solutions were centrifuged (Medifuge, Heraeus Sepatech, GmbH, Germany) at 4000 rpm during 10 min and the supernatant solution diluted with methanol (1:10) and analyzed by HPLC.

2.2.2.2.2 Microscopy analysis

Three mixtures were prepared of 10% (w/w) of the glycol in water (ethoxydiglycol – water, pentanediol / caprylocaproyl (7:3 w/w) – water and pentanediol – water) and the MF was dispersed in each mixture (0.1% w/w).

A computerized image analyzing device was used for the microscopic observations of the three mixtures, which was connected to a Polyvar microscope (Rheichart-Jung, Vienna, Austria) between crossed polars. Samples were stored at 22º C and examined 5 days after preparation at a magnification of 200x.

2.2.2.2.3 Stability of MF in the selected glycols

MF at 0.1% (w/w) was dispersed in PEG 400; pentanediol/caprylocaproyl (7:3 w/w) pentanediol and ethoxydiglycol. At predetermined times (0, 30, 60 and 90 days)

samples were collected and quantified by HPLC. Each mixture was submitted to different conditions: room temperature (22 ºC); 22 ºC at pH 4 and pH 5 and stress conditions (40 ± 2ºC; 75 ± 5% relative humidity).

2.2.2.3 Data analysis

The data was analyzed using the ANOVA test (Kaleida Graph, version 4.0, Synergy Systems) and expressed as the mean ± SD (standard deviation); $p < 0.05$ was considered to be statistically significant.

2.2.2.4 Selection of cetrimide concentration

Two concentrations of cetrimide were assessed (0.075 and 0.600 % w/w) by a test of efficacy of antimicrobial preservation [10]. Two formulations of 11 A, (one containing 0.075 and the other 0.600 % (w/w) of cetrimide), two formulations of 11 B (one containing 0.075 and the other 0.600 % (w/w) of cetrimide) and their respective placebos without cetrimide (PA and PB) were tested. Each formulation was contaminated with *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538), *Candida albicans* (ATCC 10231) and *Aspergilus niger* (ATCC 16404) obtained from the ATCC bacterial strain collection. Antimicrobial activity was measured throughout the log reduction of the colony-forming units (cfu) at 0h, 48h, 7 days, 14 days and 28 days.

2.2.3 Formulations development

38 emulsions were developed in order to select the mixture of excipients that provide the best conciliation between physical and chemical stability.

2.2.3.1 Required HLB of oil (IPM / alkyl benzoate) mixture

The selection of the emulsifier system was based on the HLB concept, where the required HLB of the oil is equivalent to the HLB of the emulsifiers. The oil is a mixture of 50% alkyl benzoate and 50% IPM. The required HLB value of this mixture was calculated using literature values for IPM (HLB = 12) [11] and experimental values derived for alkyl benzoate. To obtain the HLB of alkyl benzoate, matched pairs of Span® 80 (HLB = 4.3) and Tween® 80 (HLB = 15) were prepared in order to obtain a

range of HLB values between 6 and 13 approximately. Each mixture of 2 g of surfactants (Span® 80 and Tween® 80) was dispersed in 5 g of water and then 3 g of alkyl benzoate was added to each mixture. The resultant mixtures were homogenized in a vortex (Heidolph® Reax 2000, Germany) and the HLB value of the most stable emulsion (i.e. the emulsion that took the longest time to separate) was considered to be the HLB of alkyl benzoate.

The main surfactant was selected matching the HLB of the oils being emulsified and the surfactant.

2.2.3.2 Physical and chemical characterization of emulsions

2.2.3.2.1 Appearance and physical stability

The macroscopic appearance of each formulation was visually analyzed and used as first stability indicator. The second parameter evaluated concerning the stability was the submission of the samples under centrifugation (Sigma 112 microcentrifuge, Sigma Laborzentrifugen GmbH, Germany) during 5 min at 12000 rpm. In addition, samples were stored at 22 ± 3 ºC for 15, 30 and 60 days to examine the real time stability.

2.2.3.2.2 Determination of the pH values

The pH of the most stable emulsions was measured using a pH meter (pH Meter 744, Metrohm®, USA), with a glass electrode.

2.2.3.2.3 Assay of MF

HPLC with UV detection was used to assay MF in the formulations. The method used was adapted from [12]. A chromatograph Merck - Hitachi (diode array detector, pump and software), with an Inertsil C8 - 5μm – 4.6x150 mm column (GL Sciences, Japan) was used. The analysis was performed at room temperature. Test conditions were: mobile phase - water: methanol (30:70, v/v), flow rate – 1.5 mL/min, injection volume - 10 mL and UV detection at 248 nm.

2.2.3.2.4 Analytical centrifugation of emulsions

Emulsions of the series 9, 10, 11 and 12, were compared by analytical centrifugation (LUMiSizer®, L.U.M. GmbH, Germany). The samples were analyzed employing the

STEP-Technology®, using 2300 x g at 20 ºC, which allows the measure of the intensity of the transmitted light as function of time and position over the entire sample length simultaneously. Briefly, the light source sends out parallel NIR-light which is passed through the sample cells lying on the rotor. The distribution of local transmission is recorded over the entire sample length by the charge-coupled device line detector. The data are displayed as function of the position within the sample, as the distance from the centre of rotation (transmission profiles). This phenomenon is expressed by the value of the slope (%/hour).

2.2.4 Preparation of the final emulsions

The o/w emulsions were prepared initially by the preparation at room temperature (cold process) of an oil liquid phase (19 g), achieved by dissolving the surfactant (bis-PEG/PPG-16/16 PEG/PPG-16/16 dimethicone and caprylic/capric triglyceride) and the co-emulsifiers (PEG-20 glyceryl laurate or polyglyceryl-4-isostearate) in the oils (IPM and C12-15 alkyl benzoate) and mixing at 700 rpm (MR 3001, Heidolph, Germany) for about 15 minutes.

Next, an aqueous phase (81 g) was prepared, at room temperature, by dispersing the aqueous thickening agents (HPMC and PVM/MA) in water at 1000 rpm (MR 3001, Heidolph, Germany). The cetrimide (0.075 % w/w) and the glycol (pentanediol or ethoxydiglycol), with or without MF at 0.1% (w/w), were added to the aqueous solution and the resulting mixture was homogenized until a clear homogeneous gel was achieved.

The emulsification phase was performed at room temperature by slowly adding the oil phase to the aqueous phase with high shear mixing at a rate of 12800 rpm/min (IKA® T25 Ultra Turrax). This addition was done at uniform rate over a period of 5 minutes. The final pH was adjusted to 4 with NaOH.

3. Components of the drug product

3.1 Drug substance

MF is a synthetic, lipophilic, 16 alpha methyl analogue of beclomethasone [13] (Fig. 3.1), classified as class III (European Classification) or potent glucocorticoid for dermatological use. This topical steroid is currently available in dermatologic, nasal, and oral preparations. *In vitro* studies have shown that MF is among the most potent glucocorticoids in inhibiting cytokine production, histamine release, and eosinophil survival [14] and for binding to the glucocorticoid receptor and stimulation of gene expression associated with the anti-inflammatory response [15].

The systemic bioavailability of MF is claimed to be negligible, leading to a minimal potential for systemic adverse effects [16].

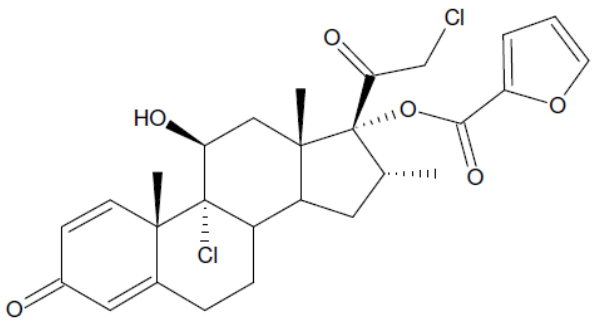


Fig. 3.1. Chemical structure of MF [17].

MF is a white or almost white crystalline powder, with the chemical name 9,21-dichloro-11β-hydroxy-16α-methyl-3,20 dioxopregna-1,4-dien-17-yl furan-2-carboxylate pregna-1,4-diene-3,20-dione, 9,21 dichloro-17-[(2-furanylcarbon-yl)oxy]-11-hydroxy-16-methyl-, (11β,16α). The main chemical-physical properties of MF are described in Table 3.1.

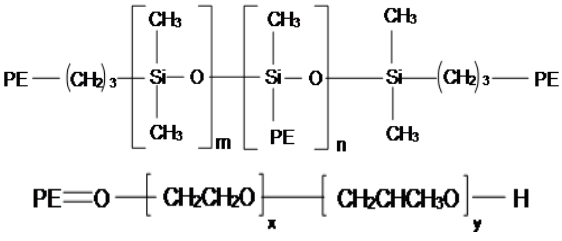
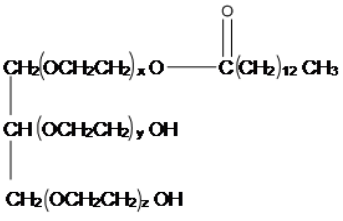
Table 3.1. Chemical and physical properties of MF according to its drug master file [17] and Index Merk [18].

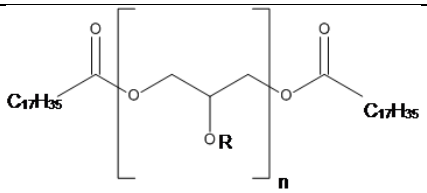
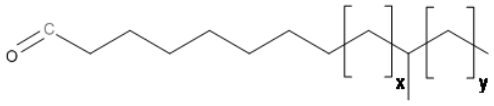
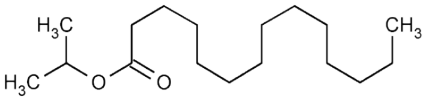
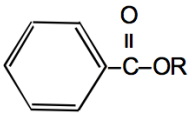
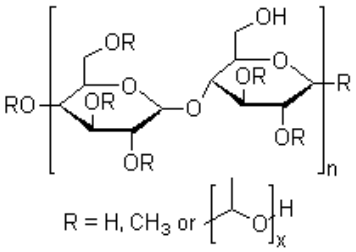
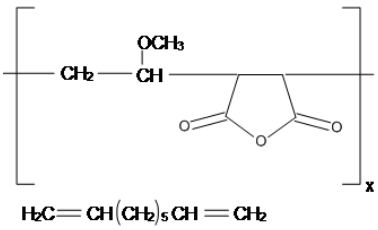
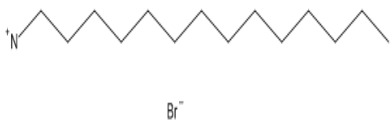
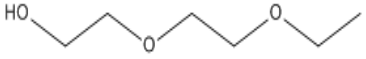
Molecular formula	$C_{27}H_{30}Cl_2O_6$
Molecular weight	521.44 g/mol
Solubility	Practically insoluble in water, soluble in acetone and in methylene chloride, slightly soluble in alcohol.
Polymorphism	No polymorphic form has been detected by X-ray diffraction and differential scanning calorimetry.
Melting point	218-220 ºC
Log P	2.81 (estimated) [19]
Maximum absorption	247 (methanol)

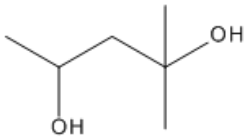
3.2 Excipients

The final developed formulations of MF contain the excipients described in Table 3.2.

Table 3.2. Excipients used in the final emulsions with their chemical structure.

Emulsion excipients	**Trade name / Abbreviation**	**Main functions / Additional functions**	**Chemical structure**
Bis-PEG/PPG-16/16 PEG/PPG-16/16 Dimethicone (and) Caprylic/Capric Triglyceride	Abil® Care 85 / Polymer modified silicone surfactant	Non-ionic surfactant / sensorial modifier (possible to use in cold processed emulsions)	
PEG-20 glyceryl laurate	Tagat® L2 / PGL	Non-ionic co-emulsifier	 Where x, y, z has an average value of 20

Polyglyceryl-4-isostearate	Isolan® GI 34 / PGIS	Non-ionic co-emulsifier	 n = 2-4 R = H or  x+y=7
Isopropyl myristate	n.a. / IPM	Oil internal phase / penetration enhancer	
C12-15 Alkyl Benzoate	Tegosoft® TN / alkyl Benzoate	Oil internal phase	 R is a C12 to C15 primary or branched alkyl group
Hydroxy propyl methyl cellulose	n.a. / HPMC	Thickening agent / Polymeric emulsifier, control of drug crystallization	
Methyl vinyl ether/maleic anhydride copolymer crosslinked with decadiene	Stabileze® QM / PVM/MA	Thickening agent / Polymeric emulsifier	
Cetrimide BP	n.a. / cetrimide	Preservative / Cationic surfactant	
Ethoxydiglycol	Transcutol® CG / n.a.	Penetration enhancers	

2-methyl-2,4-pentanediol	n.a. / pentanediol	/ solubilize poorly water soluble drugs	
Water	n.a.	Aqueous external phase	

During the pharmaceutical development a variety of excipients were reviewed and used in the formulations in order to prepare and optimize the eventual excipient mixture and the product properties.

All the above excipients are recognized as safe materials for human administration, being regarded as non-irritant and nontoxic on the amounts presented (Chapter VII).

4. Drug product

4.1 Manufacturing process

According to the report of a conference organized by the Board of Pharmaceutical Sciences of the International Pharmaceutical Federation [20] concerning the pharmaceutical sciences in 2020, the 'blockbuster' model is unlikely to be sustainable as increased R&D costs are coupled with a disproportionately lower financial yield from new pharmaceutical products. There is a need to change some drive forces that might determine how the pharmaceutical sciences will look in 2020, especially the efficiency of the manufacturing processes allowing the provision of cheaper development costs.

Regarding this scenario, the benefits of cold process emulsions are many and varied.

The structure of emulsions containing non ionic surfactants, prepared by the cold process emulsification, is easier to control. The benefits of cold processed emulsions are not limited to the ease of structure control, but they also allow a decrease in the production costs. As they are easier to process, due to the elimination of the heating and cooling down phases, the time of production can be decreased, increasing production capacity as well as decreasing the energy and water consumptions. Kurt, 2009 [21], showed that the cold process emulsification saves, on average, more than 75 % of the

energy compared to a hot process, as well as saves a significant amount of time. This process has also environmental advantages, as the use of less energy means lower emissions of CO_2.

However, only a limited number of excipients can be used in such emulsions. They need to be either liquids, or readily soluble in the oil and water phases. Wax-like materials commonly used in dermatological emulsions which need to be melted, such as cetostearyl alcohol, cannot be used.

For all these reasons, the manufacturing method selected was the cold process of emulsification and the flow chart is represented in Fig. 3.2.

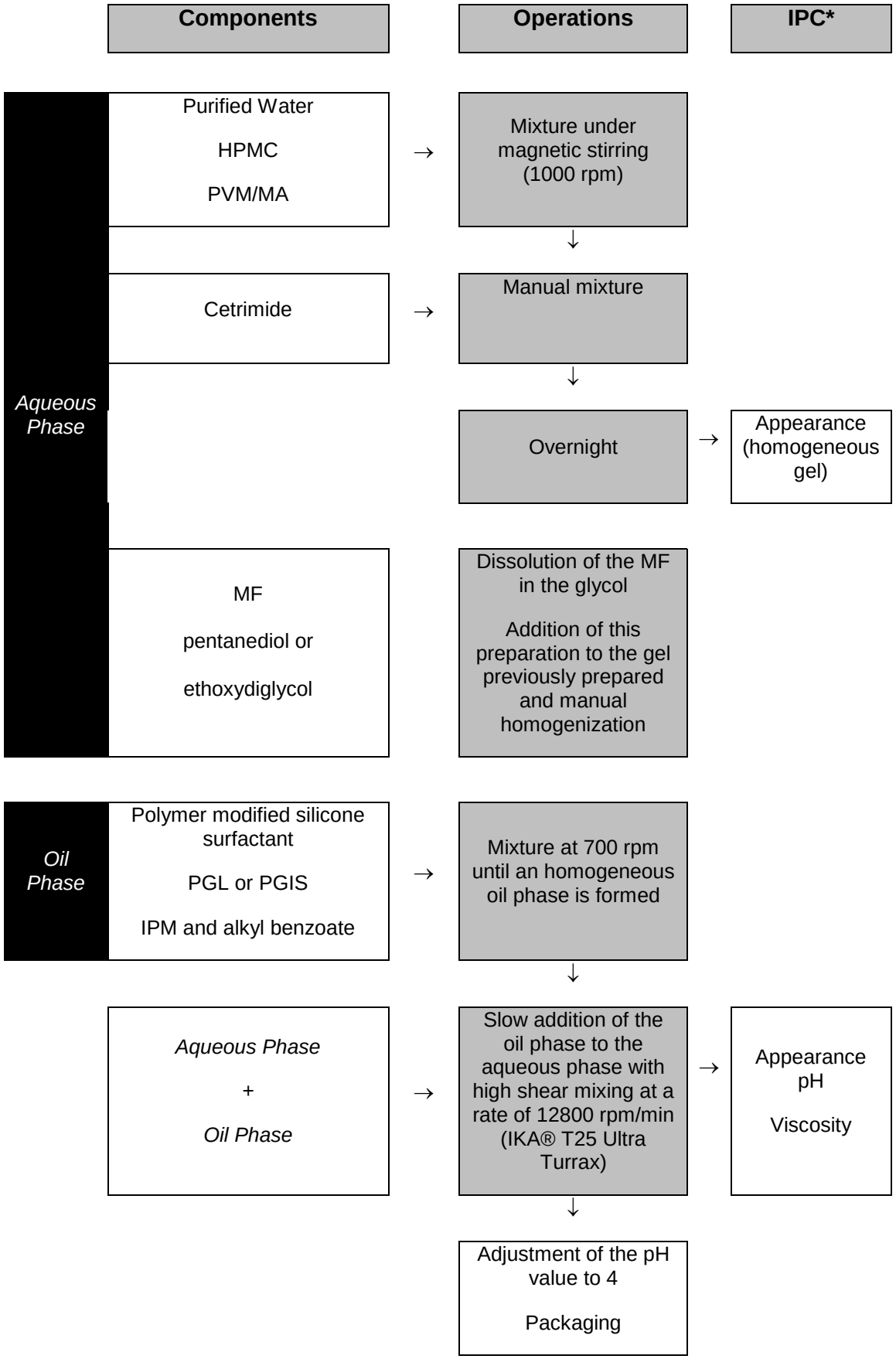


Fig. 3.2. Flow chart of the preparation of the final emulsions (*IPC: in process control).

4.2 Pre-formulation studies

4.2.1 Selection of cellulose polymers

In contrast to the traditional formulation concept, emulsions can be stabilized also by appropriate macromolecules. Polymers are frequently added to increase the stability of an emulsion by thickening and adding yield value to the continuous phase. However, it is much more effective to use surface-active polymers, such as carbomer or HPMC. Those polymers form structured interfacial films, which effectively prevent the coalescence of oil drops [6].

In this study polymers less sensitive to electrolytes and with the ability to inhibit the crystallization of the drug, like HPMC and HPC, were used.

The percentage used for each polymer was 1.5 % in water (w/w). In accordance with a previous study [22], the percentage of the polymer influences the solubility of MF in the vehicle as long as the increase of polymer amount increases the drug solubility.

The permeation profiles through silicone membrane showed no significant differences between the two gels ($p > 0.05$). However, the permeation profiles through human skin showed that the flux and permeability coefficients of MF in HPC gel was higher comparing with HPMC gels. Additionally, the analysis of variance showed significant differences between the two polymers ($p < 0.05$). The results are described in Chapter V, section 3.1.

The microscopy analysis showed that the polymers influence MF crystallization; crystals were observed, macroscopically, 15 days after the preparation in HPC gel but no crystals appeared in the presence of HPMC (Fig. 3.3). These results are in accordance to other studies, for instance for HA [23].

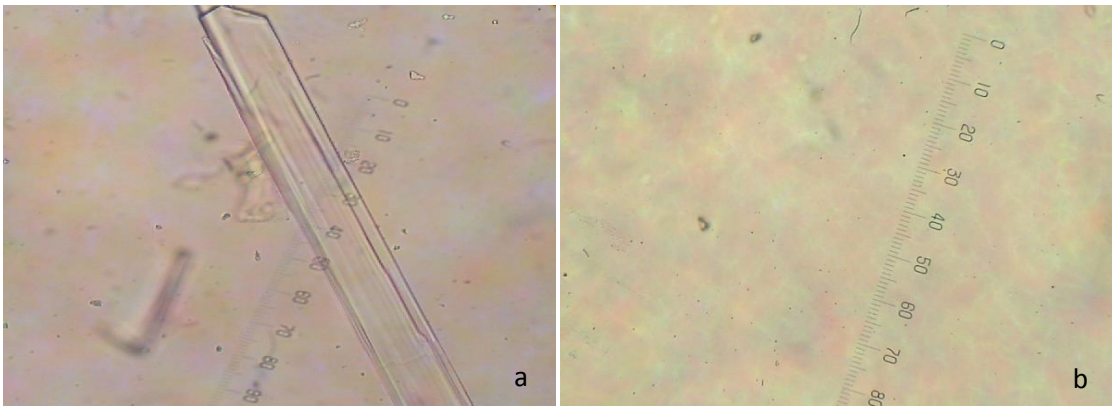


Fig. 3.3. Photomicrographs of HPC gel (a) and HPMC gel (b) after 15 days of preparation (magnification of 400x).

The mechanism of nucleation retardation by the polymers can be explained in terms of association of the drug with the polymer through hydrogen bonding. Moreover, according to Raghavan et al. [23], HPMC is adsorbed on the hydrophobic surface of the crystals, stabilizing the precipitates and increasing the thermodynamic activity of the drug.

It should be emphasized that the permeation studies were carried out 1 day after the preparation of the gels thus, as verified after microscopic analysis, the crystallization process did not occur at this time. If the permeation was repeated 15 days after the preparation, it is possible that these results might find another expression. After 15 days, crystals were observed in HPC gel which will decrease the permeation of MF.

Based on these results, the HPMC polymer was selected to be used.

4.2.2 Selection of the glycols

4.2.2.1 Solubility studies

Knowledge of the drug's solubility for each vehicle composition is necessary to avoid supersaturation (where the drug concentration is increased above its equilibrium solubility), in which the system becomes thermodynamically unstable and crystallization and precipitation of the drug in excess occurs over time. The drug solubility in the vehicle is also an important factor that influences the drug penetration across the skin or artificial membranes [24].

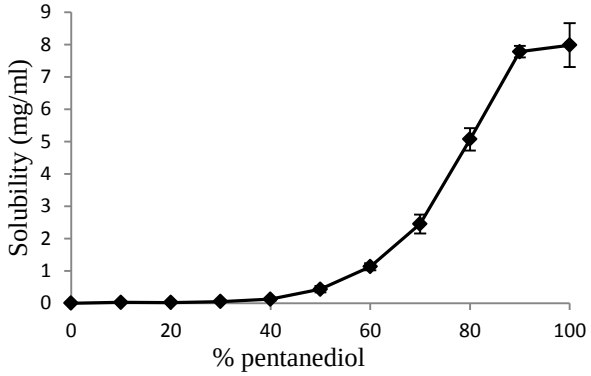
Since the solubility of a drug in its vehicle is an important factor determining availability [25], several glycols were tested concerning MF solubility (Table 3.3).

Table 3.3. MF solubility in different glycols, (n=3; mean ± SD).

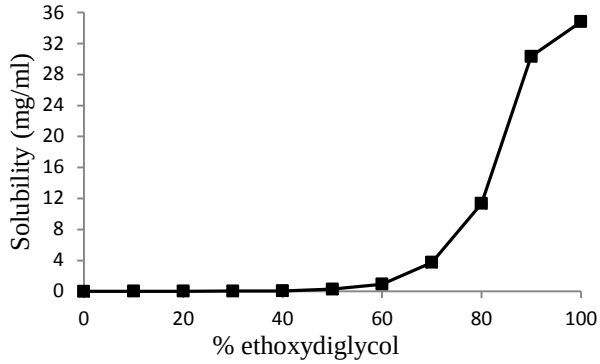
Glycol	Solubility (mg/g)
Pentanediol / caprylocaproyl (7:3 w/w)	15.97 ± 0.13
Ethoxydiglycol	34.84 ± 0.59
PEG 400	19.01 ± 3.47
Pentanediol	6.96 ± 0.76
Propilene glycol	2.02 ± 0.26
1-propanol	3.20 ± 0.10
1,4-butanediol	3.20 ± 0.20
Pentane-1,5-diol	1.90 ± 0.37

MF has a low solubility in water, but it is more soluble in glycols. The glycols in which MF presented a solubility value higher than 6.5 mg/g were considered for further studies. MF presented the highest solubility value in ethoxydiglycol, followed by PEG 400, a mixture of pentanediol/caprylocaproyl and pentanediol.

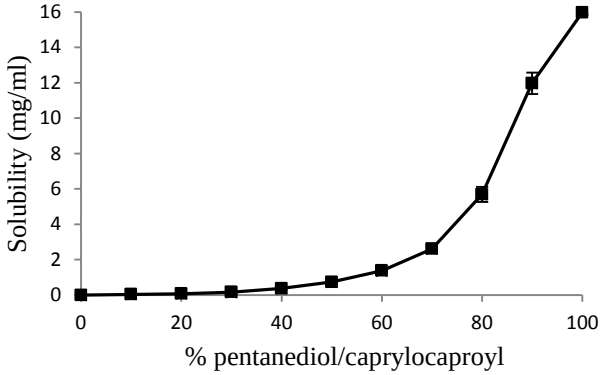
In order to understand the influence of the solubilizers in the percentage used in the emulsions, co-solvent solubility plots were built (Fig. 3.4), except for PEG 400 due to chemical instability (4.2.2.3). As demonstrated previously [23; 24], MF is insoluble in water and its solubility exponentially increases as the amount of glycol increases.



(a)



(b)



(c)

Fig. 3.4. Co-solvent solubility plot of MF in pentanediol - water mixtures (a); ethoxydiglycol - water mixtures (b) and pentanediol/caprylocaproyl - water mixtures (c) at 22ºC. Measurements were performed at least in duplicate (mean ± SD).

It was observed that, when the percentage of glycol is lower, for instance 10%, the solubility of MF in a mixture of 10 % (w/w) of pentanediol in water is almost the same when compared with the same percentage of ethoxydiglycol in water (Table 3.4). The solubility of MF in the mixture of pentanediol/caprylocaproyl at 10% (w/w) in water is almost 2-fold higher when compared with the other two glycols. The drug's solubility in glycol/water (10:90) mixtures cannot be directly extrapolated. Despite the higher solubility of MF in pure ethoxydiglycol (Table 3.3), the highest solubility of MF in these mixtures was for pentanediol/caprylocaproyl.

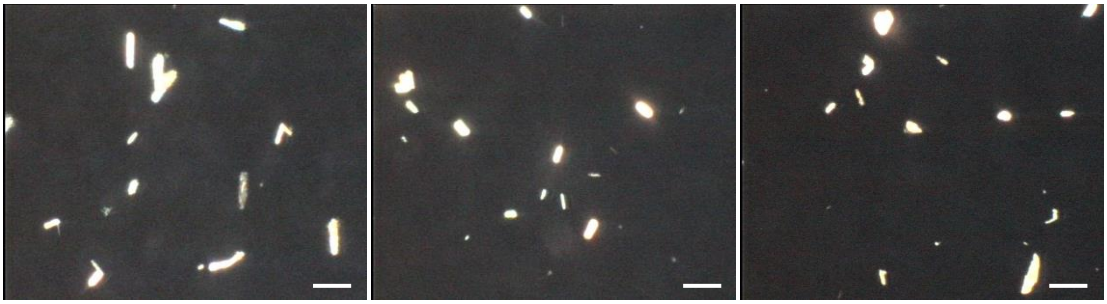
Table 3.4. Solubility of MF in 10% (w/w) glycol in water mixtures (n=2; mean ± SD).

Glycol	Solubility (mg/100g)
Ethoxydiglycol	2.83 ± 0.76
Pentanediol	2.31 ± 1.51
Pentanediol/caprylocaproyl	4.50 ± 0.15

Comparing the MF solubility values for ethoxydiglycol and pentanediol in pure mixtures, ethoxydiglycol showed 5-fold higher value for MF solubility when compared to pentanediol. However, at 10 % (w/w) of the glycol in water, ethoxydiglycol and pentanediol presented similar values regarding MF solubility (Table 3.4).

4.2.2.2 Microscopy analysis

Regarding the photomicrographs of MF solubilized in the three mixtures (glycol/water; 10:90) under study, crystals were observed in the three preparations (Fig. 3.5). The mixture pentanediol /caprylocaproyl showed less crystals of MF and this result is in accordance with the co-solvents solubility plots.



(a) (b) (c)

Fig. 3.5. MF in ethoxydiglycol (a); pentanediol (b) and pentanediol / caprylocaproyl (c), 5 days after preparation and stored at 22 ºC. Magnification of 250x (scale: 60 µm).

At 10 %, the mixture pentanediol / caprylocaproyl seems to be the most suitable glycol for the delivery of MF.

4.2.2.3 Stability of MF in the selected glycols

The glycols selected following the solubility results were tested concerning MF stability in different storage and pH conditions (Fig. 3.6). The pH values tested were in

accordance with the MF stability, in aqueous solutions. The stability of MF was found to reduce with an increase of the pH. Thus, a high pH value should be avoided in formulations containing MF. In fact, no degradation of MF was evident in acidic conditions of pH < 4 [26]. The samples containing ethoxydiglycol were tested in both pH conditions (pH 4 and pH 5). For samples with PEG 400 and pentanediol / caprylocaproyl mixture, the pH values were only adjusted to 5 because the samples, at room temperature, presented a pH value of 4. Concerning pentanediol, the pH value was only adjusted to 4 because the pH of the samples was 5.

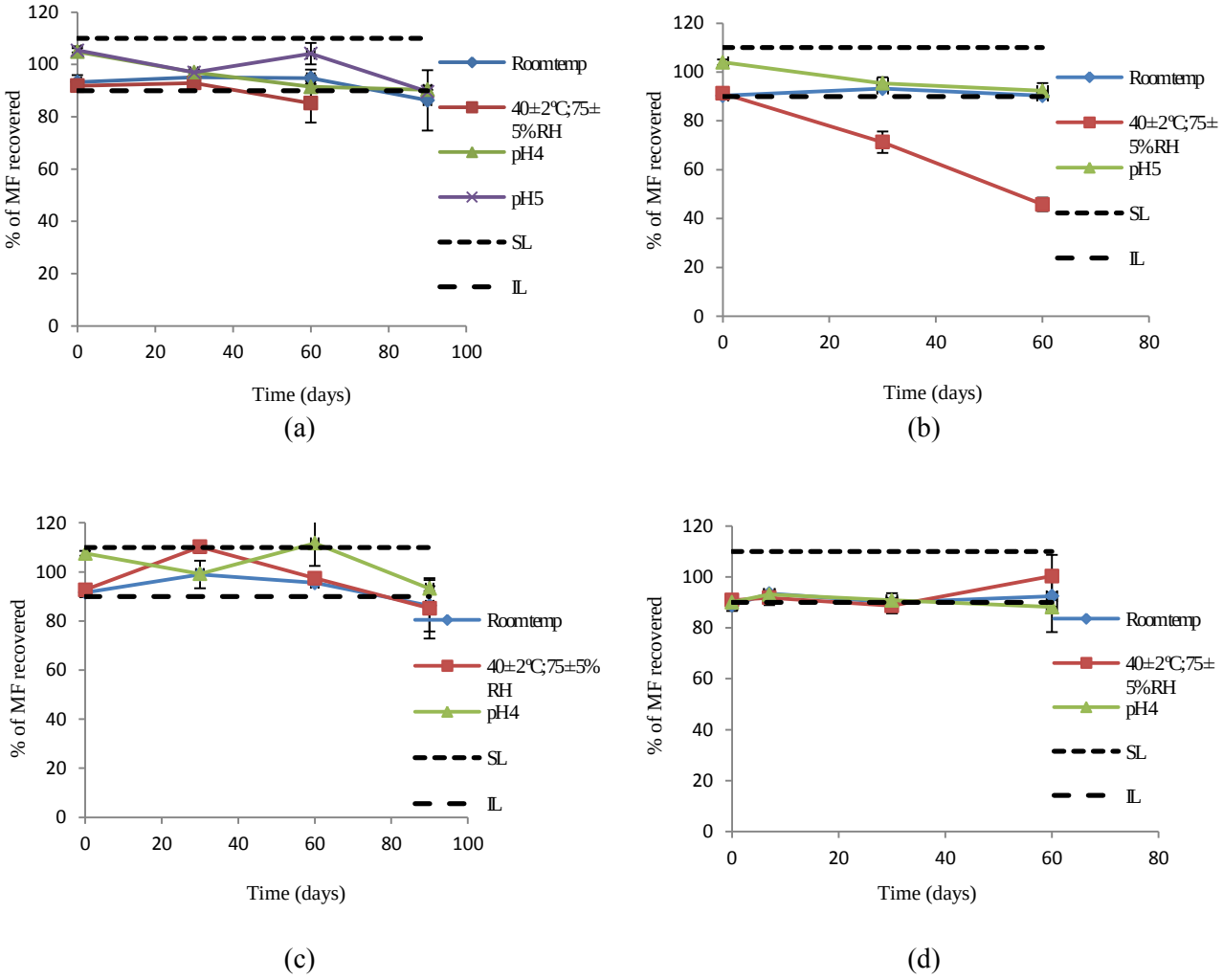


Fig. 3.6. Percentage of MF recovered as function of time in different conditions: RT, accelerated stability (40 ± 2 ºC; 75 ± 5 % relative humidity) and different pH values. The superior (SL) and inferior (IL) limits were established at 100 ± 10 %.

Ethoxydiglycol (a), PEG 400 (b), pentanediol (c), pentanediol / caprylocaproyl (d), (n= 3; mean ± SD).

After 30 days, it was observed a drastic decrease in the MF recovery under accelerate conditions in PEG 400 (Fig. 3.6b). Thus, this glycol was eliminated as a possible glycol/solvent to include in the final emulsions. Concerning the other three glycols, the stability profile of MF was satisfactory in the tested conditions.

It should be explained that the stability of MF in the glycols was assessed in parallel with the co-solvent solubility studies and the influence of the glycols on the MF crystallization. Throughout the combination of these methods, three glycols were selected: pentanediol, ethoxydiglycol and the mixture pentanediol / caprylocaproyl.

4.3 Formulation development

The first phase of development of the emulsions was the selection of the oils. The oils were selected using empirical factors. The criteria for the selection of the oils were: to be liquid at room temperature; cost effective; conferring emollience after application onto the skin and low viscosity. The oils selected based on these main characteristics were alkyl benzoate and IPM.

In addition to these features, alkyl benzoate is a solvent to lipophilic substances with a high polarity and low viscosity. IPM is also a good solvent to lipophilic substances (such as MF) and also acts as a skin enhancer [27]. The initial ratio selected for the oils was alkyl benzoate/IPM (1:1).

4.3.1 Required HLB of oil (IPM / alkyl benzoate) mixture

Different approaches for surfactant selection are available. Our strategy was to select the main surfactant, matching the HLB of the main surfactant to the required HLB of the oil mixture composed of alkyl benzoate and IPM. According to Table 3.5, the required HLB of the alkyl benzoate is around 8 since no phase separation was observed after 24 hours in this range.

Table 3.5. Physical stability of alkyl benzoate emulsions prepared with Span®80 / Tween®80 combinations.

Span® 80 / Tween® 80 content (%)	HLB	Result
80:20	6.4	No phase separation after 24h
65:35	8.0	No phase separation after 24h
50:50	9.6	Slight phase separation after 1h
40:60	10.7	Phase separation after 1h
30:70	11.8	Instantaneous phase separation
20:80	12.8	Instantaneous phase separation

Knowing that the HLB value of IPM according literature is 12, the relative percentage in the oil mixture is 50% (w/w) for each oil (IPM and alkyl benzoate) and assuming an HLB value for alkyl benzoate of 8, the required HLB for the oil mixture was calculated and the value obtained was 10.

The surfactants selected, according to the HLB concept described by Griffin [28], were two silicone-based o/w emulsifiers composed by polyether-modified silicone with HLB values of 10 and 11 (polymer modified silicone surfactant and bis-PEG/PPG-20/5 PEG/PPG-20/5 dimethicone; methoxy PEG/PPG-25/4 dimethicone; caprylic/capric triglyceride, respectively).

4.2.2 Development of the laboratory batches

The first formulations developed are presented in Table 3.6. The strategy presented here is the stabilization of the emulsion with the pre-selected ingredients. The addition of glyceryl dibehenate was done to increase the viscosity of the oil phase in order to prevent instability processes, such as coalescence.

Table 3.6. Qualitative and quantitative composition (%, w/w) of preliminary emulsions (a).

	1A	**1B**	**1C**
Oil phase			
Polymer modified silicone surfactant	2	2	2
Alkyl benzoate	3	3	3
IPM	3	3	3
Glyceryl dibehenate	2	2	2
Water phase			
HPMC	1.5	1.5	1.5
Glycol*	10	10	10
Orthophosphoric acid	pH 4	pH 4	pH 4
Purified water	q.s to 100	q.s to 100	q.s to 100

*1A: pentanediol; 1B: ethoxydiglycol; 1C: pentanediol/caprylocaproyl (7:3 w/w)

The instability could become manifest through a variety of physicochemical processes, such as creaming (or sedimentation), flocculation, coalescence or phase inversion. The stability of the emulsions was assessed by visual observation of the possible phase separation or other phenomena. After 10 days of storage, at room temperature, macroscopic observations did not reveal any sign of instability. However, the centrifugation test, that is commonly used to determine the stability of the developed emulsions, showed that all emulsions had a poor physical stability as phase separation was observed upon centrifugation. The emulsions were kept at room temperature and, in fact, it was observed macroscopic phase separation after 1 month for all formulations.

In order to increase the physical stability of the emulsions, a co-emulsifier (Tween® 80), suitable for oil in water emulsions, was added to the emulsions (Table 3.7).

Table 3.7. Qualitative and quantitative composition (%, w/w) of preliminary emulsions (b).

	2A	**2B**	**2C**
Oil phase			
Polymer modified silicone surfactant	2	2	2
Tween® 80	0.2	0.2	0.2
Alkyl benzoate	3	3	3
IPM	3	3	3
Glyceryl dibehenate	2	2	2
Water phase			
HPMC	1.5	1.5	1.5
Glycol*	10	10	10
Orthophosphoric acid	pH 4	pH 4	pH 4
Purified water	q.s to 100	q.s to 100	q.s to 100

*2A: pentanediol; 2B: ethoxydiglycol; 2C: pentanediol/caprylocaproyl (7:3 w/w)

The emulsions were submitted to a centrifuge test and after 5 min at 12000 rpm, phase separation was again observed for all of emulsions.

The emulsion stabilizers presented in the formulations revealed a lack of efficiency. In order to select co-stabilizers, additional solubility assays of the drug with co-stabilizers were done (Table 3.8).

Table 3.8. MF solubility in co-stabilizers, (n=3; mean ± SD).

	Solubility (mg/g)
PGL	21.3 ± 0.34
PEG-12 dimethicone	8.2 ± 0.30
PEG-18 glyceryl oleate/cocoate	6.6 ± 0.06

Due to the higher value of the MF solubility obtained for PGL, it was decided to add this co-surfactant to the emulsions at two different percentages (1 and 3 % (w/w) – Tables 3.9 and 3.10, respectively) and to increase the percentage of the polymer modified silicone surfactant to 3% w/w.

Table 3.9. Qualitative and quantitative composition (%, w/w) of preliminary emulsions (c).

	3A	**3B**	**3C**
Oil phase			
Polymer modified silicone surfactant	3	3	3
PGL	1	1	1
Alkyl benzoate	3	3	3
IPM	3	3	3
Glyceryl dibehenate	2	2	2
Water phase			
HPMC	1.5	1.5	1.5
Glycol*	10	10	10
Orthophosphoric acid	pH 4	pH 4	pH 4
Purified water	q.s to 100	q.s to 100	q.s to 100

*3A: pentanediol; 3B: ethoxydiglycol; 3C: pentanediol/caprylocaproyl (7:3 w/w)

Table 3.10. Qualitative and quantitative composition (%, w/w) of preliminary emulsions (d).

	4A	**4B**	**4C**
Oil phase			
Polymer modified silicone surfactant	3	3	3
PGL	3	3	3
Alkyl benzoate	3	3	3
IPM	3	3	3
Glyceryl dibehenate	2	2	2
Water phase			
HPMC	1.5	1.5	1.5
Glycol*	10	10	10
Orthophosphoric acid	pH 4	pH 4	pH 4
Purified water	q.s to 100	q.s to 100	q.s to 100

*4A: pentanediol; 4B: ethoxydiglycol; 4C: pentanediol/caprylocaproyl (7:3 w/w)

The six formulations (3A, 3B, 3C, 4A, 4B and 4C) were centrifuged for 5 min at 15000 rpm and it was observed phase separation for all of them. However, the formulations with 3% of PGL presented a less pronounced phase separation. Thus, it was selected the PGL at 3%.

The physical stability of the emulsions was not the desired, thus other stabilizers were considered. Cetrimide is a cationic surfactant and also acts as a preservative. The concentration selected was 0.075 % due to the results obtained in the antimicrobial efficacy tests (Table 3.22). Xanthan gum was also tested as it is a polysaccharide used to increase the viscosity of the external phase preventing coalescence and phase separation (Table 3.11).

Table 3.11. Qualitative and quantitative composition (%, w/w) of preliminary emulsions (e).

	5A	**5B**	**5C**	**6A**	**6B**	**6C**
Oil phase						
Polymer modified silicone surfactant	3.0	3.0	3.0	3.0	3.0	3.0
PGL	3.0	3.0	3.0	3.0	3.0	3.0
Alkyl benzoate	3.0	3.0	3.0	3.0	3.0	3.0
IPM	3.0	3.0	3.0	3.0	3.0	3.0
Glyceryl dibehenate	2.0	2.0	2.0	2.0	2.0	2.0
Water phase						
HPMC	1.5	1.5	1.5	1.5	1.5	1.5
Cetrimide	0.075	0.075	0.075	-	-	-
Xanthan gum	-	-	-	0.1	0.1	0.1
Glycol*	10	10	10	10	10	10
Orthophosphoric acid	pH 4	pH 4	pH 4	pH 4	pH 4	pH 4
Purified water	q.s to 100	q.s to 100	q.s to 100	q.s to 100	q.s to 100	q.s to 100

*5A and 6A: pentanediol; 5B and 6B: ethoxydiglycol; 5C and 6C: pentanediol/caprylocaproyl (7:3 w/w)

It was observed that, after the centrifugation at 12000 rpm for 5 min, the phases of the emulsions containing cetrimide did not separate. Thus, these formulations were reprocessed with the incorporation of the drug (Table 3.12).

Table 3.12. Qualitative and quantitative composition (%, w/w) of preliminary emulsions (f).

	7A	**7B**	**7C**
Oil phase			
Polymer modified silicone surfactant	3.0	3.0	3.0
PGL	3.0	3.0	3.0
Alkyl benzoate	3.0	3.0	3.0
IPM	3.0	3.0	3.0
Glyceryl dibehenate	2.0	2.0	2.0
Water phase			
cetrimide	0.075	0.075	0.075
HPMC	1.5	1.5	1.5
MF	0.1	0.1	0.1
Glycol*	10.0	10.0	10.0
Orthophosphoric acid	pH 4	pH 4	pH 4
Purified water	q.s to 100	q.s to 100	p.s to 100

*7A: pentanediol; 7B: ethoxydiglycol; 7C: pentanediol/caprylocaproyl (7:3 w/w)

After 30 days, it was observed phase separation, macroscopically, with a deposit in the bottom of the flask. The solubility of the glyceryl dibehenate in the oil phase was tested and, it was concluded, that this ingredient is not soluble in this phase, at room temperature. The solubilization was only achieved when the oil phase was heated. Following these results, glyceryl dibehenate was eliminated from the formulations. The emulsions presented in Table 3.13 were reprocessed without glyceryl dibehenate.

Table 3.13. Qualitative and quantitative composition (%, w/w) of preliminary emulsions (g).

	8A	**8B**	**8C**
Oil phase			
Polymer modified silicone surfactant	3.0	3.0	3.0
PGL	3.0	3.0	3.0
Alkyl benzoate	3.0	3.0	3.0
IPM	3.0	3.0	3.0
Water phase			
Cetrimide	0.075	0.075	0.075
HPMC	1.5	1.5	1.5
MF	0.1	0.1	0.1
Glycol*	10.0	10.0	10.0
Orthophosphoric acid	pH 4	pH 4	pH 4
Purified water	q.s to 100	q.s to 100	p.s to 100

*8A: pentanediol; 8B: ethoxydiglycol; 8C: pentanediol/caprylocaproyl (7:3 w/w)

Due to the elimination of the glyceryl dibehenate, the viscosity of the internal phase decreased and, consequently, the viscosity of the emulsions also decreased. The viscosity of the external phase controls the viscosity of the product, however, the internal phase viscosity is also responsible for changes in the viscosity of the final product. In fact, after 15 days, phase separation was observed. This phenomena was attributed to the decrease of the viscosity of the product. In order to overcome this problem, the amount of polymer (HPMC) was increased to 2% (w/w). The new formulations are described in the Table 3.14.

Table 3.14. Qualitative and quantitative composition (%, w/w) of preliminary emulsions (h).

	9A	**9B**	**9C**
Oil phase			
Polymer modified silicone surfactant	3.0	3.0	3.0
PGL	3.0	3.0	3.0
Alkyl benzoate	3.0	3.0	3.0
IPM	3.0	3.0	3.0
Water phase			
cetrimide	0.075	0.075	0.075
HPMC	2.0	2.0	2.0
MF	0.1	0.1	0.1
Glycol*	10.0	10.0	10.0
Orthophosphoric acid	pH 4	pH 4	pH 4
Purified water	q.s to 100	q.s to 100	q.s to 100

*9A: pentanediol; 9B: ethoxydiglycol; 9C: pentanediol/caprylocaproyl (7:3 w/w)

The same formulations described in Table 3.14 were prepared but, in this case, replacing the main surfactant, polymer modified silicone, to the other surfactant selected in the beginning of the pharmaceutical development: the Bis-PEG/PPG-20/5 Dimethicone; Methoxy PEG/PPG-25/4 Dimethicone; Caprylic/Capric Triglyceride (Table 3.15).

Table 3.15. Qualitative and quantitative composition (%, w/w) of preliminary emulsions (i).

	10A	**10B**	**10C**
Oil phase			
Bis-PEG/PPG-20/5 Dimethicone; Methoxy PEG/PPG-25/4 Dimethicone; Caprylic/Capric Triglyceride	3.0	3.0	3.0
PGL	3.0	3.0	3.0
Alkyl benzoate	3.0	3.0	3.0
IPM	3.0	3.0	3.0
Water phase			
cetrimide	0.075	0.075	0.075
HPMC	2.0	2.0	2.0
MF	0.1	0.1	0.1
Glycol*	10.0	10.0	10.0
Orthophosphoric acid	pH 4	pH 4	pH 4
Purified water	q.s to 100	q.s to 100	q.s to 100

*10A: pentanediol; 10B: ethoxydiglycol; 10C: pentanediol/caprylocaproyl (7:3 w/w)

The physical and chemical stability of the emulsions 9A, 9B, 9C, 10A, 10B and 10C were assessed. The emulsions were analyzed macroscopically for the presence of phase separation and by using analytical centrifugation (Fig. 3.7 and 3.8).

After 15 days of preparation, it was observed phase separation in emulsions 10A, 10B and 10C. Concerning the emulsions containing polymer modified silicone surfactant, it was observed phase separation, after 60 days, in 9A and 9B and after 30 days in formulation 9C.

The transmission profiles obtained from the analytical centrifugation showed that all formulations were physically unstable. The transmission profiles showed that the droplet migration occurred in the emulsions, which was translated by higher transmission. This mechanism occurs due to migration of the droplets from the bottom to the top of the cell (creaming) which is responsible for a sample clarification,

increasing the detected radiation. This phenomenon is translated by the value of the slope (%/hour). Higher values of slope mean less stable emulsions (Table 3.16).

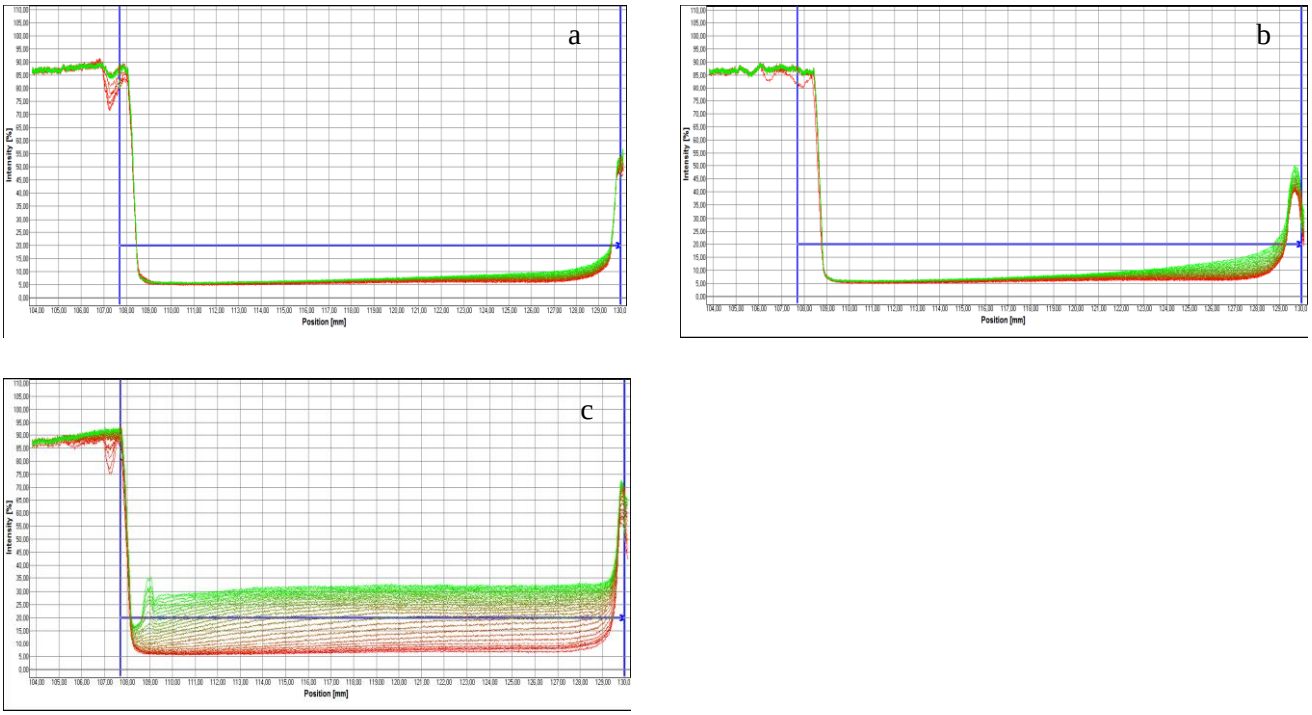


Fig.3.7. Transmission profiles of emulsion 9A (a); 9B (b) and 9C (c).

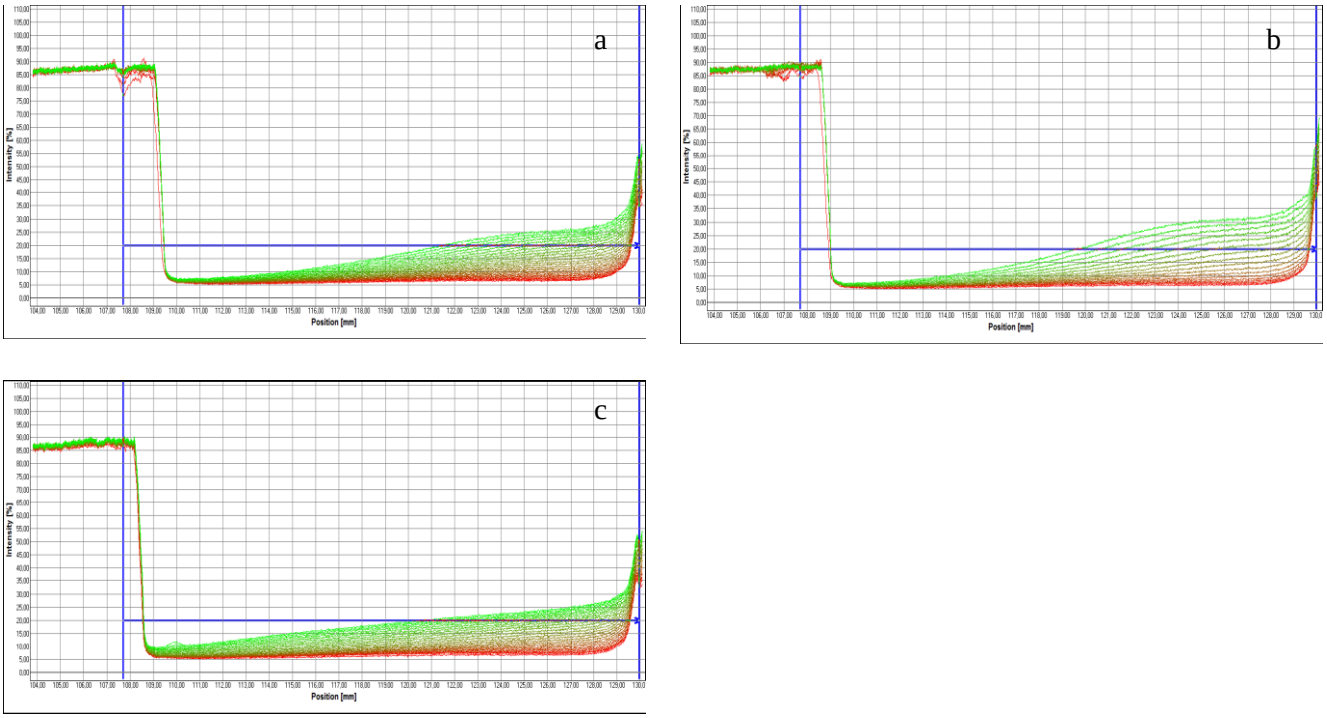


Fig. 3.8. Transmission profiles of emulsion 10A (a); 10B (b) and 10C (c).

Table 3.16. Analytical parameters obtained after the analytical centrifugation for emulsions of the series 9 and 10.

Formulation	Left border (s)	Right border (s)	Slope (%/h)	Corr. Coeff
9A	12.4	4003.2	0.952	0.981
9B	13.1	4004.0	1.327	0.992
9C	11.6	4002.5	14.949	0.985
10A	10.1	4000.9	3.135	0.992
10B	10.9	4001.7	3.667	0.980
10C	13.9	4004.8	3.526	0.985

By analyzing Fig. 3.7 and 3.8 and Table 3.14, we can conclude that formulations 9 A and 9 B are the most stable since presented the lowest slope values.

The chemical stability was assessed in terms of MF recovery over time, both at room temperature and under stress conditions (40 ± 2ºC and 75 ± 5% of relative humidity). The results are presented in Tables 3.17 and 3.18.

Table 3.17. Recover of MF (%) from formulations 9A, 9B and 9C9 during 60 days at room temperature and under stress conditions, (n=3; mean ± SD).

Time (days)	Conditions	9A	9B	9C
0	RT	99.91 ± 0.86	97.9 ± 1.18	99.56 ± 1.15
7	RT	100.79 ± 2.49	99.64 ± 0.83	95.51 ± 3.76
	40ºC	98.75 ± 0.11	98.50 ± 0.98	78.95 ± 11.01
30	RT	94.53 ± 1.06	94.28 ± 4.78	-
	40ºC	93.97 ± 1.05	66.15 ± 3.38	
60	RT	92.48 ± 0.13	-	-
	40ºC	88.33 ± 0.6		

Table 3.18. Recover of MF (%) from formulations 10A, 10B and 10C during 7 days at room temperature and under stress conditions, (n=3; mean ± SD).

Time (days)	Conditions	10A	10B	10C
0	RT	100.63 ± 2.09	105.93 ± 6.13	98.71 ± 1.56
7	RT	99.16 ± 0.82	98.10 ± 0.40	107.33 ± 9.79
	40ºC	102.12 ± 6.00	181.74 ± 11.36	75.64 ± 8.60

According to these results, the developed formulations were not physically neither chemically stable. Thus, the formulations were modified in order to improve physical and chemical stability. The selected surfactant was the polymer modified silicone surfactant because it showed a better performance in both physical and chemical stability. The mixture pentanediol / caprylocaproyl was eliminated because it seemed to be responsible for an additional destabilization of the systems.

It was demonstrated that MF degradation occurs, preferentially, in aqueous media [26], thus the approach used for the improvement of the chemical stability was the increase of the proportion of the oil phase. In order to improve the physical stability, the amounts of surfactant and co-emulsifier were increased, another co-emulsifier was tested (PGIS) and the viscosity of the external phase was increased by adding another polymer (PVM/MA) to slow down the migration of the oil droplets to the surface (Table 3.19).

Table 3.19. Qualitative and quantitative composition (%, w/w) of the preliminary emulsions (j).

	11A	**11B**	**12A**	**12B**
Oil phase				
Polymer modified silicone surfactant	5.0	5.0	5.0	5.0
PGL	4.0	4.0	-	-
PGIS	-	-	4.0	4.0
Alkyl benzoate	5.0	5.0	5.0	5.0
IPM	5.0	5.0	5.0	5.0
Water phase				
cetrimide	0.075	0.075	0.075	0.075
HPMC	2.0	2.0	2.0	2.0
PVM/MA	0.30	0.30	0.30	0.30
MF	0.10	0.10	0.10	0.10
Glycol*	10.0	10.0	10.0	10.0
NaOH 1N	pH 4	pH 4	pH 4	pH 4
Purified water	q.s to 100	q.s to 100	q.s to 100	q.s to 100

**11A and 12A: pentanediol; 11B and 12B: ethoxydiglycol.

The emulsions 11A, 11B, 12A and 12B were white glossy and pourable. After submitted to centrifuge force no phase separation was observed. The transmission profiles showed that the droplet migration was lower when compared to the emulsions 9 and 10 which was translated by lower transmission profiles (Fig. 3.9 and 3.10).

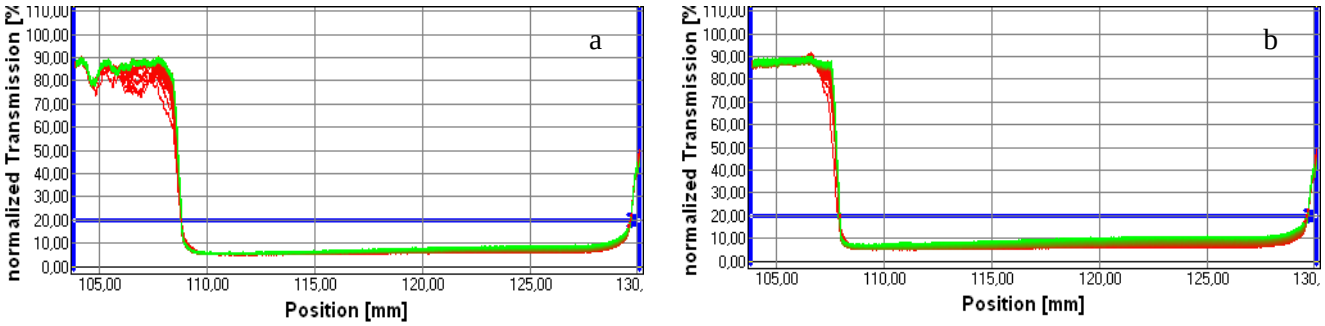


Fig. 3.9. Transmission profiles of emulsion 11A (a) and 11B (b).

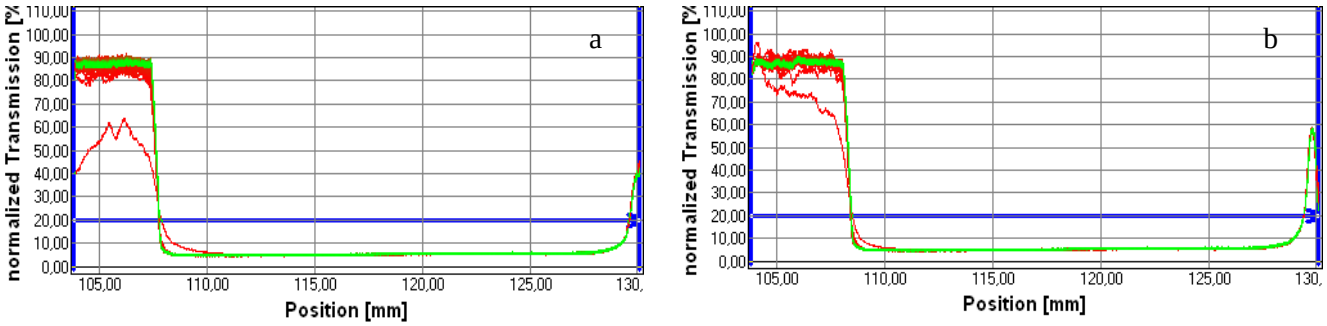


Fig. 3.10. Transmission profiles of emulsion 12A (a) and 12B (b).

In fact, as demonstrated in Chapter IV, section 3.1.2, PVM/MA is responsible for an additional stabilization that was shown by rheological analysis.

The pH values for each formulation were determined during 60 days (Table 3.20). The results showed that the pH values are maintained at around 4 during 60 days.

Table 3.20. pH values for the emulsions during 60 days storage at 22 ºC, (n=3; mean ± SD).

Formulation	0 days	7 days	30 days	60 days
11A	3.89 ± 0.01	3.87± 0.01	3.88 ± 0.01	3.86 ± 0.02
11B	3.97 ± 0.01	3.98 ± 0.02	3.97 ± 0.01	3.95 ± 0.02
12[a]	3.97± 0.01	3.97± 0.02	3.96 ± 0.01	3.95 ± 0.02
12B	3.92 ± 0.02	3.91± 0.01	3.89 ± 0.02	3.86 ± 0.01

The chemical stability was assessed concerning the assay of MF. The results are presented in Table 3.21.

Table 3.21. Recover of MF (%) of formulations 11A, 11B, 12A and 12B during 60 days at 22 ºC and under stress conditions, (n=3; mean ± SD).

Time (days)	Conditions	11A	11B	12A	12B
0	RT	99.87 ± 0.41	100.99 ± 0.68	97.93 ± 0.75	98.14 ± 0.54
7	RT	97.51 ± 1.51	96.71 ± 0.32	98.33 ± 2.05	95.81 ± 0.62
	40 ºC	100.79 ± 2.32	97.21 ± 0.60	92.56 ± 0.95	95.06 ± 0.32
30	RT	97.50 ± 2.35	102.16 ± 1.50	94.11 ± 0.20	95.11± 2.58
	40 ºC	98.69 ± 1.13	96.05 ± 0.24	97.62 ± 8.05	93.68 ± 1.16
60	RT	97.66 ± 0.34	97.74 ± 0.29	99.69 ± 0.93	100.35 ± 0.34
	40 ºC	96.56 ± 0.37	96.92 ± 0.20	104.43 ± 0.88	101.31 ± 1.85

These emulsions seem to provide both physical and chemical stability, in order to achieve a long shelf-life.

4.3.3 Selection of cetrimide concentration

Emulsions 11A and 11B were selected for testing cetrimide as a suitable preservative agent due to its additional contribution to the physical stability of cold processed emulsions as a cationic surfactant.

In order to select a suitable concentration of preservative, the antimicrobial efficacy of two cetrimide concentrations (0.075 and 0.600 % w/w, concentration below and within

the recommended range by the Handbook of Pharmaceutical Excipients [29], respectively) in 11A and 11B was assessed. Table 3.22 shows the results concerning the decrease on antimicrobial charge along 28 days, for each strain.

Table 3.22. Antimicrobial activity of placebos (PA and PB) and 11A and 11B at two different concentrations of cetrimide (0.075 and 0.600 % w/w).

Formulation	Strains	cfu/ml					
		Inoculum	**0h**	**48h**	**7 days**	**14 days**	**28 days**
PA	*P. aeruginosa*	$2.00x10^8$	0	0	0	0	0
	S. aureus	$4.04x10^8$	$\geq 3.0x10^6$	0	0	0	0
	C. albicans	$5.20x10^7$	$\geq 3.0x10^6$	$2.5x10^3$	0	0	0
	A. niger	$9.00x10^6$	$\geq 3.0x10^6$	$\geq 3.0x10^6$	$\geq 3.0x10^6$	$\pm 3.0x10^6$	$\pm 3.0x10^6$
PB	*P. aeruginosa*	$2.00x10^8$	0	0	0	0	0
	S. aureus	$4.04x10^8$	$\geq 3.0x10^6$	0	0	0	0
	C. albicans	$5.20x10^7$	$\geq 3.0x10^6$	$4.0x10^3$	20	0	0
	A. niger	$9.00x10^6$	$\geq 3.0x10^6$	$\geq 3.0x10^6$	$\geq 3.0x10^6$	$\pm 3.0x10^6$	$\pm 3.0x10^6$
11A – 0.075% cetrimide	*P. aeruginosa*	$2,00x10^8$	1.0×10^3	$1.0x10^3$	0	0	0
	S. aureus	$4.04x10^8$	40	0	0	0	0
	C. albicans	$5.20x10^7$	$\geq 3.0x10^6$	0	0	0	0
	A. niger	$9.00x10^6$	$\geq 3.0x10^6$	$\geq 3.0x10^6$	0	0	0
11B – 0.075 % cetrimide	*P. aeruginosa*	$2.00x10^8$	60	100	0	0	0
	S. aureus	$4.04x10^8$	880	0	0	0	0
	C. albicans	$5.20x10^7$	$\geq 3.0x10^6$	$3.0x10^3$	20	0	0
	A. niger	$9.00x10^6$	$\geq 3.0x10^6$	$\geq 3.0x10^6$	$\geq 3.0x10^6$	0	0
11A – 0.6% cetrimide	*P. aeruginosa*	$2.00x10^8$	0	0	0	0	0
	S. aureus	$4.04x10^8$	80	0	0	0	0
	C. albicans	$5.20x10^7$	$\geq 3.0x10^6$	50	0	0	0
	A. niger	$9.00x10^6$	$\geq 3.0x10^6$	10	0	0	0
11B – 0.6% cetrimide	*P. aeruginosa*	$2,00x10^8$	0	0	0	0	0
	S. aureus	$4.04x10^8$	30	0	0	0	0
	C. albicans	$5.20x10^7$	$\geq 3.0x10^6$	30	0	0	0
	A. niger	$9.00x10^6$	$\geq 3.0x10^6$	0	0	0	0

Formulations 11A and 11B containing 0.075 and 0.600 % of cetrimide revealed an antimicrobial activity which conformed to the requirements of the preservation efficacy test for topical formulations according to European Pharmacopeia [10]. The bacteria should be diminished at least by about 2 log-steps after two days, by about 3 log-steps

after 7 days and on day 28 their number must not be increased. In the case of fungi, the cfu should be reduced at least about 2 log-steps after 14 days and on day 28 the colony numbers should not be increased. Placebos are not in accordance to these requirements thus antimicrobial preservation is needed. Moreover, the concentration of 0.075 % (w/w) of cetrimide was selected for incorporation in the systems since it was sufficient to reduce the antimicrobial charge and, it was further observed that, the emulsions containing 0.600 % of cetrimide presented phase separation after 1 month. Cetrimide presents its maximum stability in acid conditions thus, cetrimide is suitable for our formulation, not only due to its antimicrobial activity, but also due to its efficacy over a large pH range.

5. Discussion

In the present study, we developed a formulation methodology that allowed the determination of suitable excipients to produce cold processed emulsions. During the product development, a variety of acceptable excipients were reviewed and used in the formulation in order to prepare and optimize the eventual mixture of excipients.

Systematic formulation developments coupled with more specific analysis were required in order to find out the best proportions of emulsifiers and polymers. A single method alone is not representative. For example, the HLB values used in this study are those published by Griffin [28], and most of which were calculated from theoretical chemical formulas or from the specifications of the manufacturers [30]. These values should be taken as guiding values. The selection of multifunctional components, decreasing the number of excipients, was the main strategy used in order to increase vehicles performance and decrease production costs. The polymer modified silicone surfactant selected as the main surfactant, was suitable for cold process emulsification and its chemical composition substantially contributed to a distinct physical stability of the emulsions. However, high surfactant levels are often not acceptable due to a lack of biocompatibility or economic reasons [4]. In order to decrease the levels of surfactant used, we tested the approach described in the introduction: maximization of the amount of surfactant in the HLB 9-13 region by introducing a co-emulsifier in the emulsion.

Two co-emulsifiers were introduced in the formulations: i) one lipophilic isostearic acid ester of an optimized polyglycerol, PGIS with a HLB value of 5, decreasing the HLB

value of the system to 7.8 and ii) PGL, with a HLB value of 15.7, increasing the HLB value of the system to 12.5. Thus, the HLB of the final emulsions was not in accordance with the calculated HLB for the oil phase (HLB = 10). Nevertheless, emulsions with a suitable physical stability were achieved because polymers (PVM/MA and HPMC) and cetrimide have an important role on the system's stability as well.

The HPMC was selected because it can be dispersed in cold water, is a polymer less sensitive to electrolytes and can avoid the crystallization of drugs, such as corticoids.

The selection of the preservative is also in accordance with our strategy of selection of multifunctional components, since cetrimide is a cationic surfactant and an antimicrobial agent with the ability to disrupt the surface membranes by a destructive interaction with the cell wall and/or cytoplasmic membranes [7].

It is known that the glycols can exert a significant inhibitory effect against the growth activity of various microbial strains [31, 32], thus, it is not surprising that in both placebos (A, containing 10% of pentanediol and B, containing 10% of ethoxydiglycol), a decrease in the antimicrobial charge has occurred. The relative high percentage of glycols was crucial in obtaining a microbial efficacy with a concentration of cetrimide below the recommended concentration by the Handbook of Pharmaceutical Excipients (0.1-1 % w/v) [29].

The excipients assembly allowed the manufacture by a cold process methodology, which has advantages over conventional emulsions.

6. Rational on pharmaceutical development

The aim of the present work was to develop pharmaceutical emulsion(s) for the delivery of MF that are physical stable at acidic pH, using safe excipients and by using as little energy as possible during their preparation, that is, by using a new cold process method of emulsification. However, only a limited number of excipients can be used in such emulsions. They need to be either liquids or readily soluble in the oil and water phases.

Since most dermatological preparations represent a mixture of several materials that are not miscible, they often form dispersed systems that are thermodynamically unstable. Various attempts to improve the physical and chemical stability of the emulsions and of the drug within the emulsions were made.

The first step was the choice of the glycols and the polymers avoiding the degradation of the MF which was a real challenge due to solubility limitations and pH stability. The second step was the stabilization of the emulsions themselves. And for that, several surfactants were tested alone or in co-mixtures. It was found that the ingredients that conferred the best physical and chemical stability were the ingredients presented in Table 3.2, and after their assembly, four emulsions were created (Table 3.19).
The vehicles developed for the delivery of MF obey to the following demands:
- use of specific ingredients: emollients and mild surfactants;
- use of liquid ingredients to allow the cold process emulsification;
- use of ingredients which are effective at a wide range of pH (preservative and surfactants);
- use of multifunctional components in order to decrease the number of ingredients: 1) cetrimide (cationic surfactant and preservative), 2) glycols (powerful solubilizing agents and penetration enhancers), 3) HPMC (additional stabilization by the addition of a yield value and prevents crystallization of poorly soluble drugs such as MF);
- emulsion stable in acidic conditions to avoid MF degradation.

Cold processed emulsions were developed with an appropriate physical stability intended for dermatological use. These systems can be used as carriers for therapeutic agents such as the MF, since they enclose suitable characteristics such as stability at low pH values. The production costs can be decreased due not only to the process itself but also by the reduction of the number of ingredients used.

In order to simplify the nomenclature in the following chapters, the final formulations 11A, 11B, 12A and 12B will be designated as A, B, C and D, respectively.

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1. Introduction

Structured emulsions containing a high number of excipients, may be composed of additional phases of oil-and-water. In aqueous systems containing surfactant/fatty alcohol combinations, the additional phases generally form when the emulsifier, in excess of that required to form a monomolecular film at the oil droplet interface, interacts with the continuous water phase to form a gel network of swollen bilayer structures [1]. The understanding and characterization of the microstructure of the structured emulsions and the mechanisms involved in it, are of crucial importance when formulating emulsions.

The currently available characterization methods give information about the structure and size distribution directly or indirectly, for example, microscopic techniques and light scattering are useful to assess droplet size analysis [2], while differential scanning calorimetry (DSC) techniques can be used as a tool to better understand emulsions morphology [3]. Viscoelastic measurements [4, 5] are useful to obtain information about viscous and the elastic behavior of the systems and, if performed within the linear viscoelastic region, a frequency sweep provides a fingerprint of a viscoelastic system under non-destructive conditions. Thus, the systems are examined in their rheological ground state without disrupting the structure as in continuous shear techniques.

The understanding of the microstructure is of major importance, not only to develop a stable formulation with a realistic shelf life, but also to control the manufacturing process and adapt it to the specific needs of the system. Each ingredient has an optimal manufacture method. Thus, several variables during the process if not understood and controlled, can compromise the product quality. These variables include the type of mixing regime, the rate of the heating or cooling cycle, or the order of mixing the components. For instance, when an emulsion is manufactured by the conventional hot process, which encompasses a heating and a cooling step, the order of mixing has a marked effect on the properties of the emulsion. In emulsions containing non ionic surfactants these effects are even more prominent, because they affect the hydration of the polyoxyethylene chains that are responsible for the structure and that play a crucial role in physical stability [6]. The structure of emulsions containing non-ionic surfactants, prepared by cold process emulsification, is easier to control.

The aim of the following work was to investigate the influence of the type of co emulsifier and the type of glycol on the microstructure of the emulsions by rheological, thermal and microscopic techniques.

2. Materials and methods

2.1 Materials

The materials used are described in Chapter III, section 2.1.

2.2 Methods

2.2.1 Preparation of the formulations

The emulsions were prepared according to the method described in Chapter III, section 2.2.4. The composition of the emulsions is described in Table 3.21.
In addition, two gels, one with 2% (w/w) of HPMC in water and the second with 2% (w/w) of HPMC and 0.3% (w/w) of PVM/MA in water, were prepared by dispersing the aqueous thickening agents (HPMC and PVM/MA) in water at room temperature until a clear homogeneous gel was achieved.

2.2.2 Influence of the inclusion of PVM/MA in the microstructure of HPMC gel

2.2.2.1 Viscoelastic measurements of gels

Non-destructive oscillatory experiments were performed with a controlled stress Carri-Med CSL^2 100 Rheometer (TA Instruments, Surrey, UK) using cone and plate geometry (truncated cone angle 2.1° and radius 6 cm). Oscillation frequency sweep tests were performed over a frequency range from 0.01 to 1 Hz. Viscoelastic experiments for both gels were obtained by the exposure of the samples to a forced oscillation, and the transmitted stress was measured.
Each test was performed at least in duplicate using new samples for each measurement.

2.2.3 Droplet size analysis

The size distribution of the emulsions was measured by light scattering using a Malvern Mastersizer 2000 (Malvern Instruments, Worcestershire, UK) coupled with a Hydro S accessory. For a correct turbidity, about 0.5 g of each formulation (A, B, C and D), corresponding to an obscuration between 25% - 28%, was added in the sample chamber containing 150 ml of water using a stirrer at 700 rpm. Data are expressed in terms of relative distribution of volume of particles in the range of size classes (results displayed as mean ± SD; n=5). Measurements were performed at three different time points: just after the preparation, at day 7 and day 30, stored at 22 ºC.

2.2.4 Structure analysis of emulsions

2.2.4.1 Flow curves

Shear rate against shear stress measurements were obtained at 25 °C using a Carri-Med CSL^2 100 Rheometer (TA Instruments, UK) using cone and plate geometry (truncated cone angle 1.58° and radius 4 cm). All measurements were carried out at a temperature of 22 °C. Continuous flow measurements were performed by increasing the shear rate from 0.5 to 700 s^{-1} over 5 min, followed by decreasing the shear rate from 700 to 0.5 s^{-1} over 5 min. The resulting shear stress was measured and apparent viscosity at apex of loop determined. Each test was performed at least in duplicate using samples for each measurement

2.2.4.2 Viscoelastic experiments

Viscoelastic experiments were obtained according to section 2.2.2.2.1. All samples were tested one month after preparation and stored at 22 °C.

2.2.4.3 Thermoanalytical measurements and hot stage microscopy

Thermoanalytical measurements were performed with a Mettler DSC 822^e system (Mettler, Greifensee, Switzerland) with a sample robot TS0801RO (Mettler, Greifensee, Switzerland). The sample and the reference (air) were placed in hermetically sealed pans. A scan speed of 10 °C/ min and 10-20 mg of sample gave the best compromise between resolution, temperature, accuracy and attenuation.

A Polyvar microscope (Rheichart-Jung, Vienna, Austria) equipped with a TMS91 (Linkam, Surrey, England) hot stage was used to visualize the behavior of the emulsions under stress conditions. Contact thermal microscopy was conducted by heating the samples from 22 ºC to 260 ºC using a 10 °C/min heating rate. All samples were tested one month after preparation and stored at 22 °C.

2.2.4.4 Microscopy analysis

A computerized image analysis device was used for the microscopic observations, connected to a Polyvar microscope in bright field. Samples were examined one month after preparation, storage at 22 ºC, at a magnification of 250 x.

3. Results

3.1 Influence of the inclusion of PVM/MA in the microstructure of HPMC gel

3.1.1 Viscoelastic measurements of gels

After the fluid's linear viscoelastic region has been defined by a strain sweep (data not shown), the structure was further characterized using a frequency sweep at a strain below the critical strain γ_c.

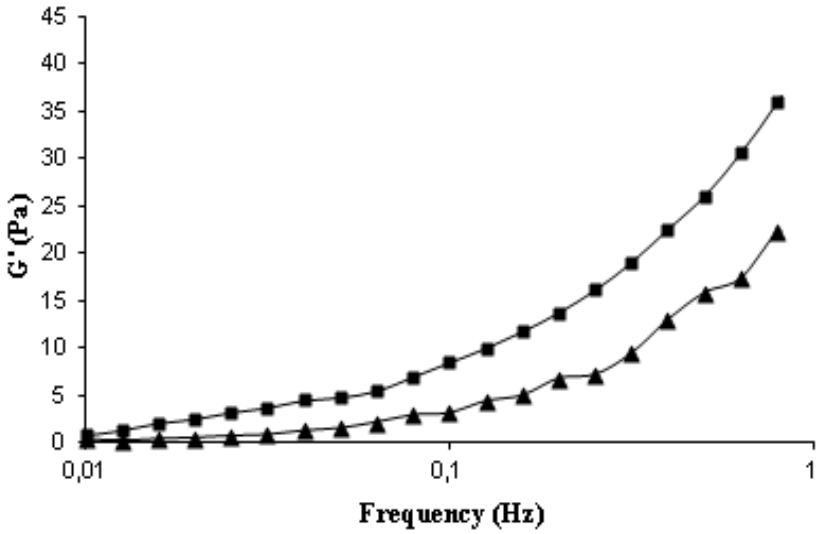


Fig. 4.1. Influence of 0.3% (w/w) of PVM/MA on the storage modulus-G' of HPMC gel (▲) and PVM/MA/HPMC gel (■).

Fig. 4.1 shows the comparison between two gels, one with HPMC and other with PVM/MA/HPMC, both in water. Given the same frequency sweep, the value of the storage modulus (G') was increased in the last. The value of G', which indicates the elastic properties, is about two-fold higher (23.9 to 42.1 Pa, respectively, measured at 1 Hz). It demonstrates that HPMC alone forms a weaker structure with a more liquid character.

3.2 Droplet size analysis

The co-emulsifier influences the droplet size distribution. In emulsion A, containing PGL, the system presents a bimodal population and in C containing PGIS, a monomodal population (Fig. 4.2). The droplet size (90% of the droplets) immediately after preparation is similar for both emulsions, (24.45 ± 2.53 µm and 22.31 ± 6.67 µm, for emulsion A and C respectively). However C seems to present a higher droplet size dispersion, after 30 days of storage at 22 ºC (32.86 ± 3.69 µm and 37.82 ± 15.66 µm, for emulsion A and C respectively), which was translated by a higher standard deviation. These results are not conclusive thus further studies to analyze the structure of the emulsions were carried out.

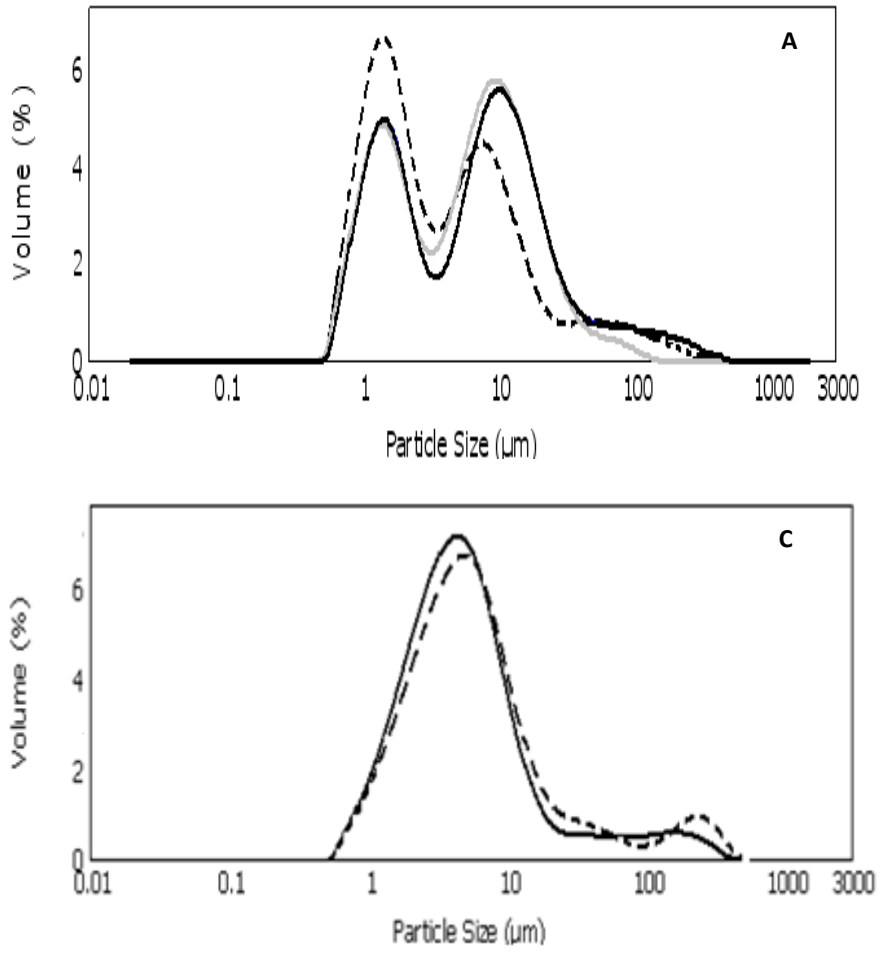


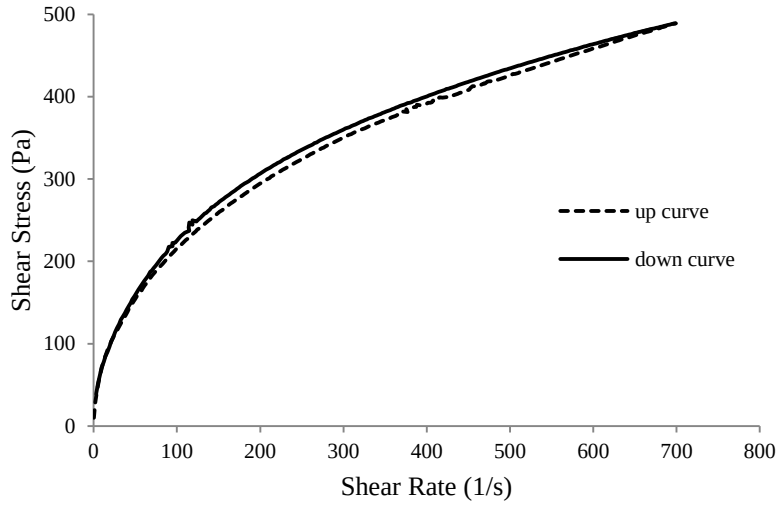
Fig. 4.2. Droplet size distribution of emulsion A and C after 0 days (dashed line), 7 days (grey line) and 30 days (black line) storage at 22 ºC.

3.3 Structure analysis of the emulsions

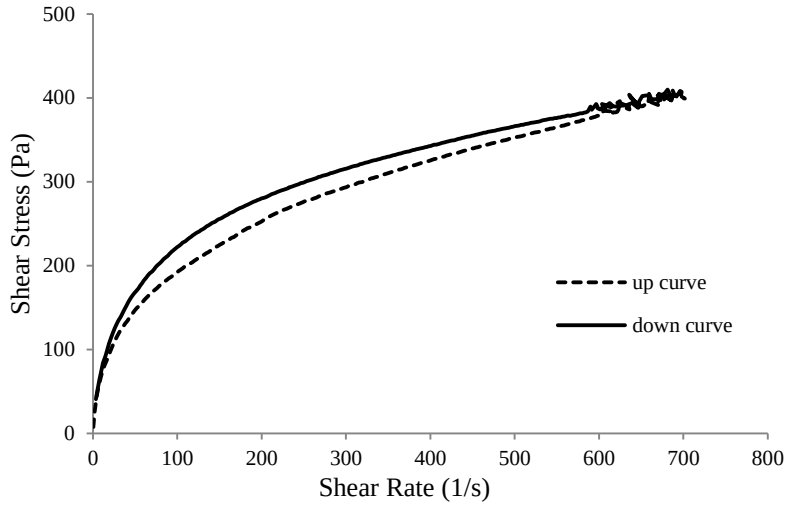
3.3.1 Flow curves

Continuous shear experiments measure the ability of each system to resist structural breakdown during the standardized shearing procedure.

Representative flow curves are shown in Fig. 4.3 with apparent viscosity values calculated at the apex of the loop (Table 4.1).



(a)



(b)

Fig. 4.3. Flow curves. Shear Stress as function of Shear Rate of A (a) and C (b); n=2.

The results show that the apparent viscosity decreases concomitantly with the increase in shear rate. The fluid is considered shear thinning (pseudoplastic), i.e., the input shear energy tends to align anisotropic molecules or particles and disaggregate any large clumps of particles. Thereby, there is a reduction of the overall hydrodynamic drag, which in turn reduces the dissipation of energy in the fluid and the viscosity.

All samples showed a small loop of anti-thixotropic or rheopectic. This behaviour occurs because the entities in the fluid referred to as “flocs” tend to disassemble or assemble when stress is applied.

Table 4.1. Apparent viscosity values calculated at the apex of the loops (698 s^{-1}).

Emulsion	Apparent Viscosity (Pa.s) at 698s^{-1} mean ± SD (n=2)
A	0.7 ± 0.1
B	0.7 ± 0.1
C	0.6 ± 0.2
D	0.6 ± 0.2

Apparent viscosity values provide a comparison of the resistance to structural breakdown between the emulsions and the loop areas compare the amount of structure that fractures in the standardized cycle.

The inclusion of PGL seems to slightly increase the resistance to structural breakdown when compared with PGIS, while the glycol essentially does not influence this parameter (Table 4.1).

3.3.2 Viscoelastic experiments

Davis [7] has proposed the plot of tan (δ) versus frequency in log form ('consistency spectrum') as the most convenient tool for the comparison of viscoelastic properties of semisolids.

A value for tan (δ) greater than unity indicates more "liquid" properties, whereas one lower than the unity means more "solid" properties, regardless of the viscosity [8].

Given that, at a low frequency range (0.01-0.1 Hz), the emulsions with PGL present a lower tan (δ) value compared to the emulsions containing PGIS, indicating that the latter have a more weak gel structure (Fig. 4.4).

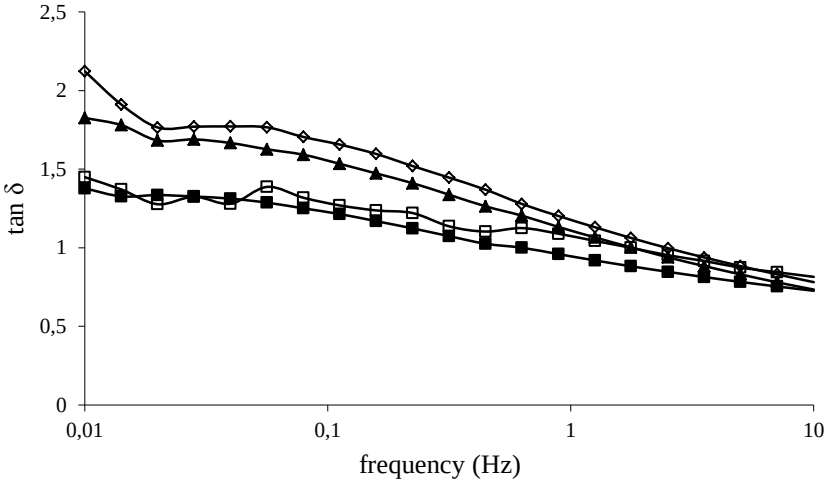
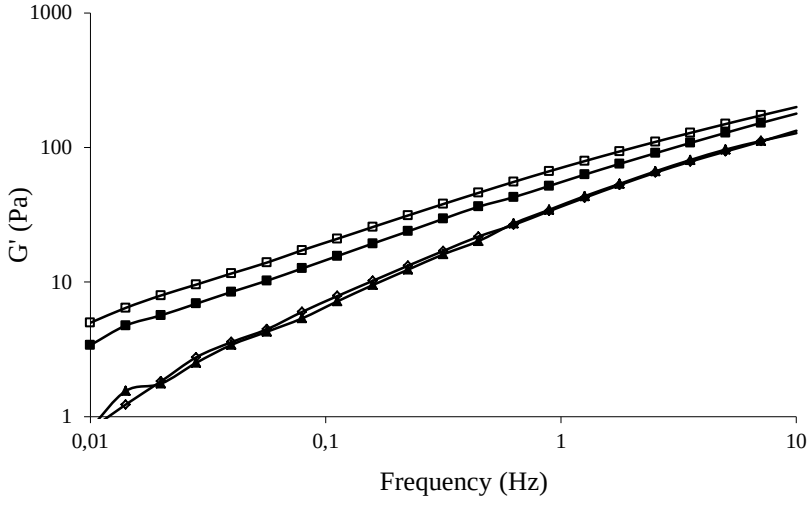
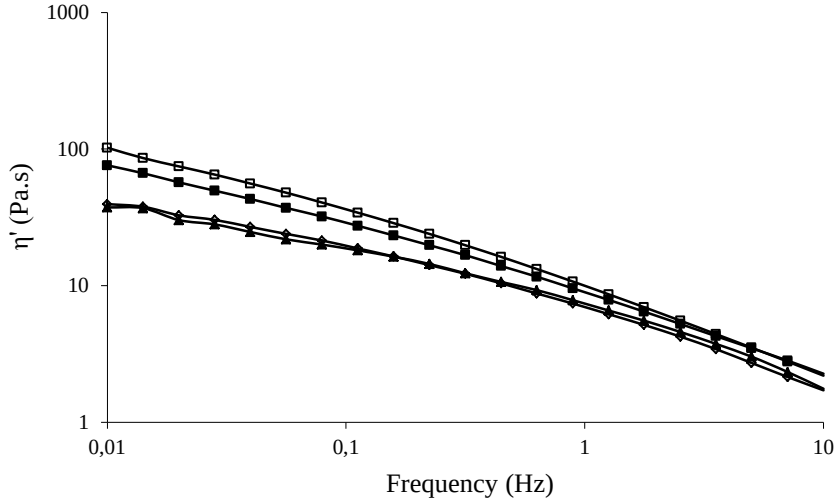


Fig. 4.4. Tan δ as function of frequency for A (■), B (□), C (▲) and D (◊), at 25 °C.

The results obtained in Fig. 4.5, show the variation of the storage modulus (G', characterizing the elastic behavior) and the dynamic viscosity (η') with the frequency. For each one, G' increases and η' decreases with an increase in frequency. This behaviour is typical of a viscoelastic liquid and can be described by a mechanical model made up from a combination of springs (elastic elements) and dashpots (viscous elements) [9].



(a)



(b)

Fig. 4.5. Influence of the co-emulsifier and the type of glycol on the G' (a) and on η' (b) in A (■), B (□), C (▲) and D (◊); n=2.

Fig. 4.5 indicates similarities in the shape of elastic moduli curves for emulsions, especially in the high frequency region. At the low frequency range, in which the viscous component of the complex rheological behavior is more pronounced, some differences exist, indicating that the structures of the emulsions with PGL are slightly stronger compared with the structures of emulsions containing PGIS. The presence of different glycols did not produce any influence on this parameter.

3.3.3 Thermoanalytical measurements and hot stage microscopy

DSC thermograms can elucidate the strength of existing structures within the emulsion. DSC thermograms between 22 ºC and 260 ºC for emulsions were obtained. A representative thermogram of A is shown in Fig. 4.6. An endotherm peak at 110-116 ºC was observed for all samples.

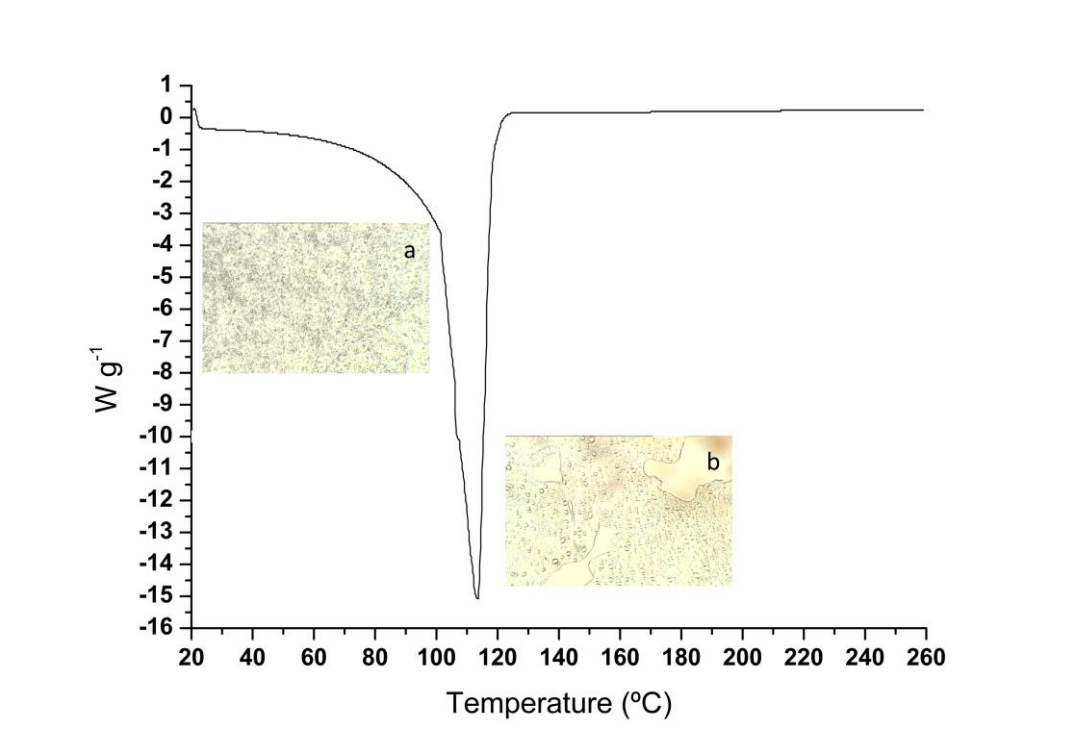


Fig. 4.6. Thermogram of emulsion A. With photomicrographs of emulsion A during a heating program with 10°C/min between 25°C and 260°C. At 25 ºC (a) and at 112 ºC (b), (magnification 100×).

Thermo-microscopic investigations of the emulsions with a scanning rate of 10 °C/min as illustrated in Fig. 4.6 show that at approximated 112 °C, (which corresponds to the main DSC peak), a dramatic change in structure starts with the loss of the initial structure. Rearrangements of the oil droplets can occur and this phenomena is associated with water evaporation and subsequent emulsion destabilization.

The kinetic parameters of the main peak, calculated by DSC software analysis are given in Table 4.2. The integral value that corresponds to the area under the curve gives the extent of the total enthalpy change [2] and can be correlated with physical stability of the systems.

Table 4.2. Calorimetric parameters of emulsions.

Sample	T, onset (°C)	T, endset (°C)	T, peak (°C)	Integral value (W °C g^{-1})
A	105.39	121.21	116.50	-224.37
B	102.85	123.64	110.67	-183.62
C	102.23	121.59	116.00	-188.17
D	104.18	121.59	115.17	-169.64

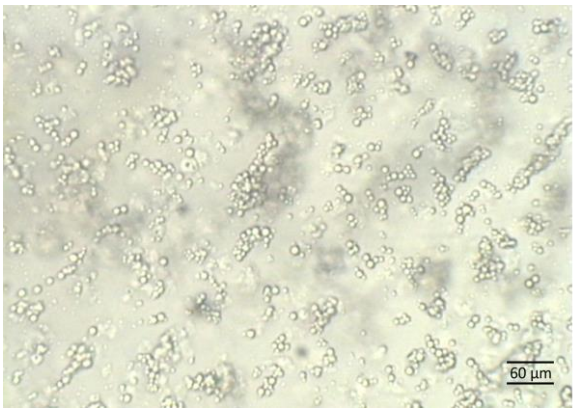
The analysis of the results above allows the assumption that, A (PGL / pentanediol) corresponds to the strongest structure since the energy released to the structure breakdown is the highest. Moreover, it can be seen that the emulsions containing pentanediol correspond to the strongest structures (A and C). Correlating these values with the results showed before, we can conclude that A corresponds to the strongest structure.

3.3.4 Microscopy analysis

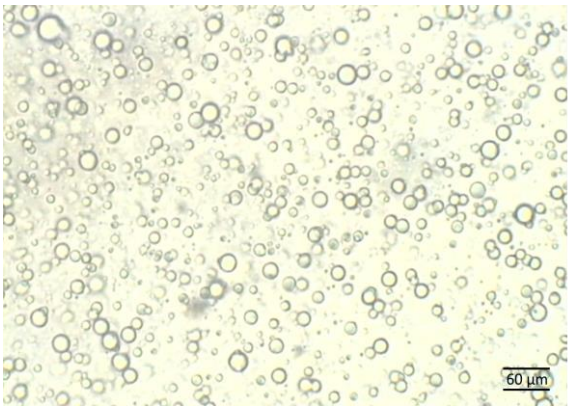
The light microscopy images revealed that the size of the droplets and the micro structure of the systems depended on the co-emulsifier used.

In emulsions with PGL, several small inner drops of oil were observed in the water phase. The droplets presented a smaller and homogeneous size (Fig. 4.7a).

In the emulsions with PGIS, greater inner oil droplets with a non homogenous size were seen. The droplet size differences between these emulsions are probably due to a coalescence phenomenon occurring in PGIS emulsions, leading to long term instability (Fig. 4.7b).



(a)



(b)

Fig. 4.7. Photomicrographs of A (a) and C (b) after 1 month of preparation (magnification 250 x).

Concerning the glycols, the results did not show a significant influence on the microstructure of the emulsions.

The microscopy analysis is not in accordance with droplet size distribution since the results showed that both systems have a similar droplet size after one month.

4. Discussion

Systematic formulation developments coupled with structure analysis work were required in order to find out the best proportions of emulsifiers and polymers. A single method alone is not representative, however when supported by different methods (rheology, microscopy, thermal analysis) they give important directives about the stability of the systems.

The polymer modified silicone surfactant selected as the main surfactant, substantially contributed to the distinct physical stability of emulsions.

The mixture of polymers selected (PVM/MA and HPMC) allowed the formation of a stronger structure as demonstrated by oscillatory methods.

The formation and stability of the present o/w emulsions is not expected to be affected by the pH and/or ionic strength of the aqueous phase. This property can be beneficial for drugs and other molecules that can be incorporated exhibiting a poor stability at low pH.

The selection of ingredients such as co-emulsifiers and glycols, can only be achieved using more specific methods. In this study we used light scattering, rheology, DSC and microscopy. The microscopy results showed that emulsions containing PGL presented smaller and more homogeneous droplets compared with emulsions containing PGIS. Moreover, the photomicrograph of the PGL emulsion (Fig. 4.7a) showed that the droplets are more aggregated which can explain the droplet size results that could be not able to distinguish between two or more droplets giving a higher droplet size. The results of microscopy analysis are in accordance with rheology and DSC results showing that emulsions containing PGL presented a stronger structure and the glycols had a minor influence on this parameter.

A hypothetical explanation might be a different cross-linking with the gel base from the co-emulsifiers. HPMC has been reported to possess surface active properties, therefore, HPMC occupies some area at the interface favoring an interaction between HPMC and co emulsifiers that is likely to take place in the polar region of the emulsion (close to the surface). As described in Table 3.2 (Chapter III), the chemical structures of these components namely the co emulsifiers, are very complex, thus, it is difficult to predict the interactions between them.

Fig. 4.8 describe a hypothetical model in which the microstructure of the cold process o/w emulsion consists in tree main effects, 1) the presence of a silicone based surfactant

combined with a PEG based co emulsifier; 2) the presence of a mixture of polymers, one with surface active properties and the second with swollen microgels; and 3) the presence of a cationic surfactant in a minimum concentration.

It is generally known that, if a silicone based surfactant and a monoglyceride emulsifier are both present in an o/w emulsion, interfacial stabilizing layers are formed around the oil droplets, since monoglycerides, such as glyceryl laurate, are surface active ingredients, as they have polar functional groups as well as nonpolar hydrocarbon chains [10]. The primary surfactant is responsible for the main decrease in the surface energy, stabilizing the oil droplets; the co-emulsifier is responsible for an additional stabilization, as the presence of the hydrophilic PEG groups are likely to stabilize the interfaces [11]. It is assumed that the orientation of polymer modified silicone surfactant is with the apolar triglycerides orientated to the oil phase and the polar PEG/PPG groups to the bulk phase, the same schematic representation for PGL. The PEG groups are with an optimal position for the interaction with the bulk phase, i.e. with the aqueous gel network (Fig. 4.8c).

PVM/MA is optimally crossed-linked so it will disperse in cold water but not fully dissolve. Consequently, its solutions are able to stabilize the dispersed phase in oil-in-water emulsions. The mechanism of suspension is believed to be related to the presence of swollen microgels, as represented in Fig. 4.8(a), which bear an overall negative charge (Fig. 4.8b). They help the oil droplets, which due to cetrimide are positively charged, to repel one another and so prevent coalescence [12, 13].

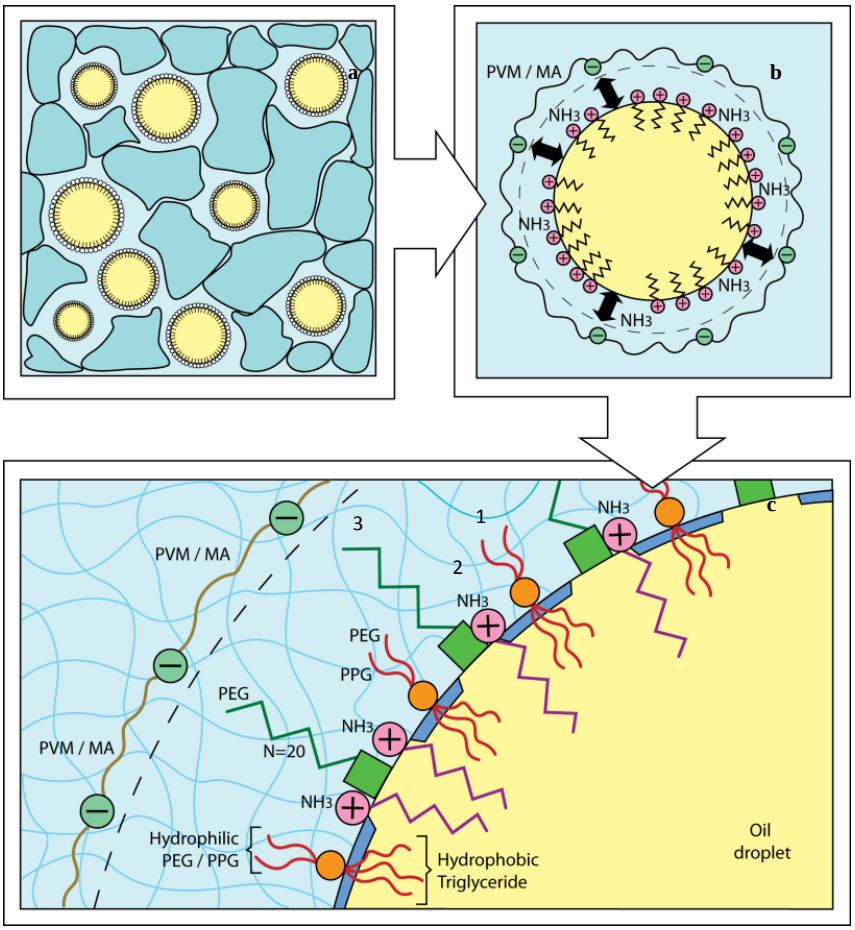


Fig. 4.8. Possible schematic representation for the structure of the cold process o/w silicone based emulsion. a) General representation of an o/w emulsion containing swollen microgels in the water phase; b) schematic representation of an oil droplet; c) schematic representation of the molecules involved in the interfacial phenomenon, polymer modified silicone surfactant (1); cetrimide (2); PGL (3).

5. Conclusions

Structure and microscopic analysis demonstrated that the emulsions containing polymer modified silicone surfactant and PGL as co-emulsifier, presented a stronger microstructure which is a good indicator for stability.

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1. Introduction

The main barrier to the percutaneous absorption of topically applied drugs is the SC. Due to the barrier nature of the SC, topically applied compounds may accumulate, i.e., the SC may serve as a reservoir from which substances can be subsequently absorbed over long periods of time [1, 2].

TG are the most frequently prescribed drugs by dermatologists. Their clinical effectiveness in the treatment of psoriasis and atopic dermatitis is related to their vasoconstrictive, anti-inflammatory, immunosuppressive and anti-proliferative effects. The treatment with TG formulations is effective, easy to administer, acceptable to patients and safe when used correctly. Since their introduction in the early 1950s they have revolutionized treatment of inflammatory skin disease [3, 4]. MF is a potent corticosteroid which presents an improved risk/benefit ratio. It is therefore of great value for inflammatory skin diseases, showing a strong anti-inflammatory action, rapid onset of action and low systemic bioavailability after topical application [5, 6].

The target cells for TG are the keratinocytes and fibroblasts within the viable epidermis and dermis, where the glucocorticoid receptors are located. The transport across the cell membrane of TG is a non-mediated passive diffusion process related to drug lipophilicity. After reach the glucocorticoid-receptor, the complex is translocated into the nucleus to either stimulate or inhibit transcription and regulate thereby the inflammatory process. The inhibition of IL-1 α in keratinocytes has anti-inflammatory effects, whereas the same inhibition in fibroblasts has anti-proliferative and atrophogenic effects [3, 7].

Over the years, research has focused on strategies to increase benefit/risk ratio of corticoids. Complex approaches, such as iontophoresis, electroporation, eutectic mixtures [8], supersaturated systems [9, 10], lipid nanocarriers [11, 12, 13], classical or deformable liposomes [14, 15], have been studied. However vehicles intended for TG delivery with an improved benefit/risk ratio are still on demand. Several approaches have been made in order to improve stability, drug release, permeation and the benefit/risk ratio of topical corticosteroid treatment. The pharmaceutical development of such carriers represents an enormous challenge and during the past 10–15 years, numerous formulations and drug delivery concepts emerged for enhanced therapeutic

applications [16], however the cost-benefit of these new galenic forms are often difficult to anticipate.

The aim of this chapter was the *in vitro* and *in vivo* characterization of cold processed oil-in-water emulsions intended for MF delivery to induce glucocorticoid targeting to upper skin strata, decreasing adverse effects of TG.

2. Materials and methods

2.1 Materials

The materials used are described in Chapter III, section 2.1. Elocon® (Schering-Plough, EUA) was purchased from a local pharmacy.

2.2 Methods

2.2.1 Preparation of the formulations

The emulsions were created according the method described in Chapter III, section 2.2.4. The composition of the emulsions is described in Table 3.21. The gels were prepared according the method described in Chapter III, section 2.2.2.1.

2.2.2 HPLC method for the determination of MF

A Hitachi Elite Lachrom System (VWR, USA) equipped with four Pumps L-2130, an autosampler L-2200, a column oven L-2300, an UV Detector L-2400 and a software EZ Chrom Elite Version 3.2.1. were used in all chromatographic analysis. An analytical reversed-phase (RP) Lichrospher 100 RP18, (125mm x 4mm, 5µm, Merck) was used.

The method used an isocratic gradient mobile phase with 70% (v/v) methanol and 30% (v/v) water. A flow rate of 1.5 mL/min was used with a 10 µL injection volume. The auto sampler chamber was maintained at 4 ºC and the eluted peaks were monitored at excitation and emission wavelengths of 248 nm. The run time was 11 min.

The experimental validation of the analytical method was performed in accordance with the CPMP/ICH/381/95 guideline [17] (Annex 1).

2.2.3 *In vitro* permeation of MF from HPMC and HPC gels

The permeation of MF from HPC and HPMC gels were measured in infinite dose conditions using a silicone membrane (Sil-Tec, reff 500-2, 0.002"), obtained from Technical Products Inc. of Georgia (USA) with a diffusion area of 1cm^2 and human skin obtained from a surgical intervention to reduce abdominal mass in a healthy Caucasian female of 54 years of age, after ethical approval and informed consent. The silicone membranes were washed and equilibrated with ethanol/water (1:1) during 30 minutes and then mounted between the donor and receiver compartments on static vertical Franz diffusion cells (receptor volume: 3 mL, permeation area: 1 cm^2). The skin was removed from the involucre, placed in ≈ 60 ºC isotonic phosphate buffer at pH 7.4 until thawed. The excess of fat was carefully removed and the SC was gently separated from the remaining tissue. The SC was visually inspected for any defects, and then cut into sections, large enough to fit on Franz Cells.

Ethanol/water (1:1) was used as receptor phase to assure perfect *sink conditions* in the whole experiment. It was constantly stirred with a small magnetic bar (200 rpm) and thermostated at 32 ± 0.5 °C throughout the experiments. The samples were then applied (0.2 to 0.4 g) evenly on the surface of the membrane in the donor compartment and sealed by Parafilm® immediately to prevent water evaporation.

Samples were collected from the receptor fluid at pre-determined time points - 1, 2, 4, 6, 8 and 24 h for silicone membrane and 1, 2, 4, 6, 8, 24, 32 and 48 h for human skin membrane and replaced with an equivalent amount (200 µL) of receptor medium. The drug content in the withdrawn samples was analyzed by HPLC. Repeated measures, using at least six replicated cells for each formulation, were used.

2.2.4 *In vitro* release of MF from A, B, C and D emulsions

Release of MF from A, B, C and D emulsions was measured in infinite dose conditions using a hydrophilic polysulfone membranes filters 0.45µm (Tuffryn®) from Pall Corporation (USA) with a diffusion area of 1cm^2. The membranes were washed and equilibrated with ethanol/water (1:1) during 30 minutes and then mounted between the donor and receiver compartments on static vertical Franz diffusion cells (receptor

volume: 3 mL, permeation area: 1 cm^2). The conditions were the same described in section 2.2.3. The time points for sample collections were, 1, 2, 3, 4, 5 and 6h.

The data obtained from *in vitro* release studies were fitted to two different kinetic models:

1). zero order

$$Q_t = Q_0 + K_0 t \quad \text{(Eq. 5.1)}$$

Where, Q_t is the amount of drug dissolved in the time t and K_0 is the zero order release constant.

2). Higuchi model:

$$Q_t = k\sqrt{t} \quad \text{(Eq. 5.2)}$$

where, Q is the amount of drug released in time t and k is the release constant.

The coefficient of determination (R^2) was determined for each model as it is an indicator of the model's suitability for a given dataset.

2.2.5 *In vitro* permeation of MF from A, B, C and D emulsions and commercial cream

Permeation of MF from A, B, C and D emulsions and commercial cream was measured in infinite dose conditions using a silicone membrane (Sil-Tec, reff 500-2, 0.002"), obtained from Technical Products Inc. of Georgia (USA) with a diffusion area of $1cm^2$. Permeation of MF from A, B, C and D emulsions was also measured through human skin obtained from a surgical intervention to reduce abdominal mass in a healthy Caucasian female of 54 years of age, after ethical approval and informed consent. The procedure was the same described in section 2.2.3. with the difference that the time points of sample collection for permeation through human skin were: 1, 2, 4, 6, 8, 24, 30 and 48 h.

2.2.5.1 Comparison between the permeation profile of emulsion A and commercial cream through silicone membrane

The two profiles were compared based on the similarity factor (*f2*) expressed by the equation 5.3.

$$f_2 = 50 \times \log\left\{\left[1 + (1/n)\sum_{j=1}^{n}|R_j - T_j|^2\right]^{-0.5} \times 100\right\} \quad \text{(Eq. 5.3)}$$

Where, *n* is the sampling number, *R* and *T* are the percent dissolved of the reference and test products at each time point *j*.

2.2.6 Skin permeation parameters

The cumulative amount of MF permeated (Qt) through excised human skin was plotted as function of time and determined based on the following equation:

$$Qt = \frac{Vr \times Ct + \sum_{t=0}^{t-1} Vs \times Ci}{S} \quad \text{(Eq. 5.4)}$$

Where, C_t is the drug concentration of the receiver solution at each sampling time, C_i the drug concentration of the sample applied on the donor compartment, and Vr and Vs the volumes of the receiver solution and the sample, respectively. S represents the skin surface area (1 cm^2).

The slope and intercept of the linear portion, between 8 and 30 h for A and B emulsions and using the all points for HPC and HPMC gels, of the plot were derived by regression using the Prism1, V. 3.00 software (GraphPad Software Inc., San Diego, CA, USA). MF fluxes (J, µg $cm^{-2}h^{-1}$) through the skin were calculated from the slope of the linear portion of the cumulative amounts permeated through the human skin per unit surface area versus time plot. The permeability coefficients (Kp, cm h^{-1}) were obtained by dividing the flux (J) by the initial drug concentration (C_0) in the donor compartment applying the Fick's 2nd law of diffusion (Eq. 5.5), and it was assumed that under *sink conditions* the drug concentration in the receiver compartment is negligible compared to that in the donor compartment.

$$M(t) = k\ l\ c_0 \left[\frac{D\ t}{l^2} - \frac{1}{6} - \frac{2}{\pi^2} \sum_{n=1}^{\infty} \frac{(-1)^n}{n^2} \exp(-Dn^2\ \pi^2\ \frac{t}{l^2})\right] \quad \text{(Eq. 5.5)}$$

2.2.7 *In vitro* tape stripping of emulsion A

Three individual tape stripping experiments were performed for emulsion A on human skin. The human skin was obtained from a surgical intervention to reduce abdominal mass in a Caucasian female of 54 years of age, after ethical approval and informed consent. The tissue was treated as described in section 2.4. After the fat being discarded, the two outermost skin layers (epidermis and dermis) were mounted between the donor and receiver compartments of static vertical Franz diffusion cells. The procedure adopted was the same described before. The formulations (300 µg) were kept in contact with the human skin for 24 h, and then the skin samples were rinsed to remove the excess of formulation.

The adhesive films employed to remove the superficial SC layers were Scotch® tape (3M, UK). Pressure was applied with the thumb covered in a vinyl glove to ensure a rolling movement and thus minimizing the influence of wrinkles. After applying pressure for 3 s, the tape was removed in a single rapid movement.

Fifteen sequential tape strips were used to separate the SC from epidermis and dermis; the 15 pieces were placed in conical tubes containing 50 mL of tetrahydrofuran/water (75:25, v/v). The remaining skin (viable epidermis and dermis) was cut in small pieces and also placed into conical tubes with 50 mL tetrahydrofuran/water (75:25, v/v). The tissues were left in contact with the extraction solvent for 24 h, then the SC and epidermis and dermis samples were homogenized using a hand-held tissue homogenizer (Polytron PT3000, Kinematica AG, Switzerland; 16.6 x 1000rpm) for 3 min, sonicated for 20 min, centrifuged for 5 min at 3000 rpm, filtered through a 0.45 µm pore membrane and assayed for MF content by HPLC. The amount of MF rinsed from the donor compartment was also quantified by HPLC.

2.2.8 *In vivo* anti-inflammatory activity studies

The croton oil-induced ear inflammation model for investigating anti-inflammatory effects of nonsteroidal and steroidal compounds [18] was tested in female NMRI mice

(23–25 g) purchased to Charles River (Cerdanyola del Vallés, Spain). Mice were used after 1-week acclimatization to the laboratory environment. All animal experiments were carried out with the permission of the local animal ethical committee in accordance with the EU Directive (2010/63/UE), Portuguese law (DR 129/92, Portaria 1005/92) and all the following legislations. The experimental protocol was approved by Direcção Geral de Veterinária.

The inside of an animal ear was challenged with 10 μL of 5% croton oil dissolved in acetone and left to dry. After the challenge, each animal was kept individually in a separate cage. One hour after the challenge, tested formulations (A and B emulsions, 1 month after preparation and commercial cream with the same MF dosage) were applied (10 μL) on the challenged area and left to dry. The resulting edema was determined 16 h later. The ear thickness was measured with a Mitutoya® micrometer with three readings per ear. The degree of edema inhibition was calculated as a percentage of inhibition, determined by comparing the drug treated group with untreated controls. Three mice were used per group.

2.2.9 Mouse ear histology

In order to evaluate the surroundings of the site of application, animals were sacrificed 16 h after the treatment with emulsion A and commercial cream. An ear challenged with 10 μL of 5% croton oil dissolved in acetone was used as positive control and a native ear as negative control. The ears were resected and fixed in 10% buffered formalin solution. Tissue samples were processed for embedding in Parafin wax by routine protocol. 5μm thick sections were stained with hematoxylin and eosin (H&E). The slides were examined using a light microscopy (Axioscope camara Leica Software Leica Image Manage IM 50) and 400x magnification images were aquired using Microsoft Image Composite Editor. The histopathological appearance of tissues was compared for structure changes and cell infiltration.

2.2.10 *In vitro* cytotoxicity

A spontaneously immortalized human keratinocyte cell line, HaCaT (CLS, Germany) and mouse embryonic fibroblast cell line, NIH 3T3 - (ATCC CRL-1658) were grown in

RPMI-1640®(Gibco, UK) medium supplemented with 10% (w/v) fetal bovine serum (FCS, Life Technologies, Inc., UK), penicillin (100 IU/mL), and streptomycin (100 μg/mL) in a humidified 95% O_2/5% CO_2 environment at 37 ºC. For the subculture, cells growing as monolayer were detached from the tissue flasks by treatment with 0.05% (w/v) trypsin/EDTA (Invitrogen, UK).

To determine *in vitro* drug effects on cell viability, cells (cultured in 96-well microplates) were incubated with MF in solution (DMSO), A and B emulsions and MF solubilized in ethoxydiglycol and pentanediol for 72 h. The cell viability was determined with the 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay as described in detail elsewhere [19, 20].

The inhibitory concentration (IC_{50}) for the drug in DMSO was calculated using Graphpad Prism software v5.0 (GraphPad Software, Inc., USA) by the sigmoidal curve fitting method. The IC_{50} obtained was used to determine the cell viability of the cells incubated with A and B emulsions and MF solubilized in ethoxydiglycol and pentanediol.

2.2.11 Data analysis

According the method described in Chapter III, section 2.2.2.3.

3. Results

3.1 *In vitro* permeation of MF from HPMC and HPC gels

Fig. 5.1 and 5.2 shows the permeation profiles of MF from HPC and HPMC gels through silicone membrane and human skin, respectively, presenting the cumulative amounts of drug permeated as a function of time, during 24 hours.

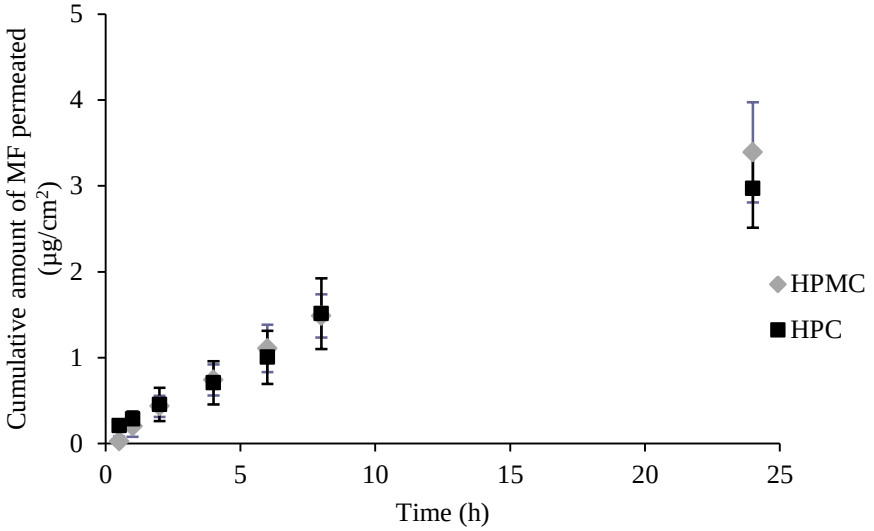

Fig. 5.1. Permeation profile of MF from the HPMC and HPC gels through silicone membrane, (mean ± SD, n=6).

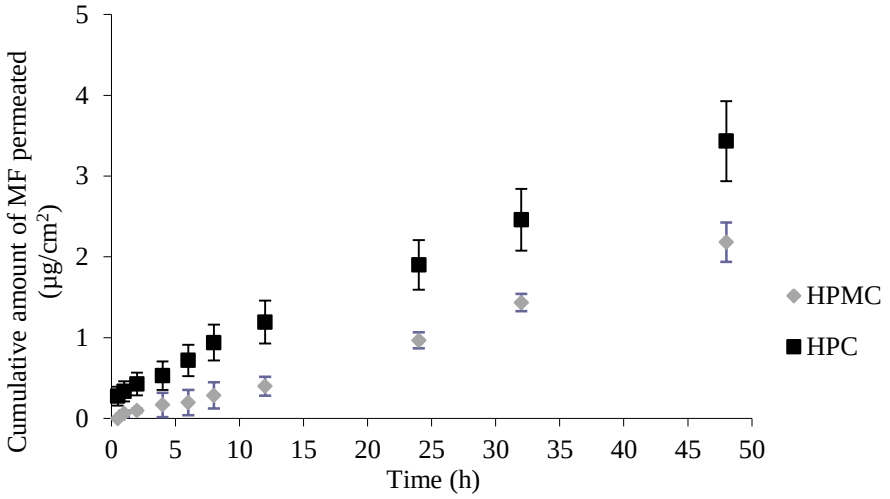

Fig. 5.2. Permeation profile of MF from HPMC and HPC gels through human skin, (mean ± SD, n=6).

The fluxes and Kp (Table 5.1), where obtained fitting the single curves of the permeation profiles in the linear region, between 0 and 48 h.

Table 5.1. Flux and permeability coefficient (Kp) of MF through skin membrane (mean ± SD; n= 6).

	HPC Gel	**HPMC Gel**
Flux (µg cm^{-2} h^{-1})	0.066	0.046
Kp (cm h^{-1})	$7.11x10^{-5}$	$5.78x10^{-5}$

The permeation profiles through silicone showed no significant differences among the two gels ($p > 0.05$), however the permeation profiles through human skin showed that the flux and permeability coefficients of MF in HPC gel was higher comparing with HPMC gels, furthermore analysis of variance showed significant differences among the two profiles ($p < 0.05$).

3.2. *In vitro* release and permeation of MF from emulsions

The results of the amount of MF released through the Tuffryn® membrane are shown in Fig. 5.3. The *in vitro* data were fitted to different equations and kinetic models to explain the profiles.

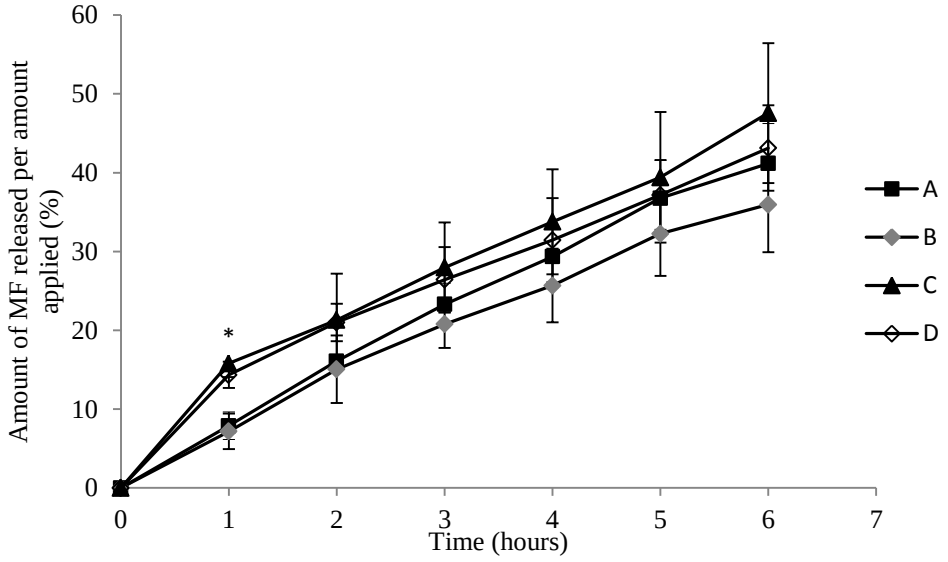

Fig. 5.3. Release profile of MF from A, B, C and D emulsions through Tuffryn® membrane, (*significantly different; mean ± SD, n=6).

From the ANOVA statistical analysis, there were significant differences only at 1h between the formulations containing PGL and formulations containing PGIS ($p < 0.05$). Regression analyses of each line were performed and the respective rates of release determined from the lines slopes and are presented in Table 5.2. The release profiles were expressed by zero order (Eq. 1) and Higuchi model (Eq. 2).

Table 5.2. Kinetic parameters obtained after fitting the release data from the formulations to different release models, where K is the release rate constant, b is the intercept and R^2 the coefficient of determination.

	Zero order			**Higuchi**		
	k	**b**	**R^2**	**k**	**b**	**R^2**
A	6.95	1.23	0.994	17.36	-4.79	0.939
B	6.02	1.49	0.991	15.14	-3.87	0.948
C	7.00	4.86	0.969	18.64	-2.29	0.976
D	6.62	4.91	0.964	17.21	-1.85	0.985

Model fitting showed that formulations A and B followed a zero order model and formulations C and D followed the Higuchi model, which had the highest values for R^2 (Table 5.2) and thus, statistically described best the drug release mechanism. Fig. 5.3 shows that for formulations C and D a burst release occurs between 0 and 1h and after that the slope is constant.

Fig. 5.4 shows the permeation profiles of MF from A, B, C and D through silicone membrane, presenting the cumulative amounts of drug permeated as a function of time, during 24 hours.

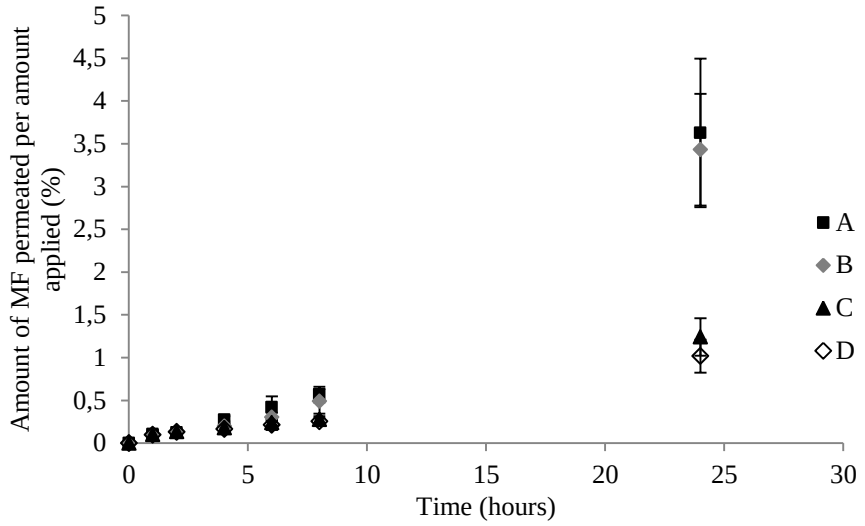

Fig. 5.4. Permeation profile of MF from A, B, C and D through silicone membrane, (mean ± SD, n=6).

Permeation profiles through silicone membrane showed that, after 6 h formulations A and B are statistically different from formulations C and D, thus, the co-emulsifier influence the permeation of MF. Due to a better permeation profile and a stronger structure (Chapter IV) we selected formulations containing PGL (A and B emulsions) to continue this work.

Fig. 5.5 shows the comparison between the permeation profiles through silicone membrane of formulation A and commercial cream.

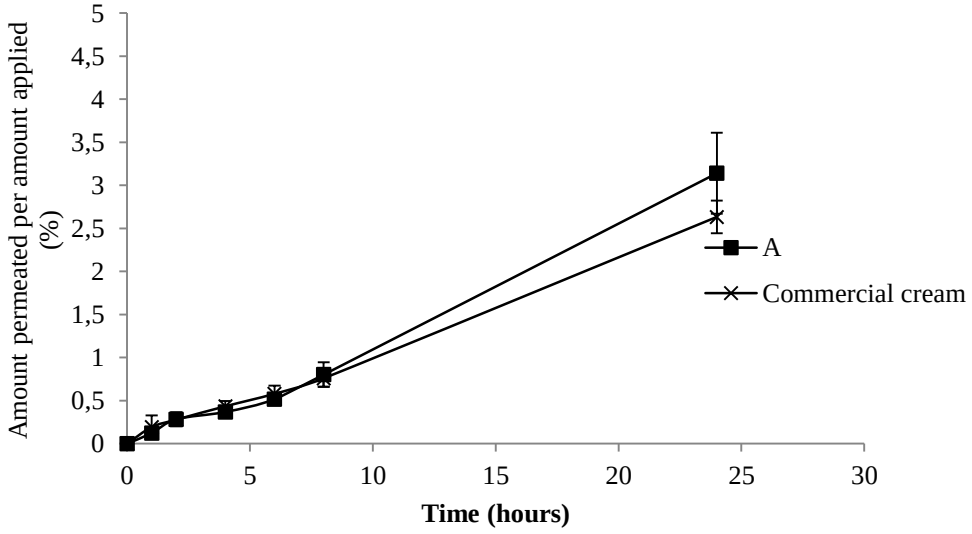

Fig. 5.5. Permeation profile of MF from A and commercial cream through silicone membrane, (mean ± SD, n=6).

Applying the eq. 5.3, the value obtained for f_2 was 99.52.

Fig. 5.6. shows the permeation profiles of MF from A and B through human skin, presenting the cumulative amounts of drug permeated as a function of time, during 48 hours.

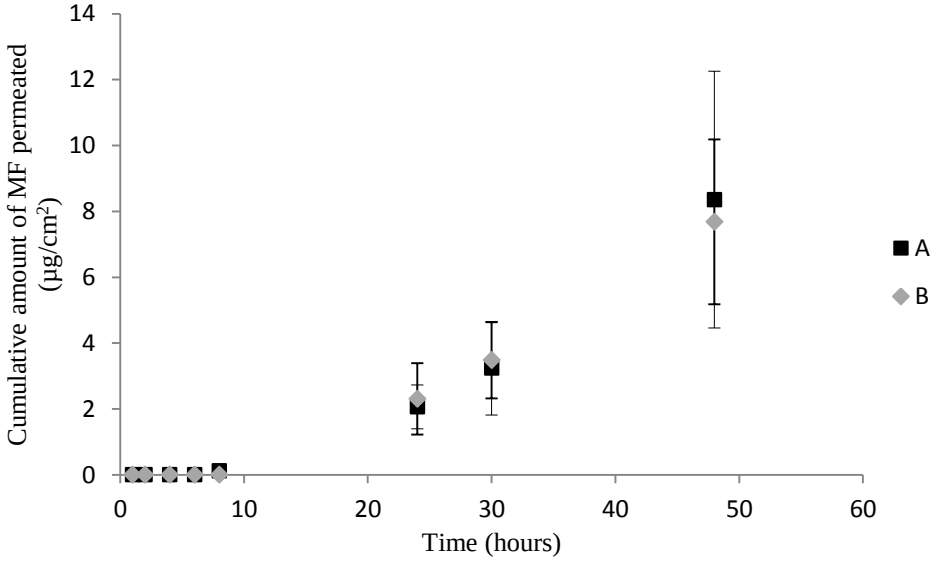

Fig. 5.6. Permeation profile of MF from A and B through human skin, (mean ± SD, n=6).

The fluxes, permeability coefficients and lag time (Table 5.3) where obtained fitting the single curves of the permeation profiles in the linear region (between 8 and 30 h).

Table 5.3. Flux, Kp and lag time of MF through skin membrane (mean ± SD, n= 6) for A and B formulations.

Formulation	Flux (µg cm^{-2} h^{-1})	Kp (cm h^{-1})	Lag time
A	0.14 ± 0.055	$5.20x10^{-4}$ ± $2.05x10^{-4}$	7.31 ± 1.36
B	0.15 ± 0.056	$6.30x10^{-4}$ ± $2.94x10^{-4}$	8.55 ± 0.30

There was no significant differences among A and B concerning the amount of MF permeated ($p > 0.05$).

3.3 *In vitro* tape stripping of emulsion A

An extraction of the drug from the skin by tape stripping was performed for A emulsion; the results are showed in Fig. 5.7.

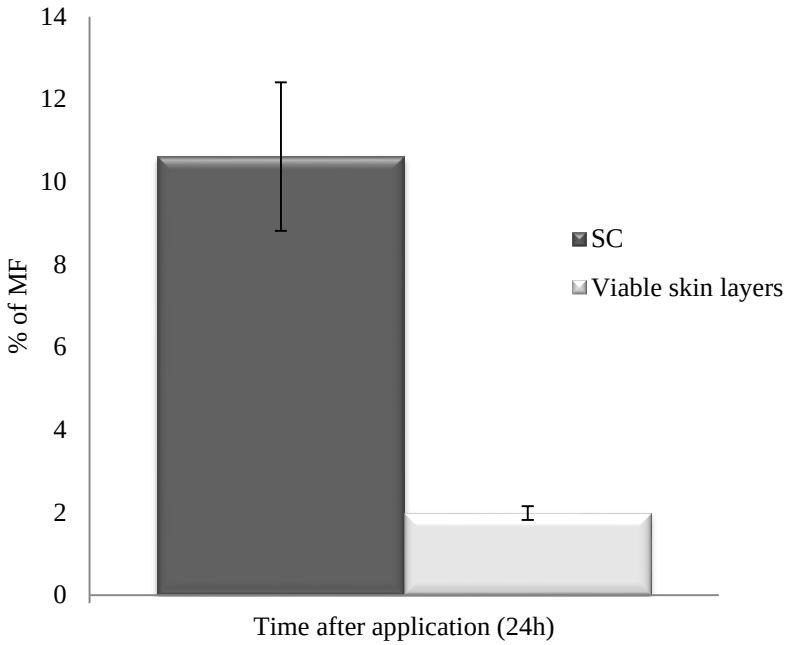

Fig. 5.7. Penetration of MF in the SC and viable skin layers (epidermis and dermis) after 24h.

The amount for MF extracted in the SC, in viable skin layers (epidermis and dermis) and in the donor compartment was analyzed by HPLC. The values of MF obtained were 30.82 μg (10.61%) in the SC and 5.72 μg (1.99%) in the viable skin layers (epidermis and dermis) and 232.01 μg (80.56 %) in the donor compartment.

3.4 *In vivo* anti-inflammatory activity studies

Application of croton oil (5% in acetone) induced erythema and edema which was remarkably attenuated by the local application of both tested formulations (A and B) containing 0.1% of MF (Fig. 5.8). The degree of edema inhibition is proportional to the anti-inflammatory activity of the formulation. Moreover, when cold processed emulsions were compared with the commercial cream containing the same amount of

drug (0.1%), the results showed that there were no significant differences between the anti-inflammatory actions ($p > 0.05$).

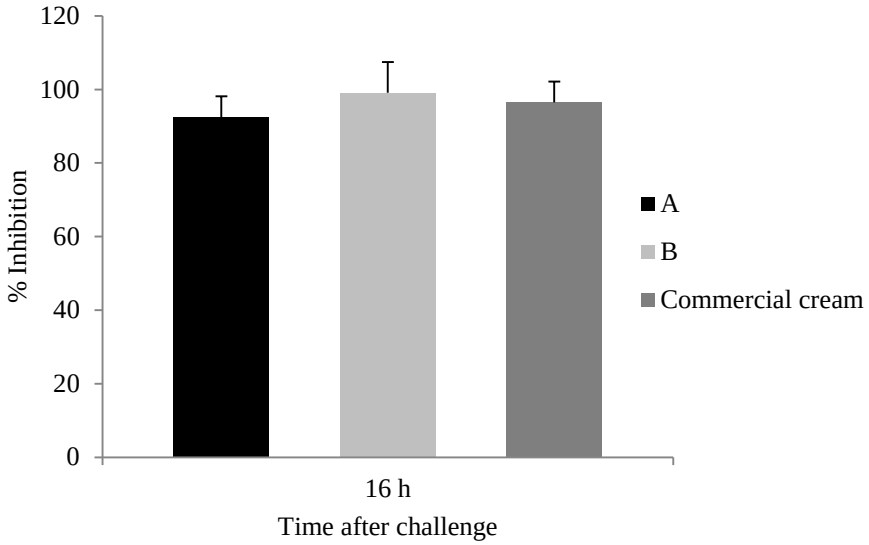

Fig. 5.8. Effect of treatment with A, B and MF commercial cream on the % of inhibition of the edema on a mouse ear, challenged with croton oil, (mean ± SD, n=3).

3.5 Mouse ear histology

Histological analysis of the mice ear skin did not reveal morphological tissue changes neither cell infiltration signs after the application of the A emulsion. The structure of the SC, epidermis and dermis were preserved, as observed in Fig. 5.9c. Moreover, comparing the A emulsion with the commercial cream, the same trend is observed, the edema (observed after the application of croton oil, Fig. 5.9b) decreased significantly after the application of both formulations.

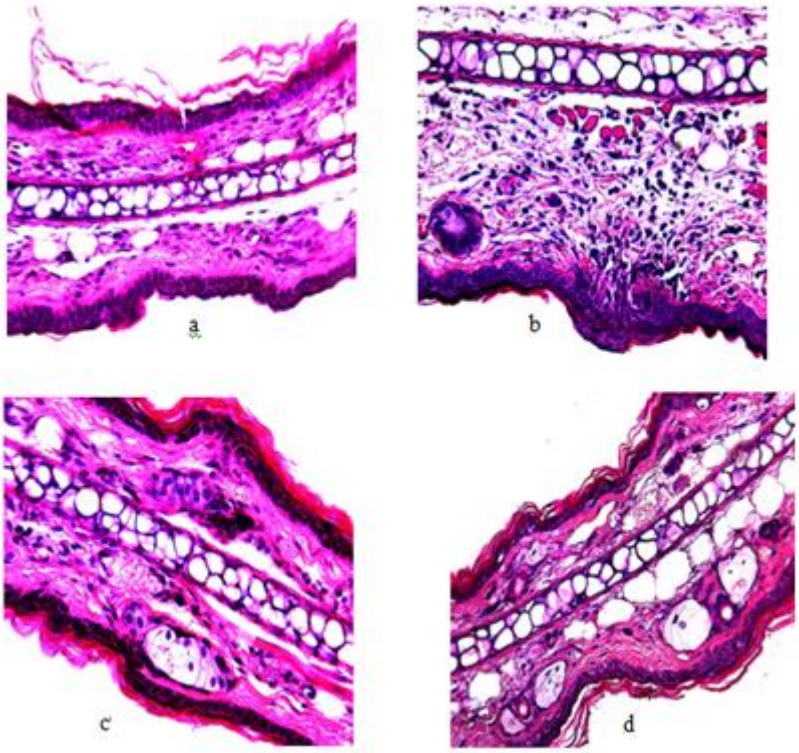

Fig. 5.9. Effect of MF on croton oil-induced inflammatory cell infiltration of mouse ear. H&E-stained histological sections were prepared from ears resected 16 h after challenge: (a) unchallenged ear; (b) ear from mouse challenged with croton-oil in the absence of any treatment; (c) ear from mouse challenged with croton-oil post-treated with PT emulsion; (d) ear from mouse challenged with croton-oil post-treated with commercial cream. Magnification: 400x.

3.6 *In vitro* cytotoxicity

To investigate the potential cytotoxicity of the MF - loaded cold processed oil-in-water emulsions, the cell viability was evaluated using NIH 3T3 and HaCaT cell lines in a MTT assay. Firstly, the IC_{50} of the drug in solution (DMSO) was determined by non linear regression analysis, and subsequently, the concentration found was used to determine the cell viability after application of A and B emulsions. The cytotoxicity of MF in ethoxydiglycol and pentanediol was also analyzed.

The MF solubilized in the glycol or formulated in the emulsions presented different behaviors in terms of cell toxicity. It was found that the IC_{50} of the MF in DMSO was $5.1x10^{-6}$ g/mL, using the same concentration it was observed that the glycols increased

the cytotocixity of MF, which was more evident in NIH 3T3 cell line. However, it was found that the 2 emulsions were not cytotoxic in both NIH 3T3 and HaCaT cells on the concentration tested (Table 5.4).

Table 5.4. Cell viability in NIH 3T3 and HaCaT cell lines after 72h of incubation with A and B emulsions and MF solubilized in ethoxydiglycol and pentanediol (mean ± SD, n = 6).

	Cell viability (%)		
	MF conc. (g/mL)	**NIH 3T3**	**HaCat**
A	$5.1x10^{-6}$	96.5 ± 3.6	98.3 ± 1.4
B		96.7 ± 11.6	104.7 ± 2.3
MF + pentanediol		19.5 ± 3.2	46.2 ± 18.2
MF + ethoxydiglycol		19.6 ± 2.7	48.2 ± 17.6

The incorporation of MF into the emulsions did not increase the cytotoxicity, in contrast to what happens when MF was solubilized in the glycol alone. Finally, the incorporation of the drug in the emulsion protects cell lines, mouse fibroblasts and human keratinocytes, from the cytotoxicity of the drug.

4. Discussion

In this study we demonstrated that the release and permeation profiles of MF from the emulsions were not influenced by the glycol used which was in accordance with the solubility results (Chapter III, section 4.2.2.1.). The drug solubility in the vehicle is an important factor for drug penetration across the skin or artificial membranes since; the solubility of a drug in its vehicle is an important factor determining availability [21].

As demonstrated in previous studies [22, 23], MF is insoluble in water and exponentially increases as the amount of glycol is increased. It was shown that in co-solvent mixtures the solubility of MF was similar for the two glycols, around 3 mg/100mL. As the solubility of MF was the same in pentanediol and ethoxydiglycol at 10% (w/w) and the concentration of MF is the same in the two emulsions, different release profiles were not expected. However the co emulsifier seems to influence the release of MF from the vehicle since formulations A and B followed a zero order model

and formulations C and D the Higuchi model. Nevertheless, the results were not very different. The permeation results through the silicone membrane from the emulsions reflected better these differences. In fact, after 6h, the MF permeation was higher in emulsions containing PGL when compared with emulsions containing PGIS. The different permeation rates can be explained regarding the different natures of the co emulsifiers. PGL is a hydrophilic emulsifier whereas PGIS is a lipophilic emulsifier, as MF presents a higher affinity for lipophilic substances, thus the permeation of the drug from the emulsions containing PGIS is retarded. This mechanism is explained by the following equation:

$$K_{sc/formulation} = C^{penetrant} \text{ in the SC} / C^{penetrant} \text{ in formulation} \quad \text{(Eq. 5.6)}$$

in which $C^{penetrant}$ represents the solubility of the penetrating molecule in the SC relative to that in the formulation. Therefore, the quantity of molecules penetrating into the SC can be increased by increasing the solubility of the penetrating molecule in the SC or by reducing its affinity in the formulation [24]. Thus, increasing the affinity of the MF in the formulation, the penetration decreases.

The plot obtained of the cumulative drug amount in the acceptor compartment versus time shows an increase with time, which approaches a constant slope as the experiment continues, this profile is typical in infinite dose experiments where the applied dose is so large that the depletion of the permeant in the donor chamber caused by evaporation or diffusion into and through the barrier is negligibly small [25].

The fluxes, permeability coefficients and lag time where obtained fitting the single curves of the permeation profiles in the linear region. The last point was not considered because, according the OECD guidance [26], at this time the skin barrier cannot be regarded as intact anymore. Moreover as the receptor fluid is composed by water:ethanol (1:1), it is possible that evaporation of the receptor fluid occurs explaining the high standard deviations. Although co-solvents added to the receptor solution can back diffuse and alter the structure of the skin, it has been shown that the penetration of a model compound was the same using, as receptor solution, 50% ethanol or 4% bovine serum albumin, suggesting that ethanol:water does not modify the skin barrier function in a significant way [27].

Potts and Guy [28] elegantly demonstrated that the permeability of a chemical from an aqueous solution through the SC could be estimated from only two parameters, the log P coefficient and the MW. Of the two, the partition coefficient had a bigger influence as evidenced by the weighting factors in the formula they derived:

$$\text{Log Kp (cm s}^{-1}) = -6.3 + 0.71 \log K_{oct/water} - 0.0061\ MW \qquad \text{(Eq. 5.7)}$$

Briefly, this formula indicates that when the lipophilicity of a penetrating molecule (expressed in the log P) increases, Kp increases and when its MW increases, the Kp decreases.

The LogP obtained by the Virtual Computational Chemistry Laboratory for MF was 2.81 [29] and the MW for this drug is 521.44 [30]. Applying the Pots Guy Eq. (5.7) the value obtained for Kp is 3.27×10^{-8} cm s^{-1}, that is 1.18×10^{-4} cm h^{-1}.

The enhancement ratio between 2.7-7.8 comparing the theoretical and experimental values of Kp for MF emulsions can be explained regarding the excipients used in these emulsions. In fact, we used co-solvents (ethoxydiglycol and pentanediol) which, in addition to affecting the drug solubility in the vehicle, may alter the structure of the skin and modify the penetration rate [31]. Moreover, the presence of IPM, a well-known penetration enhancer contributes to the percutaneous penetration of MF. It was demonstrated that the use of co solvent in combination with a potential penetration enhancer may offer synergistic enhancement [32]. Arellano et al. [33] demonstrated that the combination of propylene glycol and IPM significantly increased the percutaneous absorption of diclofenac sodium through rat skin. This enhancement effect was superior when compared with the enhancement caused by the propylene glycol alone, moreover it was demonstrated that an increase on the amount of propylene glycol resulted in the decrease in the flux due to an increase of the drug affinity to the vehicle.

Not only the presence of the polymer, as explained before, contributed to the increase on Kp, but also the presence of surfactants. Surfactants can also act as penetration enhancers due to their potential for solubilizing SC lipids besides their capacity to interact with keratin, resulting in a disruption of order within the corneocytes [34]. Moreover, it was demonstrated that glyceryl laurate enhanced the penetration of drugs through cadaverous skin and hairless rat skin *in vitro* [35]. The assemblement of these ingredients was translated in an enhancement of the Kp for MF through human skin.

The low amount of permeation (less than 4.0% after 48h for A and B emulsions) can be explained by the reservoir effect largely described for the SC, which can have advantages for clinical applications of topical steroids. It was found that the applied steroid could persist in the skin for a significant period of time (days) [1, 2].

The results obtained confirm the reservoir effect theory for SC, which can be positive for the treatment of skin diseases avoiding the adverse effects caused by the action of corticoids on fibroblasts receptors on dermis.

The results obtained in *in vivo* studies showed that the tested emulsions had, at least, the same efficacy when compared to the commercial cream. Moreover, it was demonstrated that emulsion A and commercial cream were similar regarding the permeation profiles.

The f_2 has been adopted by the Center for Drug Evaluation and Research and by Human Medicines Evaluation Unit of the European Agency for the Evaluation of Medicinal Products (EMEA), as a criterion for the assessment of the similarity between two *in vitro* dissolution profiles. Two profiles are considered similar when f_2 value is close to 100, in general values higher than 50 show the similarity of two profiles. This evaluation is based in the following conditions: a minimum of three time points, not more than one mean value of more than 85 % dissolved, 12 individual values for every time point that the SD of the mean should be less than 10 % from the second to the last time point [36, 37]. The value obtained for f_2 concerning the *in vitro* profiles of emulsion A and commercial cream was around 100 % suggesting that the two profiles are similar. However, only the two first conditions were accomplished in this study thus, the assay should be repeated with 12 replicates of each sample. Concerning the SD, we obtained values higher than 10 %, however it should be taken into account that these conditions are optimized for dissolution profiles and not for release and permeation profiles. The EMA website provides a Guidance document regarding generic topical active pharmaceutical ingredient formulations [38]. If what is sought is a generic substitution then a therapeutic equivalence study may be needed to establish essential similarity to the original topical product [39].

Cytotoxicity results showed that, the MF solubilized in the glycols, presents a higher cytotoxicity, comparing to the free drug in both cell lines which could be explained by the enhancement effect of these glycols in the release of the MF. However when incorporated in the emulsions the cell viability increased to more than 90%. Moreover, according to the relevant OECD guideline nº 439 [40], an irritant substance is predicted

if the mean relative tissue viability is found below 50% of the mean viability of the negative controls for a 15-60 min exposition time. In the present assay, cells were exposed to test samples for 72 h with the cell viability above 50%. Thus, the formulations can be considered non-irritant.

The *in vivo* results for Placebo A (emulsion A without MF) (Chapter VII, section 3.4) showed a drastic increase on the skin lipids after the application of Placebo A. It was demonstrated that the amount of sebum affects the permeability of skin to molecules and the presence of sebum on the forehead or forearm increased the diffusion of both hydrophilic and lipophylic molecules through the human skin [41]. The increase of the amount of lipids in the SC can thus, explain the formulation effect on the enhancement of MF permeation across the human SC.

5. Conclusions

This study reports the *in vitro* and *in vivo* studies of MF emulsions obtained by cold process preparation method. *In vitro* release and permeation studies revealed that the glycols used had no influence on the release and permeation profiles of MF which was in agree with the solubility results for the two glycols. Moreover, it was demonstrated that these emulsions are suitable vehicles for the delivery of MF containing ingredients which are responsible for a drastically increased on the Kp of MF. Permeation parameters through human skin showed that the amount of drug that reach the viable skin layers is very low. The results were confirmed by the reservoir effect observed after tape stripping of the SC. It was demonstrated an epidermal targeting for emulsion A decreasing the adverse effects wildly described for topical corticoids.

The formulation A has, at least, the same efficacy than the commercial cream as demonstrated by in vivo anti-inflammatory studies. Concerning the similarity factor, the study should be repeated with at least 12 units; however, the results obtained so far suggest that emulsion A and the commercial cream are similar. The emulsion A was selected as the final formulation.

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1. Introduction

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies over time under the influence of a variety of environmental factors such as the temperature, humidity, and light, and to establish a re-test period for the drug substance or a shelf life of the drug product and to recommended the most suitable storage conditions [1, 2]. The stability of a dosage form is defined by the USP 31 [3] as the maintenance of the chemical and physical integrity of the preparation, and when applied, the capacity of maintaining the microbiological quality. During the formulation development, the main objective of the stability testing is to prove the compatibility between the drug and the excipients and between the excipients themselves and/or to define adequate the packaging conditions in order to establish a suitable shelf life [4].

In general, the stability tests performed during the formulation development are done in accordance to the ICH, particularly the ICH Q1A (R2) which has been adopted by the CPMP (CPMP/ICH/2736/99) in March of 2003. This guideline defines the storage conditions during the test according to the climatic zones (Table 6.1).

Table 6.1. General case for stability testing in climatic zones I and II.

Study	Storage condition	Minimum time period covered by data at submission
Long term	25 ± 2 °C / 60 ± 5% RH or 30 ± 2 °C / 65 ± 5% RH	12 months
Intermediate	30 ± 2 °C / 65 ± 5% RH	6 months
Accelerated	40 ± 2 °C / 75 ± 5% RH	6 months

*RH – relative humidity

The long term testing should cover a minimum of 12 months and include at least three primary batches at the time of submission, and should be continued for a period of time sufficient to cover the proposed shelf life.

These requirements are applicable to the market introduction authorization (MIA) and not to the stability tests during the clinical trials neither to the stability tests during the formulation development.
It is important to underline that it is usually during the phase III of the clinical trials that the stability tests to support the MIA begin.

2. Materials and Methods

2.1 Materials

The materials used are described in Chapter III, section 2.1.

2.2 Methods

2.2.1 Production of three batches of emulsion A for stability assessment

Three batches of 1500 g each were produced using a miniplant reactor system (IKA® LR 2 ST) (pilot lab-scale). Briefly, the aqueous phase was prepared at room temperature by dispersing the aqueous thickening agents (HPMC and PVM/MA) in water at 150 rpm, inside the reactor vessel. Afterwards, the cetrimide and the drug (0.1 % w/w) dispersed in the pentanediol were added to the reactor vessel. The resulting mixture was homogenized until a clear and homogeneous gel was obtained. The oil phase was prepared, at room temperature, by mixing the polymer modified silicone surfactant with the co-emulsifier (PGL) and the oils (alkyl benzoate and IPM). The resultant mixture was added to the reactor vessel and the system was homogenized at 250 rpm during 30 min using an anchor stirrer.

2.2.2 Physical and chemical stability of emulsion A

The three batches of emulsion A were stored during 12 months at room temperature (25 ± 2 °C / 60 ± 5 % RH), intermediate conditions (30 ± 2 °C / 65 ± 5 % RH) and under accelerated conditions (40 ± 2°C / 75 ± 5 % RH). Samples were analyzed for macroscopic appearance, pH, apparent viscosity and MF chemical stability before the

storage period and on months 1, 3, 6, 9 and 12 of storage. At every evaluation time points, an accelerated stability test was also performed centrifuging the samples during 15 min at 5000 rpm (Medifuge, Heraeus Sepatech, GmbH, Germany). Macroscopic appearance was assessed by visual inspection. The pH was measured by immersing the glass electrode directly into the sample using a pH meter (Metrohm® pH Meter 744). The apparent viscosity was measured using a viscometer (Brookfield® RV DV-II, SSA, spindle SC4-27). HPLC was used to assay the stability of MF.

2.2.2.1 Microbiological stability of the emulsion A

The microbiological stability assessment was performed according to the Portuguese Pharmacopoeia 9 edition [5].

2.2.2.2 Droplet size analysis

The size distribution of the droplets was measured according the method described in Chapter IV, section 2.2.3.

2.2.3.3 HPLC conditions for the assessment of MF stability

The method used was the same described in Chapter V, section 2.2.2.

2.2.3 Production of one batch of placebo A

A batch of 15 kg of placebo A was produced in a Dumek® Dumoturbo 25 (pilot industrial-scale). The water was introduced in the reactor and the polymers (HPMC and PVM/MA) were dispersed by using a planetary mixing device during 15 min at 30 rpm and a homogenizer system during 5 min at 1498 rpm and followed for another 10 min at 2610 rpm. Afterwards, the cetrimide was added and left homogenized for 10 min with the planetary mixing device for 30 rpm and the homogenizer system at 2610 rpm. The system was kept under vacuum for 12 h at 40 cmHg. The pentanediol was introduced in the system and mixed using the planetary mixing device at 30 rpm and the homogenizer device at 1498 rpm during 15 min under vacuum. Finally the oil phase (polymer modified silicone surfactant, PGL, alkyl benzoate and IPM), previously mixed at room temperature, was added to the aqueous phase under constant mixing (30 rpm). The obteined mixture was homogenized during 19 min at 2610 rpm.

2.2.4 Physical and microbiological stability of placebo A

One batch of the emulsion A without MF (placebo A) was produced and stored for 12 months at room temperature (real-time, 25 ± 2 °C / 60% ± 5% RH), intermediate conditions (30 ± 2 °C / 65% ± 5% RH) and at accelerated aging conditions (40 ± 2 °C / 75% ± 5% RH). Samples were taken for analysis at the end of the following time periods: 0, 1, 3, 6 and 12 months and assessed in terms of macroscopic organoleptic characteristics, pH values (pH meter Metrohm® 827 pH Lab) and apparent viscosity (Brookfield® DV-I+, spindle SC4-21).

The microbiological stability assessment was performed according to the Portuguese Pharmacopoeia 9 edition [5].

2.2.4.1 Droplet size analysis of the placebo A

The size distribution was measured for every evaluation time points by light scattering using the method described in Chapter IV, section 2.2.3.

2.2.4.2 Cetrimide assay

It was used a Beckman system, equipped with a system gold solvent module, a Midas Spark 1.1 autoinjector (Spark®, AJ Emmen, Netherlands), a UV 166 detector (Beckman Instruments®) and a 32 Karat Software (Beckman Instruments®, Palo Alto, CA, USA), in all chromatographic analysis. The chromatographic analysis was performed at RT on an analytical reversed-phase (RP) Waters Spherisorb CN_RP (250mm x 4.6mm).

The optimized method used an isocratic gradient mobile phase with 50% (v/v) of acetonitrile and 50% (v/v) of sodium chloride buffer (0.05M). A flow rate of 1.0 mL/min was used and the volume of sample injected was 20 µL. The auto sampler chamber was maintained at RT and the eluted peaks were monitored at excitation and emission wavelengths of 210 nm. The solvent used to prepare the samples was acetonitrile. Each run lasted for 12 min.

3. Results

3.1 Physical, chemical and microbiological stability of the emulsion A

The stability of the emulsion in terms of drug content, pH values, apparent viscosity and macroscopic characteristics was assessed during 12 months at room temperature, intermediate conditions and under accelerate conditions.

The emulsion remained white with a creamy and homogeneous aspect after 12 months. The stability of the emulsion was assessed by visual observation for the possible phase separation or other instability phenomena and also after submission to centrifuge force. At every evaluation time points, and for the three different storage conditions, macroscopic observations did not reveal any sign of instability. The results after centrifugation were in accordance to the latter because the emulsion did not present any sign of phase separation after submission to the centrifugation.

The MF presents its maximum stability in acidic conditions [6]. In order to assess the stability of the pH of the emulsion, this parameter was evaluated during 12 months (Tables 6.2, 6.3 and 6.4).

Table 6.2. Stability test results for batch 1 during 12 months at 25 ºC, 30 ºC and 40 ºC. (n=3; mean ± SD).

Conditions of storage	**25 ºC**		**30 ºC**		**40 ºC**	
Time (months)	pH	Apparent viscosity (Pa.s)	pH	Apparent viscosity (Pa.s)	pH	Apparent viscosity (Pa.s)
0	3.89 ± 0.01	15.23 ± 0.55	3.89 ± 0.01	15.23 ± 0.55	3.89 ± 0.01	15.23 ± 0.55
1	3.84 ± 0.01	12.40 ± 0.90	3.82 ± 0.01	12.80 ± 0.26	3.79 ± 0.01	11.43 ± 0.51
3	3.76 ± 0.01	13.33 ± 0.15	3.73 ± 0.01	12.80 ± 0.35	3.67 ± 0.02	11.97 ± 0.58
6	3.68 ± 0.01	13.30 ± 0.15	3.62 ± 0.01	14.27 ± 0.21	3.67 ± 0.02	10.33 ± 0.25
9	3.64 ± 0.01	13.50 ± 0.36	3.56 ± 0.01	13.50 ± 0.25	3.37 ± 0.01	10.20 ± 0.15
12	3.63 ± 0.01	13.20 ± 0.25	3.39 ± 0.01	12.80 ± 0.36	3.34 ± 0.01	9.90 ± 0.25

Table 6.3. Stability test results for batch 2 during 12 months at 25 ºC, 30 ºC and 40 ºC. (n=3; mean ± SD).

Conditions of storage	**25 ºC**		**30 ºC**		**40 ºC**	
Time (months)	pH	Apparent viscosity (Pa.s)	pH	Apparent viscosity (Pa.s)	pH	Apparent viscosity (Pa.s)
0	3.76 ± 0.01	16.90 ± 0.40	3.76 ± 0.01	16.90 ± 0.40	3.76 ± 0.01	16.90 ± 0.40
1	3.67 ± 0.02	14.97 ± 0.40	3.74 ± 0.01	16.33 ± 0.55	3.80 ± 0.01	14.73 ± 0.25
3	3.52 ± 0.01	15.07 ± 0.35	3.55 ± 0.01	14.27 ± 0.38	3.57 ± 0.01	13.03 ± 0.32
6	3.44 ± 0.01	15.27 ± 0.70	3.40 ± 0.01	14.20 ± 0.10	3.51 ± 0.01	10.33 ± 0.58
9	3.44 ± 0.01	15.05 ± 0.36	3.45 ± 0.01	13.95 ± 0.36	3.32 ± 0.01	10.20 ± 0.15
12	3.42 ± 0.01	14900 ± 250	3.39 ± 0.01	13500 ± 358	3.30 ± 0.01	9750 ± 152

Table 6.4. Stability test results for batch 3 during 12 months at 25 ºC, 30 ºC and 40 ºC. (n=3; mean ± SD).

Conditions of storage	**25 ºC**		**30 ºC**		**40 ºC**	
Time (months)	pH	Apparent viscosity (Pa.s)	pH	Apparent viscosity (Pa.s)	pH	Apparent viscosity (Pa.s)
0	3.96 ± 0.01	13.70 ± 0.40	3.96 ± 0.01	13.70 ± 0.40	3.96 ± 0.01	13.70 ± 0.40
1	3.89 ± 0.01	12.60 ± 0.74	3.88 ± 0.01	13.60 ± 0.52	3.86 ± 0.01	13.10 ± 0.78
3	3.84 ± 0.01	13.27 ± 0.40	3.78 ± 0.01	13.50 ± 0.50	3.72 ± 0.01	12.40 ± 0.10
6	3.68 ± 0.01	15.70 ± 0.10	3.67 ± 0.01	14.87 ± 0.21	3.73 ± 0.01	11.43 ± 0.15
9	3.69 ± 0.01	15.05 ± 0.26	3.64 ± 0.01	13.90 ± 0.35	3.47 ± 0.01	10.90 ± 0.15
12	3.50 ± 0.01	14.75 ± 0.25	3.41 ± 0.01	13.20 ± 0.23	3.41 ± 0.01	10.10 ± 0.28

The pH was acidic in all the freshly prepared formulations. The results showed that the pH values tend to slightly decrease along the time.

Concerning the apparent viscosity, it was seen that the values are constant over the time, after 1 month, for the batches stored at 25 ºC and 30 ºC. For the samples stored at 40 ºC the apparent viscosity tends to decrease with the time.

By analysing Fig. 6.1, 6.2 and 6.3 it was observed that the MF recovery tends to decrease with time. Moreover, after 9 months of storage under the three different conditions, the MF recovery of at least one batch is above 90%.

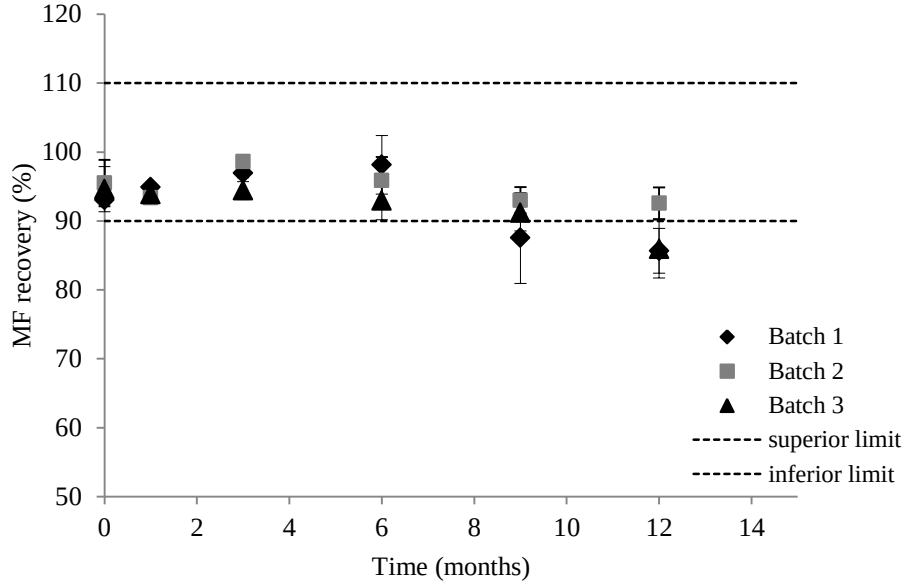

Fig 6.1. Percentage of MF recovered in batches 1, 2 and 3 stored at 25°C ± 2 °C / 60 % RH ± 5 % RH over 12 months, (mean ± SD, n=3).

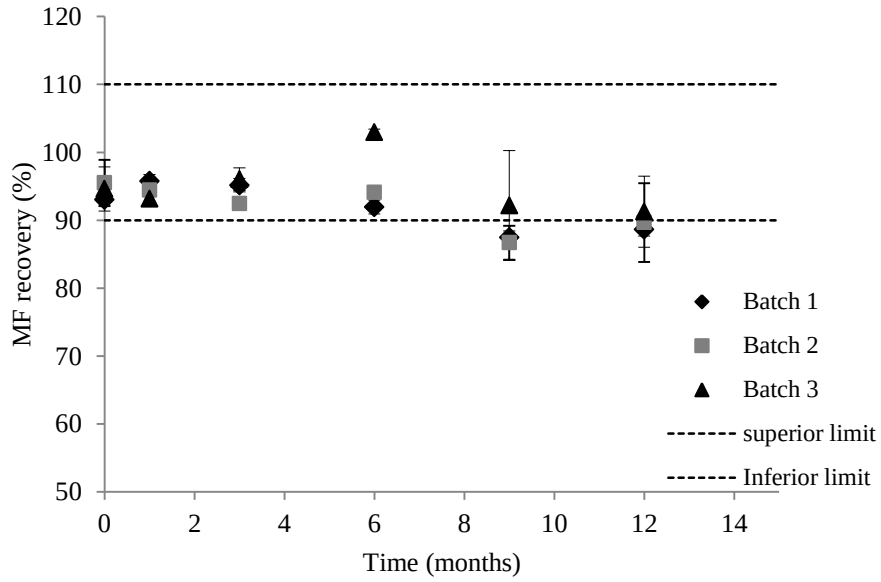

Fig 6.2. Percentage of MF recovered in batches 1, 2 and 3 stored at 30 °C ± 2 °C / 65 % RH ± 5 % RH over 12 months, (mean ± SD, n=3).

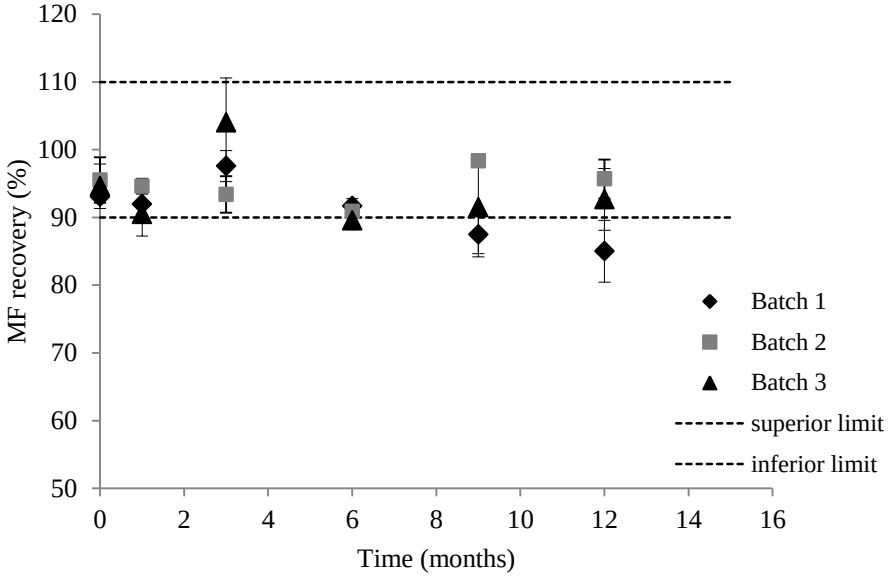

Fig 6.3. Percentage of MF recovered in batches 1, 2 and 3 stored at 40 °C ± 2 °C / 75 % RH ± 5 % RH over 12 months, (mean ± SD, n=3).

As the initial percentage of MF assayed was 93.03, 95.52 and 94.61 % for batches 1, 2 and 3 respectively, we considered these values as 100% and the other percentages were adjusted. The results are presented in Fig 6.4, 6.5 and 6.6, for 25 ºC, 30 ºC and 40 ºC, respectively. For every evaluation time points the MF assay is within the pre-established limits – 90 – 110%.

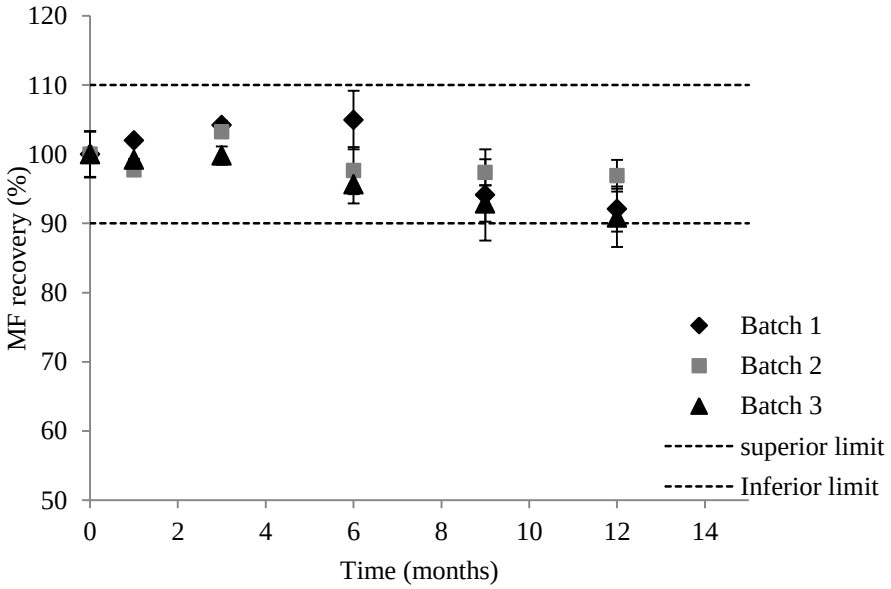

Fig 6.4. Percentage of MF recovered in batches 1, 2 and 3 stored at 25 °C ± 2 °C / 60 % RH ± 5 % RH over 12 months and considering t_0 as 100%, (mean ± SD, n=3).

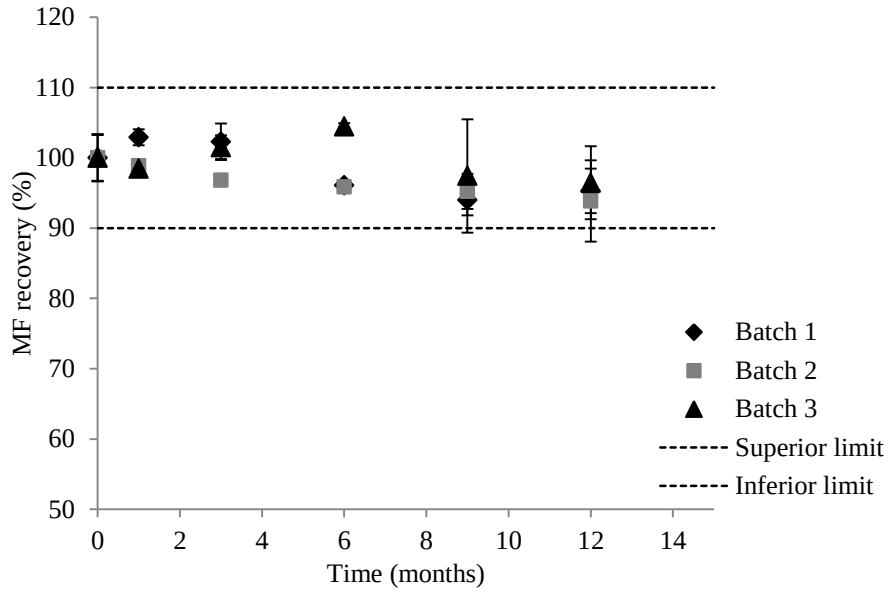

Fig 6.5. Percentage of MF recovered in batches 1, 2 and 3 stored at 30 °C ± 2 °C / 65 % RH ± 5 % RH over 12 months and considering t_0 as 100%, (mean ± SD, n=3).

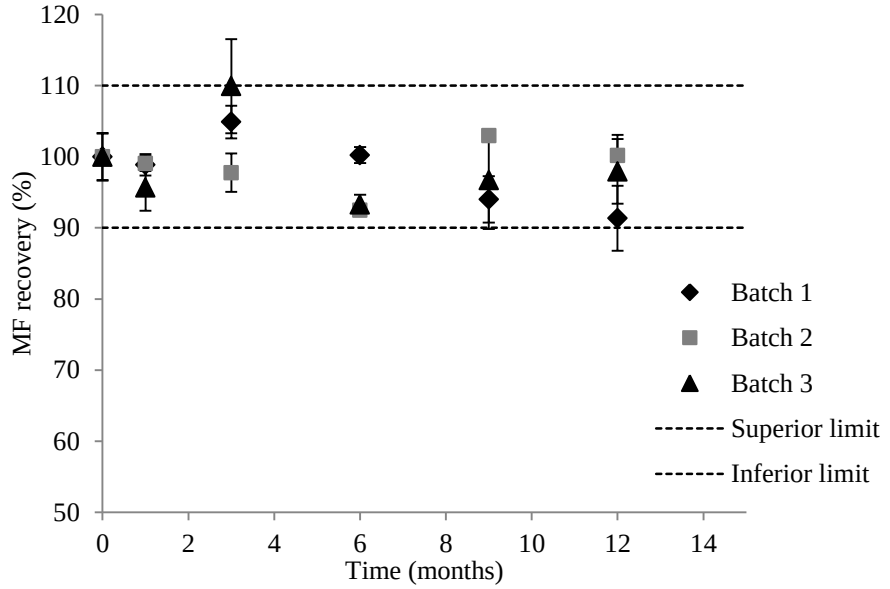

Fig 6.6. Percentage of MF recovered in batches 1, 2 and 3 stored at 40 °C ± 2 °C /75 % RH ± 5 % RH over 12 months and considering t_0 as 100%, (mean ± SD, n=3).

Table 6.5 shows the microbiological results for batch 1. According to these results, it was observed that the product is microbiological stable for 12 months as the total aerobic microbial count, the yeast and mould count and the specific microorganisms count (*E. coli*, *P. aeruginosa* and *S. aureus*) presented values inside of the established

criteria. The results for the other 2 batches were also according the specifications (data not shown).

Table 6.5. Microbiological stability of batch 1.

Time (0, 1, 3, 6, 9, 12 months)	Total aerobic microbial count		Yeast / mould count	*E. coli* *P. aeruginosa* *S. aureus*
	30 ºC	37 ºC		
25 ºC	Conform	Conform	Conform	Conform
30 ºC / 65 % RH	Conform	Conform	Conform	Conform
40 ºC / 75 % RH	Conform	Conform	Conform	Conform

The droplet size for the emulsions was also assessed during the stability evaluation time points.

The emulsion presented a bimodal population. The droplet size for the emulsion stored at 25 ºC did not vary over time (Fig. 6.7). However, the emulsion stored at 30 ºC and 40 ºC, presented an alteration in its droplet size distribution after 12 and 9 months, respectively indicating physical instability (Fig. 6.8 and 6.9). The results presented below concerns to batch 1. The same trend was observed in the other two batches (results not shown).

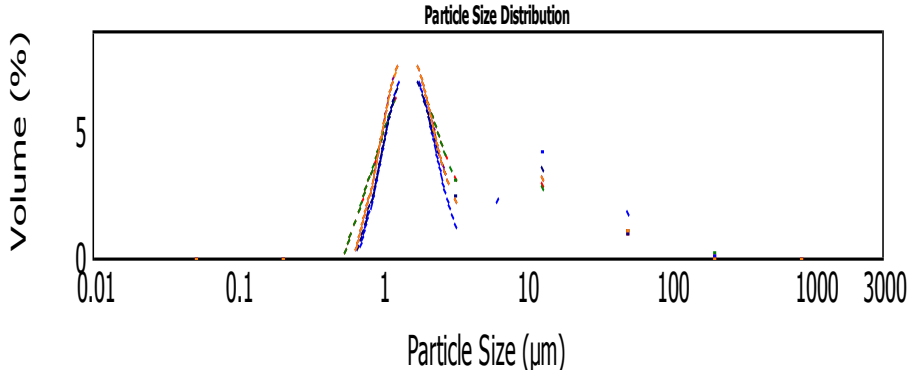

Fig 6.7. Droplet size distribution of the batch 1 stored at 25 ºC 0 (red line), 1 (green line), 3 (blue line), 6 (black line), 9 (violet line) and 12 (orange line) months after the preparation.

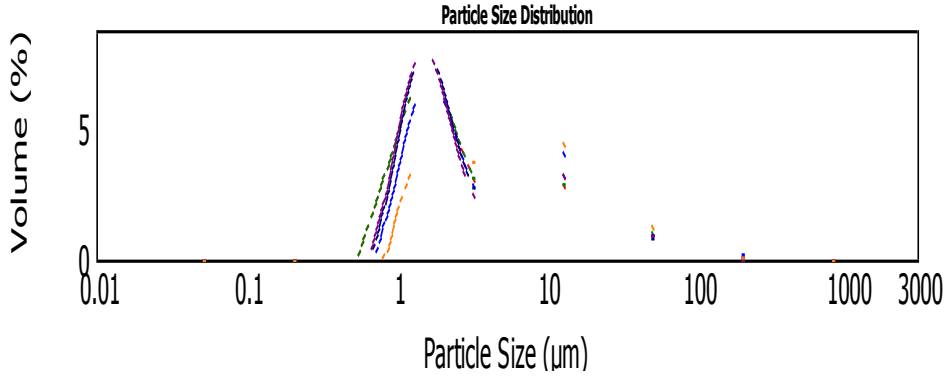

Fig 6.8. Droplet size distribution of the batch 1 stored at 30 ºC, 0 (red line), 1 (green line), 3 (blue line), 6 (black line), 9 (violet line) and 12 (orange line) months after the preparation.

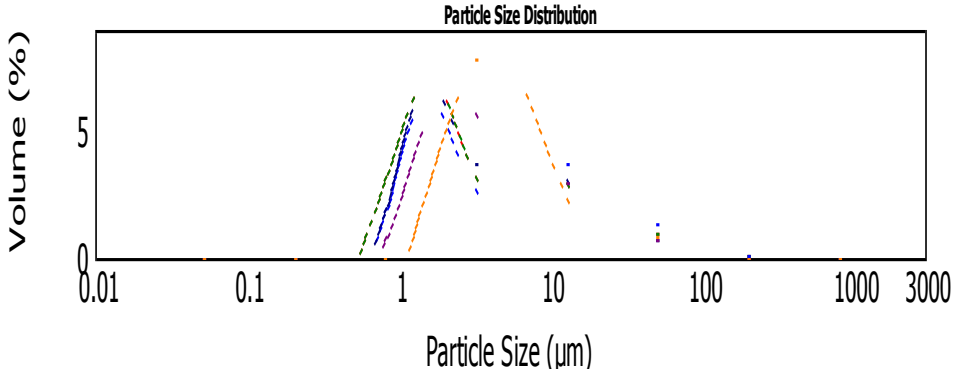

Fig 6.9. Droplet size distribution of the batch 1 stored at 40 ºC, 0 (red line), 1 (green line), 3 (blue line), 6 (black line), 9 (violet line) and 12 (orange line) months after the preparation.

Table 6.6. Droplet size distribution of emulsion A (batch 1) immediately after preparation and after 1, 3, 6, 9 and 12 months of storage at 25 ºC, (n=5, mean ± SD).

Time (months)	10% of the droplets (µm)	50% of the droplets (µm)	90% of the droplets (µm)
0	1.03 ± 0.002	2.77 ± 0.003	23.08 ± 0.234
1	1.15 ± 0.001	3.71 ± 0.007	28.06 ± 0.785
3	1.17 ± 0.061	4.71 ± 0.018	30.06 ± 0.321
6	1.16 ± 0.070	3.69 ± 0.014	25.55 ± 0.073
9	1.10 ± 0.007	2.72 ± 0.014	20.64 ± 0.250
12	1,52 ± 0.005	3.44 ± 0.020	23.35 ± 0.325

Table 6.7. Droplet size distribution of emulsion A (batch 1) immediately after preparation and after 1, 3, 6, 9 and 12 months of storage at 30 ºC, (n=5, mean ± SD).

Time (months)	10% of the droplets (µm)	50% of the droplets (µm)	90% of the droplets (µm)
0	1.03 ± 0.002	2.77 ± 0.003	23.08 ± 0.234
1	1.07 ± 0.001	3.55 ± 0.004	25.98 ± 0.065
3	1.19 ± 0.018	5.39 ± 0.002	27.87 ± 0.060
6	1.15 ± 0.005	2.93 ± 0.017	18.35 ± 0.152
9	1.12 ± 0.005	2.86 ± 0.015	18.88 ± 0.189
12	1.65 ± 0.015	3.56 ± 0.020	21.66 ± 0.350

Table 6.8. Droplet size distribution of emulsion A (batch 1) immediately after preparation and after 1, 3, 6, 9 and 12 months of storage at 40 ºC, (n=5, mean ± SD).

Time (months)	10% of the droplets (µm)	50% of the droplets (µm)	90% of the droplets (µm)
0	1.03 ± 0.002	2.77 ± 0.003	23.08 ± 0.234
1	1.80 ± 0.005	3.80 ± 0.018	27.76 ± 0.180
3	1.19 ± 0.003	4.87 ± 0.016	28.69 ± 0.250
6	1.17 ± 0.015	3.52 ± 0.020	16.06 ± 0.175
9	1.41 ± 0.020	3.91 ± 0.025	16.60 ± 0.289
12	2.05 ±0.010	4.74 ± 0.018	17.03± 0.390

3.2 Physical, chemical and microbiological stability of the placebo A

The placebo A was white glossy and pourable and uniform in appearance in all time points. The stability of the placebo A was assessed by visual observation for detection of phase separation or other instability phenomena. In all test times, macroscopic observations did not reveal any sign of instability. Equally, the centrifugation test, which is commonly used to evaluate the stability of the developed emulsions, showed that the placebo had a good physical stability because no phase separation, creaming, cracking or precipitation signs were observed upon centrifugation.

The pH values (Table 6.9) did not significantly vary over time.

Table 6.9. Stability test results of placebo A stored for 12 months at 25 ºC, 30 ºC and 40 ºC.

Conditions of storage	**25 ºC**		**30 ºC**		**40 ºC**	
Time (months)	pH	Apparent viscosity (Pa.s)	pH	Apparent viscosity (Pa.s)	pH	Apparent viscosity (Pa.s)
0	4.38	12.42	4.38	12.42	4.38	12.42
1	4.38	17.49	4.49	19.89	4.16	16.44
3	4.45	20.40	4.48	18.51	4.20	16.80
6	4.47	20.61	4.31	16.55	4.34	14.82
12	4.44	22.20	4.20	19.62	4.43	17.28

As shown in Table 6.9, the viscosity of the placebo A increased during the first months and after that it remained stable. The microbiological studies (Table 6.10) showed that the results were within the recommended limits of the specifications. These results indicated that the placebo A is physical and microbiological stable during 12 months.

Table 6.10. Microbiological stability of the placebo A.

Time (0, 1, 3, 6, 12 months)	Total aerobic microbial count		Yeast / mould count	*E. coli* *P. aeruginosa* *S. aureus*
	30 ºC	37 ºC		
25 ºC	Conform	Conform	Conform	Conform
30 ºC / 65 % RH	Conform	Conform	Conform	Conform
40 ºC / 75 % RH	Conform	Conform	Conform	Conform

The placebo A presented a monomodal population as the scale up influenced the emulsion characteristics (Chapter VIII, section 3.2). The storage time and the temperature did not interfere in the droplet size of the placebo (Fig. 6.10, 6.11 and 6.12 and Tables 6.11, 6.12 and 6.13) which remained physical stable over the test period.

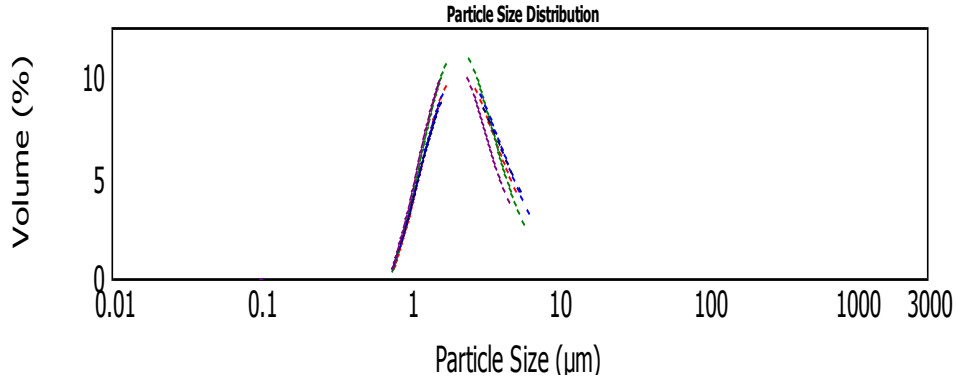

Fig 6.10. Droplet size distribution of the placebo stored at 25 ºC, 0 (red line), 1 (green line), 3 (blue line), 6 (black line) and 12 (violet line) months after production.

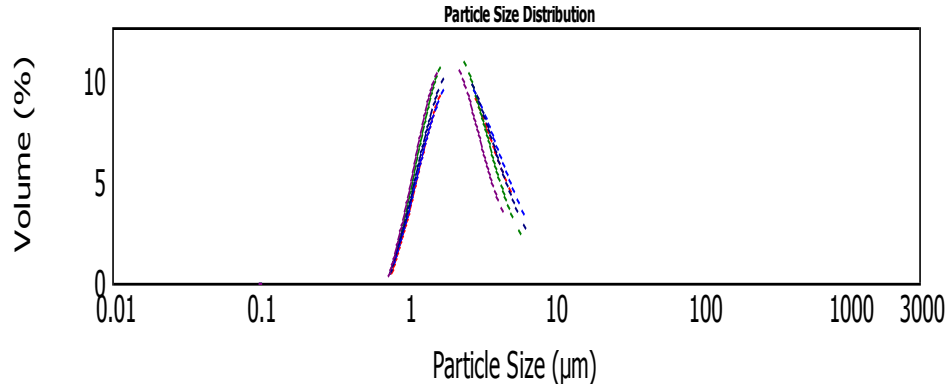

Fig 6.11. Droplet size distribution of the placebo stored at 30 ºC 0 (red line), 1 (green line), 3 (blue line), 6 (black line) and 12 (violet line) months after production.

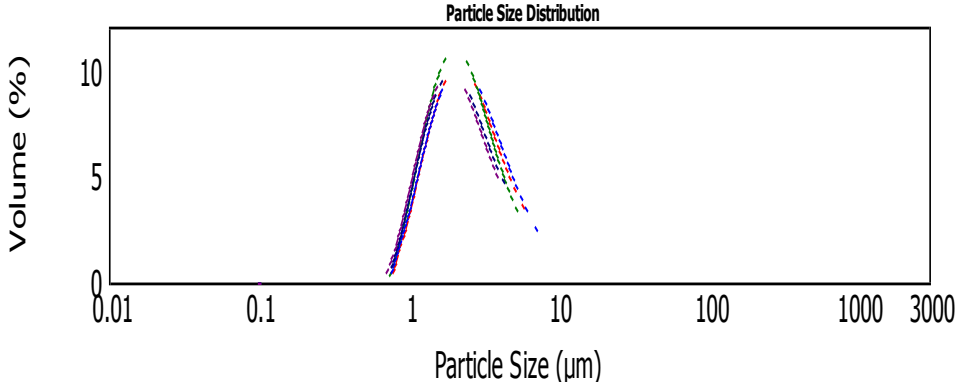

Fig 6.12. Droplet size distribution of the placebo stored at 40 ºC 0 (red line), 1 (green line), 3 (blue line), 6 (black line) and 12 (violet line) months after production.

Table 6.11. Droplet size distribution of placebo A immediately after preparation and after 1, 3, 6 and 12 months of storage at 25 ºC, (n=5, mean ± SD).

Time (months)	10% of the droplets (μm)	50% of the droplets (μm)	90% of the droplets (μm)
0	1.26 ± 0.001	2.46 ± 0.003	6.38 ± 0.026
1	1.23 ± 0.002	2.29 ± 0.003	4.95 ± 0.007
3	1.24 ± 0.002	2.44 ± 0.008	5.69 ± 0.026
6	1.24 ± 0.002	2.49 ± 0.011	6.25 ± 0.158
12	1.20 ± 0.001	2.27 ± 0.003	6.71 ± 0.050

Table 6.12. Droplet size distribution of placebo A immediately after preparation and after 1, 3, 6 and 12 months of storage at 30 ºC, (n=5, mean ± SD).

Time (months)	10% of the droplets (μm)	50% of the droplets (μm)	90% of the droplets (μm)
0	1.26 ± 0.001	2.46 ± 0.003	6.38 ± 0.026
1	1.20 ± 0.0004	2.22 ± 0.002	4.96 ± 0.013
3	1.20 ± 0.0008	2.23 ± 0.005	5.10 ± 0.003
6	1.22 ± 0.065	2.35 ± 0.006	5.35 ± 0.056
12	1.17 ± 0.003	2.19 ± 0.003	6.97 ± 0.039

Table 6.13. Droplet size distribution of placebo A immediately after preparation and after 1, 3, 6 and 12 months of storage at 40 ºC, (n=5, mean ± SD).

Time (months)	10% of the droplets (μm)	50% of the droplets (μm)	90% of the droplets (μm)
0	1.26 ± 0.001	2.46 ± 0.003	6.38 ± 0.026
1	1.20 ± 0.002	2.25 ± 0.002	5.28 ± 0.066
3	1.21 ± 0.001	2.30 ± 0.001	6.24 ± 0.051
6	1.38 ± 0.065	3.76 ± 0.452	6.55 ± 0.214
12	1.15 ± 0.014	2.34 ± 0.004	7.36± 0.331

3.2.1 Cetrimide assay

It should be clarified why the assay of the cetrimide was done for time 12 months. According to the applicable legislation for medicinal products, the preservatives should be assayed during the stability process. However, the main role of cetrimide in this formulation is to provide an additional physical stability to the system and not as a

preservative. For that reason we questioned the Portuguese national authorities in order to clarify this matter, however the response was not available. Thus, we decided to develop a method for the assay of cetrimide and additionally, the amount of cetrimide was quantified in the placebo after 12 months of storage at 25 ºC. The results obtained were 104.4 ± 2.3 % (mean ± SD, n=3).

4. Discussion

The instability of the emulsions could arise through a variety of physicochemical destabilizing processes, such as creaming (or sedimentation), flocculation, coalescence or phase inversion [7]. The stability of the emulsion A was assessed by visual observation in order to detect the occurrence of phase separation or other instability phenomena, before and after centrifugation. At evaluation time points of 9 and 12 months, the emulsion stored at 40 ºC showed a slight phase separation after the centrifugation whereas placebo did not. These results can be explained by the different production processes as demonstrated in Chapter VIII. The droplet size analysis was in accordance to the latter observations as the emulsion A, stored at 40 ºC, showed a significant change in droplet size distribution after 9 months, which was even more pronounced after 12 months. The modifications detected seemed to be coalescence of the droplets, since the two populations of droplets tended to form one single population. It is expected that the physical stability of the emulsion produced in the industrial equipment will be improved, as a monomodal population was achieved.

The apparent viscosity values showed that, in general, this parameter did not suffer any significant changes over time, with the exception of the samples stored at 40 ºC. At this temperature, the apparent viscosity tended to decrease, which was expectable since higher temperatures is a factor for physical instability in emulsions. These results found another expression for the placebo A. In general, the apparent viscosities for the placebo A were higher comparing with the emulsion A, indicating that the structure of the placebo is more resistant to the structural breakdown. Moreover, the apparent viscosities had a tendency to slightly increase along the time, especially in the first evaluation time points. This phenomenon can be explained by rearrangement processes that occur in the emulsions, especially those produced by non-ionic surfactants with PEG chains. The

PEG chains need time to elongate and hydrate. Thus, the free water decreases and the apparent viscosity increases which is more evident when the batch size increases.

Regarding the pH values in both, emulsion A and placebo A, the values were acidic. These observations were in accordance to the maximum stability of the drug. The presence of MF seemed to influence this parameter since the pH values of the emulsion slightly decreased along the time and the pH values of the placebo were constant during the 12 months. Despite the decrease of the pH values of the emulsion A, it did not cause any physical or chemical instability.

Concerning the MF assay, the low percentage of recovery in time 0 could be explained by the production process. The MF was suspended in pentanediol and this suspension was added to the reactor vessel with inevitable losses of drug. It should be emphasizes that the validation of the method took place during the stability tests, which can explain some variations observed in the MF assay. However, when it was considered that the recovery percentage of MF was 100% for time 0, the MF assay along the time remained between 90-110%. It was observed a trend for the degradation of MF along the time. However, the analysis of the chemical stability of MF in the emulsion A should be repeated under industrial conditions.

Considering the intermediate and accelerate storage conditions, the ICH guideline recommends at least 6 months of storage at the submission data. In our case, we have results for a period of 12 months, which is a good predictor for a suitable chemical stability.

5. Conclusion

It was demonstrated that the emulsion A is physical and microbiological stable for a period of 12 months. Concerning the chemical stability, the tests should be repeated in industrial conditions. However, the results obtained so far positively support the further scale-up and stability assessment of this emulsion. Additionally, the acidic values required to the stability of the drug did not influence the physical stability of the emulsion.

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1. Introduction

Emulsion systems used in dermopharmacy have to fulfill a number of requirements, e.g. acceptable physical stability, chemical inertness, satisfactory safety profile and efficacy, reaching at the same time optimal sensory attributes [1]. In order to provide all of these attributes to emulsions, several excipients have to be used [2] such as surfactants, co-emulsifiers, polymers, preservatives, emollients and solubilizers. It is of crucial importance to evaluate the safety profile of the ingredients used in such vehicles, especially if those vehicles are intended to be applied in damaged skin.

Dermatological emulsions, without drug, are considered cosmetic products falling under the general requirements of the EC Cosmetics Directive 76/768 [3] regarding their safety. This directive will be replaced stepwise by the new EC Cosmetics regulation 1223/2009 [4]. Under both regulations, the toxicological profile of all used ingredients and detailed knowledge of the product-specific exposure are required as fundamental for the safety assessment [5]. As imposed by the legislation, cosmetics are considered to be safe for the consumer. Although this appears to be self-evident, there is a whole scientific exercise preceding this "obvious" conclusion [6]. The safety of a cosmetic product is determined based on the safety assessment of its ingredients which is done using literature data, *in vitro* tests and human tests since, in EU, finished cosmetic products are no longer tested in animals.

There are ingredients of special concern in terms of safety assessment, such as, preservatives, solubilizers and surfactants. Concerning the surfactants, most of them are based on ethoxylated non-ionic emulsifiers or their mixtures with long chain fatty alcohols (so called mixed emulsifiers). While, vehicles based on these mixed emulsifiers meet general requirements for pharmaceutical bases, their use may be accompanied by adverse skin reactions [7].

Although human external contact with a substance rarely results in its penetration through the skin and significant systemic exposure, skin care products produce local exposure. Therefore, human systemic exposure to their ingredients can rarely be completely excluded [8].

The key factors in the management of atopic dermatitis are not only related to the use of effective topical anti-inflammatory agents but also in providing skin hydration and barrier repair [9]. The ingredients selected to such vehicles are of extremely importance

and should present a suitable equilibrium between safety and efficacy [10]. Emollients or moisturizers are often used in the treatment of atopic dermatitis and other inflammatory dermatitis with the aim of improving skin hydration and mitigating xerosis. Given their resemblance to the lipids in the SC, beneficial effect of skin lipid supplementation both in composition and in the structuring of topical formulations for skin repair was already described [11, 12].

In this chapter, we aimed to evaluate the safety profile and biological effects of placebo A, using literature data and a systematic approach for the safety assessment, comparing it with *in vitro* and *in vivo* data obtained by the methods of skin bioengineering and by tests assessed on human volunteers, respectively. It should be referred that the placebo A (without MF), can be market as a cosmetic product thus, it is of great importance to study its safety profile and biological effects according to the European Cosmetic Regulation.

2. Materials and methods

2.1 Materials

The materials used are described in Chapter III, section 2.1.

2.2 Methods

2.2.1 Preparation of placebo A

Placebo A was prepared according the method described in Chapter III, section 2.2.4. The composition of the placebo A is described in Table 3.21 (same composition of emulsion A but without MF).

2.2.2 Safety assessment of placebo A

The safety evaluation of the placebo A was conducted according to the SCCS's Notes of Guidance for Testing of Cosmetic Ingredients and their Safety Evaluation [13].

For each ingredient it was collected information acquired from ingredient's supplier and publicly available information.

2.2.2.1 Hazard identification

Based on the results of *in vivo* tests, *in vitro* tests, clinical studies and human epidemiological studies, the intrinsic physical, chemical and toxicological properties of each ingredient under consideration was studied to identify whether the substance has the potential to damage human health.

2.2.2.2 Exposure assessment

The amount and the frequency of human exposure to the placebo A were determined. The systemic exposure dose (SED) was calculated to each ingredient, according to the equation 7.1.

$$SED = A\ (mg/kg/bw/day) \times C\ (\%)\ /100 \times DA\ (\%)\ /\ 100 \qquad \text{(Eq. 7.1)}$$

Where, A is the estimated daily exposure to a cosmetic product per Kg body weight, based upon the amount applied and the frequency of application; C the concentration of the ingredient under study in the finished cosmetic product and DA the dermal absorption expressed as a percentage of the test dose assumed to be applied in real life conditions.

2.2.2.3 Dose-response assessment

The relationship between the toxic response and the exposure was studied. Public data was used to find out the No Observed (Adverse) Effect Level (NOAEL), which is the highest dose or exposure level where no adverse treatment-related findings are observed.

2.2.2.4 Risk characterization

The probability that the substances under investigation causes damage to human health and the level of risk, were examined. In the case of a threshold effect, the margin of safety (MoS) was calculated according to the equation 7.2.

$$MoS = NOAEL / SED \quad \text{(Eq.7.2)}$$

2.2.3 EpiSkin™ assay

The validated reconstructed human epidermis EpiSkin™ skin irritation test method was used [14].

The EpiSkin™ tissues were supplied by SkinEthic Laboratories (www.skinethic.com) consisting in a reconstructed organotypic culture of adult human keratinocytes reproducing a multilayered and well differentiated epidermis.

The method used following the instruction of the producer, the 12 well plates, containing 12 inserts of tissues (0.38 cm^2), were transferred into 12 wells plates containing 2 mL of maintenance medium and incubated at 37 ºC (5% CO_2, >95% humidity). After 24 h, the second column of each plate was filled with maintenance medium preheated at 37 ºC.

Ten mg of placebo A were applied directly and contacted during 15 min with the epidermis samples. Phosphate buffer saline (PBS) was used as negative control and sodium dodecyl sulfate (SDS) (5% in distilled water) as positive control.

Cell viability was determined with the MTT assay. Tissues were transferred to wells containing 2 mL of a 0.3 mg/mL MTT solution and incubated for 3 h (37 ºC, 5% CO_2, 95% humidified atmosphere). After incubation, the epidermis tissues were contacted with acidic isopropanol (0.5 mL/tube) to extract the intracellular formazan.

The tubes were incubated for 4 h in dark with periodic vortexing, after that, a duplicate of 200 µL was transferred to a 96-well flat bottom microtiter plate. Absorbance was read at 570 nm with acidified isopropanol as blank and viability was calculated considering 100% for the negative control.

2.2.4 Human repeat insult patch test (HRIPT)

A safety evaluation study was performed on placebo A, using a Marzully&Maibach [15] HRIPT protocol. In brief, the product was applied on the back of 50 healthy volunteers that gave their informed written consent. Subjects with dermatological or other medical or physical conditions precluding topical application of the test material were excluded, along with pregnant and nursing women. For the induction period, a series of nine patches (Finn Chamber standard) were performed over a period of 3 weeks. At the product site, an occlusive patch containing 20 mg of the formulation was applied to the left side of the back where it remained for 48 hours. After that period, the patch was removed, the skin was evaluated and a new patch was applied. Reactions after patching were scored according to International Contact Dermatitis Research Group [16].

A 2 weeks rest period was observed without application of the test material. During the challenge period, new patches were prepared and fixed in the same manner as in the induction period, but also on the right side of the back (ie, a virgin site).

The patches were removed after 48 hours and scoring of skin reactions was performed in the same manner as before at 48, 72, and 96 hours after patching using the same International Contact Dermatitis Research Group scoring system.

The protocol was approved by the local Ethical Committee and respected the Helsinki Declaration and the AFSSAPS regulations on performed HRIPT studies on cosmetic products. The study was conducted under the supervision of a dermatologist who participated in the evaluation of irritation/allergic reactions to the placebo A.

2.2.5 Biological effects of placebo A

The trans-epidermal water loss (TEWL), epidermal capacitance and skin surface lipids for the placebo A was evaluated with a Tewameter TM 210, Corneometer CM 820 and a Sebumeter SM 810 (C+K Electronics GmbH, Germany) respectively, during 21 days.

TEWL was measured with an evaporation meter. Data are expressed in $g/m^2/h$.

The Corneometer CM 825 determines the hydration level of the SC by measuring electrical capacitance. Alterations of epidermal skin hydration lead to a change in capacitance of the measuring condensator. The probe is applied to the skin for 1 second

at a pressure of 7.1 N/cm^2. The degree of skin capacitance is indicated in system specific units, arbitrary units (AU). One unit represents a water content of SC of 0.02 mg/cm^2, at a measuring depth of 20 nm.

The Sebumeter SM 810 was used for quantitative measurements of skin surface lipids composed of sebum and corneal lipids. It consists of a fat-stain photometer that measures the level of light transmission of a plastic sheet coated with sebum. The method is insensitive to humidity. A probe is pressed on the skin region under investigation for 30 seconds at a constant pressure of 9.4 N/cm^2. The change in sheet transparency is computed, and the results displayed in units that are then converted into μg/cm^2.

An uniform volunteers panel was chosen (n=10, young healthy females - 18-25 y.a., same professional activity) and subjects included in the study after written and informed consent. The formulation was applied in the forearm and the results were compared with a defined control area (anatomically equivalent and without product) on the same forearm. The protocol was approved by the local Ethical Committee. Data were compared using a two-way ANOVA (95% confidence level). Results are expressed as mean ± SD.

Measurements were performed under standardized conditions, at room temperature according the rules of Good Clinical Practices.

2.2.6 Data Analysis

According the method described in Chapter III, section 2.2.2.3.

3. Results and Discussion

3.1 Safety assessment of placebo A

3.1.1 Hazard identification

It is important to know the physic and chemical properties of each ingredient to predict in which extend it is expected skin permeation (Table 7.1) [17]. Firstly, molecules must be in the liquid form, molecules in the solid state are not absorbed.

As a general rule, chemicals with a molecular weight greater than 500 Da do not penetrate the skin. This is known as the 'rule of 500' [23]. This upper limit on molecular size mainly results from the physical arrangement of lipids between adjacent corneocytes of the SC. Considering the MW of the ingredients presented in Table 7.1, it is concluded that both polymers (HPMC and PVM/MA) and the polymer silicone based surfactant are not able to penetrate the SC.

The relationship between solubility and the rate of skin absorption stems primarily from the ability of a chemical to partition into the SC. If a chemical is excessively hydrophilic, it will not partition into the predominantly lipid environment of the SC. In contrast, if a chemical is too strongly lipophilic, it will readily partition into the SC but will not partition out into the predominantly hydrophilic environment of the underlying epidermal tissue. Thus, in order to penetrate the skin, the solubility of a chemical requires a balance between these two extremes. In general, a Log P between 1 and 3 is considered to be optimal for skin absorption [24]. Considering the molecular weight and the Log P values, the ingredients of most probability to penetrate into the SC are cetrimide and pentanediol and PGL.

Table 7.1. Chemical properties of the ingredients presented in the placebo A.

Ingredient	CAS number	Molecular weight (g/mol)	Impurities	Log P
Aqua	7732-18-5	18.02	n.a.	-
Polymer modified silicone surfactant	n.a.	> 10000 [18]	n.a.	n.a.
PGL	59070-56-3	362.50 [19]	Ethylenoxide < 1 ppm Dioxane < 5 ppm	3.70 [19]
IPM	110-27-0	270.45 [19]	Ash < 0.10 % Water content < 0.10 %	7.02 [19]
Alkyl Benzoate	68411-27-8	290.44 [19]		7.16 [19]
HPMC	9004-65-3	> 13000 < 200000 [20]	n.a.	-2.34 [19]
PVM/MA	136392-67-1	> 1000000 [21]	Cyclohexane and Ethyl acetate < 0.75 % Maleic Anhydride negative	n.a.
Cetrimide	1119-97-7	364.45 [19]	Free amines < 0.15% Amine HBr < 0.3 % Sulphated ash < 0.5%	1.86 [19]
Pentanediol	111-29-5	104.15	n.a.	0.58 [22]

The biological safety evaluation requires that cytotoxicity, sensitization and irritation or intracutaneous reactivity are determined and the risk of chronic toxicity, carcinogenicity, reproductive/development toxicity or other organ-specific toxicities based on specific nature and duration of exposure of the product are assessed (Table 7.2) [25].

Table 7.2. Summary of the biological safety of the ingredients.

Ingredient	Acute toxicity	Dermal Irritation	Ocular irritation	Sensitization	Genotoxicity / carcinogenicity	Ref
Polymer modified silicone surfactant	n.a	n.a	n.a	n.a	n.a	-
PGL	Rat (oral) LD_{50} > 48 ml/kg	No irritant	Rabbit: no irritant	n.a	n.a	26
IPM	Rat (oral) LD_{50} > 5000 mg/kg	Rabbit (undiluted): mild irritant	Rabbit: minimally irritant	Guinea pig: non sensitizer Human: non sensitizer	n.a	27, 28
Alkyl Benzoate	Rat (oral) LD_{50} > 2000 mg/kg Rabbit (dermal) LD_{50} > 2000 mg/kg	Rabbit: no irritant	Rabbit: no irritant	Guinea pig: non sensitizer	n.a	29, 30
HPMC	Oral LD_{50} > 10000 mg/kg	Can cause irritation	Can cause irritation	Guinea pig: non sensitizer	n.a	20
PVM/MA	Rat (oral) LD_{50} > 1500 mg/kg Rat (oral), 1% in solution LD_{50} >5000 mg/kg	Rabbit: slightly irritant	May cause irritantion	Human patch test: non sensitizer (2% gel)	*In vitro* gene mutation in bacteria: negative	31
Cetrimide	Rat (oral) LD_{50} > 400 < 600 mg/kg	Rabbit: Irritant	Potent irritant	Sensitizer	*Salmonella Typhimurium*: negative	32
Pentanediol	Rat (oral) LD_{50} 10000 mg/kg Rabbit (dermal)LD_{50} > 19800 mg/kg	Rabbit: no irritant	Rabbit: no irritant	n.a	Ames test: negative	33

Emulsifiers are of particular concern due to their skin irritative potential [34, 35] and, because, they have the potential to act as penetration enhancers by decreasing surface tension and conditioning the SC and hence may enable or enhance diffusion of other molecules through the skin [36]. The main emulsifier present in placebo A is a polymer modified silicone surfactant containing PEG chains as hydrophilic part and medium-chain triglycerides as lipophylic part. Due to the absence of data in literature for this emulsifier, we decompose this ingredient in three parts: PEG, silicone and medium-chain triglycerides, and we assessed the safety profile of the individual ingredients.

PEGs and PEG fatty esters were not or very slightly irritant to the skin of rabbits and humans [37]. However, independent of the erythema, increased TEWL was induced by some of the emulsifiers, indicating an invisible impairment of the SC barrier function [7].

Clinical and animal absorption studies reported that dimethicone was not absorbed following oral or dermal exposure. Dimethicone was not acutely toxic following oral exposure. No adverse reactions were found in rabbits following short-term dermal dosing with 6% to 79% dimethicone. Most dermal irritation studies using rabbits, classified dimethicone as a minimal irritant. Dimethicone (tested undiluted and at 79%) was not a sensitizer in four assays using mice and guinea pigs. Moreover, it was not a sensitizer at 5.0% in a HRIPT using 83 panelists. Most ocular irritation studies using rabbits, classified dimethicone as a mild to minimal irritant. Dimethicone was tested in numerous oral-dose (using rats) and dermal-dose (using rats, rabbits, and monkeys) reproductive and developmental toxicity studies. Dimethicone was negative in all genotoxicity assays. It was negative in both an oral (tested at 91%) and dermal (tested at an unknown concentration) dose carcinogenicity assay using mice [38].

Medium-chain triglycerides exhibit very low levels of toxicity in a variety of laboratory animals and in humans when administered orally, parenterally or by the dermal route [39].

Based on these results, concerning PEGs, dimethicone and medium-chain triglycerides, we can predict that, the polymer modified silicone surfactant pose no consumer risk in the concentration used.

Concerning the PGL, it was demonstrated that glyceryl monoesters have little acute or short-term toxicity in animals, and no toxicity was noted following chronic administration of a mixture consisting mostly of glyceryl di- and mono- esters. Glyceryl laurate was not classified as ocular irritant in rabbits. Undiluted glyceryl monoesters may produce minor skin irritation, especially in abraded skin, but in general these ingredients are not irritating at concentrations used in cosmetics. Glyceryl monoesters are neither sensitizers nor photosensitizers. At concentrations higher than used in cosmetics, glyceryl laurate did cause moderate erythema in HRIPT studies. Based on these data, the Cosmetic Ingredient Review Expert Panel found that these glyceryl monoesters are safe as cosmetic ingredients in the present practices of use and concentration [36].

Based on these data, the ingredients of special concerns are cetrimide and pentanediol because they present suitable physical characteristics to penetrate the skin, the glycol is present in the formulation in a relatively high concentration and cetrimide showed to be irritant to the skin and it is a sensitizer.

3.1.2 Exposure assessment

placebo A is intended for use on intact skin of adults. It can be used as an adjuvant in corticoid therapy. It is applied to the affected area in the desired quantity once or twice a day with a soft massage to enhance the product absorption.

It will be supplied for use as a leave-on cosmetic product which is intended to stay in prolonged contact with the skin.

According to the Scientific Committee on Consumer Safety [13], the human surface area is 15670 cm^2. The placebo A will be considered as a body cream, thus, the estimated daily amount applied for a body cream is 7.82 g/day and the frequency of application is 2.28 / day which is translated in a daily exposure of 123.2 bw/day. Applying the equation 7.1 the SED values were calculated for each ingredient (Table 7.3).

Table 7.3. Exposure data of formulation ingredients.

Ingredient	**Daily exposure (bw/day)**	**% in the final product**	**Dermal Absoption* (%)**	**SED (mg/kg/bw/day)**
Polymer modified silicone surfactant	123.2	5.0	100	6.16
PGL	123.2	4.0	100	4.93
IPM	123.2	5.0	100	6.16
Alkyl Benzoate	123.2	5.0	100	6.16
HPMC	123.2	2.0	100	2.46
PVM/MA	123.2	0.3	100	0.37
Cetrimide	123.2	0.075	100	0.09
Pentanediol	123.2	10.0	100	12.32

*when no permeation data is available, the value considered is 100%.

3.1.3 Dose-response assessment

The NOAEL is mainly derived from repeated dose animal studies (90 day, developmental toxicity studies).

As far as the determination of critical effects in repeated dose toxicity studies is concerned, the available repeated dose toxicity data should be evaluated in detail for a characterization of the health hazards upon repeated exposure. The NOAEL values found out for cetrimide and pentanediol were 20 and 450 mg/kg/day, respectively [22, 40].

3.1.4 Risk characterisation

The MoS is used to extrapolate from a group of test animals to an average human being, and subsequently from average humans to sensitive subpopulations. The world health organization proposes a minimum value of 100, and it is generally accepted that the MoS should at least be 100 to declare a substance safe for use.

The value of 100 consists of a factor 10 for the extrapolation from animal to man and another factor 10 taking into account the inter-individual variations within the human population.

The MoS for the two ingredients of special concerns (cetrimide and pentanediol) were calculated according Eq. 7.2. The MoS value obtained for cetrimide was 222.22 which is above of the threshold value of 100 suggesting that the ingredient can be considered to pose no consumer risks on systemic toxicity effects. Concerning pentanediol the value obtained was 36.53, however it should be emphasize that this is a very conservative approach. In fact, the actual safety margins of cosmetic ingredients tend to be higher than theoretical values, since calculated MoS data represents a worst-case scenario. For example, it was considered a skin penetration of 100%, which not corresponds to the reality. In this case *in vitro* and *in vivo* tests will be useful to decide about the safety of this ingredient.

3.2 EpiSkin™ assay

The safe topical use of the placebo A was tested on reconstituted human epidermis. The Episkin™ model mimic morphologically and biochemically living skin and is useful to classify skin irritants able to produce a decrease in cell viability, evaluated by a MTT assay [41]. The tissue viability measured as optical density by the MTT assay and calculated as percentage of cytotoxicity compared to the negative control (PBS), was 92.0 ± 6.0 %, whereas in the positive control (SDS) it was 30.0 ± 4.0 %. A product is considered an irritant when viability is reduced by 50%.

The absence of skin-irritant effects at the concentrations tested indicated that placebo A could be safe for topical use.

3.3 HRIPT

During the HRIPT study no reactions were observed in the initial 3 weeks contact or after the final challenge contact.

Therefore, the repeated application of the product did not induce any sensitization on the skin of the volunteers and the formulation presented very good skin compatibility.

3.4 Biological effects of placebo A

The skin is often exposed to surface-active agents like soaps, which may affect the skin barrier. Differences in the effects of surfactants have been investigated previously, e.g. using biophysical instruments [7, 42]. These investigations showed that surfactants exert strong effects in experimental settings. Sodium lauryl sulfate (SLS), a surfactant with a carbon chain length of 12, is ranked as the most irritating [43]. An increased TEWL is a sensitive measure of barrier damage [44, 45] and an indication of the skin permeability [46]. Fig. 7.1 shows the comparison between placebo A and control area in terms of TEWL during 21 days. The placebo A did not significantly increased TEWL compared to the control ($p > 0.05$).

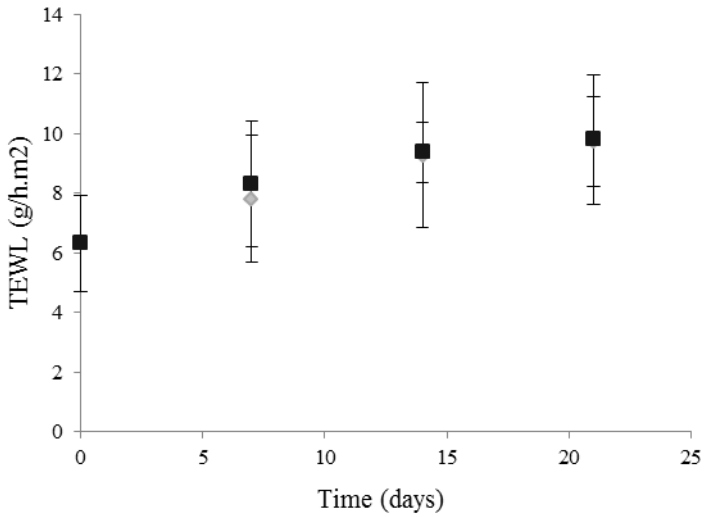

Fig. 7.1. Comparison of TEWL during 21 days between placebo A (black bars) and control (grey bars), (mean ± SD, n = 10).

SC water retention and skin surface lipids properties are a crucial factor in keeping the skin supple and flexible and influence skin permeability to molecules. The methodological procedure chosen, allowed the identification of positive results regarding skin water dynamics, expressed in terms of epidermal capacitance changes (Fig. 7.2) and skin lipids expressed in terms of sebum (Fig. 7.3).

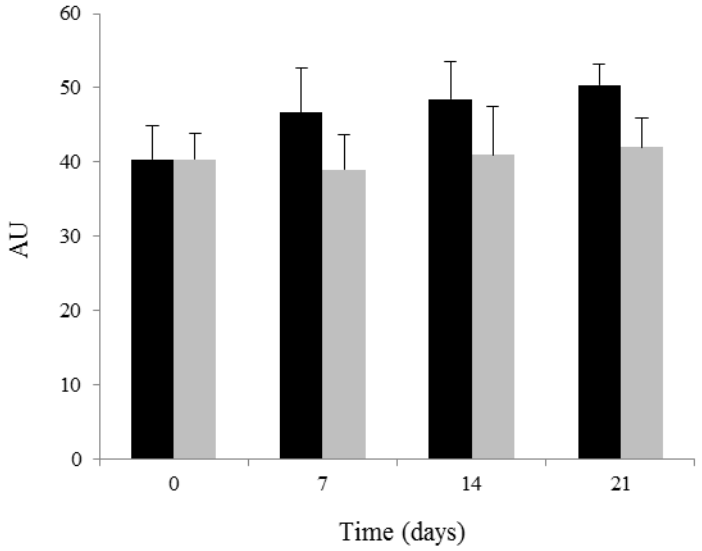

Fig. 7.2. Comparison of skin hydration values in terms of capacitance during 21 days between placebo A (black bars) and control (grey bars), (mean ± SD, n = 10).

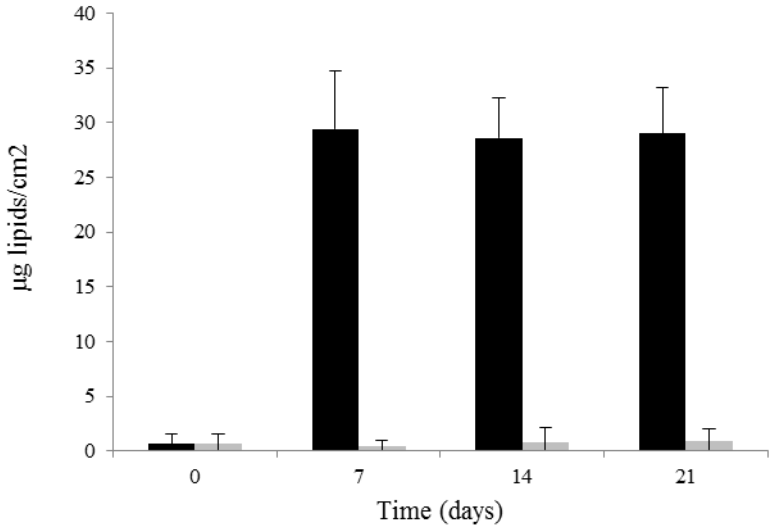

Fig. 7.3. Effect of the application of placebo A on the skin surface lipids. placebo A (black bars) and control (grey bars), (mean ± SD, n = 10).

The *in vivo* studies for human skin hydration showed a slight increase after application of placebo A when compared to the control area ($p > 0.05$). The principal mechanisms of hydration are humectancy, emolliency, and occlusion. The hydration provided by emulsion is attributed to humectants (pentanediol) and emollients (PEG based surfactants, IPM and alkyl benzoate). In fact, humectants promote water retention within the SC, whereas emollients smooth the skin by filling spaces between skin flakes and adding a complementary occlusive activity which contributes to SC hydration [47]. In this formulation occlusive substances are not present.

On the other hand, a drastic increase on the skin lipids occurred after application of placebo A, these results can be explained by the mechanism of action of emollients as a role substitution of skin lipids by lipid ingredients from the formulation. The increase on skin lipids is of great importance in impaired eczematous skin due to the ability to restore the lipid barrier, ability to attract, retain, and redistribute water. These findings suggest that not only the anti-inflammatory agent (MF) itself but also the formulation will play an important role in the treatment of skin disorders like atopic dermatitis.

4. Conclusion

Considering the composition of the product and the physico chemical characteristics of the ingredients, the toxicological profile of the ingredients, the risk characterization, the *in vitro* and *in vivo* results, the formulation can be considered safe in the normal and reasonably foreseeable use. Additionally, placebo A demonstrated to contribute to restore the skin barrier by increasing the amount of lipids within the skin.

A suitable equilibrium between safety and efficacy was demonstrated.

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Isotridecanoate/Stearate/Adipate, Glyceryl Oleate SE, Glyceryl Oleate/Elaidate, Glyceryl Palmitate, Glyceryl Palmitate/Stearate, Glyceryl Palmitoleate, Glyceryl Pentadecanoate, Glyceryl Polyacrylate, Glyceryl Rosinate, Glyceryl Sesquioleate, Glyceryl/Sorbitol Oleate/Hydroxystearate, Glyceryl Stearate/Acetate, Glyceryl Stearate/Maleate, Glyceryl Tallowate, Glyceryl Thiopropionate, and Glyceryl Undecylenate. Int J Toxicol 2004;23(2):55-94

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1. Introduction

Introducing a pharmaceutical product on the market involves several stages of research. During the development stage, a series of refinements in the formulation is achieved progressively, including the optimization of the manufacturing processes. The scale-up stage comprises the integration of the previous phases of development, as well as the transfer of technology to fabricate a given product.

This stage is extremely important since many process limitations arise, which were not detectable on the small scale, and become significant on the transposition to a larger scale. In practice, the transition from a laboratory production system to an industrial production is not direct, and the product is usually manufactured on intermediate scales, larger than the initial ones, but smaller than the industrial scale.

Basically, the idea is to simulate production as much as possible and to optimize the operating parameters before a large-volume work is performed. A scale-up procedure based on a well design and prepared technical transfer will assure the quality of the product, an overall economy of resources and a timely and readiness achievement of the markets [1, 2].

The role of the pilot scale batches is to provide predictive data of the production scale product. It may be necessary to further develop and optimize the manufacturing process using several pilot scale batches. The pilot batch therefore provides the link between the process development and the industrial production of the final product.

The purpose of the pilot batch is to challenge the method proposed for routine production, identifying and analyzing the difficulties and the critical points of the manufacturing process.

The pilot batch size should correspond to, at least, 10% of the production scale batch, that is such that the multiplication factor for the scale-up does not exceed 10 [3].

2. Materials and Methods

2.1 Materials

The materials used are described in Chapter III, section 2.1.

2.2 Methods

2.2.1 Lab-scale

The emulsion A was prepared according to the method described in Chapter III, section 2.2.4.

2.2.2 Pilot lab-scale production

The scale-up production of the emulsion A was carried out by increasing in ten-fold the volume of the lab scale using a miniplant reactor system (IKA® LR 2 ST), according to the method described in Chapter VI, section 2.2.1.

2.2.3 Pilot industrial-scale production

A batch of 15 kg of placebo A was produced in a Dumek® Dumoturbo 25 as described in Chapter VI, section 2.2.2. Fig. 8.1 presents the flow chart of the industrial scale production.

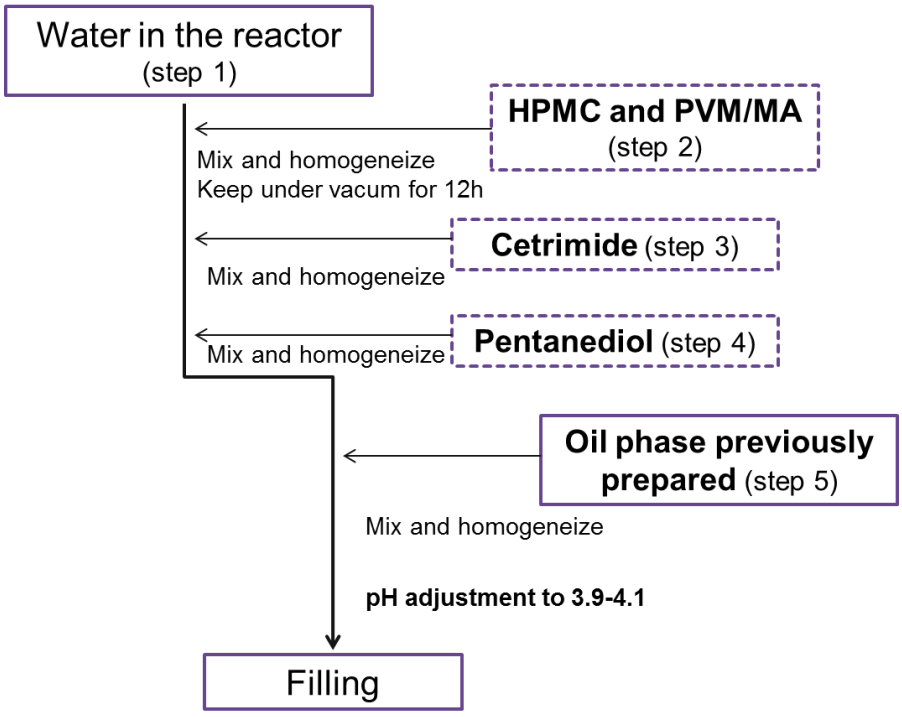

Fig. 8.1. Flow chart of the placebo A industrial scale production.

2.2.4 In-process tests in pilot industrial-scale production

The pH of the placebo, during the industrial-scale production, was measured using a pH meter (Metrohm® pH Meter 744), with a glass electrode in step 1, 2, 3, 4 and 5 (Fig. 8.1).

2.2.5 Droplet size analysis

The size distribution of the droplets of the three samples of placebo, produced by the three different scales, was measured by light scattering following the method described in Chapter IV, section 2.2.3.

2.2.6 Flow curves

Rheograms were determined using a Brookfield® viscometer, model RV DV II, at 22 ºC. A sample, of each scale, was placed into an appropriate container and the rheograms were obtained by submitting the samples to growing shear rates (from 0.6 to 122 s^{-1}) during 30 s at each shear rate value. Spindle SC4-27 was used for each sample. The samples were analyzed 1 month after production.

2.2.7 Comparison between cold and hot processes concerning the production costs

The manufacturing process (cold process) was compared with a conventional hot process, considering that after the introduction of the water phase the reactor is heated to 80 ºC; afterwards, the oil phase is heated to 80 ºC prior to the introduction in the reactor and, after the homogenization of both phases, the reactor is programmed to decrease the temperature to 25 ºC which takes approximately 1h considering 15 kg of product.

The total production costs were calculated taking into account the electrical and water expenditures.

3. Results

3.1 In-process tests in pilot industrial-scale production

The pH is a critical parameter since MF presents its maximum stability below pH 4 [4]. Thus, it is critical to assess the pH during the production to avoid drug instability.
According to Table 8.1, the pH of the water was within the specifications (5.0-7.0). However, after the inclusion of both polymers (HPMC and PVM/MA) and cetrimide the pH drastically decreased. In step 4, which was the phase where MF was added dispersed in the glycol, the pH was acidic. Thus, the degradation of the drug is not expected during the production process. At the end of the process the pH was adjusted with NaOH to 4 to avoid emulsion instability that can occur at low pH values.

Table 8.1. pH values in process control for placebo A during the industrial pilot scale production.

Step	pH value
1	5.30
2	2.72
3	2.04
4	2.12
5	2.72

3.2 Droplet size analysis

The production scale influences the droplet size distribution. In the two lab scales (lab-scale and pilot lab-scale), the placebo A presented a bimodal population whereas after the industrial pilot-scale production, the placebo A presented a monomodal population (Fig. 8.2). The mean droplet size (90% of the droplets) for the two lab scales, immediately after preparation, was similar (23.23 ± 3.89 μm and 18.42 ± 5.76 μm, for lab scale and pilot-lab scale, respectively). The mean droplet size (90% of the droplets) for pilot-industrial scale presented a smaller mean droplet size dispersion (6.37 ± 2.49 μm).

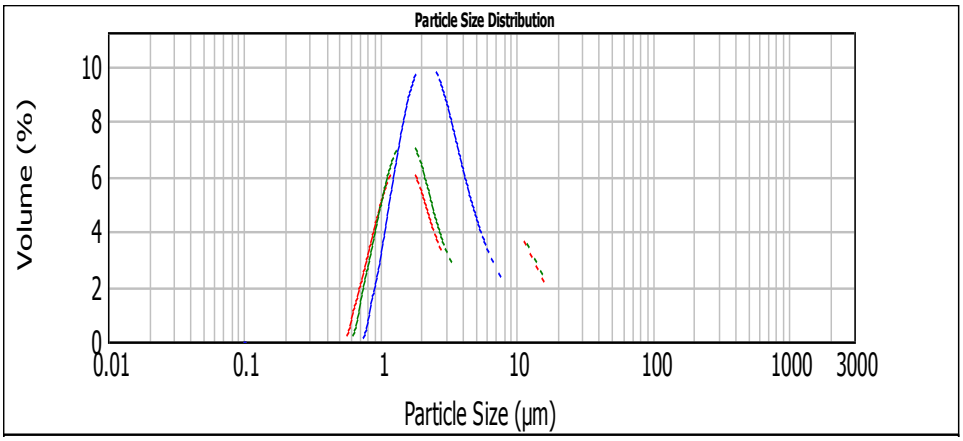

Fig. 8.2. Droplet size distribution of lab-scale (red line), pilot lab-scale (green line) and industrial pilot-scale (blue line) batches, stored at 25 ºC and 1 month after production.

3.3 Flow curves

Representative flow curves are shown in Fig. 8.3 with apparent viscosity values calculated at the apex of the loop (Table 8.2).

The results show that the apparent viscosity decreases concomitantly with the increase of the shear rate in the three scales.

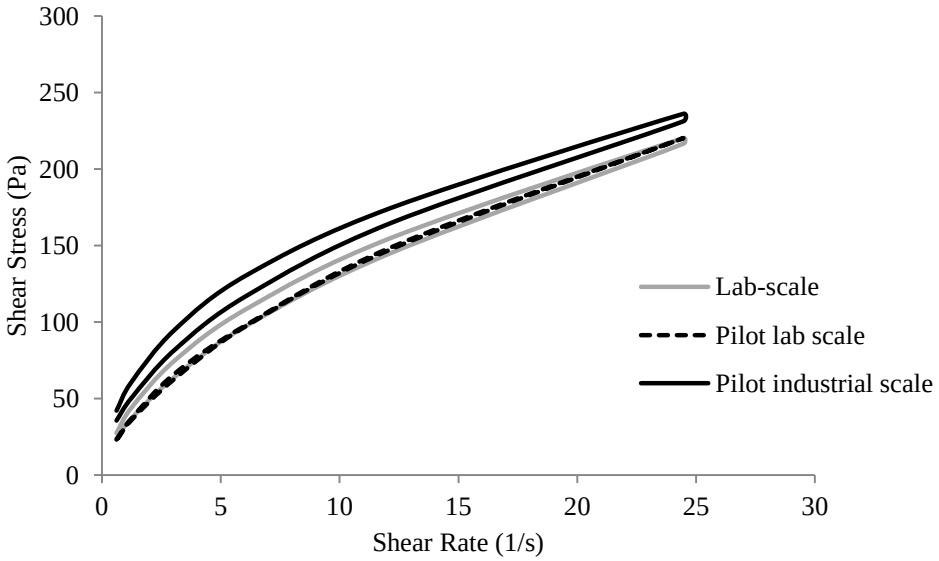

Fig. 8.3. Flow curves. Shear stress as function of shear rate of lab-scale (grey line), pilot lab-scale (dashed line) and pilot industrial-scale (black line).

The apparent viscosity values provide a comparison of the resistance to structural breakdown between the emulsions, and the loop areas compare the amount of structure that fractures in the standardized cycle.

As we increase the production scale, the resistance to the structural breakdown slightly increases. The emulsion produced in pilot industrial-scale presented the highest value of apparent viscosity at the apex of the loop.

Table 8.2. Apparent viscosity values calculated at the apex of the loops (24.47 s^{-1}).

	Apparent Viscosity (Pa.s) at 24.47s^{-1}
Lab-scale	8.85
Pilot lab-scale	9.00
Pilot industrial-scale	9.65

3.4 Comparison between cold and hot processes concerning the production costs

In the lab-scale the emulsification was achieved by high shear homogenization during 5 min at room temperature. In processing 15 kg, it was found that the combination of a lower shear and a longer time of homogenization were the most suitable conditions for the emulsification phase. However, at the end of the process, it was observed that the temperature in the reactor increased to 43 ºC, thus the refrigerator system was needed. According to Table 8.3, despite the energy produced by the high shear of homogenization, the water and energy consumption were decreased in 36.7 and 67.0 %, respectively, compared to a conventional hot process. The cold process method used in the preparation of the emulsion allowed a decrease in the total production costs of more than 17 %. This value is obtained taking into account the differences in the total production costs between the two processes. These differences arise from the equipment costs per hour in terms of water and electrical costs (Table 8.3), reactor amortization, equipment availability and human resources costs per hour. As the production is faster, the costs related to human resources are lower, the amortization of the equipment

decreases and the availability increases as more batches are produced. Other factors such as costs related to the raw materials, packaging, quality control (in process and final product), validation methods and inspections are similar between the two processes.

Table 8.3. Comparison between the cold and hot processes in terms of production costs for placebo A.

	Electrical costs (€/uni)	**Water costs (€/uni)**	**Total production (€/uni)**
Cold process	0.001	0.002	0.58
Hot process	0.003	0.003	0.70
Savings (%)	67.0	36.7	17.1

4. Discussion

It is known that problems associated with costs and time consuming of industrial processes may be avoided if key parameters are previously studied. Industrial processes are usually designed through a gradual increase of the manufactured batch size.

The processing conditions required to produce a high quality product with the desire characteristics vary according to the type of ingredients used and the manufacture conditions.

It is known [5] that for emulsions structured by fatty alcohol/non-ionic surfactants, the key points are the cooling rate and the extent of mixing step. As we are operating in cold processed conditions, our attention was focused on the extent of the mixing step.

Polymers, such as HPMC, need relatively mild conditions of temperature and agitation to prevent depolymerization, whereas, surfactant and fatty alcohol blends need to be processed under high shear. That is the reason we operated firstly at lower shear to disperse the polymers and, after the addition of the oil phase containing the non ionic surfactants, under a high shear.

The in-process tests, as presented in the guideline ICH Q6A [6], must be performed during the manufacture of the drug product, rather than as part of the formal battery of tests which are conducted prior to release.

The pH determination during processing, had the objective to identify and optimize the operating parameters since it is a critical point to the drug stability.

The drastic decrease of the pH value after the inclusion of both polymers is attributed to the PVM/MA polymer. The monoesters of PVM/MA contain two essential components: a hydrophobic ester group and a solubilizing carboxylic group. Due to the acidic carboxylic groups, the pH at the surface of the PVM/MA matrix is low, decreasing the bulk pH [7].

The droplet size of the emulsion significantly decreased when the production scale was increased. Moreover, the industrial pilot-scale produced an emulsion with a monomodal population (Fig. 8.2). The apparent viscosity values are in accordance with the latter results. The emulsion produced in the industrial pilot-scale seemed to be more structured. However, it should be taken in account that only one batch was produced. The variations batch to batch that usually occur, were not analyzed. Nevertheless, the results suggested that the emulsion produced in industrial pilot-scale had a better physical stability.

These results were related to the type of homogenizer of the different equipments. Geometric similarities of the agitation systems used at lab and pilot-scales for the emulsification step should be maintained at all production scales in order to obtain a similar fluid motion. However, it was not possible to use devices with the same geometry in the three scales. In the lab-scale, it was used manual agitation followed by rotor stator homogenization, in the pilot lab-scale the homogenization was achieved using an anchor stirrer and in the pilot industrial-scale a turbine stator and a universal rotor helix shaped.

Concerning the batch size of the industrial pilot-scale production, we decided to produce 15 kg of placebo A because, according to the CPMP/QWP/848/96 [3], the batch size should correspond to, at least, 10 % of the production scale batch.

5. Conclusion

The scale-up process led to more significant alterations on the rheological profile and on the droplet size distribution of the placebo produced by the industrial-scale than the lab-scale production. Moreover, it was observed that a scale-up procedure must be designed according to a robust technology of technical transfer in order to assure product quality, an overall reduction of the production costs and readiness achievement of the markets.
The risks associated to the process of scale-up were minor. However, three batches of emulsion A (containing MF), should be produced to perform the process validation.
The cold process method of production allowed a total savings of more than 17% when compared to the traditional hot process.

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1. Conclusions

The development of advanced delivery systems for topical administration of corticoids is a demanding task that involves not only the improvement of stability, but also the optimization of drug release, permeation, and accumulation.

Published data do not allow a straightforward comparison between formulation factors, making it difficult to clearly identify the critical physicochemical factors when designing drug delivery systems dedicated to dermal application of corticoids.

New technologies (lipid nanoparticles, foams, liposomes) have been developed for topical glucocorticoids (TG) delivery. However, most studies are based on *in vitro* results, which are often not reproducible in *in vivo* testing, and studies are currently underway in order to obtain improved benefit / risk ratio.

A rationale development approach that integrates simple and cost effective formulations easily transposable to the industry and with therapeutic efficacy were performed in this thesis. In summary, cold process emulsions were developed with an appropriate physical, chemical and microbiological stability at acidic pH intended to the delivery of mometosone furoate (MF).

The structure analysis demonstrated that the co-emulsifier containing polyethylene glycol (PEG) chains formed stronger structures indicating a better physical stability.

In vitro release and permeation studies revealed that the glycols used had no influence on the release and permeation profiles of MF which was in agree with the solubility results for the two glycols. Moreover, it was concluded that these emulsions are suitable vehicles for the delivery of MF containing ingredients which were responsible for a drastically increase on the permeability coefficients of MF.

It was demonstrated an epidermal targeting for the final emulsion decreasing the adverse effects wildly described for topical corticoids.

Additionally, the placebo (emulsion without MF) contributed to restore the skin barrier by increasing the amount of lipids within the skin with a suitable safety profile.

Preliminary studies on the scale-up of the placebo showed that the risk associated to the scale-up procedure was not relevant and thus, three batches of emulsion A should be produced.

The production costs were decreased due to, not only the process itself but also but also by the minimization of the ingredients used. For that, multifunctional ingredients were selected such as the polymer, the glycol and the cationic surfactant.

The main goal of this thesis was achieved. The further objective will be the introduction on the market of the new developed topical formulation containing MF for that, some pharmaceutical challenges are still to be met.

TABLE OF CONTENTS:

1. Introduction

Mometasone furoate has the chemical name 9,21-dichloro-11β-hydroxy-16α-methyl-3,20 dioxopregna-1,4-dien-17-yl furan-2-carboxylate pregna-1,4-diene-3,20-dione, 9,21 dichloro-17-[(2-furanylcarbon-yl)oxy]-11-hydroxy-16-methyl-, (11β,16α), with the empirical formula $C_{27}H_{30}Cl_2O_6$, and a molecular weight of 521.44 g/mol [1, 2]. It is a highly potent synthetic chlorinated glucocorticoid with a favorable ratio between local and systemic side-effects. Its effectiveness has been shown in the treatment of glucocorticoid responsive dermatological disorders as topical formulations of ointments, creams and lotions [3]. In clinical studies, MF exhibits strong anti-inflammatory activity, rapid onset of action and low systemic bioavailability [4].

The development of topical products for dermatological diseases represents an untapped opportunity for clinical pharmacology since they represent the most widely used preparations in dermatology [5].

In order to fully characterize candidate formulations or delivery systems such as cold process oil in water emulsions, suitable and validated quantification methods are required to assess critical pharmaceutical parameters such as drug content, release or stability.

United States Pharmacopoeia 2006 [6] has described a procedure for the assay of raw material and MF cream by HPLC using a mixture of methanol and water (65:35 v/v) as mobile phase at a flow rate of 1.7 mL/min, and a stainless steel column (4.6 × 250 mm) containing L7 packing, with the detector wavelength set at 254 nm.

The determination of FA raw material, cream, and eye drops has also been described in the British Pharmacopoeia 2005 [7] by HPLC using a mixture of methanol, 0.05M orthophosphoric acid, and acetonitrile (10:40:50 v/v) as mobile phase at a flow rate of 2.5 mL/min, and a stainless steel column (4.0 × 125 mm) containing lichrospher 100 RP-18 packing, with the detector wavelength set at 235 nm. However, these methods usually require long run times and the extraction solvent doesn´t allow an efficient extraction of the API in complex matrixes.

In this annexe, a new rapid reversed-phase HPLC method for the determination of MF from cold process emulsions made from silicone surfactants and PEG based co surfactants is described and validated according the ICH Q2 (R1) [8].

2. Materials and Methods

2.1. Equipment

2.1.1 Chromatographic Systems

System: HPLC 1

VWR – Hitachi Elite Lachrom Organizer;
VWR – Hitachi Elite Lachrom UV Detector L-2400;
VWR – Hitachi Elite Lachrom Column Oven L-2300;
VWR – Hitachi Elite Lachrom Autosampler L-2200;
VWR – Hitachi Elite Lachrom Pump L-2130;
Software EZ Chrom Elite Version 3.2.1.

System: HPLC 2

VWR – Hitachi Elite Lachrom Organizer;
VWR – Hitachi Elite Lachrom UV Detector L-2400;
VWR – Hitachi Elite Lachrom Column Oven L-2300;
VWR – Hitachi Elite Lachrom Autosampler L-2200;
VWR – Hitachi Elite Lachrom Pump L-2130;
Software EZ Chrom Elite Version 3.2.1.

2.1.2 Other Equipment

Analytical Balance *Mettler Toledo AG204 (d=0,1mg);*
Water bath WBU 45 *Memmert;*
Ultrasonic Bath *Branson 8210*;
Vacum Drying Over JP Selecta

2.2. Reagents, Reference Substances and Samples

2.2.1 Reagents

Methanol HPLC Grade, *Panreac*
Tetrahydrofuran HPLC grade, *Sigma Aldrich*

Purified water (by the *Millipore* system)

Sodium hydroxide 0.1N, *Merck*

Hydrochloric Acid 37% reagent grade, *Scharlau*

Hydrogen Peroxide 30% reagent grade, *Merck*

2.2.2 Reference Substances

Mometasone furoate, Batch nº U0264/1 11010 *Crystal pharma*.

Assay (as is): 100.6%; Loss on drying: 0.4%. Re-test: 02/2017.

2.2.3 Samples

Emulsion A; Laboratory Scale Batch

Placebo A; Laboratory Scale Batch

Table 1. Formulation of emulsion A

	Excipients	Concentration (%, w/w)
Oil Phase	Bis-PEG/PPG-16/16 PEG/PPG-16/16 Dimethicone (and) Caprylic/Capric Triglyceride	5
	PEG-20 glyceryl laurate	4
	C12-15 Alkyl Benzoate	5
	Isopropyl myristate	5
Water Phase	Hydroxy propyl methyl cellulose	2
	Methyl vinyl ether/maleic anhydride copolymer crosslinked with decadiene	0.3
	2-methyl-2,4-pentanediol	10
	Cetrimide BP	0.075
	Mometasone Furoate	0.1
	Water	68.53

2.3. Analytical Conditions

Column: Lichrospher 100 RP18, 125x4mm, 5µm “Merck”

Detection: UV at 248nm

Injection volume: 10µl

Column Oven temperature: 40 ºC

Auto Sampler temperature: 4 ºC

Flow Rate: 1.5ml/min

Mobile phase: Methanol:Water (70:30; v/v)

Solvent: Tetrahydrofuran:Water (75:25; v/v)

Run time: 11 minutes

Working concentration

Sample

Solutions containing mometasone furoate at 25.0 µg/ml.

Reference

Solution containing mometasone furoate at 25.0 µg/ml.

2.4. Preparation of Solutions

2.4.1. Selectivity

Standard Solution

About 12.5 mg of mometasone furoate reference substance, accurately weighed, were dissolved in tetrahydrofuran/water using the ultrasonic bath for about 10 minutes and diluted to 100 ml with the solvent.

An aliquot of 2 ml from the stock solution was accurately measured and diluted to 10ml with the solvent. It was filtered by Puradisc 0.45µm PVDF (Whatman) membrane before injected (~25 µg/ml).

Sample Solution

About 0.5 g of mometasone furoate fluid emulsion (equivalent to 500 µg of mometasone furoate), accurately weighed were dissolved in 20 ml of solvent, using a vortex during 60 seconds, then the solution has been transferred to the ultrasonic bath for about 10 minutes. When it got room temperature, it was filtered by Puradisc 0.45µm PVDF (Whatman) membrane before injected (~25 µg/ml).

Placebo Solution

Placebo solution at a concentration equivalent to the sample solution prepared as the sample solution.

Solvent

Tetrahydrofuran for HPLC and Water (75:25; v/v).

Mobile phase

Methanol:Water (70:30; v/v).

2.4.2. Linearity

Stock Solution

About 12.5 mg of mometasone furoate reference substance, accurately weighed, were dissolved in solvent using the ultrasonic bath for about 10 minutes and diluted to 100 ml with the solvent (0.125 mg/ml).

50% Standard Solution

An aliquot of 2 ml from the stock solution was accurately measured and diluted to 20 ml with the solvent (12.50 µg/ml).

75% Standard Solution

An aliquot of 3 ml from the stock solution was accurately measured and diluted to 20 ml with the solvent (18.75 µg/ml).

100% Standard Solution

An aliquot of 2 ml from the stock solution was accurately measured and diluted to 10 ml with the solvent (25 µg/ml).

125% Standard Solution

An aliquot of 5 ml from the stock solution was accurately measured and diluted to 20 ml with the solvent (31.25µg/ml).

150% Standard Solution

An aliquot of 3 ml from the stock solution was accurately measured and diluted to 10 ml with the solvent (37.50 µg/ml).

Table 2. Solutions for the assessment of Linearity

Solutions[(a)]	Stock Solution (µg/ml)	Aliquot (ml)	Dilution Volume (ml)	Concentration (µg/ml)
50%	133	2	20	13.70
75%	133	3	20	20.55
100%	133	2	10	27.40
125%	133	5	20	34.25
150%	133	3	10	41.10

(a) Percentage of the test concentration

2.4.3 Accuracy

Sample Solutions (50%, 100% and 150%)

Three replicates of the placebo spiked with known amounts of mometasone furoate solution over 3 concentration levels covering the working range (50%, 100% and 150% of the test concentration), completing a total of 9 determinations.

Solutions at 50 % (MF at about 12.5 µg/ml)

12.5 mg of mometasone furoate reference substance, accurately weighed, were dissolved in solvent using the ultrasonic bath for about 10 minutes and diluted to 100 ml with the solvent (0.125 mg/ml).

To a 20ml volumetric flask an aliquot of 2 ml from the previous solution was accurately addicted to 0.5 g of Placebo and 20ml of solvent – about 12.5 µg/ml.

The solutions were prepared as described in 2.4.1. (Selectivity).

Solutions at 100 % (MF at about 25 µg/ml)

12.5 mg of mometasone furoate reference substance, accurately weighed, were dissolved in solvent using the ultrasonic bath for about 10 minutes and diluted to 100 ml with the solvent (0.125 mg/ml).

To a 20ml volumetric flask an aliquot of 4 ml from the previous solution was accurately addicted to 0.5 g of Placebo and 20ml of solvent – about 25 µg/ml.

The solutions were prepared as described in 2.4.1. (Selectivity).

Solutions at 150 % (MF at about 37.5 µg/ml)

12.5 mg of mometasone furoate reference substance, accurately weighed, were dissolved in solvent using the ultrasonic bath for about 10 minutes and diluted to 100 ml with the solvent (0.125 mg/ml).

To a 20ml volumetric flask an aliquot of 6 ml from the previous solution was accurately addicted to 0.5 g of placebo and 20 ml of solvent – about 37.5 µg/ml.

The solutions were prepared as described in 2.4.1. (Selectivity).

2.4.4. Precision

2.4.4.1. Suitability test

Six replicate injections of a reference solution containing mometasone furoate at about 25 µg/ml (test concentration) were prepared as described in 2.4.1. (Selectivity).

2.4.4.2. Analysis Repeatability

Standard Solution

A reference solution containing mometasone furoate at about 25 µg/ml (test concentration) was prepared as described in 2.4.1. (Selectivity).

Sample Solutions

Six replicates of sample solution containing mometasone furoate at about 25 µg/ml (test concentration) were prepared as described in 2.4.1. (Selectivity).

2.4.4.3. Intermediate Precision

Two analysts prepared individually, on different days and using different equipment systems and different columns, 6 replicates of sample solution and a reference solution, as described in 2.4.1. (Selectivity).

2.4.5. Solutions Stability

Reference Standard Solution

It was prepared a mometasone furoate reference solution at about 25.0 µg/ml, as described in 2.4.1. (Selectivity). After that, the referred solution was maintained at 5 ± 3 ºC, protected from light, in the initial volumetric flask and in sealed capsules , and also in the same vial used in the first analysis into the auto sampler at 4 ºC. The reference solution was kept for 24 and 48 hours in these conditions.

Test Solutions

It was prepared a sample solution at test concentration (25.0 µg/ml), as described in 2.4.1. This solution was analyzed at the preparation day. After that, the referred solution was maintained at 5 ± 3 ºC, protected from light, in the initial volumetric flask and in sealed capsules, and also in the same vial used in the first analysis into the auto sampler at 4 ºC. The test solution was kept for 24 and 48 hours in these conditions.

2.4.6. Stress Studies

Reference solution submitted to stress conditions

Stock solution containing mometasone furoate at about 25.0 µg/ml

12.5 mg of mometasone furoate reference substance, accurately weighed, were dissolved in solvent using the ultrasonic bath for about 10 minutes and diluted to 100 ml with the solvent (0.125 mg/ml).

An aliquot of 2 ml from the stock solution was accurately measured to a volumetric flask of 10 ml.

Five replicates of this mixture were prepared, and each one was treated as follows:

Acid agent: It was added 1.0 ml of HCl 1N and the final solution was maintained for 5 days at room temperature and protected from light.

Alkaline agent: It was added 1.0 ml of NaOH 0.1N and the final solution was maintained for 1 hour at room temperature and protected from light.

Oxidant agent: It was added 1.0 ml of H_2O_2 15% and the final solution was maintained for 5 days at room temperature and protected from light.

Ultraviolet light: The sample was maintained for about 5 days at room temperature, under UV light.

Temperature: The sample was maintained for 5 days at about 60ºC, protected from light.

The five mixtures were then diluted to 10ml with the solvent and filtered by Puradisc 0.45µm PVDF (Whatman) membrane before injected (~25 µg/ml).

Sample solutions submitted to stress conditions

Five replicates of about 0.5 g of mometasone furoate fluid emulsion (equivalent to 500 µg of mometasone furoate), accurately weighed to a volumetric flask of 20 ml were prepared, and each one was treated as follows:

Acid agent: It was added 1.0 ml of HCl 1N and the final solution was maintained for 5 days at room temperature and protected from light.

Alkaline agent: It was added 1.0 ml of NaOH 0.1N and the final solution was maintained for 1 hour at room temperature and protected from light.

Oxidant agent: It was added 1.0 ml of H_2O_2 15% and the final solution and was maintained for 5 days at room temperature and protected from light.

Ultraviolet light: The sample was maintained for about 5 days at room temperature, under UV light.

Temperature: The sample was maintained for 5 days at about 60ºC, protected from light.

The samples were then dissolved in 20 ml of solvent, using a vortex during 60 seconds, then the solution has been transferred to the ultrasonic bath for about 10 minutes. When it got room temperature, it had been filtered by Puradisc 0.45µm PVDF (Whatman) membrane before injected (~25 µg/ml).

Placebo solutions submitted to stress conditions

Five replicates of about 0.5 g of fluid emulsion placebo, accurately weighed to a volumetric flask of 20 ml were prepared, and each one was treated as follows:

Acid agent: It was added 1.0 ml of HCl 1N and the final solution was maintained for 5 days at room temperature and protected from light.

Alkaline agent: It was added 1.0 ml of NaOH 0.1N and the final solution was maintained for 1 hour at room temperature and protected from light.

Oxidant agent: It was added 1.0 ml of H_2O_2 15% and the final solution was maintained for 5 days at room temperature and protected from light.

Ultraviolet light: The sample was maintained for about 5 days at room temperature, under UV light.

Temperature: The sample was maintained, after extraction procedure, for 5 days at about 60ºC, protected from light.

The samples were then dissolved in 20 ml of solvent, using a vortex during 60 seconds, then the solution has been transferred to the ultrasonic bath for about 10 minutes. When it got room temperature, it had been filtered by Puradisc 0.45µm PVDF (Whatman) membrane before injected (~25 µg/ml).

3. Results

3.1. Selectivity

Selectivity was evaluated by the analysis of the solvent, the mobile phase and a placebo solution at a concentration equivalent to the sample solution, a reference solution containing mometasone furoate (25 µg/ml) and a sample solution containing mometasone furoate (25 µg/ml)

Acceptance Criteria

Chromatographic peaks resulting from the solvent, the mobile phase and the placebo should not interfere with the analyte of interest. Any peak corresponding to the analyte should be separated from any other peaks and separated from each peak. The resolution factors should be at least 1.5 between peaks of interest.

The results are shown in the table below.

Table 3. Selectivity - Chromatographic peaks

Solution	Retention time (min)	Peaks	Relative Retention Time concerning MF	Resolution Factor
Solvent	0.673		0.090	-
	0.800		0.107	1.380
	1.407		0.188	-
	1.527		0.204	-
	1.773		0.236	-
	1.833		0.244	-
	1.913		0.255	-
	2.553		0.340	-
	2.833		0.378	-
	5.627		0.750	-
	5.860		0.781	-
Mobile Phase	0.667		0.089	-
	0.793		0.106	1.624
	2.553		0.340	13.75
	2.827		0.377	-
Placebo	0.620		0.083	-
	0.833		0.111	2.018
	1.400		0.187	-
	1.527		0.204	-
	1.767		0.236	1.716
	1.833		0.244	0.798
	1.913		0.255	-
	2.547		0.340	-
	2.820		0.376	1.42
	3.907		0.521	5.169
	5.627		0.750	-
	5.860		0.781	-
Reference solution	0.667		0.089	-
	0.800		0.107	1.837
	1.113		0.148	1.540
	1.347		0.180	-
	1.467		0.196	-
	1.687		0.225	-
	1.773		0.236	-
	3.920		0.522	-
	5.747		0.766	7.078
	6.033		0.804	0.914

	7.500	MF	1.000	3.272
Sample solution	0.653		0.087	-
	0.833		0.111	1.898
	1.353		0.180	-
	1.473		0.196	-
	1.693		0.226	1.841
	1.773		0.236	-
	3.940		0.525	-
	5.760		0.768	7.865
	6.047		0.806	0.992
	7.527	MF	1.004	3.497

MF – Mometasone furoate

The chromatograms are presented in annex (chromatograms 1, 2, 3, 4, 5)

Comments

The analytical method for the determination of mometasone furoate in fluid O/W emulsions has demonstrated to be selective since the solvent, the mobile phase and the placebo do not interfere with the analyte and these are well separated from each other.

3.2. Linearity

The linearity of the analytical method was evaluated under the concentration range equivalent to 50% to 150% of the test concentration (25 µg/ml), using 5 different solutions. The regression line of the analytical response as a function of analyte concentration was calculated by the method of least squares. The correlation coefficient (r^2), Y- intercept, slope of the regression line, a plot of the data and an analysis of the deviations (D%) of the actual data points from the regression line is reported.

The deviations D(%) are expressed by:

$$D\% = C_c/C_n \times 100$$

C_c Calculated concentration from the regression line; C_n Nominal concentration

Table 4. Linearity parameters

Parameters	Results	Acceptance criteria
Range	13.70 – 41.10 µg/ml	-
Desviations (%)		-
Minimum/Maximum	99.01 - 100.54	98.0 – 102.0
Average	100.01	-
CV (%)	0.62	≤ 2.0%
r^2	0.99985	> 0.995
	Regression line	
Slope	735314.16	-
Intercept	297417.90	-

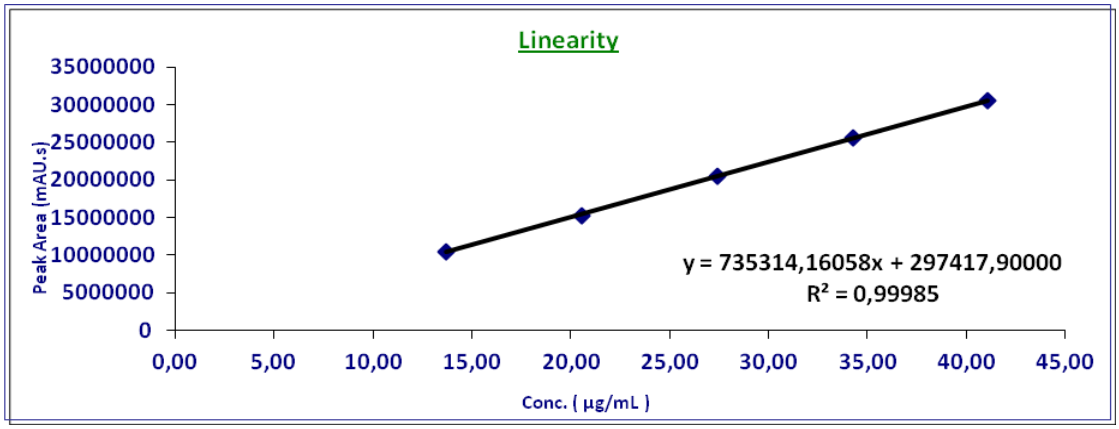

Fig. 1. Regression line plot

Comments

The results are in compliance with the established acceptance criteria, showing that the analytical method is linear in the working range.

3.2.1 Residual analysis

The residual analysis shows the residuals of experiment point's distributions. In linear function residuals must be casually distributes. The results of residuals, calculated by the following formula, are presented in Table 5.

$$\text{Residuals} = \frac{y(i) - y\,c(i)}{y\,c(i)} \cdot 100$$

Table 5. Residual Analysis for unknown related compounds

Concentration (µg/mL)	Peak Area (mAU.s) Y(i)	Estimated Peak Area (mAU.s) Yc(i)	Residuals
13,70	10425469,23	10371221,90	**0,5**
20,55	15259189,23	15408123,90	**-1,0**
27,40	20538882,57	20445025,90	**0,5**
34,25	25524029,23	25481927,90	**0,2**
41,10	30477559,23	30518829,90	**-0,1**

Slope (b) = 735314.16

Intercept (a) = 297417.9

(r) = 0.99993

(r^2) = 0.9999

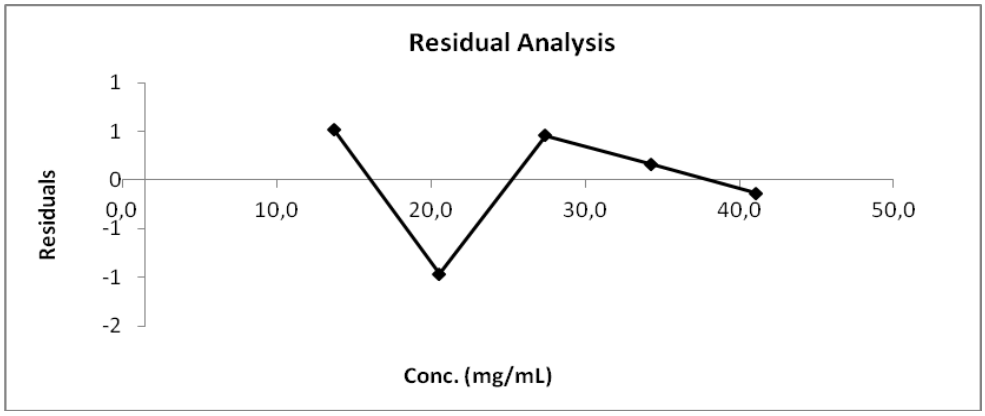

Fig. 2. Residual Analysis for unknown related compounds

Comments

The results are in compliance with the established acceptance criteria, showing that the analytical method has a random distribution of residuals. Correlation coefficient is a good correlation indicator, but not linearity indicator. Mandel test and Rikilt Test must be used to prove that linearity function can be adapted to experimental representation.

3.2.2. Mandel Test

Mandel Test uses linear and polynomial adjustment to study which function can be adapted to experimental values. In this assay a Test Value (TV) is compared with tabulated value (F). In fact polynomial adjustment is always better to describe the function. However, differences are not significant, for this reason, linear function can be used. The results are presented in Table 6.

Table 6. Mandel Test – Results / Calculations for FM

Concentration (µg/mL)	Peak Area (mAU.s) Y(i)	Linear adjustment signal yL (i)	Polinomial adjustement signal yP(i)
13,70	10425469,23	10371221,90	10363375,23
20,55	15259189,23	15408123,90	15412047,23
27,40	20538882,57	20445025,90	20452872,57
34,25	25524029,23	25481927,90	25485851,23
41,10	30477559,23	30518829,90	30510983,23

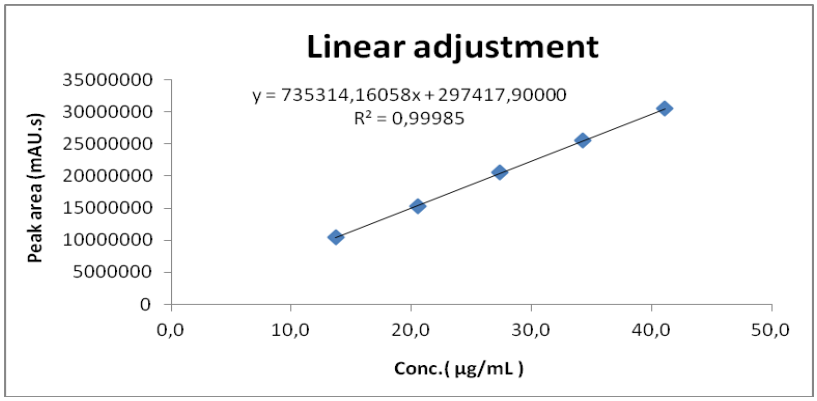

Fig. 3. Linear Adjustement – Mandel test

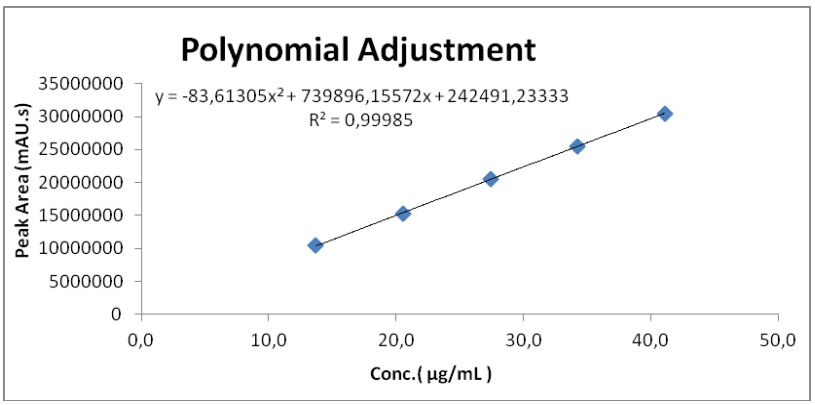

Fig. 4. Polynomial Adjustement – Mandel test

Table 7. Linear Adjustement

(i)	(y-yi)^2
1	2.9E+09
2	2.2E+10
3	8.8E+09
4	1.8E+09
5	1.7E+09
Soma =	**3.7E+010**
N-2 =	**3**
$S_{y/x}$	**111667.92**

Table 8. Polynominal Adjustement

(i)	(y-yi)^2
1	3.9E+09
2	2.3E+10
3	7.4E+09
4	1.5E+09
5	1.1E+09
Soma =	**3.7E+010**
N-2 =	**2**
$S_{y/x}$	**136370.22**

DS^2 parameter, calculated according following formula was 2.155E+8

$$DS^2 = (N-2).S^2_{Y/X} - (N-3).S^2_{Y/X(2^o)}$$

The Test Value (TV), obtained using the equation presented below, was 0.01

$$VT = \frac{DS^2}{S^2_{Y/X(2^o)}}$$

The tabulated F factor, for a confidence range of 95% and N-3 degrees of freedom, is 18.51. As TV < F, linear function can be used for experimental representation.

3.2.3. RIKILT Test

Table 9. Rikilt Test – Results / Calculations for MF

Concentration (µg/mL)	Peak Area (mAU.s) Y(i)	IFi (yi/xi)	RFi / average RF x 100	Acceptance Criteria
13.70	10425469.23	760983.16	101.70	98.0 – 102.0
20.55	15259189.23	742539.62	99.30	
27.40	20538882.57	749594.25	100.20	
34.25	25524029.23	745227.13	99.60	
41.10	30477559.23	741546.45	99.10	
	Average	747978.12	100.00	98.0 – 102.0
	Standard deviation	7909.72	1.057	-
	RSD	1.06	1.06	< 2.0 %

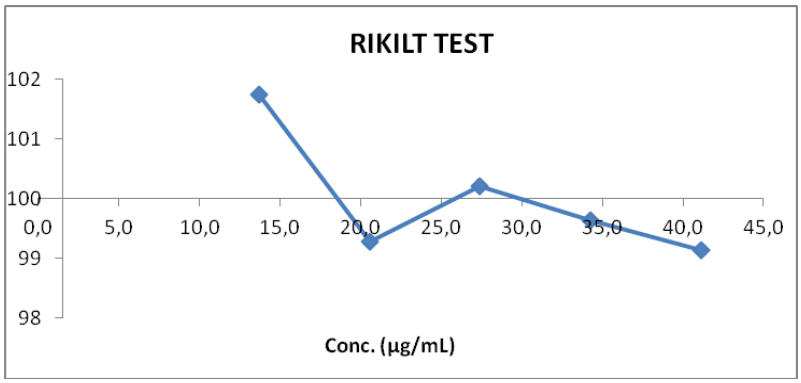

Fig. 5. Response factor analysis for MF

Comments

The results are in compliance with the established acceptance criteria, showing that the analytical method is linear in the working range. Response factor can also be used in this linear function (routine work).

3.3. Accuracy

The accuracy was evaluated by the variability of the assay of 9 determinations, prepared of the placebo spiked with known amounts of the analyte over 3 concentration levels, covering the working range (50%, 100% and 150% of the test concentration). There were 3 replicates to each concentration level and the accuracy was reported as percent recovery (ratio between experimental concentration and theorical concentration).

The results are presented in table 10.

Table 10. Accuracy Results

Sample Solutions	Theorical Concentration (µg/ml)	Experimental Concentration (µg/ml)	Recovery (%)	Average Recovery (%), n=3	Acceptance Criteria (%)	Comment
50%	13.20 13.20 13.20	13.20 13.26 13.19	100.0 100.4 99.9	100.1	97.0-103.0	Complies
100%	26.60 26.60 26.60	26.52 26.63 26.92	99.7 100.1 101.2	100.3		Complies
150%	39.90 39.90 39.90	39.86 40.11 40.14	99.90 100.50 100.60	100.3		Complies

Comments

The results are in compliance with the established acceptance criteria, showing that the analytical method is accurate in the working range.

3.4. Precision

3.4.1. Suitability tests

System repeatability was evaluated by the variability of the analytical response of a reference solution at 100% of the test concentration (~25 µg/ml). The results obtained are presented in Table 11.

Table 11. System repeatability

Concentration (µg/ml)	Average Response (n=6)	RSD (%)	RT-Average (min)	RT-RSD (%)	Acceptance Criteria (RSD)	Results
26.6	1863546.7	0.43	7.515	0.0036	≤ 2.0%	Complies

3.4.2. Analysis Repeatability

Analysis repeatability was evaluated by the variability of the assay of 6 replicates of the O/W fluid emulsion, prepared as described in 2.4.1 (selectivity - sample solution). The results obtained are presented in Table 12.

Table 12. Analysis repeatability

Assay (%)	Average (%) n=6	RSD (%) n=6	Acceptance Criteria (RSD %)	Comment
98.11 97.87 97.43 97.53 97.65 97.60	97.70	0.257	≤ 3.0%	Complies

3.4.3. Intermediate Precision

Two analysts prepared individually, on different days, in different equipment systems, 3 replicates of sample solution and a reference solution, as described in 2.4.3.2 (Analysis Repeatability).

Table 13. Method Intermediate Precision

Parameters	Results Test I	Results Test II	Acceptance Criteria
% Recovery	98.11 97.87 97.43 97.53 97.65 97.60	99.48 101.42 101.37 100.65 102.27 101.15	90% – 110%
Average (%)	97.69	101.06	
RSD (%)	0.255	0.924	≤ 3.0%
Average pooled (%)	99.377		-
RSD pooled (%)	0.688		≤ 3.0%

Comments

The results are in compliance with the established acceptance criteria, showing that the analytical method is precise in the working range.

3.5. Solutions Stability

The stability of a reference solution containing mometasone furoate at 25.0 µg/ml and a sample solution containing mometasone furoate at 25.0 µg/ml maintained at 5 ± 3 ºC protected from light, maintained at 5 ± 3 ºC protected from light in sealed capsules and kept in the autosampler at 5 ºC was evaluated throughout the determination of the variation of the concentration of the active, expressed by:

Variation (%) = $(C_f – C_i)/C_i$ *100

C_i – Inicial concentration (Day 0)
C_f – Final concentration (after 24 h and 48h)

The results are presented in Table 14.

Table 14. Solutions Stability

Solution	Solution	Ci (µg/ml)	Cf (µg/ml)	Variation (%)	Acceptance crieteria
Reference	Same vial kept in autosampler during 24 h	25.80	27.13	5.16	Variation ≤ 2.0 %
	New vial, from the solution kept in fridge during 24 h	26.60	27.15	2.08	
	New vial, from the solution kept in fridge during 24 h in sealed capsules	25.80	25.70	0.37	
	Same vial kept in autosampler during 48 h	25.80	28.94	12.19	
	New vial, from the solution kept in fridge during 48 h	26.60	27.01	1.54	
	New vial, from the solution kept in fridge during 48 h in sealed capsules	25.80	25.89	0.34	
Sample	Same vial kept in autosampler during 24 h	26.64	27.37	2.76	Variation ≤ 2.0 %
		29.27	30.65	4.73	
		29.88	31.27	4.65	
	New vial, from the solution kept in fridge during 24 h	26.09	27.27	4.51	
		25.75	27.04	5.13	
		24.37	25.39	4.19	
	New vial, from the solution kept in fridge during 24 h in sealed capsules	26.64	26.54	0.35	
		29.27	29.38	0.39	
		29.88	30.11	0.77	
	Same vial kept in autosampler during 48 h	26.64	28.52	7.07	
		29.27	31.94	9.12	
		29.88	33.32	11.21	
	New vial, from the solution kept in fridge during 48 h	26.09	26.98	3.41	
		25.72	26.66	3.65	
		24.37	25.34	4.05	
	New vial, from the solution kept in fridge during 48 h in sealed capsules	26.64	26.79	0.58	
		29.27	29.50	0.79	
		29.88	30.20	1.04	

Comments

The results for reference solution of mometasone furoate and for sample are in compliance with the established acceptance criteria only for the solutions kept in the fridge in sealed capsules, showing that the solutions are stable during the period of analysis in those conditions.

3.6. Stress Studies

Table 15. Stress studies for mometasone furoate fluid emulsion

Stress Studies for Mometasone furoate fluid emulsion				
Stress Condition	RT	Individual	Total	Assay
Mometasone furoate standard solution + HCl 1N for 5 days at room temperature	-	-	-	108.87
Mometasone furoate standard solution + NaOH 0.1N for 1 hour at room temperature	0.827	18.54	77.73	38.85
	2.707	1.95		
	3.180	1.31		
	4.233	1.94		
	4.687	15.14		
Mometasone furoate standard solution + H_2O_2 15 V for 5 days at room temperature	-	-	-	104.55
Mometasone furoate standard solution + UV for 5 days at room temperature	-	-	-	106.74
Mometasone furoate standard solution at 60 ºC during 5 days	-	-	-	110.34

Table 16. Stress studies for mometasone furoate reference solution

Stress Studies for Mometasone furoate standard solution				
Stress Condition	RT	Individual	Total	Assay
Mometasone furoate standard solution + HCl 1N for 5 days at room temperature	-	-	-	93.96
Mometasone furoate standard solution + NaOH 0.1N for 1 hour at room temperature	0.840	31.25	80.69	19.85
	1.040	11.58		
	1.247	1.35		
	2.707	1.80		
	3.247	7.36		
	4.687	7.30		
	5.513	0.20		
Mometasone furoate standard solution + H_2O_2 15 V for 5 days at room temperature	-	-	-	97.23
Mometasone furoate standard solution + UV for 5 days at room temperature	3.800	5.091	94.64	89.55
Mometasone furoate standard solution at 60 ºC during 5 days	1.447	17.66	100.94	83.29

4. Analytical Procedure for Assay of MF in emulsion A

Analytical Conditions

Column: Lichrospher 100 RP18, 125x4mm, 5µm "Merck"
Column Oven temperature: 40 ºC
Auto Sampler temperature: 4 ºC
Detection: UV at 248nm
Injection volume: 10µl
Flow Rate: 1.5ml/min
Mobile phase: Methanol:Water (70 : 30; v/v)
Solvent: THF:Water (75 : 25; v/v)
Run time: 11 min

Reference Solution

Weigh accurately 12.5 mg of mometasone furoate reference substance, dissolve in the solvent using the ultrasonic bath for about 10 minutes and dilute to 100 ml with the solvent.
Take an aliquot of 2 ml from the stock solution and dilute to 10 ml with the solvent. Filter by Puradisc 0.45µm PVDF (Whatman) membrane before inject (~25 µg/ml).

Sample Solution

Weight accurately 0.5 g of mometasone furoate fluid emulsion (equivalent to 500 µg of mometasone furoate), dissolve in 20 ml of solvent, using a vortex during 60 seconds and then transfer the solution to the ultrasonic bath for about 10 minutes. When the solution is at room temperature, filter by Puradisc 0.45µm PVDF (Whatman) membrane before inject (~25 µg/ml).

Procedure

Inject 3 times both reference and sample solutions and record the chromatograms. The retention time of the analyte peak is approximately 7.5 minutes.

The CV between replicate injection should be not more than 2.0%

Determine the mometasone furoate content, expressed in percent, by the following expression:

$$C = Rs/Rr \times Pr/Ps \times fds/fdr \times P/Ad$$

C – Content in mometasone furoate (%);

Rs – Analytical response of the sample solution;

Rr - Analytical response of the reference solution;

Pr – Weight of the reference solution (mg);

Ps – Weight of the sample solution (mg);

fdr – Dilution factor of reference solution;

fds – Dilution factor of sample solution;

Ad – Amount of mometasone furoate per dosage (1 mg/g);

P – Purity/Content of mometasone furoate in the standard substance (%)

Determine the average value of the two samples solutions.

5. Conclusions

The analytical methodology of High Pressure Liquid Chromatography with ultra-violet detection for the determination of mometasone furoate in the fluid emulsion containing 0.1% of mometasone furoate has been developed and validated.
The method has demonstrated to be selective concerning mometasone furoate, solvents and excipients, linear, accurate and precise within the working range.
The method is also suitability for monitoring the stability of the product.

6. References

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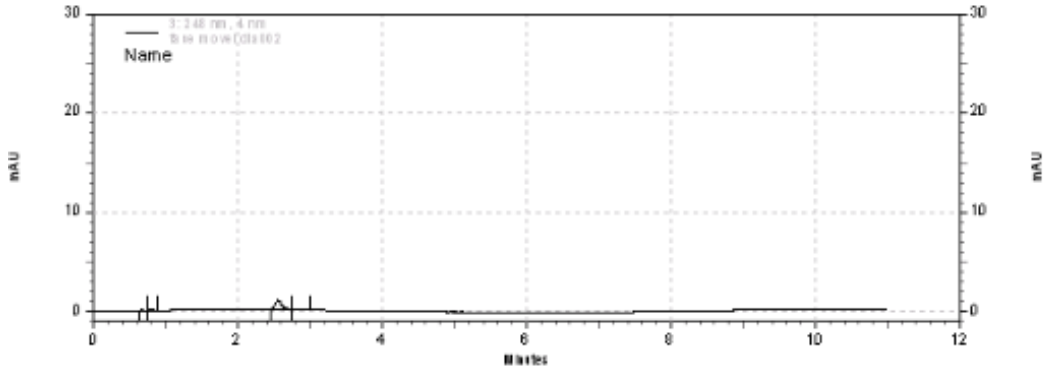
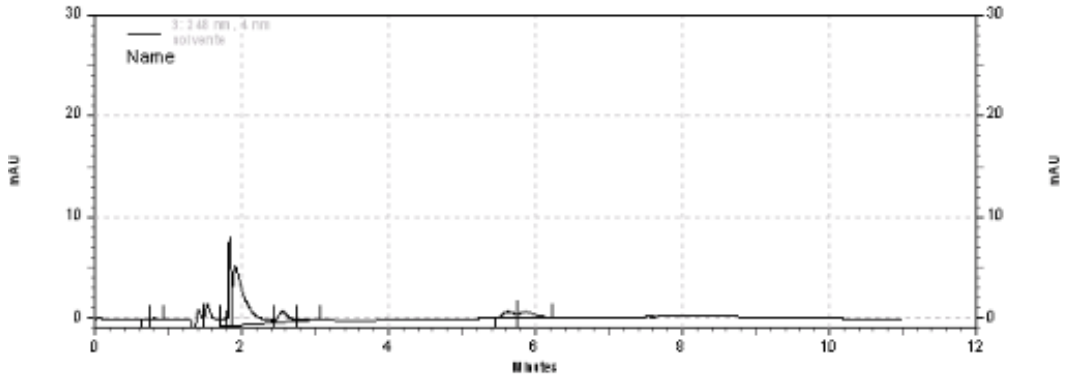
7. Annexes

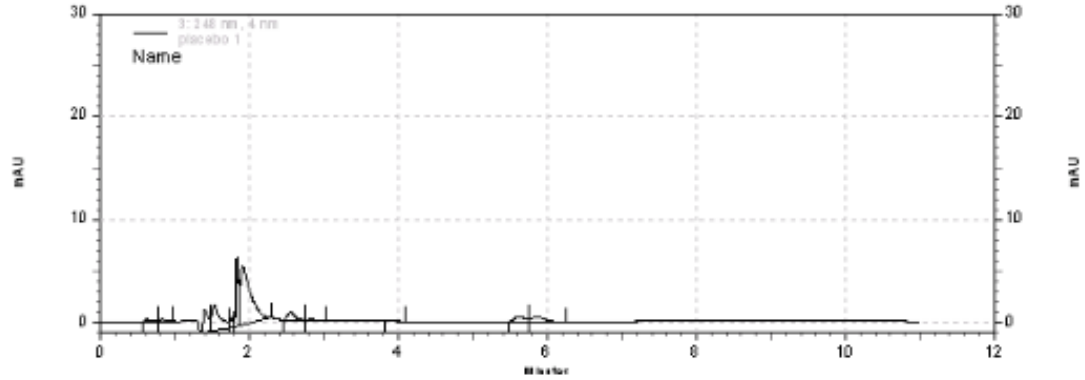
Chromatogram 1: Mobile phase



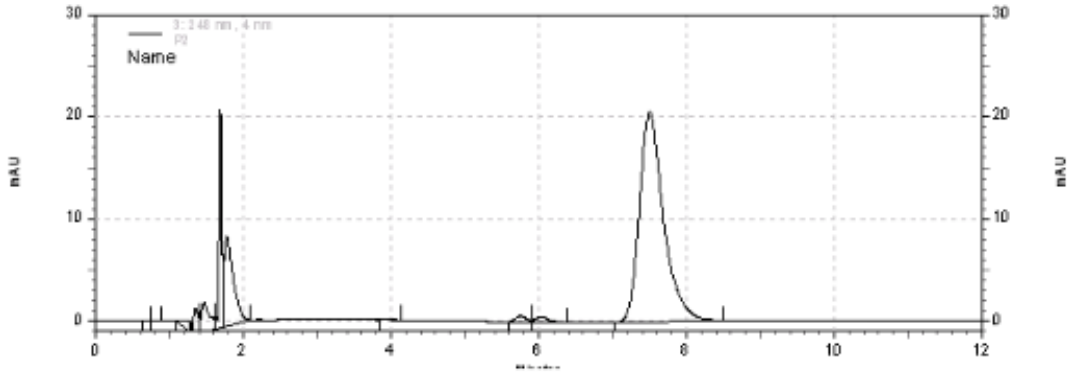
Chromatogram 2: Solvent



Chromatogram 3: Placebo



Chromatogram 4: Reference Solution



Chromatogram 5: Sample solution 100%