Strategies for the delivery of insoluble drugs to the eye

Rodriguez Aller, Marta Ruth

UNIVERSITÉ DE GENÈVE FACULTÉ DES SCIENCES

Section des sciences pharmaceutiques

Pharmacie galénique Professeur Robert Gurny
Chimie analytique pharmaceutique Professeur Jean-Luc Veuthey

Strategies for the delivery of insoluble drugs to the eye

THÈSE

présentée à la Faculté des Sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences, mention Sciences Pharmaceutiques

par

Marta Ruth RODRIGUEZ ALLER

de

Espagne

Thèse N° 4741

Genève
Atelier de reproduction Repromail
2015

UNIVERSITÉ
DE GENÈVE

FACULTÉ DES SCIENCES

Doctorat ès sciences
Mention sciences pharmaceutiques

Thèse de *Madame Marta Ruth RODRIGUEZ ALLER*

intitulée :

"Strategies for the Delivery of Insoluble Drugs to the Eye"

La Faculté des sciences, sur le préavis de Monsieur R. GURNY, professeur honoraire et directeur de thèse (Section des sciences pharmaceutiques), Monsieur J.-L. VEUTHEY, professeur ordinaire et codirecteur de thèse (Section des sciences pharmaceutiques), Monsieur J.-L. WOLFENDER, professeur ordinaire (Section des sciences pharmaceutiques), Madame M. J. ALONSO, professeure (Center for Research in Molecular Medicine and Chronic Diseases, University of Santiago de Compostela, Spain), Monsieur C. SCHOCH, docteur (Schoch Pharma Consulting, Muttenz, Basel, Switzerland), et Madame K. LANDAU, professeure (UniversitätsSpital Zürich, Augenklinik und Poliklinik, Zürich, Schweiz), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 28 novembre 2014

Thèse - 4741 -

Le Doyen

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

To my family,

Acknowledgements

I would like to start these acknowledgements sincerely thanking my PhD directors, Prof. Robert Gurny and Prof. Jean-Luc Veuthey, for having made possible this PhD work through their support, trust and enthusiasm. Thank you both for giving me the space I needed to learn how to construct my own ideas and acquire independence. Your careers have been very inspirational to me. Thank you Robert for accepting me as your "very last PhD student", for your time, enthusiasm and inestimable advices. Thank you Jean-Luc for your availability, openness, positivism and priceless tips. My warm thanks also go to Dr. Davy Guillarme. Thanks Davy for your constant support and good mood, your extraordinary efficiency, pragmatism and for being an inexhaustible source of solutions for any kind of analytical issue. I cannot be more grateful to the three of you for allowing me to do my PhD in such exceptional conditions.

My acknowledgements also go to the jury members: Prof. Maria José Alonso, Prof. Klara Landau, Dr. Christian Schoch and Prof. Jean-Luc Wolfender for accepting judging this work, the valuable discussions, comments, advices and contagious positive energy.

I would also like to thank Prof. Serge Rudaz for his scientific advices and for accepting to be my "administrative tutor" during these last years. Thanks also to Dr. Béatrice Kaufmann, Dr. Cinzia Stella, Dr. Pascal Furrer and Dr. Claudia di Tommaso for their contribution to the first part of the prodrug project. I would also like to thank Sylvie Guinchard for her exceptional work and expertise. My acknowledgements go to Prof. Francine Behar-Cohen, Dr. Fatemeh Valamanesh, Mrs. Ikram El Zaoui and Mr. Mohamed El Sanharawi from the University Paris Descartes for the nice collaboration on in vivo experiments with prodrug formulations. I would also like to express my gratitude to Dr. Yan Guex-Crosier, Dr. Simone Eperon and Dr. Konstantinos Balaskas from the Jules Gonin Eye Hospital in Lausanne for the pleasant collaboration related to intraocular implants. The scientists from Ophthalmopharma, Dr. Randall Gatz and Dr. Motti Geron, are also acknowledged for the instructive partnership. Thanks also to Dr. Damien Jeannerat and Mrs. Marion Pupier for their valuable help with structural determinations through 2D-NMR. I would also like to thank Patrick and Baptiste from the slaughterhouses of Meinier and Loëx.

Many thanks to all the members of the pharmaceutical technology and analytical chemistry laboratories for the incredible working environment, the animated lunch breaks and sports activities. Thank you Myrtha, the kindest fire spitting dragon ever! I am also grateful to my office and lab mattes Marieke, Ludmila, Béatrice, Betty, Aline, Isa, Greg, Steph, Vincent, Yoric, Aurélie, Alex, Béné and Sema for the permanent optimism and sharing. Thanks also to all the students, postdocs and university members I was lucky enough to share the PhD experience with!

My acknowledgements also go to all the people who have participated in my learning journey from preschool to PhD, making it fascinating.

Finally, I would like to warmly thank my family for making possible this adventure and teaching me the most important things in life. Gracias por vuestro apoyo incondicional. ¡Sin vosotros nunca habría podido llegar hasta aquí! Gracias Abuelitas por transmitirme vuestra paz y vuestro coraje. Gracias Papá por enseñarme lo fascinante que es la naturaleza, el valor de las cosas bien hechas y ser un modelo de esfuerzo y dedicación. Gracias Mamá por contagiarme la fuerza y determinación que siempre has demostrado tener, aun sin darte cuenta. Gracias Nuria por la complicidad, los consejos y por ser un ejemplo a seguir. Gracias Iván por tu buen humor a prueba de bombas, por tu escucha y comprensión, y por acompañarme en esta y tantas otras increíbles aventuras. Gracias Javier y Daniel por enseñarme tanto, por demostrarme que los sueños se cumplen y por llenar de alegría cada día.

Table of contents

Foreword

Foreword

Life is closely related to water. Cells consisted of an internal aqueous cytosol isolated from the external environment by a phospholipidic membrane. Cells can organize forming tissues, organs, systems and complex individual organisms containing a high proportion of water. Inside the human body, a number of strategies are required to cope with poor water-soluble molecules. Three well-known examples that can be cited are chaperones, albumins and cholic salts that allow the handling of poorly water-soluble molecules in the aqueous environments of the cytosol, the blood stream and the gastrointestinal tract, respectively.

Similarly, pharmaceutical strategies are highly needed to cope with poorly water-soluble drugs that represent 90% of the new chemical entities and 75% of the compounds under development [1-3]. The solubility of drugs in water is paramount since only the solubilized drug molecules can permeate physiological barriers and interact with their target. Therefore, from the formulator's standpoint, water solubility is one of the most important parameters and can determine the chances of a compound of being successfully transformed into a final drug product. The importance of a drug's water-solubility is supported by the fact that the European and US Pharmacopeias classify drugs according to their solubility, as illustrated in Figure 1 [4, 5].

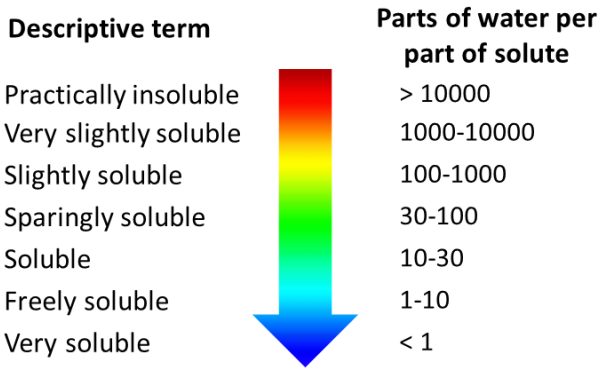


Figure 1: The European and US Pharmacopeias classification of drugs according to their water solubility.

Drug water-solubility is undoubtedly a key factor for the delivery of drugs to the eye. The eye is a unique organ with a unique functional role: vision. Of our five senses, vision is undoubtedly the most important and powerful. The eye has a complex structure, as illustrated in Figure 2A, allowing the light to travel successively through the cornea, the aqueous humor, the crystalline lens and vitreous humors to finally reach the cone and rod receptors of the retina. The visual signal is then transmitted through the optic nerve to the visual cortex. The eye needs to be in direct contact with the external environment while being protected from it. As a consequence, the eye has very efficient physiological barriers (such as the corneal and conjunctival epithelia and the ocular-blood barrier) and a number of effective protective mechanisms: eyelid movement, enzymatic activity in the tear fluid, lachrymation and naso-lachrymal drainage. Further protection of the eye relies on the extreme sensitivity of the cornea, concentrating approximately 7000 nociceptors per mm^2 ensuring immediate signaling of any potential hazard [6].

There are four main administration routes for ocular therapeutics: topical, periocular, intraocular and systemic; each involving different biological barriers, as illustrated in Figure 2B. There is a large variety of ophthalmic dosage forms (solutions, suspensions, ointments, gels, emulsions, strips, injections, inserts, lens, implants, etc.) all requiring finely tuned pH, osmolarity and sterility [4].

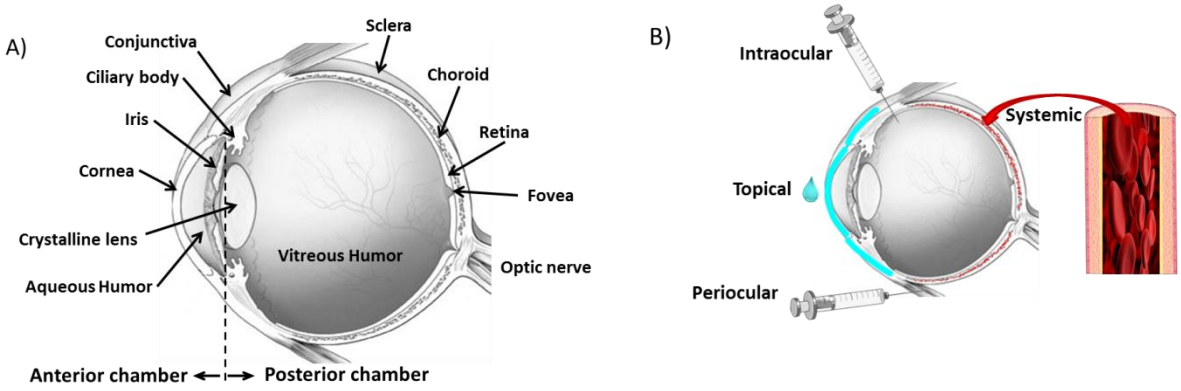


Figure 2: General characteristics of the eye with A) its structure and B) main routes for drug administration.

Topical drug delivery is the preferred route for local ocular treatment due to its non-invasiveness. However, the ocular topical route presents various challenges linked to three main factors: the rapid precorneal loss, the limited retention time and the efficient barrier function of the eye. The first factor, the rapid precorneal loss, is related to; i) lachrymation, ii) drainage, iii) tear turnover, iv) drug-protein interaction and v) drug metabolism. The second factor, the limited retention time, is linked to the fact that a large fraction of the drug is cleared by the natural defense mechanisms in the first minutes after eyedrop administration [7, 8]. The third factor, the efficient barrier function of the eye, is related to the corneal and conjunctival epithelia, which are the superficial tissues of the eye [7, 9-14]. The cornea is an avascular highly innervated stratified squamous epithelium; its superficial layer is highly lipophilic and presents an elevated number of tight junctions limiting the penetration of foreign substances. As a consequence, the corneal route is preferentially taken by small lipophilic molecules. It gives access to the anterior segment of the eye through the aqueous humor. The conjunctiva is a stratified columnar epithelium with a lower number of tight junctions than the cornea. It covers 97% of the ocular surface and presents a higher permeability compared to the cornea, allowing the penetration of large hydrophilic molecules (up to 40 kDa). The conjunctival route gives direct access to the posterior segment of the eye via the uveal tract. Drug formulation is therefore key in determining drug ocular availability and distribution as well as its ocular tolerance [15, 16].

The intraocular and periocular routes are not the preferred choice for ocular treatment since they are invasive and require an intervention that can lead to complications such as infections, inflammation and pain. Nevertheless, they bypass superficial ocular barriers and ensure patient compliance. The drug delivery profile after intraocular or periocular administration can be modulated to meet clinical requirements by using different formulation strategies.

When the use of a local ocular administration route is not possible, a systemic approach can be considered. After systemic or oral administration, high concentrations of the drug circulate in the blood leading to the desired therapeutic effect on the eye, but also causing adverse effects in the rest of the organism.

The present thesis explores three different strategies to deliver poorly water-soluble drugs to the eye based on a chemical modification, a physical modification and a changing of the route of administration. The immunosuppressant cyclosporine A (CsA) and the prostaglandin analog latanoprost were selected as model drugs since they both belong to the group of "practically insoluble" drugs (presenting water-solubilities of 0.008 mg/mL for CsA and 0.050 mg/mL for latanoprost) and their formulations present stability, safety and availability limitations. In addition, these drugs are used in the clinic to treat highly prevalent conditions such as the dry eye syndrome, uveitis, conjunctivitis, postoperative ocular inflammation or glaucoma.

The **Introduction** describes the problem of the delivery of poorly water-soluble drugs to the eye from two perspectives: the pharmaceutical technology aspect and the analytical chemistry aspect, which will be closely linked throughout this thesis. **Chapter I** reviews the pharmaceutical strategies designed to overcome poor water-solubility of drugs. **Chapter II** presents the analytical challenges related to investigating ocular formulations and describes ultra-high performance liquid chromatography coupled with a mass spectrometric detection as a powerful tool.

Part A describes the chemical modification approach based on a water soluble CsA prodrug. In **Chapter III**, the ocular tolerance, precorneal elimination and ocular distribution of a commercial 0.05% CsA formulation and its equivalent prodrug version are compared. **Chapter IV** presents the preformulation study performed for the development of an appropriate CsA prodrug formulation for preclinical investigations. Finally, the permeation and biodistribution profiles of 0.05%, 0.50% and 2.00% of conventional CsA and prodrug formulations were studied and compared in **Chapter V** to further understand the mechanisms behind their different behaviors.

Part B presents a physical modification approach based on the complexation of latanoprost with cyclodextrins (CDs) for topical ocular delivery. **Chapter VI** presents the screening of a panel of CDs towards the selection of the best preforming latanoprost-CD formulation in regards to latanoprost stability and availability. The selected formulation was further characterized regarding latanoprost solubilization, complex formation and structure as well as ocular tolerance. A commercial latanoprost product was used as a reference throughout these investigations.

Part C presents a modification of the administration strategy based on the intraocular implantation of a CsA loaded drug delivery system. **Chapter VII** presents the use of CsA implants for the management of uveitis and postoperative inflammation related to cataract surgery.

Conclusions and perspectives, followed by a **Summary**, a **Summary in French** (Résumé) and a list of **Abbreviations** close this manuscript.

REFERENCES

[1] L. Di, E.H. Kerns, G.T. Carter, Drug-like property concepts in pharmaceutical design, Current pharmaceutical design, 15 (2009) 2184-2194.

[2] L. Di, P.V. Fish, T. Mano, Bridging solubility between drug discovery and development, Drug discovery today, 17 (2012) 486-495.

[3] T. Loftsson, M.E. Brewster, Pharmaceutical applications of cyclodextrins: effects on drug permeation through biological membranes, J Pharm Pharmacol, 63 (2011) 1119-1135.

[4] European Pharmacopeia, 8th edition ed., Strasbourg, 2014.

[5] United States Pharmacopeia and National Formulary, Rockville,, 2014.

[6] L.J. Müller, C.F. Marfurt, F. Kruse, T.M.T. Tervo, Corneal nerves: structure, contents and function, Experimental eye research, 76 (2003) 521-542.

[7] K. Jarvinen, T. Jarvinen, A. Urtti, Ocular Absorption Following Topical Delivery, Advanced drug delivery reviews, 16 (1995) 3-19.

[8] A.L. Weiner, B.C. Gilger, Advancements in ocular drug delivery, Veterinary ophthalmology, 13 (2010) 395-406.

[9] B. Nichols, C.R. Dawson, B. Togni, Surface features of the conjunctiva and cornea, Investigative ophthalmology & visual science, 24 (1983) 570-576.

[10] M.A. Watsky, M.M. Jablonski, H.F. Edelhauser, Comparison of conjunctival and corneal surface areas in rabbit and human, Current eye research, 7 (1988) 483-486.

[11] D.H. Geroski, H.F. Edelhauser, Transscleral drug delivery for posterior segment disease, Advanced drug delivery reviews, 52 (2001) 37-48.

[12] A. Urtti, Challenges and obstacles of ocular pharmacokinetics and drug delivery, Advanced drug delivery reviews, 58 (2006) 1131-1135.

[13] K.M. Hamalainen, K. Kananen, S. Auriola, K. Kontturi, A. Urtti, Characterization of paracellular and aqueous penetration routes in cornea, conjunctiva, and sclera, Investigative ophthalmology & visual science, 38 (1997) 627-634.

[14] M.R. Prausnitz, J.S. Noonan, Permeability of cornea, sclera, and conjunctiva: a literature analysis for drug delivery to the eye, Journal of pharmaceutical sciences, 87 (1998) 1479-1488.

[15] M. Kuwano, H. Ibuki, N. Morikawa, A. Ota, Y. Kawashima, Cyclosporine A formulation affects its ocular distribution in rabbits, Pharmaceutical research, 19 (2002) 108-111.

[16] L. Cheeks, R.L. Kaswan, K. Green, Influence of vehicle and anterior chamber protein concentration on cyclosporine penetration through the isolated rabbit cornea, Current eye research, 11 (1992) 641-649.

INTRODUCTION

Chapter I

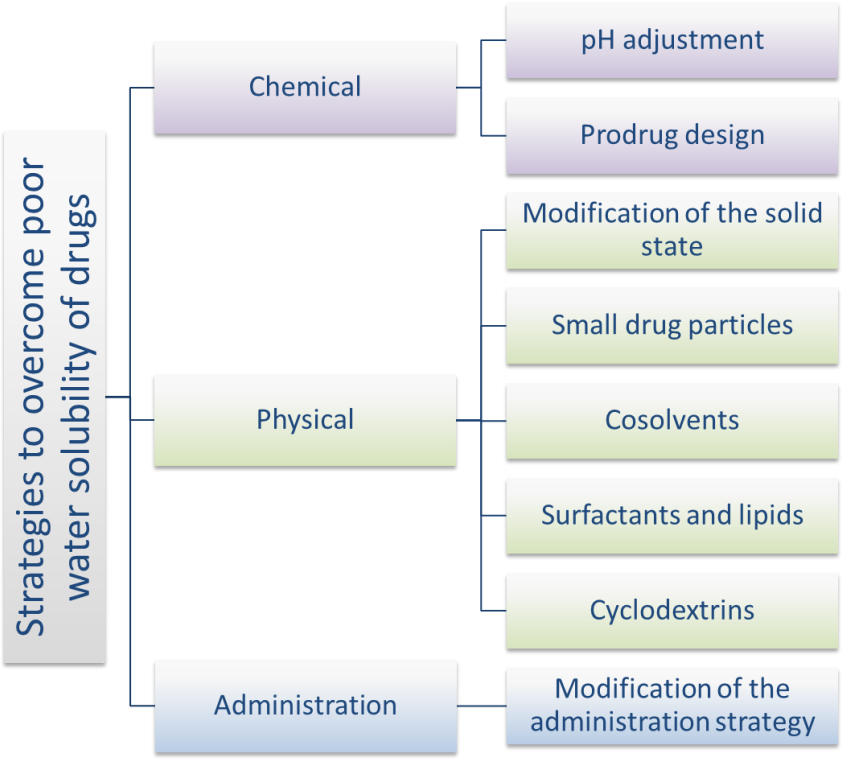
Strategies to address the poor water-solubility of drugs

Strategies to address the poor water-solubility of drugs

Marta RODRIGUEZ-ALLER, Davy GUILLARME, Jean-Luc VEUTHEY, Robert GURNY*

School of Pharmaceutical Sciences, University of Geneva, University of Lausanne,
30, Quai Ernest Ansermet, 1211 Geneva 4, Switzerland
*Correspondence: Phone: +41223793816, Fax: +41223796146, E-mail: robert.gurny@unige.ch

To be submitted.



ABSTRACT

Water solubility is a key parameter in drug formulation since it highly influences drug pharmacokinetics and pharmacodynamics. In the past decades, the challenge with poorly water soluble drugs has been growing continuously. As a matter of fact, poorly soluble compounds now represent 40% of the top 200 oral drugs marketed in the US, 33% of drugs listed in the US Pharmacopeia, 75% of compounds under development and 90% of new chemical entities. The present article presents and discusses the pharmaceutical strategies available to overcome poor water solubility in light of final drug product examples. First, chemical modifications based on the adjustment of the pH and the design of prodrugs are presented and discussed. Physical modifications based on modified solid states of the drug, small drug particles, cosolvents, surfactants, lipids and cyclodextrins are discussed in a second part. Finally, the option of modifying the route of administration is briefly presented.

Keywords: poor water solubility, pH, prodrug, emulsion, microemulsion, micelle, liposome, cosolvent, cyclodextrins, administration route.

1. INTRODUCTION

The water solubility of drugs strongly influences their pharmacokinetics and pharmacodynamics and is a key parameter for formulators. Drug solubilization is based on the breaking of some drug-drug and water-water interactions for the creation of new drug-water interactions. The strength of such interactions determines the solubility of a drug in water. Water solubility is one of the main parameters of the biopharmaceutical classification system (BCS) of drugs, as illustrated in Figure 1A [1]. Moreover, "Lipinski's rule of 5" considers the solubility of drug candidates in view of the rejection of inappropriate candidates at early stages of the drug discovery process [2].

In the past decades, the challenges linked to poor water solubility have been continuously growing. The surge of combinatorial chemistry and high throughput miniaturized screening methods for drug discovery have resulted in an increase in molecular weight and lipophilicity of drug candidates [3-5]. In addition, the push towards increasing the potency of drugs often resulted in an increase in their lipophilicity (leading to stronger interactions with their receptors). Currently, poorly soluble compounds represent approximately 40% of the top 200 oral drugs marketed in the US and other European countries (UK and Spain), as shown in Figure 1B [6]. In addition, they represent 90% of new chemical entities, 75% of compounds under development and 33% of drugs listed in the US Pharmacopeia [2, 3, 6-11].

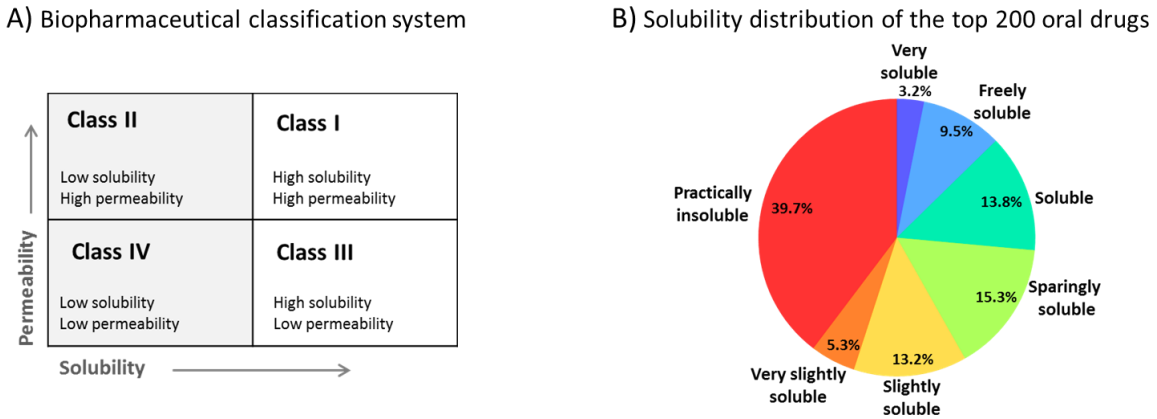


Figure 1: A) The biopharmaceutical classification system (BCS) and B) the solubility distribution of the top 200 marketed oral drugs in the US and Europe (adapted from [6]).

Interestingly, a variety of pharmaceutical strategies have been designed to address the formulation and delivery challenges presented by poorly soluble drugs, these are reviewed and discussed in the present article.

2. STRATEGIES TO ADRESS THE POOR WATER SOLUBILITY OF DRUGS

The pharmaceutical strategies to address the poor water solubility of a drug can be organized into three categories according to the nature of the modification involved: the chemical, physical and administration strategies, as illustrated in Figure 2. These approaches can of course be used separately or combined.

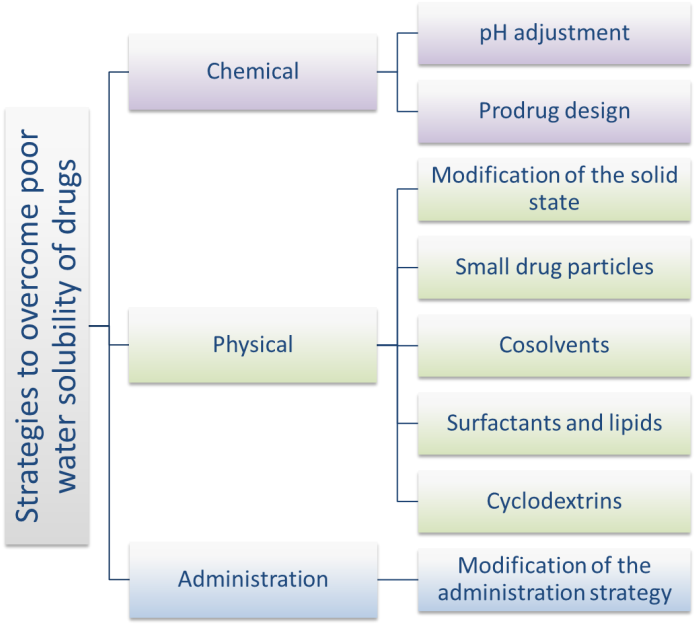


Figure 2: A schematic representation of the different strategies to overcome the poor water solubility of drugs.

Over the past decades, many efforts have been made to improve the formulation and delivery of poorly water-soluble immunosuppressants, prostaglandins and antineoplastic agents, which will often be used as examples in the following sections.

It is worth mentioning that colloidal systems represent a more recent option for the formulation of poorly water soluble drugs that can involve chemical or physical modifications [12]. Thus, colloidal systems can be found in the sections describing prodrug design, small drug particles and surfactant-lipid-based formulations. The prodrug design can include drug-polymer nanoparticles and drug covalent link to inorganic nanoparticles. The use of small drug particles can involve nanocrystals and the use of nanoparticles for drug loading or adsorption. Finally, the surfactant and lipid formulations could include nanoemulsions, micelles, liposomes or solid lipid nanoparticles.

2.1. Chemical modifications

2.1.1. pH adjustment

The pH influences the solubility of a drug by affecting its degree of ionization as a function of its pKa. In its ionized form, a drug has a higher solubility than at its neutral form. However, drugs are generally neutral at physiological pH. Thus, the pH of the formulation can be adjusted with buffering excipients to ensure the presence of the most soluble form of the poorly water-soluble drug.

For solid dosage forms, the buffering excipients control the pH of the microenvironment surrounding drug particles during *in vivo* dissolution [13]. Kranz and coworkers achieved a constant pH-independent release of the immunosuppressant, ZK811752, by adding organic acids to the final composition of the tablets [14].

The pH adjustment is a simple approach and represents a first-line strategy for the formulation of insoluble drugs. It is frequently combined with other solubilizing approaches such as surfactants, cyclodextrins or cosolvents. The pH of the final formulation is selected not only according to drug solubility, but also considering its tolerance, bioavailability, efficacy and stability, which strongly depend on the pH. In addition, the potential risk of drug precipitation after administration needs to be considered.

2.1.2. Prodrug design

A prodrug can be defined as an inactive, chemically modified version of a parent drug displaying improved physico-chemical properties and being able to generate the active parent drug through a rapid biotransformation. Two main prodrug design categories can be identified: i) carrier-linked prodrugs where the parent drug backbone is covalently linked to a prodrug moiety and ii) bioprecursor prodrugs which are modified parent drugs with functional groups requiring hydration or redox reactions, as illustrated in Figure 3A. In addition, pre-prodrugs or double prodrugs combine two prodrug design approaches in their design (carrier-linked and/or bioprecursor), one example being illustrated in Figure 3B.

The prodrug strategy has been gaining interest in the past years and today its usefulness in drug formulation is unquestionable. Prodrugs represent 10% of worldwide marketed drugs and were 33% of the small active molecules approved in 2008 [15, 16]. Prodrug design represents a versatile and powerful approach that can solve a large variety of issues related to drug solubility, absorption, distribution, metabolism, toxicity or stability, among others [17, 18].

The prodrug bioconversion is of major importance and needs to be carefully evaluated and optimized. Ideally, the prodrug should have an *in vitro* half-life one million times higher than its *in vivo* half-life. Such a difference is only possible with enzyme-based biotransformations [19].

For Anderson and Conradi, the prodrug of a poorly water soluble drug should not be limited to the covalent link of a promoiety to the parent drug, but should represent a new and optimized drug delivery system of its own [19]. In this sense, the use of prodrugs to address the challenges with poor water solubility will be discussed through a number of examples, covering both the carrier and bioprecursor approaches.

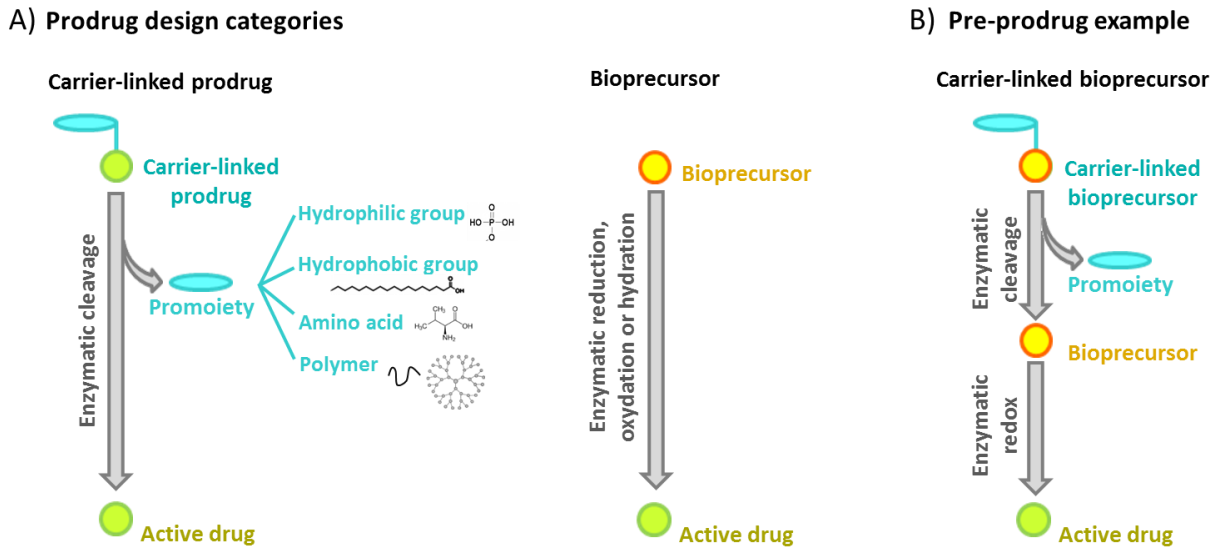


Figure 3: A) Prodrug design categories based on carrier-linked prodrugs and bioprecursors. B) Illustration of a pre-prodrug example combining the carrier-linked and bioprecursor designs.

For **carrier-linked prodrugs**, the first type of prodrug design, four main carrier moieties can be used: i) hydrophilic groups, ii) hydrophobic groups, iii) amino acids and iv) macromolecules, as illustrated in Figure 3A. Carrier-linked prodrugs are frequently used to simultaneously address the question of poor water solubility of a drug and achieve its targeted delivery.

The covalent linking of ***hydrophilic structures*** often confers a higher solubility to the parent drug. Phosphate ester prodrugs are one of the most common examples for this group. Telzir® and Lexiva® (GlaxoSmithKline, Brentford, UK) contain fosamprenavir, the phosphate ester prodrug of the HIV protease inhibitor amprenavir. Fosamprenavir displays a water solubility 10 times higher than amprenavir as well as an increased bioavailability, allowing a simplification of the dosage regimen. With this prodrug formulation, the treatment went from 8 capsules twice a day to 4 tablets once a day, which has a direct impact on patient quality of life and compliance.

Hydrophobic structures can also be used to improve the aqueous solubility of drugs. Their action is based on the disruption of some drug-drug interactions (e.g. H-bounds) resulting in a higher dissolution rate. The levodopa ethyl ester prodrug displayed a higher solubility and absorption than the parent levodopa, allowing its administration to Parkinson's disease patients as an oral solution instead of the conventional tablets with known absorption

issues [20]. Interestingly, this levodopa ethyl ester is a double prodrug, levodopa being itself a prodrug of dopamine that targets the central nervous system. The development of novel formulations for the anticancer drug 5-fluorouracil (5-FU) based on the prodrug approach has been intensively investigated to increase its solubility, plasma half-life and selectivity. Xeloda® (Hoffmann-LaRoche, Basel, Switzerland) contains capecitabine, which is a double prodrug of 5-FU displaying an oral bioavailability close to 100% thanks to its high solubility, high absorption and low affinity for the intestine thymidine phosphatases [21]. Interestingly, for capecitabine design hydrophobic hydrocarbon chains and amides were covalently linked to the doxifluridine backbone, which is already a pre-prodrug of 5-FU. After its oral absorption, capecitabine is biotransformed by carboxylesterases, deaminases and tumor-specific thymidine phosphorylases, releasing the cytotoxic 5-FU specifically in the tumor. Capecitabine combines the advantages of an enhanced oral availability with a tumor-specific activity [22-25].

The modifications with ***amino acids*** can simultaneously achieve two goals: increased water solubility and transporter-mediated absorption (using amino acid transporters). The diversity in physical properties of amino acids confers a high versatility to the approach. An interesting example is valacyclovir, the L-valyl ester prodrug of acyclovir marketed as Valtex® (GlaxoSmithKline, Brentford, UK). The bioavailability of valacyclovir is two times higher than acyclovir due to its higher solubility and active transport via amino acid receptors [26]. After intracellular absorption, valacyclovir is hydrolyzed, generating acyclovir, which requires activation by viral thymidine kinase and cellular kinases to finally inhibit herpes virus DNA polymerase. Valacyclovir can therefore also be considered a pre-prodrug.

Finally, insoluble drugs can be combined with ***macromolecules***. Drug-macromolecule conjugates can: i) assist drug solubilization, ii) decrease drug toxicity, iii) prevent drug degradation and iv) achieve drug targeting [27, 28]. Hyaluronic acid, polyethylene glycol (PEG), hydroxypropylmetacrylamide (HPMA) and polyamidoamines or nitrodiol dendrimers are used in macromolecule prodrug designs. HPMA is a versatile tool for the formulation of poorly water-soluble drugs, such as anticancer agents (e.g. daunorubicine or wortmannin), and is especially well suited for being combined with drug targeting strategies [29-32]. Various HPMA conjugates (with doxorubicin, paclitaxel, camptothecin or palatinate) have gone through clinical trials [33]. In contrast to linear polymers, dendrimers have branched structures that allow the linkage of various drug molecules. They represent an interesting

approach for the formulation and delivery of insoluble drugs. De Groot and coworkers presented nitrodiol-based dendrimers for the targeted release of the poorly water-soluble drug paclitaxel based on a single tumor specific activation that triggers a cascade of reactions towards its release [34]. The triggering reaction can be designed to occur exclusively in the target tissue, allowing site-specific drug release.

Regarding **bioprecursor** prodrugs, the second type of prodrug design, an example is the Clinoril® (Merck, New Jersey, US) oral tablets, which contain sulindac, a non-steroidal anti-inflammatory drug [35, 36]. Sulindac displays a 100-fold increased solubility and improved oral absorption compared to its parent drug [37, 38]. The reduction of its sulphoxide group is necessary to generate the active sulphide form.

The prodrug approach is therefore a powerful and versatile strategy to not only address issues with poor water solubility, but also to develop a myriad of strategies for efficient site-specific drug delivery. Nevertheless, the stability of prodrug formulations can be a hurdle since prodrugs require a high reactivity for a quick biotransformation, but also need an excellent stability for a long product shelf-life.

2.2. Physical modifications

2.2.1. Modified solid state

Modifying the solid state of a drug influences the strength of drug-drug interactions, determining its solubility and dissolution rate. In general, a higher structural disorder in the solid leads to lower drug-drug interactions and a higher solubility.

When using modified solid states of a drug, formulators need to find a tradeoff between: i) the potential increase in drug solubility, dissolution and/or availability and ii) potential stability issues. It is very important to ensure that the selected drug state is not altered during development, manufacturing and storage to guarantee that patients receive the appropriate form of the drug. Special attention needs to be paid to metastable polymorphs, salts, cocrystals and amorphous forms that tend to recrystallize into their most stable form. In addition, since the hydrated forms of a drug present a lower stability and solubility than its pure crystal, the risk of the drug form to be transformed into a hydrate needs to be evaluated. The different modified solid forms illustrated in Figure 4 can be characterized as amorphous or crystalline (pure drug crystals, polymorphs, hydrates, salts and cocrystals) according to their disordered or ordered structures, respectively.

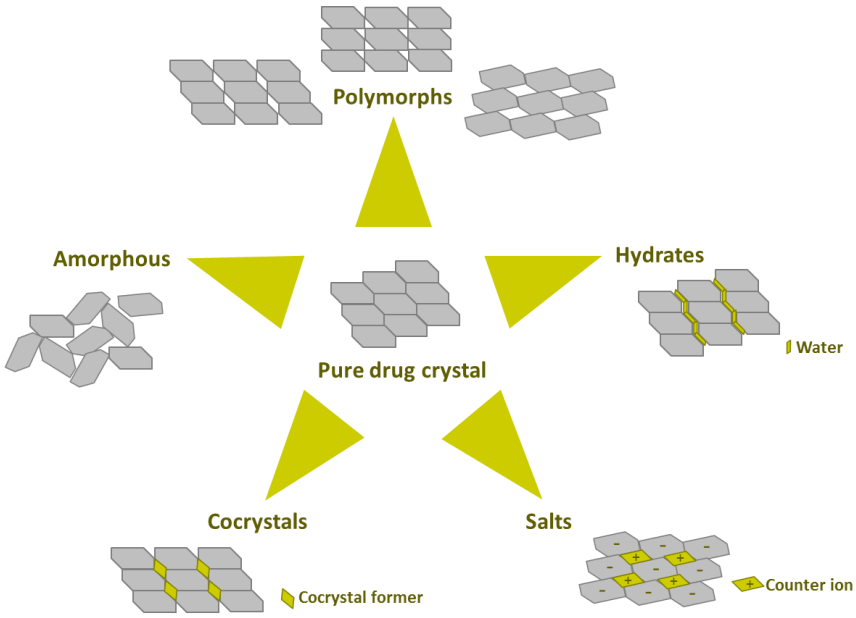


Figure 4: Different drug solid forms that can be used in formulations, including the most stable pure drug crystal, as well as polymorphs, hydrates, salts, cocrystals and amorphous forms.

Crystalline modified drug forms include polymorphs, hydrates, salts and cocrystals.

Drug crystal ***polymorphs*** are composed solely of the pure drug, but different structures can lead to different drug-drug interactions and physico-chemical properties (e.g. density, melting point, conductivity, solubility or stability). Metastable polymorphs displayed a higher dissolution rate and solubility than the most stable polymorphs having a positive impact on therapeutic efficacy, as reported for cimetidine [39, 40]. Besides, different polymorphs were demonstrated to have different degradation and thermodynamic profiles, some of which resulting in drug instability [41, 42]. A comprehensive screening and monitoring of the potential polymorphic transformations during the drug life-cycle is mandatory to ensure drug quality, safety and efficacy [43].

Hydrates are crystalline drug structures that entrap water molecules, facilitating close packaging and drug-drug interactions. Generally, hydrates present limited pharmaceutical interest due to their lower solubility, bioavailability and stability compared to the anhydrous forms [41, 44-47]. However, if a drug present a high tendency to form hydrates, the study and characterization of the hydrated forms of the drug help to determine appropriate manufacturing, packaging and storage conditions (particularly important for hygroscopic drugs).

Crystalline ***salts*** are based on a proton transfer between an ionizable group of the drug and a counter ion species. The resulting salt modifies the pH in the thin diffusion layer surrounding the drug, resulting in an increased solubility compared to the corresponding free form [48-50]. The counter ion species highly influences the solubility and must be carefully chosen [51]. Guzman and coworkers demonstrated the higher solubility and availability of the celecoxib sodium salt compared to its free form [52].

Cocrystals contain a drug and a cocrystal former, linked by H-bonds (different from salts in which a proton transfer takes place). These H-bonds decrease the strength of the drug-drug interactions compared to the pure drug crystal structure. The cocrystal approach has been successfully used for drug dissolution and bioavailability enhancement of poorly water soluble drugs [53-55]. This approach does not require the presence of ionisable groups on the drug.

Amorphous solids are partially disordered solids where the drug-drug interactions are weaker than in the crystals. They are obtained either by preventing the formation of a crystalline structure or by disrupting an already existing crystal. Interestingly, amorphous forms can theoretically present solubilities up to 1600 times higher than crystalline forms [56]. The amorphization is usually combined with other small drug particle strategies such as solid dispersions (explained in the following "small drug particle" section). The solid dispersion of amorphous CsA particles in polymeric matrices was demonstrated to lead to a higher dissolution rate and an improved bioavailability compared to crystalline CsA [57, 58]. In general, the solubility enhancement obtained with amorphization is higher than that achieved with metastable polymorphs [11]. However, amorphous forms can have a higher hygroscopicity, reactivity and instability.

2.2.2. Small drug particles

A decrease in particle size of poorly water soluble drugs allows i) the increase of the drug surface area and its dissolution rate, ii) an improved bioavailability and iii) a reduced toxicity [58-69]. This approach can be combined with any of the modified solid states detailed above, cumulating the advantages of both strategies.

Fine and ultrafine drug particles can be obtained using two types of strategies: reducing the size of preexisting drug particles or inducing drug solidification into small particles. The methods to reduce the size of preexisting drug particles are based on cuts, compression, impact, or attrition, or both impact and attrition [70]. The methods to form small drug particles are: hot-melt extrusion, hot-melt encapsulation, spray drying and supercritical fluid methods. It is also worth mentioning another approach consisting of the loading or superficial adsorption of poorly water-soluble drugs into nanoparticles [12, 71].

Small drug particles form a metastable system that needs to be further stabilized to avoid agglomeration and crystalline growth. A large number of excipients can be used as stabilizers acting by electrostatic repulsion or steric stabilization (e.g. surfactants or polymers) [11, 72, 73]. Different systems can be formed depending on the environment surrounding the small particles: suspensions and nanosuspensions are obtained when drug particles are in a liquid environment, while solid dispersions are formed when the drug particles are embedded in a solid matrix.

The advantages of using small drug particles for the formulation of poorly water-soluble actives are illustrated by the number of marketed products that employ this strategy. Three marketed formulations of the immunosuppressants sirolimus and everolimus used for the prevention of organ graft rejection can be cited as examples. Rapamune® (Pfizer, New York, US) is an oral tablet containing sirolimus nanocrystals (NanoCrystal® technology, Elan Drug Technologies, Dublin, Ireland). Solid dispersion strategies have been successfully used in the development of the everolimus formulations Certican® and Zortress® (Novartis, Basel, Switzerland).

2.2.3. Cosolvents

A cosolvent is a water-miscible organic solvent used to increase the solubility of a drug in water. This approach is based on the theory that the dissolution is enhanced when the solute and solvent have similar physicochemical characteristics. The most important factor to be considered is the polarity of the mixture (i.e. its dielectric constant). A large variety of cosolvents such as ethanol, polyethylene glycol (PEG), propylene glycol (PG), glycerin, dimethylacetamide (DMA), N-methyl-2-pyrrolidone (NMP), dimethylsolfoxide (DMSO), as well as a number of oils (e.g. peanut, corn, sesame, olive or peppermint) can be used [73, 74]. The cosolvent approach has been used for VePesid® (Bristol-Myers Squibb, New York, US) where the solubilization of the anticancer agent etoposide was achieved with a mixture of PEG 400, citric acid, glycerin and water.

This cosolvent strategy presents some limitations linked to i) cosolvent taste and stability, ii) adverse physiological effects, iii) potential modification of the pharmacokinetic profile of the drug and iv) potential drug precipitation after administration. This strategy remains a simple option frequently used in combination with other solubilizing strategies for the formulation of poorly water soluble drugs. Nevertheless, the risks of drug instability, drug precipitation and modification of the pharmacokinetic profile need to be considered.

2.2.4. Surfactants and lipids

A surfactant is a surface-active agent that can stabilize interfaces. A large variety of surfactants can be listed, from nonionics (e.g. polyoxyethylene sorbitan fatty acid esters) to amphoteric (e.g. lecithins) and anionics (e.g. soaps, phospholipids). Cationic surfactants (e.g. quaternary ammonium) are less common due to toxicity issues and incompatibilities. Generally, a surfactant presents a polar "head" and an apolar "tail". As illustrated in Figure 5, a fraction of the surfactant adsorbs to the interfaces present in the system (liquid-air or liquid-liquid interfaces), decreasing the interfacial tension and stabilizing the system. This property is used for emulsion stabilization. When the surfactant is added at a concentration above its critical micellar concentration (CMC), surfactant molecules self-assembly into micelles, liposomes or other structures [75].

Micelles present a hydrophilic spherical shell composed of polar heads and a hydrophobic core of apolar tails, creating an appropriate environment to solubilize poorly water-soluble drugs, as illustrated in Figure 5A. Polymeric micelles deserve a special mention since they display interesting properties such as: i) low CMC, ii) slow dissociation, iii) longer drug retention or potential increase in drug half-life [76-82].

Liposomes are globular bilayer formations allowing the solubilization of poorly water-soluble drug inside the bilayer, as illustrated in Figure 5B.

Emulsions are dispersions of two immiscible phases (typically an oily phase and an aqueous phase) stabilized by a surfactant, as illustrated in Figure 5C, and are appropriate for the formulation of lipophilic drugs [70]. The drug is solubilized in the oily phase, which could be the dispersed or dispersant phase, leading to oil-in-water (o/w) or water-in-oil (w/o) emulsions. Two types of emulsions can be identified: i) conventional emulsions and ii) microemulsions. A conventional emulsion is thermodynamically unstable needing an input of energy for its formation. Emulsion droplets have a diameter higher than 100 µm, conferring a milky aspect to the preparation. On the contrary, a microemulsion is thermodynamically stable and the droplets have a diameter between 6 and 80 nm, which does not affect optical transparency. The excipients and percentage of each phase play major roles in emulsion formation and stabilization. Some commonly used oily phases include vegetable oils, glycerols, fatty acids and their derivatives or glycerides. Microemulsions requiring further

stabilization include a cosurfactant or a mixture of hydrophilic and lipophilic surfactants in specific proportions.

Solid-lipid nanoparticles are another type of formulation based on surfactants and lipids, which combine the advantages of liposomes, emulsions and nanoparticles [12, 83].

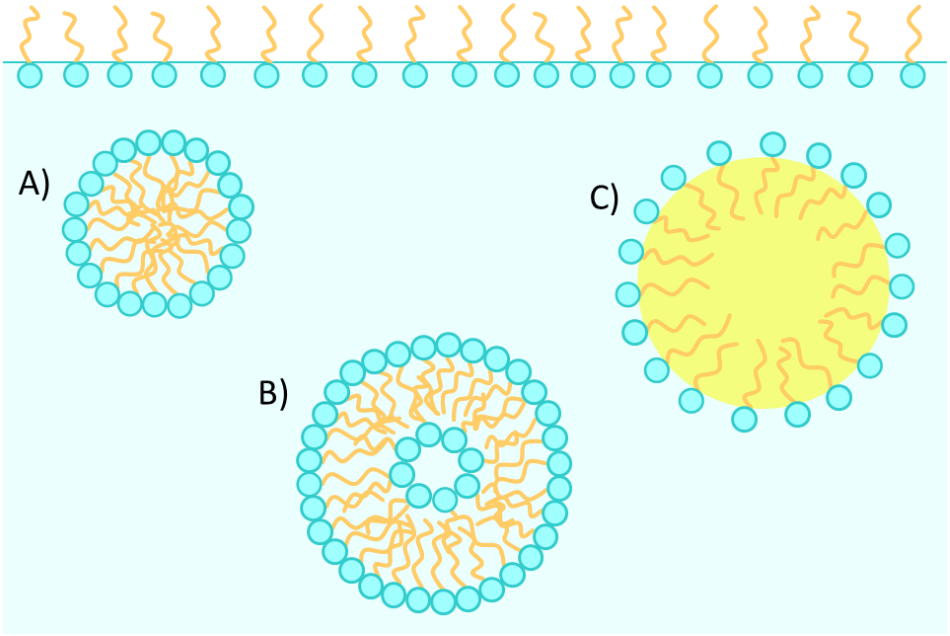


Figure 5: Surfactant distribution and self-assembly structures in aqueous environment for the formation of A) a micelle and B) a liposome and C) an emulsion droplet.

Surfactant-related formulations can be divided into three groups: i) a first group with ready-to-use formulations, ii) a second group with formulations requiring dilution in an aqueous vehicle prior to administration and iii) a third group involving "pro-formulations" that readily form the final emulsion or micelle system in contact with the biological medium. The presented groups are further discussed in light of examples of marketed products.

Ready-to-use formulations include emulsions or micelle formulations intended to be administered as such. This is the case for Restasis® (Allergan, California, US) developed for the topical treatment of dry eye syndrome.

The formulations requiring a dilution in water prior to administration lead to the formation of: i) micelles, as in the case of Sandimmune® (Novartis, Basel, Switzerland) for the iv administration of CsA micelles, ii) liposomes, as for Visudyne® (Novartis, Basel, Switzerland) leading to verteporfin liposomes or iii) emulsions, as for Rapamune® (Pfizer, New York, US) leading to a CsA o/w emulsion for oral administration.

"Pro-formulations" form their final appropriate system inside the biological medium. Micelles loaded with CsA are formed inside the gastrointestinal tract after the administration of Gengraf® (Abbott, Chicago, US). Emulsions can be obtained with self-emulsifying drug delivery systems (SEDDS), such as Neoral® and Sandimmune® (Novartis, Basel, Switzerland) leading to different CsA emulsions inside the gastrointestinal tract. Interestingly, Neoral was demonstrated to lead to a microemulsion with smaller droplet than Sandimmune after self-emulsification, causing an increase in bioavailability of 239% and an increased plasma peak concentration (C_{max}) and area under the curve of the plasma concentration-time curve (AUC) when compared to Sandimmune [84-87].
The mechanisms behind self-emulsification have been identified as follows: i) diffusion and stranding, ii) osmotic pressure and iii) changes in the characteristics of the surrounding medium (e.g. pH or ionic strength) [88-92].

These surfactant-related formulations require extensive characterizations (e.g. solubility of the drug, size of the micelle, liposome or droplet, viscosity, osmolarity or stability) and can be related to the following risks: i) toxicity of the excipients (which could represent a high percentage of the final composition), ii) drug precipitation and iii) modification of the pharmacokinetic profile and biodistribution.

2.2.5. Complexation with cyclodextrins

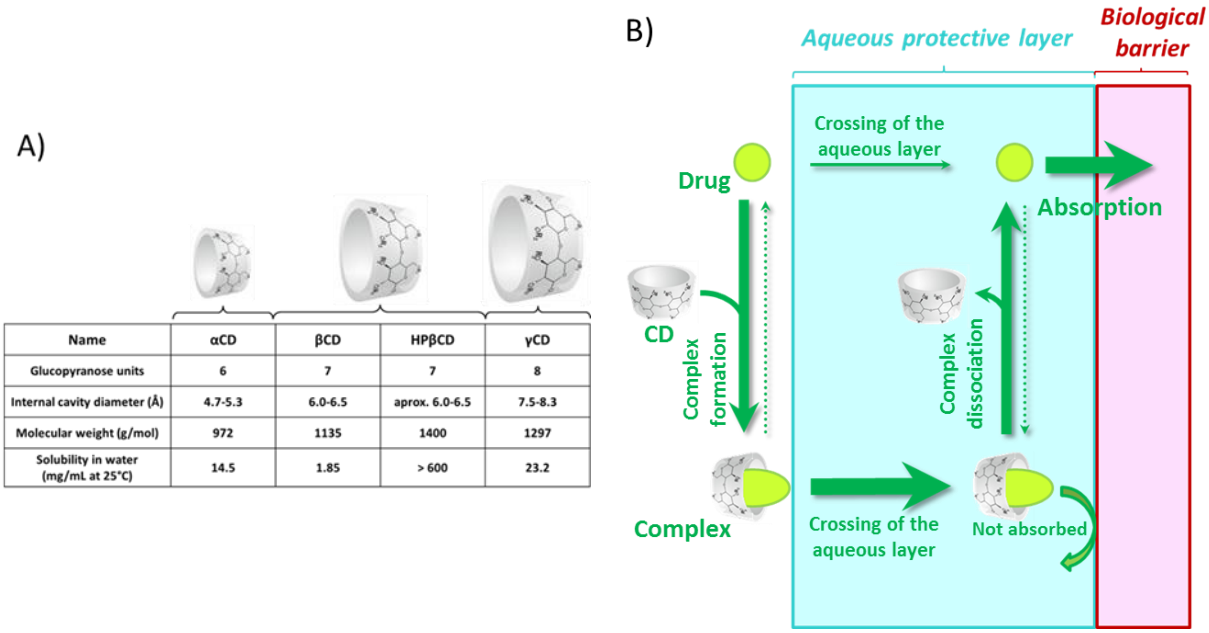
Cyclodextrins (CDs) are a family of macromolecular cyclic oligosaccharides presenting a hydrophilic surface and a hydrophobic cavity that can be classified into α, β or γ CDs according to their number of saccharide monomers. In addition to the "native non-substituted CDs", a large variety of CD derivatives are also currently available. Figure 6A illustrates the structure and characteristics of the four CDs included in the US Pharmacopeia [93]. CDs are known to act as drug solubilizers by forming dynamic inclusion complexes with poorly water-soluble drugs [94-100]. Despite the fact that the drug-CD complexation involves non-covalent interactions, it modifies the physico-chemical properties of both the CD and the drug. Complex formation and dissociation involve different mechanisms. Complex formation is linked to i) hydrophobic interactions, ii) release of the high-energy water molecules inside the CD and iii) "dissolution" of the drug inside the CD cavity. While complex dissociation is based on the continuous dilutions and displacements that take place *in vivo*, leading to an efficient release of the drug from its complex, as shown in former investigations [95].

Besides an enhanced solubility, drug-CD complexes can lead to an improved drug availability by acting as drug carriers, and to an improved drug stability by preventing drug degradation [95, 99, 101-105]. The carrier-like behavior of CDs is illustrated in Figure 6B. In addition, CDs are considered as non-toxic [103, 104, 106].
CD-based formulations require a comprehensive characterization regarding complex formation, stability and structure (e.g. complex stoichiometry, stability constants).

The complexation approach was explored for the formulation of cyclosporine A with αCD resulting in a higher penetration, higher therapeutic effect and lower toxicity than the equivalent oily formulation [107-109]. However, to date, there is no commercial formulation containing cyclosporine A and CDs. In contrast, the combination of CDs and prostaglandins (PGs) can be found in a number of marketed formulations. For example, Caverjet Dual® (Pfizer, New York, US), Prostavasin® and Prostandin 500® (Ono, Osaka, Japan) contain PGE_1 and αCD for the systemic treatment of cardiovascular-related diseases or dysfunctions. Prostarmon E® (Ono, Osaka, Japan) contains PGE_2 and βCD for the induction of uterine contractions after its oral administration.

Complexation can also be combined with i) hydrophilic polymers, ii) drug salts, iii) solid dispersion strategies or iv) cosolvents [102, 110-115]. One example is Sporanox® (Janssen, New Jersey, US) which contains itraconazole and HPβCD together with propylene glycol as cosolvent for the systemic treatment of fungal infections.

The complexation strategy has been gaining interest in the past years, in conjunction with the surge of a variety of substituted CDs. This attractive and sophisticated technology brings new opportunities for the development and patent-protection of innovative formulations. Nevertheless, its cost is still high due to added regulatory hurdles and the elevated cost of materials.



Name	αCD	βCD	HPβCD	γCD
Glucopyranose units	6	7	7	8
Internal cavity diameter (Å)	4.7-5.3	6.0-6.5	aprox. 6.0-6.5	7.5-8.3
Molecular weight (g/mol)	972	1135	1400	1297
Solubility in water (mg/mL at 25°C)	14.5	1.85	> 600	23.2

Figure 6: A) Structure and characteristics of different cyclodextrins (CDs) included in the US Pharmacopeia such as native αCD, βCD and γCD as well as the substituted hydroxypropylβCD (HPβCD) [93]. B) Potential effect of the use of a CD for the formulation of a drug that has to reach a biological barrier protected by an aqueous layer (such as mucosa or tear fluid). The CD interacts with the drug by: i) forming a complex, ii) caring the drug across the aqueous layer and iii) allowing drug release close to the biological barrier for its subsequent absorption thanks to complex dissociation. When the drug is formulated alone (upper part of the scheme), the crossing of the aqueous layer is more difficult and potentially a lower amount of drug can reach the biological barrier for its absorption.

2.3. Administration modification

Another option to circumvent possible limitations of poorly water-soluble drug formulations is to modify the administration strategy. Although the oral route seems to be the golden standard for drug administration, it presents limitations linked to drug availability and potential systemic side effects. The use of an alternative administration route can be particularly interesting considering that previously mentioned physical or chemical modifications do not always allow an appropriate drug delivery.

On one hand, alternative administration routes can be used to achieve a systemic release of a drug. Testosterone is a steroid hormone practically insoluble in water that has a rapid hepatic clearance, therefore requiring prolonged drug delivery systems or frequent administration. Intramuscular depot formulations or subcutaneous implants require invasive techniques for their administration. The transdermal and buccal routes were explored as alternative noninvasive options for the delivery of this drug. The developed transdermal and buccal formulations were demonstrated to achieve similar circulating concentrations of testosterone as the systemic options, while improving patient comfort [116, 117].

On the other hand, local administration routes can be used for the treatment of local diseases. Indeed, local routes are the best option for the treatment of local diseases, allowing a high ratio of local to systemic drug concentrations which is translated into the achievement of the pharmacological effect with minimal systemic secondary effects. In this context, implants represent an attractive option for the local prolonged release of drugs; their main application fields are ophthalmology, cancer therapy and birth control [118]. Retisert® (pSivida Corporation, Watertown, US) is an intravitreal insert delivering active concentrations of flucinolone for up to three years allowing the treatment of chronic posterior uveitis [119]. Gliadel® (Arbor Pharmaceuticals, Atlanta, US) is an intracerebral implant containing carmustine placed on the brain tissue after surgical resection of the tumor for the prevention of glioblastoma recurrence [120]. Finally, Nuvaring® (Merck, New Jersey, US) is an intravaginal implant containing estrogens for contraceptive purposes which was demonstrated to result in a lower estrogen exposure than with the use of other oral or transdermal contraceptive options [121].

3. CONCLUSION

During the last decades, a number of pharmaceutical strategies have been developed for the formulation and delivery of poorly water-soluble drugs. In this article, eight of these strategies have been presented and critically discussed in light of examples of marketed products. The presented approaches included i) chemical modifications such as the adjustment of the pH and the design of prodrugs, ii) physical modifications such as the use of modified solid states of the drug, small drug particles, cosolvents, surfactants, lipids and cyclodextrins and iii) modifications of the administration strategy such as the use of alternative local administration approaches. These strategies can be used alone or in combination and offer a panel of options for formulators to address the challenges related to poorly water-soluble drugs.

Through this article it could be seen that the development of a generic approach to solve drug solubility issues is not possible for two main reasons. The first reason is because each drug presents a different set of specific challenges. The second reason is related to the fact that the modification of the solubility of a drug could affect many drug properties such as its lipophilicity, stability, permeability, availability or elimination, which can potentially influence its *in vitro* and *in vivo* behavior in a non-predictable manner.

REFERENCES:

[1] G.L. Amidon, H. Lennernas, V.P. Shah, J.R. Crison, A theoretical basis for a biopharmaceutic drug classification: the correlation of *in vitro* drug product dissolution and *in vivo* bioavailability, Pharmaceutical research, 12 (1995) 413-420.

[2] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, Advanced drug delivery reviews, 46 (2001) 3-26.

[3] C.A. Lipinski, Drug-like properties and the causes of poor solubility and poor permeability, Journal of pharmacological and toxicological methods, 44 (2000) 235-249.

[4] M.M. Hann, T.I. Oprea, Pursuing the leadlikeness concept in pharmaceutical research, Current opinion in chemical biology, 8 (2004) 255-263.

[5] R.A. Lipper, Modern drug discovery, in: A.C. Society (Ed.), Washington, DC, 1999, pp. 55.

[6] T. Takagi, C. Ramachandran, M. Bermejo, S. Yamashita, L.X. Yu, G.L. Amidon, A provisional biopharmaceutical classification of the top 200 oral drug products in the United States, Great Britain, Spain, and Japan, Molecular pharmaceutics, 3 (2006) 631-643.

[7] L. Di, E.H. Kerns, G.T. Carter, Drug-like property concepts in pharmaceutical design, Current pharmaceutical design, 15 (2009) 2184-2194.

[8] L. Di, P.V. Fish, T. Mano, Bridging solubility between drug discovery and development, Drug discovery today, 17 (2012) 486-495.

[9] H.D. Williams, N.L. Trevaskis, S.A. Charman, R.M. Shanker, W.N. Charman, C.W. Pouton, C.J. Porter, Strategies to address low drug solubility in discovery and development, Pharmacological reviews, 65 (2013) 315-499.

[10] T. Loftsson, M.E. Brewster, Pharmaceutical applications of cyclodextrins: effects on drug permeation through biological membranes, J Pharm Pharmacol, 63 (2011) 1119-1135.

[11] R. Liu, Water-insoluble drug formulation, 2nd ed., CRC Press, Boca Raton, FL, 2008.

[12] S. Guo, L. Huang, Nanoparticles containing insoluble drug for cancer therapy, Biotechnology advances, 32 (2014) 778-788.

[13] G.A. Stephenson, A. Aburub, T.A. Woods, Physical stability of salts of weak bases in the solid-state, Journal of pharmaceutical sciences, 100 (2011) 1607-1617.

[14] H. Kranz, C. Guthmann, T. Wagner, R. Lipp, J. Reinhard, Development of a single unit extended release formulation for ZK 811 752, a weakly basic drug, European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences, 26 (2005) 47-53.

[15] J. Rautio, H. Kumpulainen, T. Heimbach, R. Oliyai, D. Oh, T. Jarvinen, J. Savolainen, Prodrugs: design and clinical applications, Nature reviews. Drug discovery, 7 (2008) 255-270.

[16] V.J. Stella, Prodrugs: Some thoughts and current issues, Journal of pharmaceutical sciences, 99 (2010) 4755-4765.

[17] B. Testa, Prodrugs: bridging pharmacodynamic/pharmacokinetic gaps, Current opinion in chemical biology, 13 (2009) 338-344.

[18] J.B. Zawilska, J. Wojcieszak, A.B. Olejniczak, Prodrugs: a challenge for the drug development, Pharmacological reports : PR, 65 (2013) 1-14.

[19] A.B. Anderson, R.A. Conradi, Application of physical organic concepts to *in vitro* and *in vivo* lability design of water soluble prodrugs, in: E.B. Roche (Ed.) Bioreversible carriers in drug design : theory and application, Pergamon Press, New York, 1987, pp. viii, 292 p.

[20] R. Djaldetti, R. Inzelberg, N. Giladi, A.D. Korczyn, Y. Peretz-Aharon, M.J. Rabey, Y. Herishano, S. Honigman, S. Badarny, E. Melamed, Oral solution of levodopa ethylester for treatment of response fluctuations in patients with advanced Parkinson's disease, Movement disorders : official journal of the Movement Disorder Society, 17 (2002) 297-302.

[21] C.M. Walko, C. Lindley, Capecitabine: a review, Clinical therapeutics, 27 (2005) 23-44.

[22] N. Shimma, I. Umeda, M. Arasaki, C. Murasaki, K. Masubuchi, Y. Kohchi, M. Miwa, M. Ura, N. Sawada, H. Tahara, I. Kuruma, I. Horii, H. Ishitsuka, The design and synthesis of a new tumor-selective fluoropyrimidine carbamate, capecitabine, Bioorganic & medicinal chemistry, 8 (2000) 1697-1706.

[23] G.V. Koukourakis, V. Kouloulias, M.J. Koukourakis, G.A. Zacharias, H. Zabatis, J. Kouvaris, Efficacy of the oral fluorouracil pro-drug capecitabine in cancer treatment: a review, Molecules, 13 (2008) 1897-1922.

[24] P.G. Johnston, S. Kaye, Capecitabine: a novel agent for the treatment of solid tumors, Anti-cancer drugs, 12 (2001) 639-646.

[25] M. Miwa, M. Ura, M. Nishida, N. Sawada, T. Ishikawa, K. Mori, N. Shimma, I. Umeda, H. Ishitsuka, Design of a novel oral fluoropyrimidine carbamate, capecitabine, which

generates 5-fluorouracil selectively in tumours by enzymes concentrated in human liver and cancer tissue, European journal of cancer, 34 (1998) 1274-1281.

[26] K.M. Huttunen, H. Raunio, J. Rautio, Prodrugs--from serendipity to rational design, Pharmacological reviews, 63 (2011) 750-771.

[27] J. Filipovicgrcic, D. Maysinger, B. Zorc, I. Jalsenjak, Macromolecular Prodrugs .4. Alginate-Chitosan Microspheres of Phea-L-Dopa Adduct, International journal of pharmaceutics, 116 (1995) 39-44.

[28] P. De Caprariis, F. Palagiano, F. Bonina, L. Montenegro, M. D'Amico, F. Rossi, Synthesis and pharmacological evaluation of oligoethylene ester derivatives as indomethacin oral prodrugs, Journal of pharmaceutical sciences, 83 (1994) 1578-1581.

[29] B. Rihova, J. Kopecek, P. Kopeckova-Rejmanova, J. Strohalm, D. Plocova, H. Semoradova, Bioaffinity therapy with antibodies and drugs bound to soluble synthetic polymers, Journal of chromatography, 376 (1986) 221-233.

[30] J. Kopecek, P. Kopeckova, T. Minko, Z. Lu, HPMA copolymer-anticancer drug conjugates: design, activity, and mechanism of action, European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V, 50 (2000) 61-81.

[31] R. Duncan, P. Kopeckova-Rejmanova, J. Strohalm, I. Hume, H.C. Cable, J. Pohl, J.B. Lloyd, J. Kopecek, Anticancer agents coupled to N-(2-hydroxypropyl)methacrylamide copolymers. I. Evaluation of daunomycin and puromycin conjugates *in vitro*, British journal of cancer, 55 (1987) 165-174.

[32] L. Varticovski, Z.R. Lu, K. Mitchell, I. de Aos, J. Kopecek, Water-soluble HPMA copolymer-wortmannin conjugate retains phosphoinositide 3-kinase inhibitory activity *in vitro* and *in vivo*, Journal of controlled release : official journal of the Controlled Release Society, 74 (2001) 275-281.

[33] R. Duncan, S. Gac-Breton, R. Keane, R. Musila, Y.N. Sat, R. Satchi, F. Searle, Polymer-drug conjugates, PDEPT and PELT: basic principles for design and transfer from the laboratory to clinic, Journal of controlled release : official journal of the Controlled Release Society, 74 (2001) 135-146.

[34] F.M. de Groot, C. Albrecht, R. Koekkoek, P.H. Beusker, H.W. Scheeren, "Cascade-release dendrimers" liberate all end groups upon a single triggering event in the dendritic core, Angewandte Chemie, 42 (2003) 4490-4494.

[35] D.E. Duggan, L.E. Hare, C.A. Ditzler, B.W. Lei, K.C. Kwan, The disposition of sulindac, Clinical pharmacology and therapeutics, 21 (1977) 326-335.

[36] D.E. Duggan, K.F. Hooke, R.M. Noll, H.B. Hucker, C.G. Van Arman, Comparative disposition of sulindac and metabolites in five species, Biochem Pharmacol, 27 (1978) 2311-2320.

[37] N.M. Davies, M.S. Watson, Clinical pharmacokinetics of sulindac. A dynamic old drug, Clinical pharmacokinetics, 32 (1997) 437-459.

[38] T.Y. Shen, C.A. Winter, Chemical and biological studies on indomethacin, sulindac and their analogs, Advances in drug research, 12 (1977) 90-245.

[39] N. Blagden, M. de Matas, P.T. Gavan, P. York, Crystal engineering of active pharmaceutical ingredients to improve solubility and dissolution rates, Advanced drug delivery reviews, 59 (2007) 617-630.

[40] M. Pudipeddi, A.T. Serajuddin, Trends in solubility of polymorphs, Journal of pharmaceutical sciences, 94 (2005) 929-939.

[41] J. Haleblian, W. McCrone, Pharmaceutical applications of polymorphism, Journal of pharmaceutical sciences, 58 (1969) 911-929.

[42] S.R. Byrn, Solid state chemistry of drugs, Academic Press, New York, 1982.

[43] G.G. Zhang, D. Law, E.A. Schmitt, Y. Qiu, Phase transformation considerations during process development and manufacture of solid oral dosage forms, Advanced drug delivery reviews, 56 (2004) 371-390.

[44] R. Suryanarayanan, A.G. Mitchell, Phase transitions of calcium gluceptate, International journal of pharmaceutics, 32 (1986) 213-221.

[45] J.W. Poole, G. Owen, J. Silverio, J.N. Freyhof, S.B. Rosenman, Physiochemical factors influencing the absorption of the anhydrous and trihydrate forms of ampicillin, Current therapeutic research, clinical and experimental, 10 (1968) 292-303.

[46] M. Stoltz, M.R. Caira, A.P. Lotter, J.G. van der Watt, Physical and structural comparison of oxyphenbutazone monohydrate and anhydrate, Journal of pharmaceutical sciences, 78 (1989) 758-763.

[47] R.K. Khankari, D.J.W. Grant, Pharmaceutical Hydrates, Thermochim Acta, 248 (1995) 61-79.

[48] A.T. Serajuddin, Salt formation to improve drug solubility, Advanced drug delivery reviews, 59 (2007) 603-616.

[49] A. Avdeef, Solubility of sparingly-soluble ionizable drugs, Advanced drug delivery reviews, 59 (2007) 568-590.

[50] P.H. Stahl, C.G. Wermuth, International Union of Pure and Applied Chemistry., Handbook of pharmaceutical salts : properties, selection, and use, VHCA ; Wiley-VCH, Weinheim ; New York, 2002.

[51] S. Li, S. Wong, S. Sethia, H. Almoazen, Y.M. Joshi, A.T. Serajuddin, Investigation of solubility and dissolution of a free base and two different salt forms as a function of pH, Pharmaceutical research, 22 (2005) 628-635.

[52] H.R. Guzman, M. Tawa, Z. Zhang, P. Ratanabanangkoon, P. Shaw, C.R. Gardner, H. Chen, J.P. Moreau, O. Almarsson, J.F. Remenar, Combined use of crystalline salt forms and precipitation inhibitors to improve oral absorption of celecoxib from solid oral formulations, Journal of pharmaceutical sciences, 96 (2007) 2686-2702.

[53] N. Schultheiss, A. Newman, Pharmaceutical Cocrystals and Their Physicochemical Properties, Crystal growth & design, 9 (2009) 2950-2967.

[54] M.S. Jung, J.S. Kim, M.S. Kim, A. Alhalaweh, W. Cho, S.J. Hwang, S.P. Velaga, Bioavailability of indomethacin-saccharin cocrystals, J Pharm Pharmacol, 62 (2010) 1560-1568.

[55] D.P. McNamara, S.L. Childs, J. Giordano, A. Iarriccio, J. Cassidy, M.S. Shet, R. Mannion, E. O'Donnell, A. Park, Use of a glutaric acid cocrystal to improve oral bioavailability of a low solubility API, Pharmaceutical research, 23 (2006) 1888-1897.

[56] B.C. Hancock, M. Parks, What is the true solubility advantage for amorphous pharmaceuticals?, Pharmaceutical research, 17 (2000) 397-404.

[57] S. Onoue, H. Sato, K. Ogawa, Y. Kawabata, T. Mizumoto, K. Yuminoki, N. Hashimoto, S. Yamada, Improved dissolution and pharmacokinetic behavior of cyclosporine A using high-energy amorphous solid dispersion approach, International journal of pharmaceutics, 399 (2010) 94-101.

[58] C. Liu, J. Wu, B. Shi, Y. Zhang, T. Gao, Y. Pei, Enhancing the bioavailability of cyclosporine a using solid dispersion containing polyoxyethylene (40) stearate, Drug development and industrial pharmacy, 32 (2006) 115-123.

[59] M. Mosharraf, C. Nystrom, The Effect of Particle-Size and Shape on the Surface Specific Dissolution Rate of Microsized Practically Insoluble Drugs, International journal of pharmaceutics, 122 (1995) 35-47.

[60] D. Horter, J.B. Dressman, Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract, Advanced drug delivery reviews, 46 (2001) 75-87.
[61] B.E. Rabinow, Nanosuspensions in drug delivery, Nature reviews. Drug discovery, 3 (2004) 785-796.
[62] P. Kocbek, S. Baumgartner, J. Kristl, Preparation and evaluation of nanosuspensions for enhancing the dissolution of poorly soluble drugs, International journal of pharmaceutics, 312 (2006) 179-186.
[63] J.P. Sylvestre, M.C. Tang, A. Furtos, G. Leclair, M. Meunier, J.C. Leroux, Nanonization of megestrol acetate by laser fragmentation in aqueous milieu, Journal of controlled release : official journal of the Controlled Release Society, 149 (2011) 273-280.
[64] D. Xia, F. Cui, H. Piao, D. Cun, H. Piao, Y. Jiang, M. Ouyang, P. Quan, Effect of crystal size on the *in vitro* dissolution and oral absorption of nitrendipine in rats, Pharmaceutical research, 27 (2010) 1965-1976.
[65] S. Onoue, H. Takahashi, Y. Kawabata, Y. Seto, J. Hatanaka, B. Timmermann, S. Yamada, Formulation design and photochemical studies on nanocrystal solid dispersion of curcumin with improved oral bioavailability, Journal of pharmaceutical sciences, 99 (2010) 1871-1881.
[66] Y. Kawabata, K. Yamamoto, K. Debari, S. Onoue, S. Yamada, Novel crystalline solid dispersion of tranilast with high photostability and improved oral bioavailability, European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences, 39 (2010) 256-262.
[67] J. Moschwitzer, R.H. Muller, New method for the effective production of ultrafine drug nanocrystals, Journal of nanoscience and nanotechnology, 6 (2006) 3145-3153.
[68] A. Scholz, B. Abrahamsson, S.M. Diebold, E. Kostewicz, B.I. Polentarutti, A.L. Ungell, J.B. Dressman, Influence of hydrodynamics and particle size on the absorption of felodipine in labradors, Pharmaceutical research, 19 (2002) 42-46.
[69] Y. Wu, A. Loper, E. Landis, L. Hettrick, L. Novak, K. Lynn, C. Chen, K. Thompson, R. Higgins, U. Batra, S. Shelukar, G. Kwei, D. Storey, The role of biopharmaceutics in the development of a clinical nanoparticle formulation of MK-0869: a Beagle dog model predicts improved bioavailability and diminished food effect on absorption in human, International journal of pharmaceutics, 285 (2004) 135-146.

[70] M.E. Aulton, Pharmacuetics, the science of dosage form design, Churchill Livingstone, New York, 1994.

[71] M. Van Speybroeck, V. Barillaro, T.D. Thi, R. Mellaerts, J. Martens, J. Van Humbeeck, J. Vermant, P. Annaert, G. Van den Mooter, P. Augustijns, Ordered mesoporous silica material SBA-15: a broad-spectrum formulation platform for poorly soluble drugs, Journal of pharmaceutical sciences, 98 (2009) 2648-2658.

[72] A. Le Hir, Abrégés de Pharmacie galénique; Bonnes pratiques de fabricatio des médicaments, 8th ed., Masson, Issy-les-Moulineaux, 2006.

[73] R.G. Strickley, Solubilizing excipients in oral and injectable formulations, Pharmaceutical research, 21 (2004) 201-230.

[74] Physicians' Desk Reference, 65th ed., Medical Economics Company, Inc., Montvale, New Jersey, 2010.

[75] R. Nagarajan, E. Ruckenstein, Theory of Surfactant Self-Assembly - a Predictive Molecular Thermodynamic Approach, Langmuir, 7 (1991) 2934-2969.

[76] K. Kataoka, G.S. Kwon, M. Yokoyama, T. Okano, Y. Sakurai, Block-Copolymer Micelles as Vehicles for Drug Delivery, Journal of Controlled Release, 24 (1993) 119-132.

[77] G.S. Kwon, T. Okano, Soluble self-assembled block copolymers for drug delivery, Pharmaceutical research, 16 (1999) 597-600.

[78] M.F. Francis, M. Cristea, F.M. Winnik, Polymeric micelles for oral drug delivery: Why and how, Pure Appl Chem, 76 (2004) 1321-1335.

[79] E.R. Gillies, J.M.J. Frechet, Development of acid-sensitive copolymer micelles for drug delivery, Pure Appl Chem, 76 (2004) 1295-1307.

[80] Y. Yamamoto, Y. Nagasaki, Y. Kato, Y. Sugiyama, K. Kataoka, Long-circulating poly(ethylene glycol)-poly(D,L-lactide) block copolymer micelles with modulated surface charge, Journal of Controlled Release, 77 (2001) 27-38.

[81] K. Mondon, M. Zeisser-Labouebe, R. Gurny, M. Moller, Novel cyclosporin A formulations using MPEG-hexyl-substituted polylactide micelles: a suitability study, European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V, 77 (2011) 56-65.

[82] C. Di Tommaso, J.L. Bourges, F. Valamanesh, G. Trubitsyn, A. Torriglia, J.C. Jeanny, F. Behar-Cohen, R. Gurny, M. Moller, Novel micelle carriers for cyclosporin A topical ocular delivery: *in vivo* cornea penetration, ocular distribution and efficacy studies, European journal

of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V, 81 (2012) 257-264.

[83] R.H. Muller, K. Mader, S. Gohla, Solid lipid nanoparticles (SLN) for controlled drug delivery - a review of the state of the art, European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V, 50 (2000) 161-177.

[84] W.A. Ritschel, Microemulsion technology in the reformulation of cyclosporine: the reason behind the pharmacokinetic properties of Neoral, Clinical transplantation, 10 (1996) 364-373.

[85] E.A. Mueller, J.M. Kovarik, J.B. van Bree, W. Tetzloff, J. Grevel, K. Kutz, Improved dose linearity of cyclosporine pharmacokinetics from a microemulsion formulation, Pharmaceutical research, 11 (1994) 301-304.

[86] E.A. Mueller, J.M. Kovarik, J.B. van Bree, A.E. Lison, K. Kutz, Pharmacokinetics and tolerability of a microemulsion formulation of cyclosporine in renal allograft recipients--a concentration-controlled comparison with the commercial formulation, Transplantation, 57 (1994) 1178-1182.

[87] P.P. Constantinides, Lipid microemulsions for improving drug dissolution and oral absorption: physical and biopharmaceutical aspects, Pharmaceutical research, 12 (1995) 1561-1572.

[88] C.W. Pouton, Formulation of self-emulsifying drug delivery systems, Advanced drug delivery reviews, 25 (1997) 47-58.

[89] M. Buchanan, L. Starrs, S.U. Egelhaaf, M.E. Cates, Kinetic pathways of multiphase surfactant systems, Physical review. E, Statistical physics, plasmas, fluids, and related interdisciplinary topics, 62 (2000) 6895-6905.

[90] T. Nishimi, C.A. Miller, Spontaneous emulsification of oil in aerosol-OT/water/hydrocarbon systems, Langmuir, 16 (2000) 9233-9241.

[91] N. Shahidzadeh, D. Bonn, J. Meunier, M. Nabavi, M. Airiau, M. Morvan, Dynamics of spontaneous emulsification for fabrication of oil in water emulsions, Langmuir, 16 (2000) 9703-9708.

[92] J.C. Lopez-Montilla, P.E. Herrera-Morales, S. Pandey, D.O. Shah, Spontaneous emulsification: Mechanisms, physicochemical aspects, modeling, and applications, J Disper Sci Technol, 23 (2002) 219-268.

[93] United States Pharmacopeia and National Formulary, Rockville,, 2014.

[94] T. Loftsson, M.E. Brewster, Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization, Journal of pharmaceutical sciences, 85 (1996) 1017-1025.

[95] R.A. Rajewski, V.J. Stella, Pharmaceutical applications of cyclodextrins. 2. *In vivo* drug delivery, Journal of pharmaceutical sciences, 85 (1996) 1142-1169.

[96] V.J. Stella, R.A. Rajewski, Cyclodextrins: their future in drug formulation and delivery, Pharmaceutical research, 14 (1997) 556-567.

[97] K. Uekama, F. Hirayama, T. Irie, Cyclodextrin Drug Carrier Systems, Chemical reviews, 98 (1998) 2045-2076.

[98] M.E. Davis, M.E. Brewster, Cyclodextrin-based pharmaceutics: past, present and future, Nature reviews. Drug discovery, 3 (2004) 1023-1035.

[99] M.E. Brewster, T. Loftsson, Cyclodextrins as pharmaceutical solubilizers, Advanced drug delivery reviews, 59 (2007) 645-666.

[100] T. Loftsson, D. Duchene, Cyclodextrins and their pharmaceutical applications, International journal of pharmaceutics, 329 (2007) 1-11.

[101] K. Jarvinen, T. Jarvinen, A. Urtti, Ocular Absorption Following Topical Delivery, Advanced drug delivery reviews, 16 (1995) 3-19.

[102] T. Loftsson, E. Stefansson, Cyclodextrins in eye drop formulations: enhanced topical delivery of corticosteroids to the eye, Acta ophthalmologica Scandinavica, 80 (2002) 144-150.

[103] T. Loftsson, M.E. Brewster, Pharmaceutical applications of cyclodextrins: basic science and product development, J Pharm Pharmacol, 62 (2010) 1607-1621.

[104] T. Loftssona, T. Jarvinen, Cyclodextrins in ophthalmic drug delivery, Advanced drug delivery reviews, 36 (1999) 59-79.

[105] T. Loftsson, Drug permeation through biomembranes: cyclodextrins and the unstirred water layer, Die Pharmazie, 67 (2012) 363-370.

[106] T. Irie, K. Uekama, Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation, Journal of pharmaceutical sciences, 86 (1997) 147-162.

[107] L. Cheeks, R.L. Kaswan, K. Green, Influence of vehicle and anterior chamber protein concentration on cyclosporine penetration through the isolated rabbit cornea, Current eye research, 11 (1992) 641-649.

[108] A. Kanai, R.M. Alba, T. Takano, C. Kobayashi, A. Nakajima, K. Kurihara, T. Yokoyama, M. Fukami, The effect on the cornea of alpha cyclodextrin vehicle for cyclosporin eye drops, Transplant Proc, 21 (1989) 3150-3152.

[109] Y. Sasamoto, S. Hirose, S. Ohno, K. Onoe, H. Matsuda, Topical application of ciclosporin ophthalmic solution containing alpha-cyclodextrin in experimental uveitis, Ophthalmologica. Journal international d'ophtalmologie. International journal of ophthalmology. Zeitschrift fur Augenheilkunde, 203 (1991) 118-125.

[110] T. Loftsson, M. Masson, Cyclodextrins in topical drug formulations: theory and practice, International journal of pharmaceutics, 225 (2001) 15-30.

[111] M.T. Faucci, P. Mura, Effect of water-soluble polymers on naproxen complexation with natural and chemically modified beta-cyclodextrins, Drug development and industrial pharmacy, 27 (2001) 909-917.

[112] E. Redenti, L. Szente, J. Szejtli, Drug/cyclodextrin/hydroxy acid multicomponent systems. Properties and pharmaceutical applications, Journal of pharmaceutical sciences, 89 (2000) 1-8.

[113] E. Redenti, L. Szente, J. Szejtli, Cyclodextrin complexes of salts of acidic drugs. Thermodynamic properties, structural features, and pharmaceutical applications, Journal of pharmaceutical sciences, 90 (2001) 979-986.

[114] M.S. Nagarsenker, R.N. Meshram, G. Ramprakash, Solid dispersion of hydroxypropyl beta-cyclodextrin and ketorolac: enhancement of in-vitro dissolution rates, improvement in anti-inflammatory activity and reduction in ulcerogenicity in rats, J Pharm Pharmacol, 52 (2000) 949-956.

[115] R. Govindarajan, M.S. Nagarsenker, Formulation studies and *in vivo* evaluation of a flurbiprofen-hydroxypropyl beta-cyclodextrin system, Pharmaceutical development and technology, 10 (2005) 105-114.

[116] S. Basavaraj, G.V. Betageri, Can formulation and drug delivery reduce attrition during drug discovery and development—review of feasibility, benefits and challenges, Acta Pharmaceutica Sinica B, 4 (2014) 3-17.

[117] G. Corona, G. Rastrelli, G. Forti, M. Maggi, Update in testosterone therapy for men, The journal of sexual medicine, 8 (2011) 639-654; quiz 655.

[118] A.C. Anselmo, S. Mitragotri, An overview of clinical and commercial impact of drug delivery systems, Journal of controlled release : official journal of the Controlled Release Society, 190C (2014) 15-28.
[119] B.Y. Shen, O.S. Punjabi, C.Y. Lowder, J.E. Sears, R.P. Singh, Early treatment response of fluocinolone (retisert) implantation in patients with uveitic macular edema: an optical coherence tomography study, Retina, 33 (2013) 873-877.
[120] T.J. Abel, T. Ryken, M.S. Lesniak, P. Gabikian, Gliadel for brain metastasis, Surgical neurology international, 4 (2013) S289-293.
[121] M.W. van den Heuvel, A.J.M. van Bragt, A.K.M. Alnabawy, M.C.J. Kaptein, Comparison of ethinylestradiol pharmacokinetics in three hormonal contraceptive formulations: the vaginal ring, the transdermal patch and an oral contraceptive, Contraception, 72 (2005) 168-174.

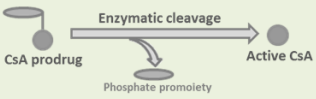
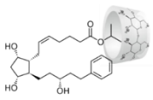
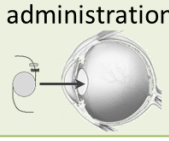
Chapter II

UHPLC-MS, a powerful tool for investigations related to ocular formulations

Analytical challenges for the quantification of cyclosporine A, latanoprost and related compounds

A variety of different experiments have been performed towards the completion of this thesis, from stability testing to *in vivo* distributions passing by *ex vivo* permeation evaluations, as illustrated in Table 1. The corner stone of these investigations was the quantification of active molecules and their related compounds (cyclosporine A prodrug, latanoprost ester, biotransformation intermediates, cyclosporine A (CsA) or latanoprost acid). As a consequence, particular attention was given to the development and optimization of the analytical methods.

Table 1: *In vitro*, *ex vivo* and *in vivo* experiments performed in the present thesis.

	In vitro	Ex vivo	In vivo
Prodrug of cyclosporine A 	*Formulation development, Stability* ✓	*Transformation, Permeation, Accumulation* ✓	*Tolerance, Elimination, Biodistribution* ✓
Latanoprost complexation 	*Formulation development, Stability, Complex structure* ✓	*Availability* ✓	*Tolerance* ✓
Cyclosporine A intraocular administration 	*Formulation development, DDS structure* ✓	*Not applicable*	*Efficacy, Distribution* ✓

A number of analytical requirements were identified prior to the development of the analytical methods. The first challenge was related to the huge number of samples that would need to be analysed in a limited time frame. For example, the *in vivo* precorneal kinetic evaluation required the collection and analysis of more than two hundred samples during the first three hours of the experiment. In addition, stability studies of the CsA prodrug and latanoprost formulations involved more than two thousand analyses. A second challenge was related to the limited amount of each sample. For example, during the precorneal kinetic evaluation, only two microliters of tear fluid could be collected at each time point. Also, for the distribution studies performed in rats, the collected ocular tissues weighted between 2 and 10 mg (rat eyes being more than four times smaller than human ones). The third challenge was related to the broad range of concentrations that had to be covered during analyses. For example, high prodrug concentrations and low CsA levels could be expected in the tear fluid immediately after topical administration of the CsA prodrug formulation. In contrast, a few minutes after the start of the biotransformation, the prodrug levels should rapidly decrease while the CsA levels should rapidly increase, involving a wide range of concentrations. The fourth challenge was linked to the complexity of the biological samples. The *in vivo* investigations involved samples of ocular tissues and fluids (cornea, conjunctiva, aqueous humor, iris-ciliary body, vitreous humor or retina) as well as serum, spleen and lymphatic ganglions. In these samples, the analytes were embedded in complex biological matrixes containing high amounts of endogenous compounds that could enhance or suppress the signals of interest. The fifth important point to consider was the presence of unstable intermediates released during the biotransformation of the CsA prodrug and latanoprost. The chemical structures of these intermediates were very similar to those of the analytes, which could lead to interferences with the signals of interest. The last challenge was related to the extremely rapid reactions of the biotransformations that needed to be halted immediately after sample collection.

The ultra-high-pressure liquid chromatography (UHPLC), which is based on the use of porous sub-2µm particles, was selected over conventional HPLC because it offered shorter analysis times and improved resolution. The higher throughput was particularly interesting for experiments requiring a high number of sample analyses, such as the *in vivo* precorneal kinetic evaluation or the stability studies. The higher resolution was of particular interest in

biodistribution studies allowing the separation of the analytes from potentially interfering compounds such as matrix components and biotransformation intermediates.

The enhanced throughput and resolution offered by UHPLC separation allowed for satisfactory analyses of samples produced during *in vitro* experiments. In addition, ultraviolet (UV) detection was a robust technique particularly adapted for stability studies. Therefore, UHPLC-UV methods were developed for the analysis of samples from *in vitro* experiments.
On the other hand, the *in vivo* and *ex vivo* experiments could be considered as high throughput microscale bioanalyses, thus requiring high sensitivity and selectivity in addition to the high throughput and resolution brought by the UHPLC separation. The UHPLC chromatographic approach was hyphenated with a tandem mass spectrometric detection based on selected reaction monitoring (SRM), the MS/MS detection providing a much higher sensitivity and selectivity than UV detection. The higher sensitivity was particularly interesting when analysing samples available in limited amounts and where the compounds of interest were expected to be at low concentrations (as in precorneal kinetic studies and biodistribution investigations). Moreover, the high selectivity could further reduce the matrix effect and potential interferences from the biotransformation intermediates.

For accurate quantifications with the presented UHPLC-MS/MS technology, the analytical methods had to be further optimized in terms of internal standards and solvents for sample dilution. Internal standard selection was of key importance for exact quantifications. Deuterated standards were chosen because they displayed the same chromatographic behaviour as the analytes of interest, while having a higher m/z signal and a different transition. Twelve times deuterated CsA, four times deuterated latanoprost and four times deuterated latanoprost acid were used as the base for CsA, CsA prodrug, latanoprost and latanoprost acid quantifications. Methanol was selected as the most appropriate solvent for sample dilution because of its ability to solubilize the molecules of interest, while stopping the biotransformation by enzyme denaturation. In addition, methanol could be used as the mobile phase. The dilution factor for the biological samples was optimized to minimize the matrix effect while limiting the dilution of the analytes.
The coupling of UHPLC separation with tandem MS detection appeared to be a powerful tool to fulfil the analytical requirements of the planned investigations.

From a more general point of view, the following publication presents and discusses the constraints related to the coupling of UHPLC separation with MS detection before reviewing the most significant applications for this technique.

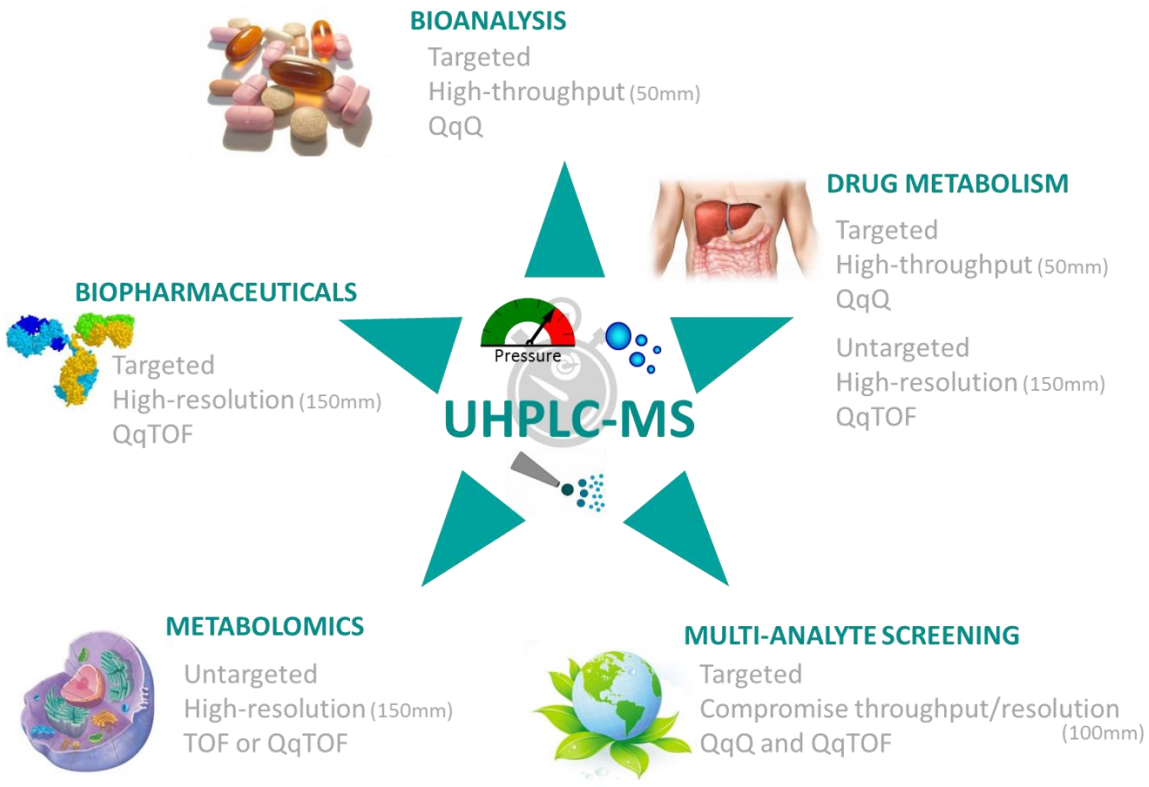
Coupling ultra high-pressure liquid chromatography with mass spectrometry: constraints and possible applications

Marta RODRIGUEZ-ALLER, Robert GURNY, Jean-Luc VEUTHEY, Davy GUILLARME*

School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland

*Correspondence: Phone: +41223793463, Fax: +41223796808, E-mail: davy.guillarme@unige.ch

Published in Journal of Chromatography A 2013;1292: 2-18.



ABSTRACT

The introduction of columns packed with porous sub-2 µm particles and the extension of the upper pressure limit of HPLC instrumentation to 1300 bar (Ultra-High Pressure Liquid Chromatography, UHPLC) has opened new frontiers in resolution and speed of analysis. However, certain constraints appear when coupling UHPLC technology with mass spectrometry (MS). First, the most significant limitation is related to the narrow peaks that are produced by UHPLC that require a fast duty cycle, which is only available on the latest generations of MS devices. Thus, certain analyzers are more readily compatible with UHPLC (e.g., QqQ or TOF/MS) than others (e.g., ion trap or FT-MS). Second, due to the reduction of the column volume, extra-column band broadening can become significant, leading to a reduction in the kinetic performance of the UHPLC-MS configuration. Third, as the mobile phase linear velocity is higher in UHPLC, the electrospray ionization source must also be able to provide high sensitivity at flow rates of up to 1 mL/min.

Despite these limitations, the UHPLC-MS(/MS) platform has successfully been employed over the last decade for various types of applications, including those related to bioanalysis, drug metabolism, multi-residue screening, metabolomics, biopharmaceuticals and polar compounds.

Keywords: UHPLC, UPLC, mass spectrometry, multi-residue screening, bioanalysis, TOF/MS

1. INTRODUCTION

The past few years have brought considerable improvements to conventional high-pressure liquid chromatography (HPLC) in terms of innovative supports and instrumentations. The goal of these new LC technologies has been either to achieve faster analyses while maintaining acceptable chromatographic performance or to attain higher resolution within a reasonable analysis time [1-3]. These advances have mainly been driven by the constant increases in the numbers of analyses or increases in sample complexity. High analysis speed is required, among others, in toxicology, environmental analysis, clinical chemistry and doping control because the response delivery time has to be reduced to its minimum [4,5]. Additionally, pharmaceutical industries are also interested in high-throughput approaches to reduce costs and enhance productivity. Highly efficient separations are also attractive, particularly because of the important development of genomics, proteomics and, more recently, metabolomics [6,7]. Indeed, in these fields of application, efficient separations are required to analyze very complex samples that potentially contain hundreds of constituents, such as tryptic digests, plant extracts or biological samples.

Conventional LC suffers from some obvious limitations, and there is a strong need for techniques that are able to separate larger numbers of compounds within acceptable timescales. Various strategies have been proposed to improve the throughput and resolution in LC [8-10].

Due to the theoretical works of Giddings or Knox during the 1970s [11-13] and the proof of concept by Jorgenson in 1997 [14,15], it is well known that columns packed with fully porous sub-2 µm particles improve chromatographic performance and throughput. However, until 2004, columns and instrumentation that could withstand pressures beyond 400 bar were not commercially available [16]. Currently, there are various UHPLC instruments that are compatible with pressures from 600 to 1300 bar, in addition to numerous column chemistries and dimensions (more than 10 providers and > 80 chemistries) [8,17]. Compared to conventional LC, UHPLC technology has increased throughput by a factor of ~ 10 for similar efficiency or improved resolution by ~ 3 under similar analysis time [18,19]. After less than 10 years, this technology is considered mature and is commonly implemented in numerous academic and industrial laboratories. Recently, columns packed with sub-2µm particles bonded with polar groups have become available for supercritical fluid chromatography

(SFC). This technology has been described as UHPSFC or UPC², depending on the provider [20]. It allows an additional gain in performance compared to UHPLC and is also applicable to a wider range of analytes, from very polar to apolar ones. This approach will most likely become the next evolution of UHPLC. One alternative to UHPLC involves the use of monoliths, which consist of a single rod of porous material that possess unique properties in terms of permeability and efficiency [21,22]. The second generation of monoliths presenting macropores of 1.2 µm and mesopores of 15 nm was released in 2011. As was recently reported [23], because of a more dense packing, this second generation of silica monoliths provides similar kinetic performance and lower backpressures compared to columns packed with porous sub-2 µm particles. However, this technology still suffers from a restricted number of column chemistries and geometries (i.e., maximal length of 100 mm) [24]. Another type of stationary phase known as "fused-core" or "core-shell" technology appeared on the market in 2007 and provides similar performance to UHPLC but with a 2-3 times lower pressure [25,26]. It consists of using columns packed with 2.6 - 2.7 µm superficially porous particles composed of a 1.7 - 1.9 µm solid inner core and a 0.35 - 0.5 µm porous outer core. Today, this type of column technology is considered to be the main competitor of UHPLC, particularly because the number of providers and column chemistries has increased exponentially over the last two years [27-31]. For additional information about these different strategies, the readers can refer to various papers that compare the performance of UHPLC, core-shell and monolithic approaches [3,9].

The LC-MS system can be improved on two levels. Improvements in the LC part are beneficial for throughput and/or resolution, while MS detection improvements have a positive impact on sensitivity and selectivity. Significant progress has been made in mass spectrometry during the last decade that has provided more sensitive, robust, user-friendly and faster (fast duty cycle) systems. The most important MS advances have been made on analyzers technology and on optics design for improved ion transmission and sensitivity. Even if the triple quadrupole (QqQ) remains the reference instrument for targeted analysis, there are various other powerful hybrid analyzers, such as the i) Q-trap [32], which combines the capabilities of a triple quadrupole and an ion trap, and the ii) QqTOF/MS which is composed of a quadrupole, a collision cell and a TOF/MS analyzer in sequence to achieve high sensitivity, mass resolution and mass accuracy [33]. In addition to these hybrid instruments, high-resolution mass spectrometry (HRMS) has also been strongly expanded in

the last few years, particularly for untargeted analysis. Such systems can achieve high resolution with excellent mass accuracy (<2 ppm) and can be classified into two categories: TOF/MS and FT-MS (including FT ion cyclotron resonance and Orbitrap) [34].

In the present review, the hyphenation of UHPLC with MS will be examined. Figure 1 illustrates the constant increase in the number of papers published in UHPLC-MS since 2004, the type of analyzers commonly used in UHPLC-MS and their most important applications. In the first part of this review, the constraints related to the coupling of UHPLC with MS will be discussed in detail, with particular emphasis on the mobile phase flow rate compatibility, duty cycle of current MS devices and additional peak broadening arising from MS. In the second part, the most significant applications of UHPLC-MS will be critically discussed into various sections, including bioanalysis, drug metabolism studies, multi-residue screening, metabolomics, biopharmaceuticals and analysis of polar compounds.

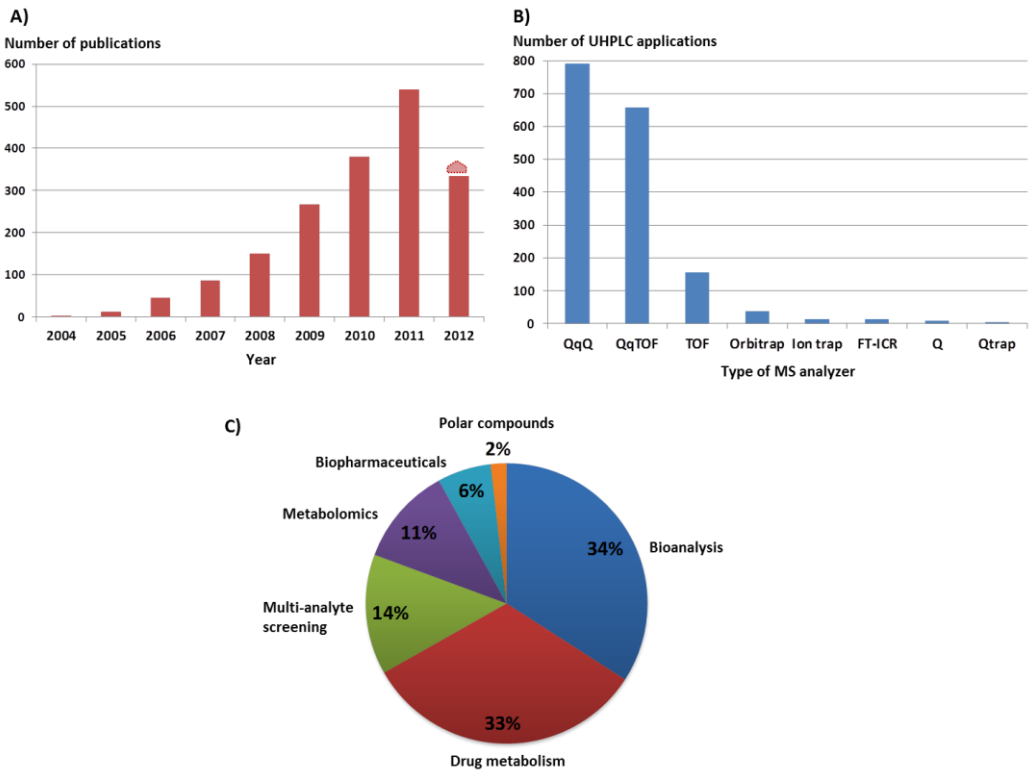


Figure 1: A) Number of papers published each year in the field of UHPLC-MS, since 2004. These numbers were obtained with the keywords "UPLC" and "UHPLC", with an additional filter (keyword "MS"), B) Number of UHPLC-MS applications classified by types of analyser across all years, C) Number of UHPLC-MS applications classified by fields of application. Source: Scifinder scholar 2007 search of the Chemical Abstracts database from 2003 to 2012. Date of information gathering: June 2012.

2. CONSTRAINTS FOR THE COUPLING OF UHPLC WITH MS

2.1. Mobile phase flow rate compatibility

Today, atmospheric pressure electrospray ionization (ESI) is considered the reference ionization technique for the coupling between LC and MS. The goal is to ionize compounds in the gas phase and to eliminate the mobile phase while maintaining a sufficient vacuum level inside the MS [35]. To achieve acceptable ionization yields and sensitivity, there is an optimum mobile phase flow rate generally comprised between 50 and 300 µL/min, that depends on the source geometry and the efficiency of the pneumatic assistance (addition of nitrogen gas at high flow rates) [36]. Higher flow rates have been reported [37], but they often have a negative impact on sensitivity. These optimal flow rate values explain why 2.1 mm I.D. columns (with their optimal flow rate of ~ 200 µL/min) are the most frequently used, while 1 mm I.D. (optimal flow rate of ~ 50 µL/min) are only occasionally employed and 4.6 mm I.D. columns (optimal flow rate of ~ 1 mL/min) are scarcely applied, except in combination with post-column flow split [38]. From a chromatographic point of view, the optimal mobile phase flow rate using columns packed with 1.7 µm particles in UHPLC-MS systems is approximately 3 times higher compared to conventional LC with 5 µm particles, because the optimal linear velocity is inversely proportional to the particle diameter [39]. The choice of the UHPLC column I.D. is also more restricted compared to conventional LC because both frictional heating effects under very high pressures [40,41] and solvent consumption [42] should be considered. Indeed, frictional heating phenomenon can be observed when using columns packed with sub-2µm particles at elevated mobile phase linear velocity, thus generating very high pressure drop. This high pressure is induced by friction of the mobile phase percolating through the stationary phase, generating heat, that can be detrimental for the separation. In this context, 2.1 mm I.D columns are the reference dimension for UHPLC operations and experiments are generally performed at flow rates between 500 and 1000 µL/min, depending on the acceptable efficiency loss and the sizes of the investigated compounds [43]. To limit the sensitivity loss that can occur in MS at elevated mobile phase flow rates, various technical solutions (modification of the source design and specifications) have been proposed by manufacturers of UHPLC-MS instruments.

As an example, Agilent Technologies (Waldbronn, Germany) recently included an additional feature in all of their ionizations sources that is called “Jet Stream thermal gradient focusing

technology" [44]. This technology consists of utilizing an additional sheath gas heated at 350°C at a flow rate of 11 mL/min. This gas flow focuses the nebulizer spray and desolvates the ions more efficiently, thus improving sensitivity even at higher flow rates. According to the provider, a 5 to 10-fold gain in sensitivity was achieved for flow rates ranging from 0.25 to 2 mL/min using this technology. Using such strategy, the consumption of gas is significantly higher compared to a conventional ESI interface. However, most of the analytical laboratories possessing MS devices are equipped with nitrogen generator and thus the associated cost is quite limited. Finally, the risk of thermal degradation of the compounds is limited because the travel time over the heated zone is less than hundreds of milliseconds. An alternative strategy based on the Z-spray source design was proposed by Waters (Milford, MA, USA). In their source configuration, the probe is perpendicular to the sampling cone, and the second extraction cone is perpendicular to the ion beam to improve the elimination of the mobile phase. In addition, the source block is heated at 150°C and the heated nebulizing gas flow and temperature can be as high as 1200 L/hr and 650°C, respectively. Under such extreme conditions, the negative impact of elevated mobile phase flow rates on sensitivity is moderate. It has been demonstrated that the sensitivity loss in UHPLC-MS between 300 and 1000 µL/min was approximately 15% for a representative set of pharmaceutical compounds [45]. However, this result must be considered with caution because it might be strongly compound-dependent, and divergent conclusions can be drawn depending on the type of analyzer employed. For UHPLC operations, AB Sciex (Concord, Canada) proposed their iondrive technology, using the IonDrive Turbo V source, which is compatible with flow rates up to 3 mL/min and is able to vaporize from 100% aqueous to 100% organic solvents. The principle is to use a nebulizer gas (up to 90 psi) and a heater gas that originates in the turbo heaters (≤ 90 psi and temperatures of up to 750°C) to efficiently nebulize the mobile phase. Then, the combination of the IonSpray effluent and the heated dry gas from the turbo sprayer are projected at a 90° angle to the ion path [46]. ThermoFisher (Palo Alto, CA, USA) also proposed the same type of innovative ionization source design for UHPLC, called the "Heated Electrospray Ionization Source (HESI-II)", which allows improved ionization efficiencies at high flow rates (> 1 mL/min). Again, the design of the heater located on the probe was improved to make it compatible with elevated flow rates.

These technical solutions proposed by providers make the MS device compatible with UHPLC columns of 2.1 mm I.D. However, these features are only available on the latest

generations of MS instruments. If UHPLC has to be coupled with older generations of MS systems, various strategies can be applied. The first one consists of employing a passive or active post-column flow split. However, it is highly recommended to evaluate the loss in chromatographic performance afforded by the split (additional source of extra-column band broadening). Alternatively, 1 mm I.D. columns can be employed in UHPLC to operate at lower flow rates (150-200 µL/min). In this case, the kinetic performance can become unacceptable in UHPLC-MS, as discussed in section 2.2. Finally, APCI can be employed instead of ESI because the ionization technique is mass-dependent. Thus, the sensitivity increases at high mobile phase flow rate. However, the sensitivity achieved in ESI *vs.* APCI also strongly depends on the nature of the selected compounds.

2.2. Band broadening associated with MS

It has recently been demonstrated that MS can contribute to a significant additional band broadening *vs*. UV detectors. In the case of UHPLC experiments, the column volume is drastically reduced compared to HPLC by a factor of 10 to 20 on average. For this reason, all of the sources of extra-column band broadening must be minimized as much as possible. This constraint is generally taken into account in the chromatographic system [47-49] but not always in the MS device. Indeed, Grata *et al.* demonstrated that a TOF/MS detector could be a significant source of band broadening [50]. For example, a peak capacity reduction by 30% was observed for a 20 min gradient run with a UHPLC column (150 x 2.1 mm, 1.7 µm) in the case of MS *vs.* UV detection at a flow rate of 400 µL/min. This important peak capacity loss was related to the extra-column dispersion, which was estimated to be 10 µL² for the UHPLC-UV and approximately 70 µL² for the UHPLC-TOF/MS. In this study [50], only long UHPLC columns (i.e., $L_{col} \geq 150$ mm) and gradient times ($t_{grad} \geq 20$ min) were considered. To better evaluate the negative impacts of the MS instrument on the UHPLC performance, Figure 2 shows the efficiency losses in UHPLC-MS that originate from the system itself for various column geometries. To construct this figure, two MS devices were considered, namely an "old-generation system", a Waters Micromass LCT Premier TOF/MS (TOF/MS) with an extra-column dispersion of 30 - 65 µL² (for flow rates between 100 and 600 µL/min) and a "new-generation device", a Waters Xevo QTOF/MS (QqTOF/MS) that generates an extra-column dispersion of 5 - 10 µL² (for flow rates between 100 and 600 µL/min). The benefits of

the new *vs.* older generations of MS instruments can most likely be explained by the improvements in the ionization source design, optics and electronic signal treatments. As illustrated in Figure 2, there are important differences between these two generations of instruments. For a retention factor of 3, the loss in efficiency for 50 to 150 x 2.1 mm columns was in the ranged from 12-32% for the most recent MS device and 48-76% for the older MS system. In the case of very fast isocratic or gradient analyses (k or k_e ~ 1), the loss in efficiency was equal to >90% for a 50 x 2.1 mm I.D. column on the "old" TOF/MS device.

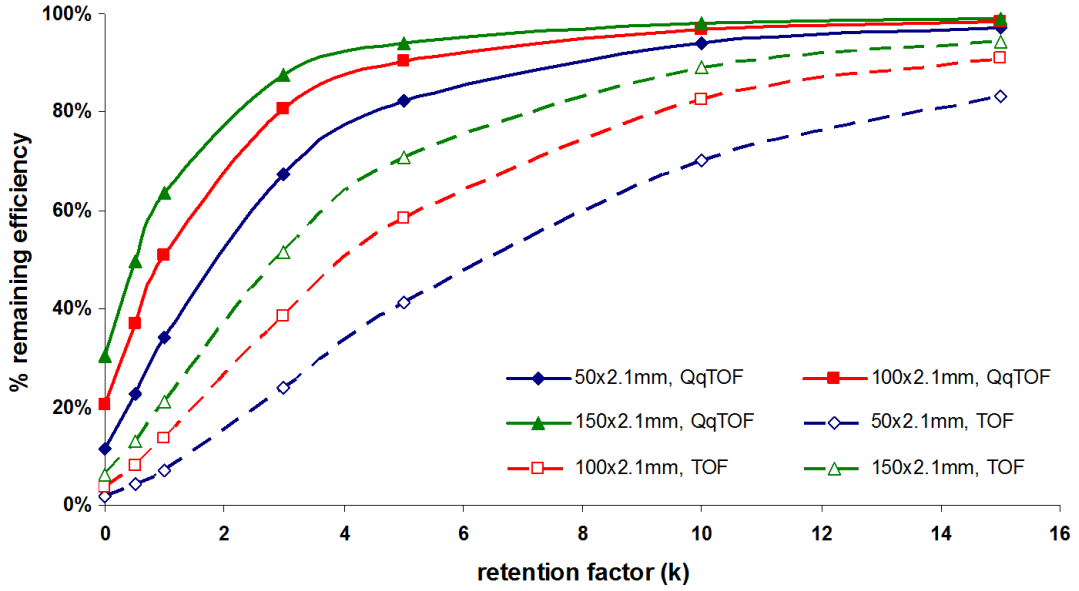


Figure 2: Percentage of remaining efficiency in UHPLC-MS for various retention factors ranging between 0.5 and 15, considering two different MS devices (TOF/MS and QqTOF/MS) and three different columns geometry (50, 100 and 150 x 2.1 mm, 1.7 µm).

Another recent study from Eugster *et al.* [51] illustrates the same type of problem encountered during the high-resolution profiling of a marine snail, *Conus Consors*, containing peptides of 1 to 5 kDa. To improve the chromatographic performance, a 3 mm I.D. UHPLC column was employed in combination with a post-column flow split. Some results from this study are presented in Figure 3 confirming that the impact of MS on chromatographic performance strongly depends on the column I.D. In the case in which MS band broadening was significant (old-generation of MS device), the best performance was achieved with a 3 mm I.D. column. In Figure 3, the maximum peak capacities that could be obtained for various column lengths

and gradient times on the *Conus* venom sample were equal to 700, 1200 and 1500 for a 1, 2.1 and 3 mm I.D. column, respectively. The observed differences in peak capacity were attributed to the contribution of the MS device to peak broadening. However, the peak capacity using a recent MS device and a UHPLC column of 2.1 mm I.D. was found to be suitable for high-resolution UHPLC-MS profiling.

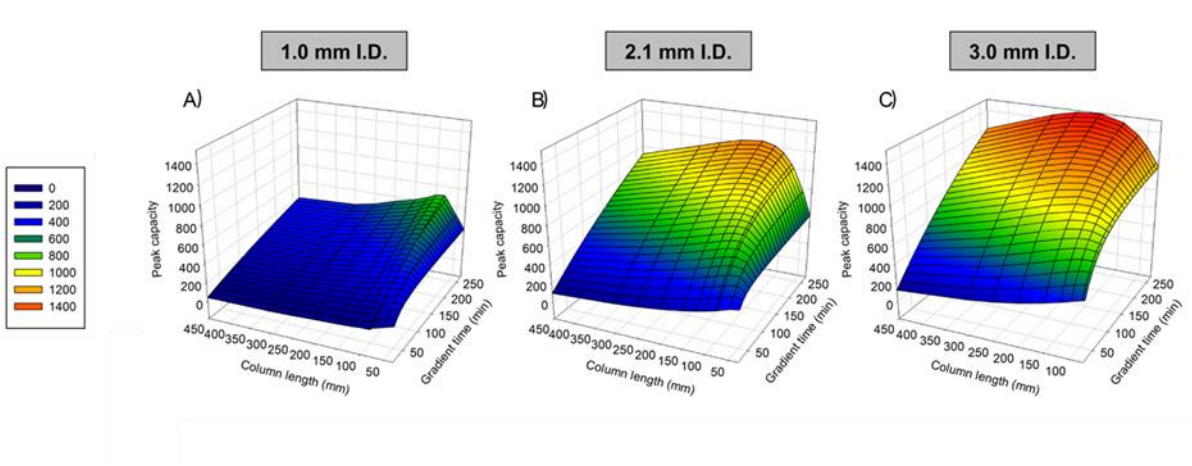


Figure 3: 3D plots of calculated peak capacity *vs.* column length and gradient time at 30°C, for peptide sample, using 3 different column internal diameters: 1.0 mm I.D. (plot A), 2.1 mm I.D. (plot B) and 3.0 mm I.D. (plot C). Flow rates were always calculated to provide the maximal backpressure (90% of ΔP_{max}). From [51], with permission.

2.3. Data acquisition rate

Another issue for UHPLC-MS coupling is related to the narrow peaks that are generated when using columns packed with sub-2 µm particles at elevated pressures. In conventional LC, the peak widths at baseline are approximately 10 s on average, while they are reduced to only 2 - 4 s in UHPLC under a gradient elution. In addition, when ultra-fast UHPLC analyses (less than 1 min) are performed, peak widths can be less than 1 s [43]. For quantitative purpose, at least 10-15 acquisition points per peak are recommended to correctly define the chromatographic peak and achieve suitable performance. This criterion can be difficult to meet, depending on the duty cycle (acquisition speed) of the employed MS device.

2.3.1. Operation in SIM, SRM and SCAN mode

The simplest MS device is a single quadrupole, which operates either in the single ion monitoring (SIM) or scan mode. With the single quadrupole, the SIM mode is generally preferred because it provides a significantly better sensitivity. In the SIM mode, the quadrupole parameters (RF and DC voltages) are adjusted to filter and select only one specific *m/z*. The period of time required to collect data at a particular mass is refered to as the dwell time. In the case in which several *m/z* values are detected, the instrument works sequentially. Starting from the lowest *m/z* to be analyzed, the instrument collects data during the programmed dwell time and then moves on to the next *m/z*. The time required to switch from one *m/z* to another is called the inter-channel delay, which should be long enough to avoid cross contamination of the *m/z* channels. For previous-generation MS devices, the minimal dwell time usually ranged between 20 and 100 ms. On modern single quadrupoles, the dwell time and inter-channel delay were reduced down to 5 ms or less to address the narrow peaks produced in UHPLC.

Today, single quadrupole instruments are not widespread in combination with UHPLC, as shown in Figure 1B. This rarity is evidently related to the limited selectivity and sensitivity provided by such MS devices. In contrast, the triple quadrupole (QqQ), which operates under similar principles, is one of the most frequently used detectors for UHPLC-MS (see Figure 1B). With a QqQ instrument, the selected reaction monitoring (SRM) mode (equivalent to the MRM mode which stands for multiple reaction monitoring, but which tends to be less applied nowadays), which consists of two mass filtration stages, is the mode of choice for powerful data acquisition. In the first stage, the precursor ion is selected by the first quadrupole. Then, fragmentation occurs in the collision cell (second quadrupole) through collisions with an inert gas. Finally, specific fragments are monitored in the third quadrupole to increase both the sensitivity and the selectivity compared to the single quadrupole operating in the SIM mode. To improve the compatibility with UHPLC, the dwell time (time spent acquiring the specific SRM transition) and inter-channel delay of QqQ instruments have decreased considerably over the last few years while maintaining acceptable sensitivity. As shown in Table 1, QqQ instruments from various providers can accommodate dwell times and inter-channel delays in the range of 1 - 5 ms. Actually, the dwell time value should be selected as a compromise between sensitivity and data acquisition rate depending on the peak widths achieved with UHPLC and the number of compounds (or number of transitions) that must be monitored. It is

also important to mention that the MS/MS method can be segmented into various time-schedule windows that contain different SRM channels and time intervals, when the number of monitored compounds is too important and the dwell time is insufficiently low.

As shown in Figure 1B, the most widely used class of MS analyzers that is combined with UHPLC involves the time-of-flight-based technologies (including TOF/MS and QqTOF/MS). With such analyzers, it is possible to quickly record and store data over a broad mass range with high sensitivity and mass accuracy. The most significant progress over the last years that has been made with TOF/MS technology has been related to the reduction of the duty cycle, the enhancement of mass accuracy and resolving power and improvements in the quantitative performance of TOF/MS. When combined with UHPLC, the potential for fast acquisition speeds is of prime interest. As illustrated in Table 2, with the last generation of TOF/MS and QqTOF/MS instruments, high resolving powers (> 10,000 FWHM) can be attained at acquisition speeds of 10 to 40 spectra *per* second. However, it is also important to consider the type of detector that is employed with a TOF/MS, which is either a time-to-digital converter (TDC) or an analog-to-digital converter (ADC). Essentially, the TDC is an ion-counting detector that can be very fast but suffers from a limited dynamic range due to its inability to properly count the events when more than one ion simultaneously hit the detector. To avoid this problem, a "pre-scan" should be performed to enhance the dynamic range, leading to a reduction of acquisition speed by a factor of 2 with the TDC vs. the ADC detector. In addition, if parallel alternating scans are acquired from a single analytical run with a QqTOF/MS, the acquisition rate is again further reduced by a factor of 2. This approach is referred to as QUAL/QUAN or MS^E approaches, in which low collision energies are used to obtain precursor information and high collision energies are used to obtain full scan accurate mass fragments. When cumulating the negative impacts of the TDC detector and MS^E experiments on the duty cycle, an acquisition speed of 10-20 spectra/s can be insufficient. To overcome this issue, the triple TOF 5600 instrument was introduced by AB Sciex. This QqTOF/MS is able to acquire data with a resolution of up to 40,000 FWHM with 10 ms accumulation times (100 Hz acquisition rate). However, when, using such extreme conditions, the sensitivity and dynamic range are probably reduced. Based on these excellent features, this instrument can be employed for UHPLC-MS QUAL/QUAN operations [52,53].

Finally, there are also a growing number of applications that deal with the coupling of UHPLC to the Orbitrap, as shown in Figure 1B. The most recent generation of Orbitrap

analyzer (Thermo Exactive Plus and Thermo Q Exactive) is remarkably fast and able to operate at acquisition rates of 12 Hz. Nevertheless, this speed is to the detriment of performance (resolving power of only 17,500 FWHM at this speed) compared to the maximal resolution that can be theoretically attained (> 140,000 FWHM at 1 Hz). For this reason, modern TOF/MS or QqTOF/MS instruments provide a comparable performance at faster acquisition speeds, lower space requirements and more favorable prices [54]. If a technical solution can be found in the future to maintain the exceptional resolution of Orbitrap at elevated acquisition speed, this analyzer will probably become the most efficient one to be combined with UHPLC technology.

Because modern quadrupole-based MS instruments possess low dwell times and inter-channel delays (as low as 1 ms), they are perfectly adapted for targeted analyses and quantitative applications. However, TOF/MS and QqTOF/MS instruments can acquire data over a wide mass range with high mass accuracy and resolving power. For these reasons, TOF/MS and QqTOF/MS instruments are particularly well suited for untargeted analyses, provided that the duty cycle is sufficiently fast to perform several types of experiments simultaneously.

Table 1: Performance comparison of the current "low resolution" MS devices currently on the market.

	Model	Maximum acquisition speed	Minimum Dwell time	Minimum Interchannel delay	Polarity switching
Triple quadrupole	AB Sciex API 5000	2400 amu/s	5 ms	2 ms	700 ms
	Agilent series 6400 (6420, 6430, 6460)	5200 amu/s	1 ms	2 ms	30 ms
	Agilent 6490	10000 amu/s	1 ms	2 ms	30 ms
	Shimadzu LCMS-8030	15000 amu/s	1 ms	1 ms	15 ms
	Shimadzu LCMS-8040	15000 amu/s	0.8 ms	1 ms	15 ms
	Shimadzu LCMS-8080	5000 amu/s	1 ms	5 ms	20ms
	Thermo TSQ Quantum Access MAX	5000 amu/s	2 ms	no information	< 25 ms
	Thermo TSQ Quantum Ultra	5000 amu/s	2 ms	no information	< 25 ms
	Thermo TSQ Vantage	5000 amu/s	2 ms	no information	< 25 ms
	Waters Xevo TQ-S	10000 amu/s	1 ms	3 ms	20 ms
	Waters Xevo TQ MS	10000 amu/s	1 ms	3 ms	20 ms
	Waters Xevo TQD	10000 amu/s	3 ms	3 ms	20 ms
	Waters TQ Detector	10000 amu/s	3 ms	5 ms	20 ms
quadrupole - ion trap	AB Sciex Qtrap 3200	2400 amu/s	5 ms	2 ms	700 ms
	AB Sciex Qtrap 4000	2400 amu/s	5 ms	2 ms	700 ms
	AB Sciex Qtrap 5500	20000 amu/s	2 ms	3 ms	50 ms
	AB Sciex Qtrap 6500	20000 amu/s	1 ms	no information	20 ms
	Thermo LTQ XL	33300 amu/s	-	-	100 ms
Ion trap	Bruker Daltonics amaZon SL	32000 amu/s	-	-	< 80 ms
	Bruker Daltonics amaZon speed series	52000 amu/s	-	-	Zero delay alterning
	Thermo LCQ Fleet	66000 amu/s	-	-	no information
	Thermo Velos Pro dual pressure	33300 amu/s	-	-	no information

Information was obtained from advertising and from providers website in June 2012. Agilent Technologies, Waldbronn, Germany / Waters, Milford, USA / Bruker Daltonics, Bremen, Germany / Thermo Finnigan, San Jose, USA / AB SCIEX, Foster City, USA / LECO Corporation, St-Joseph, USA / Shimadzu Corporation, Kyoto, Japan / Perkin Elmer, West Chester, USA

Table 2: Performance comparison of the current "high resolution" MS devices currently on the market. Information was obtained from advertising and from providers website in June 2012. (1) speed affects resolution with Orbitrap and Fourier Transform MS.

	Model	Maximum resolving power	Mass accuracy	Maximum acquisition speed
TOF/MS	Agilent series 6200	20000 FWHM	< 1 ppm	40 spectra/s
	Bruker daltonics micrOTOF II	> 16500 FWHM	< 2 ppm	20 spectra/s
	Leco Citius LC-HRT	100000 FWHM	< 1ppm	200 spectra/s
	Perkin-elmer AxION 2 TOF MS	> 12000 FWHM	< 2 ppm	70 spectra/s
	Waters Xevo G2 and G2-S TOF	22500 FWHM	< 1ppm	30 spectra/s
QqTOF/MS	AB Sciex triple TOF 4600	30000 FWHM	1 ppm	100 spectra/s (50 spectra/s in MS/MS)
	AB Sciex triple TOF 5600	40000 FWHM	1 ppm	100 spectra/s (50 spectra/s in MS/MS)
	AB Sciex triple TOF 5600+	40000 FWHM	1 ppm	100 spectra/s
	Agilent 6538 and 6540	up to 40000 FMWH	< 700 ppb	20 spectra/s (10 spectra/s in MS/MS mode)
	Agilent 6530	> 20000 FWHM	< 1 ppm	40 spectra/s
	Agilent 6550	up to 40000 FMWH	< 700 ppb	50 spectra/s
	Bruker daltonics micrOTOF-Q II	> 17500 FWHM	1-2 ppm	20 spectra/s
	Bruker MaXis 4G	60000 FWHM	< 1 ppm	20 spectra/s (10 spectra/s in MS/MS mode)
	Bruker MaXis impact	40000 FWHM	< 1 ppm	50 spectra/s
	Waters Xevo G2 and G2-S QTOF	22500 FWHM	<1 ppm	30 spectra/s
	Waters Synapt G2-S MS and HDMS	up to 50000 FWHM	< 1 ppm	30 spectra/s
Orbitrap (1)	Thermo exactive plus	> 140000 FWHM	< 1 ppm	12 spectra/s (at 17500 FWHM)
	Thermo LTQ orbitrap XL	> 100000 FWHM	< 1 ppm	1 spectra/s (at 60000 FWHM)
	Thermo orbitrap elite	> 240000 FWHM	< 1 ppm	4 spectra/s (at 60000 FWHM)
	Thermo orbitrap Velos Pro	> 100000 FWHM	< 1 ppm	1 spectra/s (at 60000 FWHM)
	Thermo Q exactive	> 140000 FWHM	< 1 ppm	12 spectra/s (at 17500 FWHM)
Fourier transform (1)	Thermo LTQ FT ultra	> 750000 FWHM	< 1 ppm	1 spectra/s (at 100000 FWHM)
	Varian 910-MS FTMS	> 400000 FWHM	< 1.5 ppm	no information

2.3.2. Polarity switching and multimode ionization

A possibility offered by recent MS detectors is to perform simultaneous detection with both positive and negative ionization during the same run [55,56]. As shown in Table 1, polarity switching times have drastically been reduced in most of mass spectrometers to as low as 15 ms to improve productivity in the cases in which it is necessary to simultaneously analyze both acidic and basic analytes. Nevertheless, because UHPLC peak widths are relatively small and the MS works sequentially, the use of polarity switching can reduce the number of data points across peaks and compromise the quantitative performance. This application has been illustrated elsewhere for a mixture of basic and acidic drugs [45]. However, polarity switching is well adapted for qualitative purposes, and only a reasonable loss in sensitivity, approximately 25%, was previously reported using this approach with UHPLC-MS [45].

Another option available on modern MS devices is the use of multimode ionization sources that are able to simultaneously acquire data in both ESI and APCI modes [57]. The time required to switch from ESI to APCI has been reduced to only 20 ms for the best instruments on the market. Again, the use of the multimode ionization source significantly improves productivity, but it compromises the sampling rate. For this reason, it should only be adapted for the qualitative evaluation of a mixture of unknown compounds.

In conclusion, modern MS devices theoretically allow the collection of data in ESI+, ESI-, APCI+ and APCI- modes to maximize the information gathered from a single run. However, this approach is hardly compatible with UHPLC because the peaks are too narrow. It is thus not advisable to use such conditions, particularly if quantitative data are required.

Finally, there are also some additional issues related to the use of UHPLC-MS platform. As example, the sizes of the inlet and outlet column frits are drastically reduced in UHPLC (~0.2 µm), compared to a conventional HPLC column (~2 µm). For this reason, there is higher risk of column clogging and failure in presence of dirty biological matrixes. It is thus advised to use a suitable sample preparation to maintain acceptable column lifetime, particularly when using plasma sample. Some pre-columns as well as in-line filters are available from most of the providers and are particularly recommended for bioanalytical applications using UHPLC-MS devices. Using a pre-column, more than 1000 injections of plasma or urine can be expected on a UHPLC stationary phase, without any loss of performance.

3. MAIN APPLICATIONS FOR UHPLC-MS

For the rest of the review dedicated to the applications of UHPLC-MS, the experiments were exclusively carried out in RPLC or HILIC modes. Size-exclusion chromatography (SEC) or Ion exchange chromatography (IEX) modes were generally not employed since there are only a limited number of commercially available phases packed with sub-2µm particles. In addition, the mobile phases employed in SEC or IEX contain non volatile salts, hardly compatible with MS device. It is finally important to notice that the Waters Acquity Shield RP18, which is a monofunctional bonded column dedicated to UHPLC operation provide a strong bleeding, that can be critical when performing untargeted analysis with a UHPLC-TOF/MS platform.

3.1. Bioanalysis

Bioanalysis involves the targeted analysis of drugs in biological systems. It includes many types of biological matrices such as: plasma, urine, saliva, tears, cerebrospinal fluid, hair, tissues and organs. The analytes of interest are typically vastly outnumbered by endogeneous compounds that can potentially interfere with the detected signal. Consequently, to minimize matrix effects, analytical procedures have been improved at various levels including the following: i) sample preparation techniques: higher sensitivity and selectivity, ii) chromatographic separations: higher resolution, limiting the likelihood of interfering compounds co-eluting with the analytes, iii) detection: more sensitive and selective based on MS/MS transitions and iv) quantification: use of internal standards to monitor potential interferences and allow accurate quantification. In this context, tandem mass spectrometric detection remains the gold standard and has been applied in combination with UHPLC to solve various bioanalytical problems [58-64]. However, other types of analyzers could be used, including Q-trap instruments [65] (for the quantification of drugs and its metabolites in plasma) or hybrid QqTOF/MS [66] (for the confirmation of illicit substance intake during sports competitions). However, there is a critical demand for high-throughput capacities due to the continuous increase in the number of samples to be analyzed and the required short response time.

Another obvious advantage of UHPLC-MS bioanalysis is related to the number of samples that can be analyzed (high throughput) and the potential to use low sample volumes. In this context of small sample volume, the use of microextraction by packed sorbent (MEPS) is a promising approach. It is fast, simple and requires low solvent and sample volumes (as low as 10 µL). The MEPS approach was successfully applied for the analysis of cholesterol-lowering drugs in plasma, serum and urine demonstrating its potential for pharmacokinetic studies [61,67]. The coupling of such a sample preparation procedure to the UHPLC-MS/MS analytical method enables accurate quantification using specific MS/MS transitions [61]. The concept of high-throughput microscale bioanalysis has gained prominence and was also recently illustrated in UHPLC-MS/MS studies evaluating the kinetic and organ distribution of an immunosuppressive drug following ocular application [58,68]. The drug was administered as an eye drop into rodent eyes, after which samples were collected and analyzed. This microscale bioanalysis was challenging due to i) limited sample weights ranging from 2 to 10 mg, ii) low expected drug concentrations, iii) the presence of complex biological matrices and iv) the considerable number of samples to be analyzed. The developed UHPLC-MS/MS method met the analytical requirements by affording high sensitivity (LLOQ of 2 ng/mL), in addition to elevated selectivity and robustness, allowing high-throughput accurate drug quantification in biological samples.

The quantification of drugs in biological samples is the cornerstone of pharmacokinetic studies (i.e., evaluation of the *in vivo* behavior of drugs) and therapeutic drug monitoring (i.e., individual patient drug-level supervision). Anti-dementia drugs represent a typical case of drug therapeutic monitoring. Noetzli and coworkers transferred a routine HPLC-MS method to UHPLC-MS/MS for simultaneous anti-dementia drug monitoring. The UHPLC-MS/MS method allowed high sensitivity and selectivity with a fast analysis and simple sample preparation [60]. The mentioned advantages led to the replacement of traditional HPLC-MS method with new UHPLC-MS/MS method for routine analyses in hospital laboratories. Another application was related to a platelet aggregation inhibitor, clopidogrel, which required systemic levels monitoring due to the variability of the therapeutic effects. Peer *et al.* developed a UHPLC-Q-trap/MS method for the study of clopidogrel in human plasma in a high-throughput manner [65].

Furthermore, it is important to be aware of the influences that physiological and environmental factors have on pharmacokinetics. Pregnancy is a unique condition in which many physiological parameters are altered, modifying drug pharmacokinetic profiles. Nevertheless, pregnant women suffering from chronic or acute diseases still need to be medicated. The study of drug transfer through the placenta is of paramount importance for the evaluation of potential consequences to the fetus. Tertti *et al.* developed a UHPLC-MS/MS method for the quantification of the transmission of a hypoglycemic agent through the placenta [59]. The experiment, conducted in placentas, demonstrated that repaglinide had a limited transfer to the fetus. Additionally, a UHPLC-MS/MS method allowed Boscarino *et al.* to study the effect of external stressors in ibuprofen pharmacokinetics. Their study showed that Afghanistan soldiers who were exposed to high levels of stress had higher ibuprofen absorption rates [63].

Finally, the evaluation of drug profiles is crucial in bioequivalence studies. Palet *et al.* developed and validated a UHPLC-MS/MS method for nifedipine quantification in plasma, allowing the comparison of a novel formulation to the marketed product [62]. The UHPLC-MS/MS method displayed high sensitivity and high-throughput capabilities, demonstrating the relevance of using this technique for bioequivalence studies.

In the case of bioanalysis, the throughput was important, because of the significant number of samples to be analyzed. For sample preparation, SPE remains the method of choice and the 96 well plates format could be of interest. From a chromatographic point of view, a UHPLC column of 50 x 2.1 mm was suitable since the number of analytes to separate was limited. A flow rate around 300-600 µL/min was generally used and the mobile phase often consisted of ACN + 0.1% formic acid and water + 0.1% formic acid. For MS detection, triple quadrupole operating in the SRM mode provide appropriate selective and dwell times were generally set in the range 5 - 20 ms, depending on the number of analytes to monitor.

3.2. Drug metabolism studies

Metabolism reactions can be divided into two categories: phase I reactions, which are mainly performed by cytochrome P450 (CYP) enzymes, and phase II reactions, which are conjugations. Drug metabolites form a heterogeneous population of structurally related compounds with divergent physicochemical properties, making their analysis challenging. Metabolism studies through LC-MS systems require high-resolution and fast-separation chromatography coupled with a highly sensitive and selective detection. These analytical needs are undoubtedly met by UHPLC-MS techniques, as illustrated by Figure 1C.

Metabolism studies are performed throughout the entire drug life cycle. First, metabolic stability should be evaluated during the early stages of the drug discovery process. For this purpose, high-throughput targeted analyses are essential due to the large number of compounds that must be screened. The UHPLC-MS approach represents an advantage that enables fast or ultra-fast separations. Xu *et al.* took advantage of the UHPLC-MS technology to develop a high-throughput metabolic stability screening using pooled samples placed into 96-well plates and a UHPLC-MS/MS method with run times of 1.3 min [69]. They were able to apply a pooling strategy due to the high resolving power and sensitivity afforded by the UHPLC-MS/MS analysis. Additionally, Plumb and coworkers observed an 80% reduction in the analysis time when using UHPLC techniques instead of conventional HPLC for metabolic stability testing [70]. A novel in-capillary approach was developed by Nicoli *et al.* that enables the analysis of the metabolic stability of a candidate compound in less than 10 min [71].

Second, the identification of drug metabolites is of major importance for the elucidation of metabolic pathways and the detection of potentially dangerous metabolites. This untargeted analysis requires high resolving powers due to the high complexity of the metabolic mixtures that include isomers and isobaric compounds. This special requirement explains the predominance of hybrid TOF detectors for this application. Particular attention is often paid to active metabolites that require readjustments to the administered drug dose. Additionally, reactive metabolites that are responsible for toxicity are intensively studied for their screening, identification and structural elucidation [72]. Liver microsomal cellular fractions are the most commonly used approach for *ex vivo* metabolic investigations [72,73]. However, the main limitation of these cellular fractions is their lack of common phase II conjugations.

Alternatively to microsomal fractions, hepatocyte cell culture assays can be performed for the determination of metabolic pathways, as proposed by Gu *et al.* for the study of an anticancer drug metabolism [74]. However, *in vivo* studies remain crucial. Shan *et al.* administered a novel cardiovascular treatment to rats and collected bile, feces, urine and plasma. UHPLC-QqTOF/MS enabled the identification, characterization and structural elucidation of the drug metabolites [75]. As shown in Figure 4, this study determined the drug metabolism profiles of each sample type. It should be noted that the continual improvement of analytical techniques allow disclosure of unreported metabolites even years after drug approval [76].

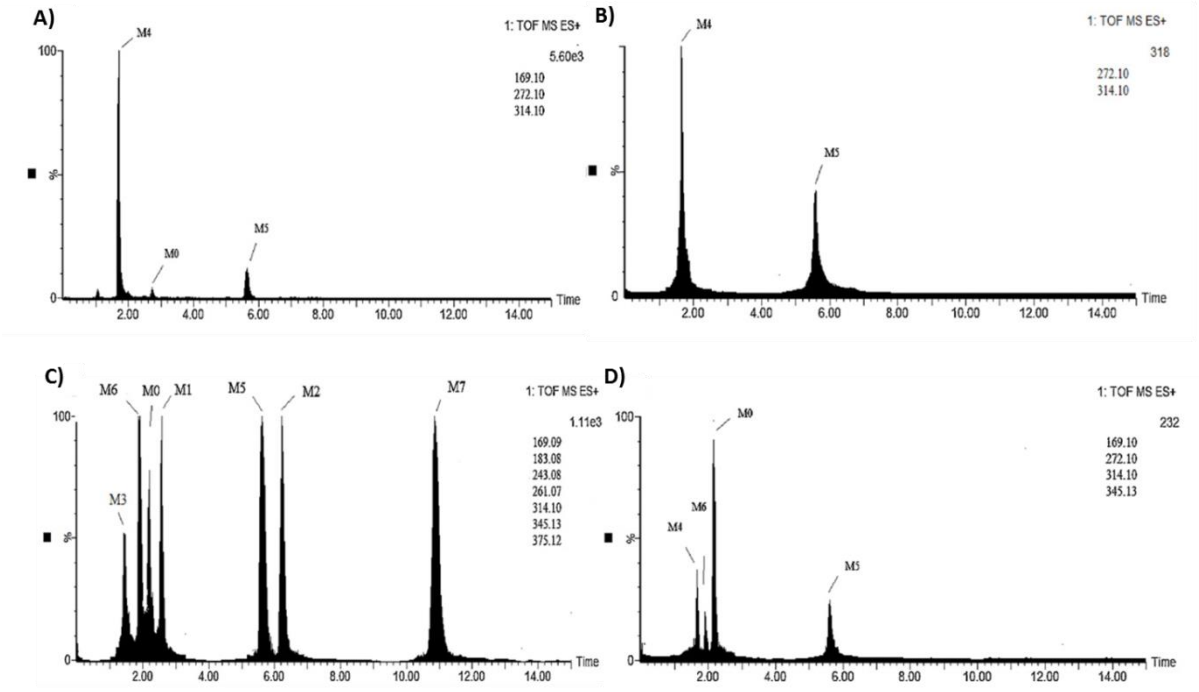


Figure 4: UHPLC-QqTOF/MS extracted ion chromatograms of rat bile, feces, urine and plasma samples after i.v. administration of liguzinediol. A) Bile samples, B) feces samples, C) urine samples, D) plasma samples. From [75], with permission.

Later in the drug life cycle, the activity of CYP enzymes should be monitored for various purposes, including i) to study the induction or inhibition effects of drugs during their development or ii) to measure the CYP activity in patients (e.g., during clinical trials). In this context, high-throughput and quantitation capabilities are critical. Interestingly, Petsalo *et al.* developed a new strategy allowing the activity evaluation of the nine most important CYP

isoenzymes *in vivo* using a UHPLC-MS/MS method [77]. An interesting approach for the *in vitro* evaluation of CYP activity was developed by Curcio *et al.* based on a 1.4 µL capillary loaded with CYP enzyme baculosomes and the candidate drug solution [78]. After incubation, UHPLC-MS/MS was used for the quantification of parent drugs and metabolites. This low-cost and versatile approach showed similar results when compared to classical incubations.

Finally, metabolite quantification is required throughout the entire drug discovery process, from drug characterization to final approval. This quantification is particularly demanded for active, reactive and major drug metabolites. The quantification of metabolites may also be of interest for the understanding of observed inter-individual variabilities in therapeutic responses and secondary effects among patients. Precht *et al.* used a UHPLC-MS/MS technique to quantify an anti-cancer drug and two of its metabolites in plasma, allowing the elucidation of the mechanisms behind the observed variabilities in the drug metabolism, toxicity and treatment efficiency [79].

Data processing represents the main bottleneck of UHPLC-MS metabolism analyses. There is a serious need for automated data treatment techniques. Complex software programs are today capable of hypothesizing metabolite compositions and structures as well as biotransformation sites and mechanisms.

In the case of drug metabolism studies two situations can be found. The first one is the analysis of known or predicted compounds in a targeted manner. These type of analyses is related to metabolic stability profiles, assessment of CYP activity or quantification of metabolites. These analyses involve an important number of samples, thus the throughput is important. A UHPLC column of 50 x 2.1 mm heated at 50°C is typically used. A flow rate around 500 µL/min and a mobile phase of ACN + 0.1% formic acid and water + 0.1% formic acid is often employed. For MS detection, triple quadrupole operating in the SRM mode with dwell times of 5 - 20 ms is generally used. The second one is the identification of drug metabolites and metabolic pathways. It requires an untargeted analysis with high resolution power. It is frequently based on 100 or 150 x 2.1 mm UHPLC columns heated at 40°C and a mobile phase composed of ACN + 0.1% formic acid and water + 0.1% formic acid at flow rates ranging from 300 to 500 µL/min. The detection is often performed with a hybrid TOF/MS.

3.3. Multi-analyte / multi-residue screening

Multi-residue screening aims to allow the evaluation of the presence of a large number of compounds in a cost-efficient manner. The potential for high-throughput is essential for this application, which involves the analysis of numerous complex samples, such as food, environmental or biological matrices. Special efforts should be made to lower the risk of false positive and false negative results. The analytical procedure of a multi-residue screening involves different steps, including screening, quantification and confirmation. This approach faces analytical challenges related to i) the high number of compounds of interest that need to be monitored, ii) the very low concentrations of analytes, iii) the need for confirmation, structural elucidation and/or identification and iv) the complexity of the matrices containing the analytes. In this context, the UHPLC-MS technology represents a serious advantage over HPLC-MS, allowing the reduction of the response delivery time throught the significant increase in throughput.

Nevertheless, sample preparation is a key step that often represents a limitation to the whole analytical process. SPE is still the most widely used technique in multi-analyte screening, even though it is hardly compatible with high throughput. Many efforts have been dedicated to the development of faster sample preparation techniques in line with the excellent performance of UHPLC-MS instruments. Two examples are i) the "dilute and shoot" approach used for the screening of veterinary drugs [80] or doping agents in urine [81,82] and ii) the protein precipitation method applied for the screening of veterinary drugs in milk [83]. Interestingly, microextraction procedures are a new trend in sample preparation procedures but are not yet widely applied in combination with UHPLC-MS.

It is worth mentioning that the UHPLC-MS technology is particularly interesting to limit matrix effect. As example, Van De Steene *et al.* demonstrated that the UHPLC-MS allows a reduction or even elimination of the matrix effects compared to conventional HPLC-MS for the analysis of aqueous environmental samples [84]. UHPLC-MS undoubtedly represents a powerful tool for multi-residue analysis, considering the high resolution and fast separation provided by the LC system together with the high sensitivity and selectivity afforded by the MS detector. In this context, the choice of the mass spectrometer is of major importance.

For quantification and targeted analysis, it is generally accepted that triple quadrupole instruments are the gold standard. Many multi-residue analyses have been conducted with QqQ instruments. For example, tandem mass spectrometry was used for the analysis of

perfluorinated compounds, sulfamides and veterinary drugs in milk and fish samples [85-87] and for the simultaneous determination of more than 90 compounds with a LOQ equal to or below 10 µg/kg within a single 13 min run, as illustrated in Figure 5 [88]. The excellent quantitative capabilities of QqQ instruments allow the quantification of 25 analytes at ultratraces levels (LOQ between 0.002 and 0.013 µg/kg) in a 9 min run [85]. Beyond quantification, QqQ instruments are sometimes limited for confirmatory purposes. Indeed, two different MS/MS transitions were found to be insufficient to unequivocally prove the presence of sebuthylazine in tarragon [89]. In addition, QqQ detectors are highly challenged when the number of analytes exceeds 200 due to limitations linked to minimal dwell times (see Table 1) and retention time variabilities when using time-schedule windows. An impressive example of an analytical method based on a triple quadrupole detection for multi analyte screening is the one developed by Mol *et al.* for the analysis of 258 compounds in food matrices [90]. Currently, there is a new trend in this field to use HRMS, such as TOF/MS or even hybrid TOF/MS technologies, among others. Many studies have been conducted to compare the performances of the above-mentioned mass spectrometer detectors [80,91-95]. These investigations have demonstrated the advantages of using HRMS which i) allow the screening of an unlimited number of residues, ii) provide evidence for confirmatory analyses or structural elucidations and identifications, iii) enable non-targeted or post-targeted analyses, iv) display high flexibility and versatility (i.e., no need for reference material for each compound) and v) afford high sensitivity. Such HRMS detectors make possible the screening of an elevated number of compounds with good sensitivities. Lacina *et al.* took advantage of the TOF technology for the screening of 212 pesticides with a limit of detection equal to or lower than 10 µg/kg [96]. An hybrid TOF/MS was used by Grimalt *et al.* to screen 423 pesticides with a sensitivity of 300 fg [95]. When compared with QqQ, the QqTOF/MS represents a good trade-off between the number of screened compounds and the sensitivity. Indeed it allowed the screening of a high number of compounds (up to 423 compounds) with a reasonable sensitivity (LOQ only 3 times higher than the QqQ).

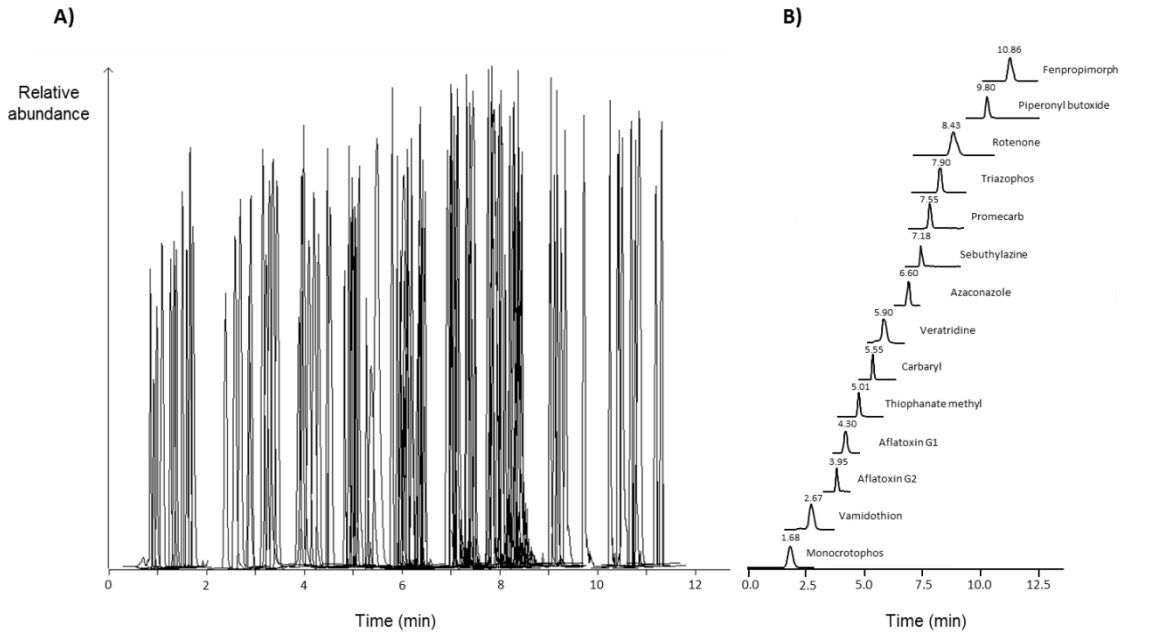


Figure 5: A) Combined UHPLC–MS/MS chromatogram from a blank wheat sample spiked at 50 μg.kg^{-1}, based on quantifying MS/MS transitions, and B) selected UHPLC–MS/MS chromatograms. From [88], with permission.

The screening capabilities of TOF/MS instruments have also been highlighted in many studies. The accurate mass measurements together with the high sensitivity of full spectrum acquisition modes make TOF/MS a valuable tool for screening a large number of residues [83,96]. Wang *et al.* took advantage of hybrid QqTOF/MS performances for the analysis of estrogens, progesterones and phenols in potable and river water of Shanghai, China [97]. Interestingly, the screening was performed through a targeted analysis of 21 known compounds. Following the targeted analysis, a post-targeted analysis based on the search and identification of additional analytes was performed without supplementary experiments. This second approach allowed the detection of an unexpected estrogen metabolite at 5 ng/L in the river water. This work highlighted the great potential of QqTOF/MS instruments for simultaneous screening, confirmation and structural elucidations and post-targeted analyses. Another attractive option afforded by the QqTOF/MS instrument is the MS^E mode, allowing simultaneous screening, confirmation, identification and elucidation, as demostrated by Hernandez *et al.* [98]. Nevertheless, a genuine non-targeted analysis has not yet been achieved, partially due to data treatment issues. It is indeed challenging to identify and extract

interesting information from the overwhelming amount of generated data. Hence, there is an urgent need for software programs that allow a faster analysis of the obtained high-quality data. The main needs are for the deconvolution of signals prior to abundance ratio searches, easier file handling and new diagnostic tools and algorithms.
Finally, it is worth mentioning that the Orbitrap HRMS detector has also opened new possibilities for multi-analyte screening, despite a relatively slow duty cycle, which is illustrated in Table 2. The Orbitrap was found to be particularly well suited for multi-residue screening in complex matrices, such as honey and liver samples [92]. This new MS detector also enabled the analysis of marine toxins in shellfish, which involved both screening and construction of compound libraries [99,100].

In the case of multi-analyte / multi-residue screening an equilibrium between high throughput and high resolution has to be found. The most widely used sample preparation technique is SPE. The posterior targeted analysis often involves UHPLC column of 50 to 100 mm long and 2.1 mm internal diameter. A flow rate around 400 µL/min and a mobile phase of ACN + 0.1% formic acid and water + 0.1% formic acid are often employed. The elution is performed in the gradient mode and takes from 5 to 20 min. The MS detection can be performed either with a triple quadrupole operating in the SRM mode with very short dwell times (down to only 1 ms in some case) or with a QqTOF instrument.

3.4. Metabolomics

The field of metabolomics fulfills the need of understanding the cellular metabolic signature that genetics, environment, diseases or interventions leave behind. This approach provides a snapshot of the cellular activity at the moment of analysis. The analytes of interest are embedded in complex biological matrices, such as cells from tissues, fluids or organs, which represents the major challenge of metabolomics. Thus, it is crucial to achieve high-resolution separations that prevent endogenous interferents from suppressing or enhancing the ionization of the molecules of interest. In addition, elevated peak capacity and high sensitivity are required to detect and quantify multiple low-abundance metabolites that are often closely chemically related. Moreover, a detector that can provide high accuracy is necessary for the identification. Thus, the coupling of UHPLC to MS detection represents a powerful tool for metabolomic studies, shortening the chromatographic runs from hours to minutes compared to conventional LC.

3.4.1. Human and animal studies

Metabolomic investigations performed in animals and humans typically have the following two purposes: i) the understanding of the pathologic mechanisms behind certain diseases or ii) identification and quantification of biomarkers for diagnostic or prognostic purposes. Depending on the desired application, a compromise must be found between the quantitative and qualitative approaches. Detectors with high resolving power and high accuracy are preferred for identification, while high sensitivity and selectivity are ideal for quantification. In most cases, a TOF/MS analyzer with fragmentation capabilities is the detector of choice. However, it is not uncommon to utilize QqQ analyzers for targeted metabolomic studies [101,102] or even inductively coupled plasma mass spectrometry for metabolic profiling studies [103].

The resolving chromatographic power is essential to separate as many metabolites as possible from complex biological matrices, such as plasma, cerebrospinal fluid, amniotic fluid, urine, dried blood spots, bile, liver tissue and tumors. The use of sub-2µm particles represent an advantage in this field allowing the separation of a higher number of compounds compared to conventional HPLC. This was evaluated in a former study where a 20% increase in the number of detected peaks was reported when UHPLC and HPLC conditions were compared [104].

Many studies have been conducted to characterize diseases through the analysis of biomarkers. The UHPLC technology was found to be a tool particularly well suited for such investigations. Former studies performed by Lame and co-workers demonstrated the excellent characteristics of UHPLC/MS analysis when compared to currently used immunological methods for the evaluation of Alzheimer's disease through biomarkers [101]. In this study, the achieved sensitivity was as low as the one obtained with ELISA. In addition UHPLC/MS analysis was found to: i) allow a higher throughtput, ii) be less labor intensive, iii) present less variability, iv) allow simultaneous quantification and v) be more economical. On the other hand, several UHPLC/MS methods were at the basis of the improvement of fundamental knowledge on renal failure mechanism [105] or the effect of age on liver metabolism [106].

Finally it is important to note that the hydrophilic interaction liquid chromatography (HILIC) is a mode frequently used in metabolomic studies. The HILIC mode was demonstrated to have an orthogonal selectivity compared to RP mode; together improving the metabolomic coverage [107]. Chen et al. took advantage of the combination of RP and HILIC modes associated with either a positive or a negative ion detection for the detailed characterisation of the metabolic abnormalities behind the acute renal graft rejection [108]. The same strategy was applied for the study of biomarkers in amniotic fluid and urine of pregnant women for the evaluation of foetal malformations or the prediction of pre-preterm delivery [109].

3.4.2. Plant studies

Plants are used as a source of nutrients and/or therapeutics. The study of their metabolism can help to better understand such applications and provide new information about their metabolic pathways.

Within this context, UHPLC-MS is a valuable tool for the discovery of new chemical entities that exhibit a positive effect on health. Wolfender *et al.* pointed out the benefits of using a UHPLC-TOF/MS platform for fingerprinting, profiling, isolating and identifying of natural product drugs [110], as illustrated in Figure 6.

UHPLC-TOF/MS platforms have not only allowed the analysis of medicinal plants such as ginseng or hypericum [111,112] but also the profiling of tomatoes, berries, apples, strawberries, raspberries, cherries, wine and tea [113,114]. In addition, the study of fundamental vegetal metabolism can be achieved with the model plant, *Arabidopsis thaliana*. For example Glauser *et al.* identified a novel jasmonate glucoside that was produced in response to a wound, allowing a better understanding of the process of responses to wounding in plants [115].

In the case of metabolomic investigations, the resolution is paramount. This untargeted analyses often involves 150 x 2.1 mm UHPLC columns used in RP or HILIC mode. The mobile phase is composed of different proportions of ACN + 0.1% formic acid and water + 0.1% formic acid (depending on the selected mode) at a flow rate of 500 µL/min with gradient times of 10 min. A highly accurate detection based on TOF or QqTOF instruments is of major importance for identification purposes.

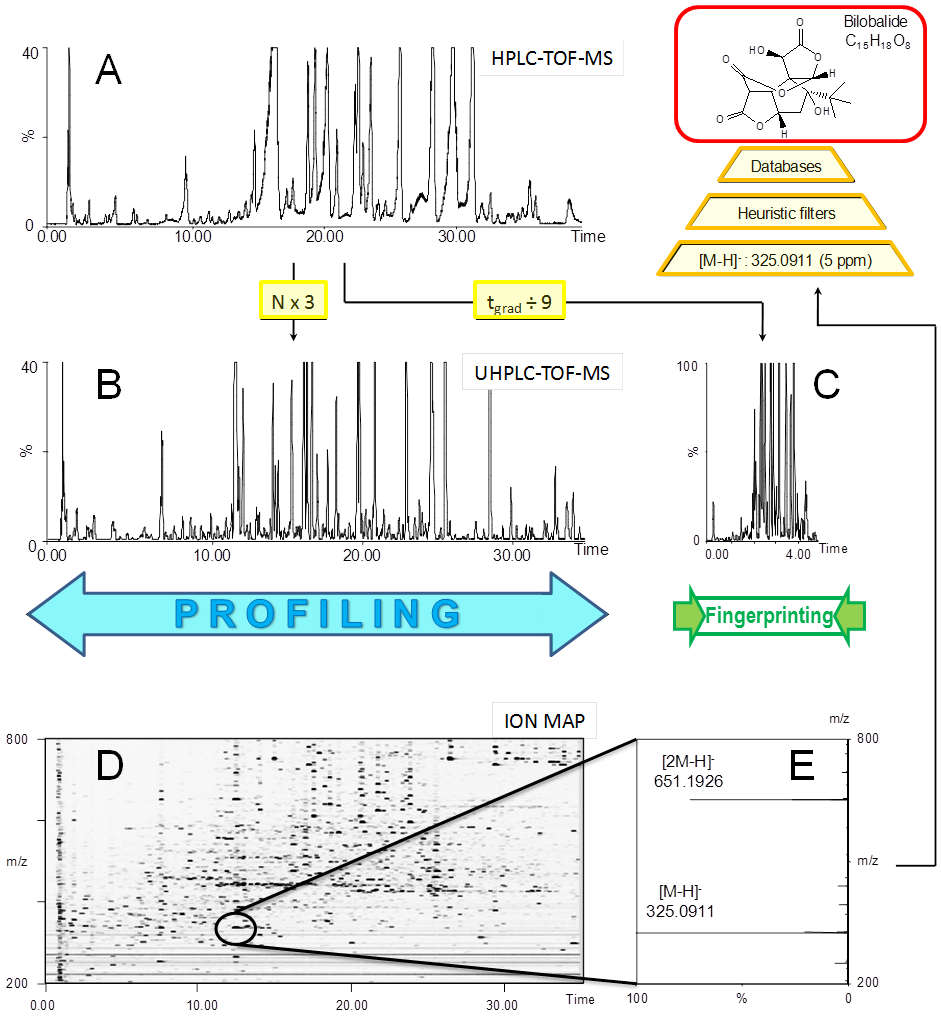


Figure 6: *Ginkgo biloba* metabolomics. Profiling by (A) Classical HPLC or (B) UHPLC with a 150 mm column and fingerprinting (C) UHPLC with a 50 mm column. (D) UHPLC-TOF/MS 2D ion map of the separation and (E) TOF-MS spectrum for final formula assignment. Adapted from [110], with permission.

3.5. Biopharmaceuticals

The biopharmaceutical field has gained much interest over the last few decades. Today, biopharmaceuticals are considered to be commonplace therapeutics, i.e., insulin, human growth hormone or immunoglobulins. Nevertheless, biopharmaceuticals are challenging analytes due to their complexity and microheterogeneity, which are increased by slight potential variations during production, extraction, purification, formulation and storage. Consequently, a large number of complementary techniques are required for biopharmaceutical analyses. Recently, new RPLC-MS/MS approaches found their application in this field due to their high chromatographic performance, high-resolution and throughput, and the identification capacity conferred by mass spectrometric detection.

A large proportion of biopharmaceutical products are therapeutic proteins or peptides that can be analyzed either in their intact form or as smaller peptides using a bottom-up approach following trypsin digestion of the protein. In this context, the resolving power of the chromatographic separation must be carefully optimized because there is a linear correlation between the peak capacity and the number of peptides that can be identified [116].

However, the chromatographic analysis of therapeutic peptides and proteins can be challenging due to their inherent characteristics, including their i) high resistance to mass transfer because of small diffusion coefficients, ii) high tendency to interact with residual silanols and iii) high adsorption potential. These characteristics explain their chromatographic behavior which is observed as poor peak shapes, severe band broadening and low resolution. Different strategies have been designed to confront these issues, including the use of sub-2 µm particles, addition of ion-pairing agents, application of higher column temperatures, and development of more adequate stationary phases. The more adequate stationary phases include those with reduced silanol activity (to limit slow secondary ionic interactions between positively charged biomolecules and weakly acid silanols), or those with improved particle morphology (e.g. core-shell type particle, wide pore size) [28,117]. According to the Van Deemter equation, the use of sub-2 µm particles improves the interaction kinetics, which is critical for large molecules. These effects are emphasized by using column temperatures between 35 and 50°C [117-120] and up to 90°C [28,121], allowing a lower resistance to mass transfer while preventing the analytes from degradation. Another strategy consists of adding an ion-pairing reagent to the mobile phase to prevent interactions with residual silanols and encourage the elution of the analytes as symmetric, narrow peaks. Even if trifluoroacetic acid

has shown excellent properties for this use, formic acid is preferred when using MS detection. Thus, 0.1% formic acid is commonly employed for the analysis of biopharmaceuticals [118-120,122,123]. Finally, new UHPLC instruments dedicated to the analysis of large biomolecules, known as bio-inert systems, have been developed to address the adsorption issue. If suitable analytical conditions are employed for peptides and proteins analysis, peak widths at baseline of 2-4 seconds can be achieved in UHPLC, as shown elsewhere [124].

Another issue related to the analysis of biopharmaceuticals, including peptides, intact proteins and tryptic digest of proteins is the lack of sensitivity. To improve limits of detection, nano columns of 75 µm I.D. packed with sub-2µm particles can be employed using dedicated nano UHPLC system. With such column dimensions, it is possible to significantly improve the sensitivity by injecting relatively large amount of sample, leading to small elution volumes increasing analyte concentrations. However, there are a number of issues with a nano-UHPLC configuration, including large gradient delay volume, significant extra-column volume contributions from the instrument, possible concerns when coupling to MS devices, lower pressure tolerability of the capillary column frits, limited overall robustness… To resolve these problems, some providers now propose all-in-one integrated strategy which replaces traditional fittings, columns, and emitters typically used for nano separations. Such devices commercialized under the names Waters Trizaic or Agilent Technologies chip cube, provide a more efficient and improved workflow for proteomics.

A recent study compared the performance of standard-flow UHPLC device and nano-flow LC system, using the same MS/MS device for the quantitation of biomarkers proteins [124]. The following conclusions were drawn: both systems gave reproducible and reliable results. However, if there are sufficient sample, the standard flow UHPLC-based platform can be used with no loss of sensitivity. This platform should be selected because of its superior robustness and ease of use. However, for the analysis of very small amounts of sample, the use of a nano-flow system with its smaller sample loading, would still be advantageous.

The numbers of biosimilarity studies have increased as the expirations of various patents of marketed products are approaching. The comparison of innovative products with biosimilar candidates is a complicated task, and it requires complementary analytical methods. Regulatory authorities identified three main properties that have to be investigated in biosimilarity studies: i) post-transcriptional modifications, ii) three-dimensional structures and iii) protein aggregation [125]. The onset of the UHPLC-MS technology was one of the major

technological breakthroughs making possible to face the challenge of investigating biosimilars [126]. A UHPLC-MS/MS method allowed Xie and coworkers to identify an unexpected change in the peptide sequence of a biosimilar candidate of a monoclonal antibody [127], as illustrated in Figure 7. Moreover, monoclonal antibodies are extensively studied with regard to their modifications (i.e., glycosylation, deamination, isomerization and/or oxidation) [118,123,127], and their *in vivo* concentrations (e.g., for preclinical studies) [120,122]. The quantitative performance of the UHPLC-MS methods has been continuously improved, and the limits of quantification today are as low as those attained with immunoassays such as ELISA [120].

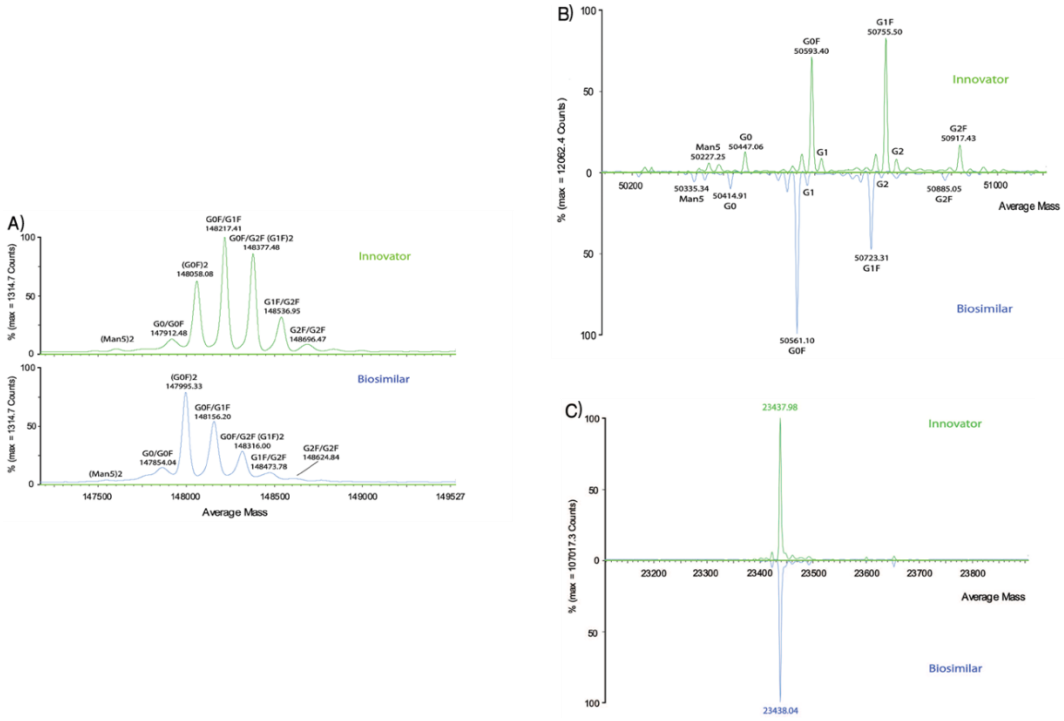


Figure 7: Comparison of deconvoluted masses between innovator and biosimilar antibodies. A) intact antibodies, B) mirror plot of heavy chain and C) mirror plot of light chain. From [127], with permission.

Beyond the analytical performance, data treatment and interpretation remain crucial for biopharmaceuticals. Intact protein analysis requires advanced software for the deconvolution of the complicated *m/z* signal, while the peptide-mapping strategy requires a large biological database to deduce the protein structure from the digested peptides.

In the case of biopharmaceuticals, the resolution is of major importance. This targeted analyses often involves 150 x 2.1 mm UHPLC columns heated at temperatures above room temperature but reasonable to ensure the analytes stability. The mobile phase is often composed of ACN + 0.1% formic acid and water + 0.1% formic acid. The detection is often based on a QqTOF instruments for high accuracy and resolution.

3.6. Analysis of polar compounds

Hydrophilic interaction liquid chromatography (HILIC) is an attractive analytical strategy for polar compounds, which are challenging analytes for RPLC systems. This technique presents a number of advantages, including i) improved retention of polar compounds, ii) improved peak shapes, iii) improved sensitivity due to the higher percentage of organic solvent in the mobile phase which enhances the ESI ionization process and iv) different selectivity than RPLC, being an orthogonal method. This new field combines the advantages of UHPLC-MS/MS with those that are related to HILIC, and it enables the analysis of polar compounds with higher resolution, sensitivity and selectivity.

The HILIC-UHPLC-MS/MS technique was shown to lead to higher peak capacity and higher resolution than HPLC-MS/MS; as demonstrated in extensive comparisons [128,129]. In addition, HILIC-UHPLC-MS/MS displayed better performance than RP-UHPLC-MS/MS for the analysis of polar compounds with its improved retention and peak shape, together with its different selectivity. Nováková *et al.* demonstrated that the HILIC technology afforded slightly higher sensitivity (LOQ of 0.1 pg compared to other RP methods with sensitivities between 0.8 and 2 pg), better linearity and higher accuracy compared to UHPLC-MS/MS [130].

Additionally, the composition of sample dilution media is of major importance under HILIC conditions and must be similar to the mobile phase, which contains at least 70% organic solvent [131]. This can have a positive impact on sample preparation. Indeed, the “dilute and shoot” approach involves the direct dilution of a sample using appropriate media for the subsequent analysis. This strategy was successfully implemented by adding acetonitrile to urine samples placed in a 96-well plate prior to their analysis for nicotine quantification. This sample treatment method allowed for high throughput, which is necessary for anti-doping applications, and an improved sample clean-up using to protein precipitation [132].

Additionally, using SPE, the use of an eluent with the same composition as the mobile phase avoided the evaporation/reconstitution step, simplifying and accelerating the sample preparation [130,133]. Nevertheless, special attention must be paid to the solubilities and compatibilities of analytes with organic solvents, particularly in the case of proteins for which natural folding can be altered.

HILIC has also been useful tool for the quantification of drugs in biological matrices. Nováková and coworkers developed a HILIC-UHPLC-MS/MS method for the analysis of entecavir in rat urine [130]. Additionally, HILIC-UHPLC-MS/MS makes possible the simultaneous quantification of nicotine and its metabolites in human urine for regular anti-doping controls in sports [132]. A similar approach was reported by Dobrinas *et al.* for the plasma quantification of varenicline, a drug prescribed for smoking cessation, and nicotine during anti-smoking campaigns, which allowed the confirmation of abstinence and the evaluation of plasmatic drug levels in patients [133]. The HILIC approach was also utilized in the forensic field to analyze of seized drugs, which made it possible to screen and profile impurities [134]. The main constraints of HILIC is related to the lack of retention for acidic as well as neutral compounds, which are not too polar.

One of the latest HILIC applications pertains to the analysis of glycans. Glycans are hydrophilic polysaccharides that play major biological roles. Indeed, the glycosylation of biological molecules influences their final locations and their functionalities whitin the cell. Recent studies have successfully characterized the glycosylation patterns of biopharmaceuticals. For example, Gilar et al. analyzed a humanized monoclonal antibody (Trastuzumab)[135]. In this study, Transtuzumab was characterized regarding its glycosilation profile and a relative quantification of peptide glycoforms performed. It is worth mentioning that these results were similar to the ones obtained using a labelling probe and a fluorescent detector. An *et al.* studied the hemagglutinin that was contained in an influenza vaccine and extensively compared and discussed the transfer of conventional RPLC-MS to UHPLC-HILIC for the study of glycosylated antibodies [136], as illustrated in Figure 8.

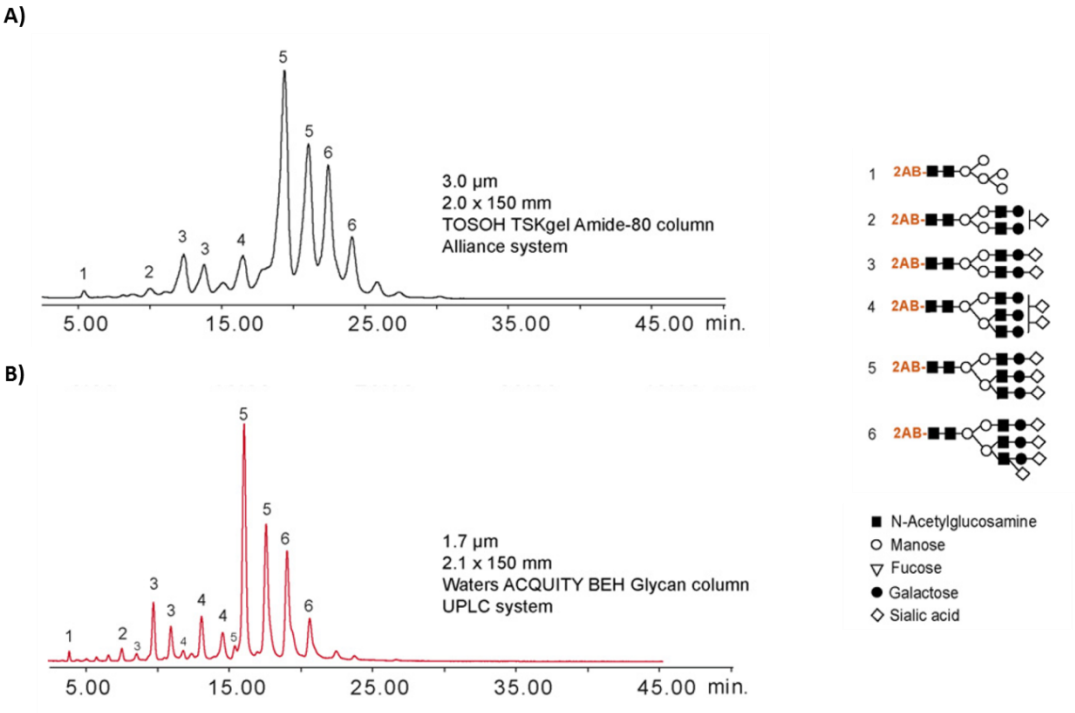


Figure 8: Separation of labeled glycans from IgG with A) HPLC and B) UHPLC, both in HILIC conditions. From [128], with permission.

4. CONCLUSION

As summarized in the present review, the UHPLC-MS technology presents some obvious advantages (i.e., reduction of matrix effects, improvement in sensitivity, higher throughput and better resolution). As example, matrix effects were reduced in UHPLC because of the narrower peaks that can be attained, leading to an improved separation between the compounds of interest and the endogenous constituents of the matrix. Nevertheless, there are also some technical issues that must be considered. First, the narrow peaks produced by UHPLC (down to 1 s in some cases) can be detrimental to MS data integrity. Thus, MS devices must be capable of short dwell times and fast duty cycles. The latest generations of instruments meet these requirements. Indeed, the most powerful QqQ devices are able to perform SRM experiments in just 1 ms. Slower instruments present the possibility of segmenting the MS/MS method into various time-schedule windows that contain different SRM channels at different time intervals. These approaches make it possible to perform the targeted analyses of dozens to hundreds of compounds, without compromising the qualitative or quantitative performance. For untargeted analysis, QqTOF/MS is the best choice due to its full scan capability at high resolution, speed and sensitivity. With the best QqTOF/MS instruments on the market, *m/z* ratios from 100 to 1000 can be scanned in only 10 ms with a resolution of ~ 40,000 FWHM. Second, another possible issue with UHPLC-MS is related to the reduction of column dimensions (reference UHPLC column dimension is 50 x 2.1 mm, V_0 of 120 µL). Indeed, it has been demonstrated that the MS detector itself could represent a significant source of additional band broadening. With old-generation MS devices, the loss in chromatographic efficiency could be detrimental (up to 90%), while with modern instruments and 2.1 mm I.D. UHPLC columns, the chromatographic efficiency remains acceptable. The third limitation of UHPLC-MS is related to the compatibility of ESI/MS with the mobile phase flow rate of UHPLC, which generally ranges from 500 to 1000 µL/min. MS providers propose complementary technical solutions to the flow-splitting strategy to make possible ESI-MS with flow rates up to 2 - 3 mL/min with minimal impact on sensitivity. Therefore, it is worth mentioning that it is possible to maintain adequate qualitative and quantitative performances with UHPLC-MS that possesses a fast duty cycle, limited contribution to band broadening and compatibility with high mobile phase flow rates. With such an analytical platform, the most important constraints of the whole analytical process shifts to sample

preparation and data treatment. The former should to be performed preferentially in 96-well plates in an automated manner, while the latter is a time-consuming task due to the significant amount of data generated by the UHPLC-MS system.

A large variety of applications can be performed using UHPLC-MS, as illustrated in Figure 9. When dealing with targeted analysis (i.e., the determination of a limited number of known substances), short UHPLC columns of 50 mm should be employed in combination with analysis times of less than 3 min. Generally, QqQ is the reference instrument because it provides a suitable quantitative performance. However, when untargeted analysis must be performed (i.e., the determination of unknown compounds in a complex matrix), long UHPLC columns (150 mm) and analysis times (30 min) combined with MS devices that achieve high resolution with good mass accuracy, such as TOF/MS, QqTOF/MS or Orbitrap, are recommended. In multi-residue screening, the number of compounds that need monitoring can be very high, e.g., several hundreds of possible residues for pesticides, which approaches the limits of QqQ. Thus, QqTOF/MS is now gaining in popularity for the screening, confirmation, and structural elucidation of unknown compounds in complex matrices. Additionally, biopharmaceuticals and polar compounds are two recent applications of the UHPLC-MS technology, broadening its domain of application.

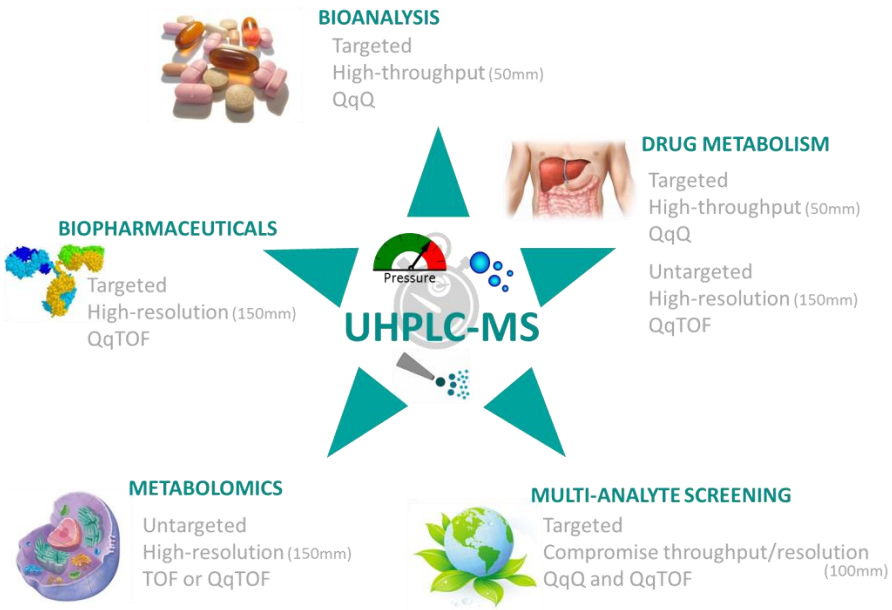


Figure 9: Main fields of applications of UHPLC-MS. Three analytical features are illustrated for each application: i) the type of analysis (targeted or untargeted), ii) the main LC requirement and corresponding column length and iii) the type of MS detector most frequently used.

ACKNOWLEDGMENTS

The authors wish to thank Dany Spaggiari and Philippe Eugster (University of Geneva, Switzerland) for providing extra-column band-broadening measurements used for the construction of Figure 2. Davy Petit (Waters, Belgium) is also acknowledged for his help in the preparation of Tables 1 and 2.

REFERENCES

[1] L. Novakova, D. Solichova, P. Solich, Journal of Separation Science 29 (2006) 2433.

[2] D. Guillarme, D.T.T. Nguyen, S. Rudaz, J.L. Veuthey, Journal of Chromatography A 1149 (2007) 20.

[3] P.W. Carr, D.R. Stoll, X. Wang, Anal Chem 83 (2011) 1890.

[4] S.A.C. Wren, P. Tchelitcheff, Journal of Chromatography A 1119 (2006) 140.

[5] V. Viette, D. Guillarme, R. Mylonas, Y. Mauron, M. Fathi, S. Rudaz, D. Hochstrasser, J.L. Veuthey, Clinical Biochemistry 44 (2011) 32.

[6] E. Grata, J. Boccard, D. Guillarme, G. Glauser, P.A. Carrupt, E.E. Farmer, J.L. Wolfender, S. Rudaz, Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 871 (2008) 261.

[7] E.F. Petricoin, L.A. Liotta, Trends in Molecular Medicine 10 (2004) 59.

[8] P.J. Eugster, D. Guillarme, S. Rudaz, J.L. Veuthey, P.A. Carrupt, J.L. Wolfender, Journal of Aoac International 94 (2011) 51.

[9] D. Guillarme, J. Ruta, S. Rudaz, J.L. Veuthey, Analytical and Bioanalytical Chemistry 397 (2010) 1069.

[10] K. Broeckhoven, D. Cabooter, S. Eeltink, G. Desmet, Journal of Chromatography A 1228 (2012) 20.

[11] J.C. Giddings, Anal Chem 37 (1965) 60.

[12] J.H. Knox, M. Saleem, J Chromatogr Sci 7 (1969) 614.

[13] J.H. Knox, J Chromatogr Sci 15 (1977) 352.

[14] J.E. MacNair, K.C. Lewis, J.W. Jorgenson, Anal Chem 69 (1997) 983.

[15] J.E. MacNair, K.D. Patel, J.W. Jorgenson, Anal Chem 71 (1999) 700.

[16] J.R. Mazzeo, U.D. Neue, M. Kele, R.S. Plumb, Anal Chem 77 (2005) 460a.

[17] L. Novakova, H. Vlckova, Analytica Chimica Acta 656 (2009) 8.

[18] L. Novakova, L. Matysova, P. Solich, Talanta 68 (2006) 908.

[19] D. Guillarme, D.T.T. Nguyen, S. Rudaz, J.L. Veuthey, European Journal of Pharmaceutics and Biopharmaceutics 68 (2008) 430.

[20] T.A. Berger, Chromatographia 72 (2010) 597.

[21] F. Svec, J.M.J. Frechet, Anal Chem 64 (1992) 820.

[22] H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka, N. Tanaka, Anal Chem 68 (1996) 3498.

[23] K. Hormann, T. Mullner, S. Bruns, A. Holtzel, U. Tallarek, Journal of Chromatography A 1222 (2012) 46.

[24] R.W. Brice, X. Zhang, L.A. Colon, Journal of Separation Science 32 (2009) 2723.

[25] S. Fekete, J. Fekete, K. Ganzler, J Pharm Biomed Anal 49 (2009) 64.

[26] J. Ruta, D. Zurlino, C. Grivel, S. Heinisch, J.L. Veuthey, D. Guillarme, Journal of Chromatography A 1228 (2012) 221.

[27] G. Guiochon, F. Gritti, Journal of Chromatography A 1218 (2011) 1915.

[28] S. Fekete, E. Olah, J. Fekete, Journal of Chromatography A 1228 (2012) 57.

[29] E. Olah, S. Fekete, J. Fekete, K. Ganzler, Journal of Chromatography A 1217 (2010) 3642.

[30] A. Liekens, J. Denayer, G. Desmet, Journal of Chromatography A 1218 (2011) 4406.

[31] F. Gritti, I. Leonardis, J. Abia, G. Guiochon, Journal of Chromatography A 1217 (2010) 3819.

[32] G. Hopfgartner, E. Varesio, V. Tschappat, C. Grivet, E. Bourgogne, L.A. Leuthold, Journal of Mass Spectrometry 39 (2004) 845.

[33] H.R. Morris, T. Paxton, A. Dell, J. Langhorne, M. Berg, R.S. Bordoli, J. Hoyes, R.H. Bateman, Rapid Communications in Mass Spectrometry 10 (1996) 889.

[34] A. Makarov, Anal Chem 72 (2000) 1156.

[35] R.D. Voyksner, Combining Liquid Chromatography with Electrospray Mass Spectrometry, Wiley-Interscience, New York, 1997.

[36] Electrospray Ionization Mass Spectrometry: Fundamentals, Instrumentation and Applications, Wiley-Interscience, New York, 1997.

[37] N.B. Cech, C.G. Enke, Mass Spectrometry Reviews 20 (2001) 362.

[38] C.G. Herbert, R.A.W. Johnstone, Mass Spectrometry Basics, CRC press, Boca Raton, 2003.

[39] D.T.T. Nguyen, D. Guillarme, S. Rudaz, J.L. Veuthey, Journal of Separation Science 29 (2006) 1836.

[40] L. Novakova, J.L. Veuthey, D. Guillarme, Journal of Chromatography A 1218 (2011) 7971.

[41] A. de Villiers, H. Lauer, R. Szucs, S. Goodall, P. Sandra, Journal of Chromatography A 1113 (2006) 84.

[42] E. de Hoffmann, V. Stroobant, Mass Spectrometry: Principles and Applications, Wiley-Interscience, New York, 2003.

[43] D.T.T. Nguyen, D. Guillarme, S. Heinisch, M.P. Barrioulet, J.L. Rocca, S. Rudaz, J.L. Veuthey, Journal of Chromatography A 1167 (2007) 76.

[44] A. Mordehai, J. Fjeldsted, in Agilent technical note 5990-3494 EN, 2009.

[45] J. Schappler, R. Nicoli, D. Nguyen, S. Rudaz, J.L. Veuthey, D. Guillarme, Talanta 78 (2009) 377.

[46] B.M. Huyghues-Despointes, J.M. Scholtz, C.N. Pace, Nat Struct Biol 6 (1999) 910.

[47] D. Guillarme, D.T.T. Nguyen, S. Rudaz, J.L. Veuthey, European Journal of Pharmaceutics and Biopharmaceutics 66 (2007) 475.

[48] F. Lestremau, D. Wu, R. Szucs, J Chromatogr A 1217 (2010) 4925.

[49] S. Fekete, J. Fekete, J Chromatogr A 1218 (2011) 5286.

[50] E. Grata, D. Guillarme, G. Glauser, J. Boccard, P.A. Carrupt, J.L. Veuthey, S. Rudaz, J.L. Wolfender, Journal of Chromatography A 1216 (2009) 5660.

[51] P.J. Eugster, D. Biass, D. Guillarme, P. Favreau, R. Stocklin, J.L. Wolfender, J Chromatogr A (2012) in press.

[52] G. Hopfgartner, D. Tonoli, E. Varesio, Analytical and Bioanalytical Chemistry 402 (2012) 2587.

[53] D. Tonoli, E. Varesio, G. Hopfgartner, Chimia (Aarau) 66 (2012) 218.

[54] L. Novakova, M. Holcapek, R. Jirasko, M. Lisa, in D. Guillarme, J.L. Veuthey (Editors), UHPLC in life sciences, Royal Society of Chemistry, Cambridge, 2012, p. 186.

[55] K. Deventer, P. Van Eenoo, F.T. Delbeke, Rapid Commun Mass Spectrom 19 (2005) 90.

[56] B. Prasad, A. Garg, H. Takwani, S. Singh, Trac-Trends in Analytical Chemistry 30 (2011) 360.

[57] M. Balogh, A High Speed Combination Multi-mode Ionization Source for Mass Spectrometers, PCT Int. Appl., WO 2003102537 A2 20031211 (2003) 33 pp.

[58] M. Rodriguez-Aller, B. Kaufmann, D. Guillarme, C. Stella, P. Furrer, S. Rudaz, I. El Zaoui, F. Valamanesh, C. Di Tommaso, F. Behar-Cohen, J.L. Veuthey, R. Gurny, Eur J Pharm Biopharm 80 (2012) 544.

[59] K. Tertti, A. Petsalo, M. Niemi, U. Ekblad, A. Tolonen, T. Ronnemaa, M. Turpeinen, T. Heikkinen, K. Laine, Eur J Pharm Sci 44 (2011) 181.

[60] M. Noetzli, N. Ansermot, M. Dobrinas, C.B. Eap, J Pharm Biomed Anal 64-65 (2012) 16.

[61] H. Vlckova, M. Rabatinova, A. Miksova, G. Kolouchova, S. Micuda, P. Solich, L. Novakova, Talanta 90 (2012) 22.

[62] D.P. Patel, P. Sharma, M. Sanyal, P. Singhal, P.S. Shrivastav, Biomed Chromatogr (2012) in press.

[63] C. Boscarino, A.N. Edginton, H. Peng, K.W. Riggs, A. Szeitz, B. Cheung, Eur J Clin Pharmacol (2012) in press.

[64] L. Goldwirt, F. Lemaitre, N. Zahr, R. Farinotti, C. Fernandez, J Pharm Biomed Anal 66 (2012) 325.

[65] C.J. Peer, S.D. Spencer, D.A. VanDenBerg, M.A. Pacanowski, R.B. Horenstein, W.D. Figg, J Chromatogr B Analyt Technol Biomed Life Sci 880 (2012) 132.

[66] E. Grata, L. Perrenoud, M. Saugy, N. Baume, Forensic Sci Int 213 (2011) 104.

[67] H. Vlckova, D. Solichova, M. Blaha, P. Solich, L. Novakova, J Pharm Biomed Anal 55 (2011) 301.

[68] C. Di Tommaso, F. Valamanesh, F. Miller, P. Furrer, M. Rodriguez-Aller, F. Behar-Cohen, R. Gurny, M. Moller, Invest Ophthalmol Vis Sci 53 (2012) 2292.

[69] R. Xu, M. Manuel, J. Cramlett, D.B. Kassel, J Chromatogr A 1217 (2010) 1616.

[70] R.S. Plumb, W.B. Potts, 3rd, P.D. Rainville, P.G. Alden, D.H. Shave, G. Baynham, J.R. Mazzeo, Rapid Commun Mass Spectrom 22 (2008) 2139.

[71] R. Nicoli, R. Curcio, S. Rudaz, J.L. Veuthey, J Med Chem 52 (2009) 2192.

[72] J.E. Barbara, J.M. Castro-Perez, Rapid Commun Mass Spectrom 25 (2011) 3029.

[73] B. Bonn, C. Leandersson, F. Fontaine, I. Zamora, Rapid Commun Mass Spectrom 24 (2010) 3127.

[74] D. Gu, Y. Ma, G. Niu, Y. Yan, L. Lang, H.A. Aisa, H. Gao, D.O. Kiesewetter, X. Chen, Amino Acids 40 (2011) 669.

[75] C.X. Shan, W. Li, H.M. Wen, X.Z. Wang, Y.H. Zhu, X.B. Cui, J Pharm Biomed Anal 62 (2012) 187.

[76] D.J. Crockford, A.D. Maher, K.R. Ahmadi, A. Barrett, R.S. Plumb, I.D. Wilson, J.K. Nicholson, Anal Chem 80 (2008) 6835.

[77] A. Petsalo, M. Turpeinen, O. Pelkonen, A. Tolonen, J Chromatogr A 1215 (2008) 107.

[78] R. Curcio, R. Nicoli, S. Rudaz, J.L. Veuthey, Analytical and Bioanalytical Chemistry 398 (2010) 2163.

[79] J.C. Precht, B. Ganchev, G. Heinkele, H. Brauch, M. Schwab, T.E. Murdter, Analytical and Bioanalytical Chemistry 403 (2012) 301.

[80] A. Kaufmann, P. Butcher, K. Maden, M. Widmer, Analytica Chimica Acta 586 (2007) 13.

[81] F. Badoud, E. Grata, L. Perrenoud, L. Avois, M. Saugy, S. Rudaz, J.L. Veuthey, J Chromatogr A 1216 (2009) 4423.

[82] F. Badoud, E. Grata, L. Perrenoud, M. Saugy, S. Rudaz, J.L. Veuthey, J Chromatogr A 1217 (2010) 4109.

[83] D. Ortelli, E. Cognard, P. Jan, P. Edder, J Chromatogr B Analyt Technol Biomed Life Sci 877 (2009) 2363.

[84] J.C. Van De Steene, W.E. Lambert, J Am Soc Mass Spectrom 19 (2008) 713.

[85] O. Lacina, P. Hradkova, J. Pulkrabova, J. Hajslova, Journal of Chromatography A 1218 (2011) 4312.

[86] M.E. Dasenaki, N.S. Thomaidis, Analytica Chimica Acta 672 (2010) 93.

[87] J.L. Martinez Vidal, A.G. Frenich, M.M. Aguilera-Luiz, R. Romero-Gonzalez, Analytical and Bioanalytical Chemistry 397 (2010) 2777.

[88] R. Romero-Gonzalez, A. Garrido Frenich, J.L. Martinez Vidal, O.D. Prestes, S.L. Grio, J Chromatogr A 1218 (2011) 1477.

[89] A. Schurmann, V. Dvorak, C. Cruzer, P. Butcher, A. Kaufmann, Rapid Commun Mass Spectrom 23 (2009) 1196.

[90] H.G. Mol, P. Plaza-Bolanos, P. Zomer, T.C. de Rijk, A.A. Stolker, P.P. Mulder, Anal Chem 80 (2008) 9450.

[91] M.J. Taylor, G.A. Keenan, K.B. Reid, D.U. Fernandez, Rapid Commun Mass Spectrom 22 (2008) 2731.

[92] A. Kaufmann, P. Butcher, K. Maden, S. Walker, M. Widmer, Rapid Commun Mass Spectrom 25 (2011) 979.

[93] A. Kaufmann, S. Walker, Rapid Commun Mass Spectrom 26 (2012) 1081.

[94] A. Kaufmann, Analytical and Bioanalytical Chemistry 403 (2012) 1233.

[95] S. Grimalt, J.V. Sancho, O.J. Pozo, F. Hernandez, Journal of Mass Spectrometry 45 (2010) 421.

[96] O. Lacina, J. Urbanova, J. Poustka, J. Hajslova, Journal of Chromatography A 1217 (2010) 648.

[97] H.X. Wang, Y. Zhou, Q.W. Jiang, Microchemical Journal 100 (2012) 83.

[98] F. Hernandez, L. Bijlsma, J.V. Sancho, R. Diaz, M. Ibanez, Analytica Chimica Acta 684 (2011) 96.

[99] A. Gerssen, P.P.J. Mulder, J. de Boer, Analytica Chimica Acta 685 (2011) 176.

[100] P. Blay, J.P.M. Hui, J.M. Chang, J.E. Melanson, Analytical and Bioanalytical Chemistry 400 (2011) 577.

[101] M.E. Lame, E.E. Chambers, M. Blatnik, Anal Biochem 419 (2011) 133.

[102] M.E. Lassman, T.M. McLaughlin, E.P. Somers, A.C. Stefanni, Z. Chen, B.A. Murphy, K.K. Bierilo, A.M. Flattery, K.K. Wong, J.M. Castro-Perez, B.K. Hubbard, T.P. Roddy, Rapid Commun Mass Spectrom 26 (2012) 101.

[103] D.F. Thompson, F. Michopoulos, C.J. Smith, C.J. Duckett, R.W. Wilkinson, P. Jarvis, I.D. Wilson, Mol Biosyst 7 (2011) 1149.

[104] A. Nordstrom, G. O'Maille, C. Qin, G. Siuzdak, Anal Chem 78 (2006) 3289.

[105] Y.Y. Zhao, J. Liu, X.L. Cheng, X. Bai, R.C. Lin, Clin Chim Acta 413 (2012) 642.

[106] N. Son, H.J. Hur, M.J. Sung, M.S. Kim, J.T. Hwang, J.H. Park, H.J. Yang, D.Y. Kwon, S.H. Yoon, H.Y. Chung, H.J. Kim, J Proteome Res 11 (2012) 2551.

[107] H.G. Gika, G.A. Theodoridis, I.D. Wilson, Journal of Separation Science 31 (2008) 1598.

[108] J. Chen, H. Wen, J. Liu, C. Yu, X. Zhao, X. Shi, G. Xu, Mol Biosyst 8 (2012) 871.

[109] G. Graca, B.J. Goodfellow, A.S. Barros, S. Diaz, I.F. Duarte, K. Spagou, K. Veselkov, E.J. Want, J.C. Lindon, I.M. Carreira, E. Galhano, C. Pita, A.M. Gil, Mol Biosyst 8 (2012) 1243.

[110] J.L. Wolfender, P.J. Eugster, N. Bohni, M. Cuendet, Chimia (Aarau) 65 (2011) 400.

[111] H.M. Zhang, S.L. Li, H. Zhang, Y. Wang, Z.L. Zhao, S.L. Chen, H.X. Xu, J Pharm Biomed Anal 62 (2012) 258.

[112] M.A. Farag, L.A. Wessjohann, Planta Medica 78 (2012) 488.

[113] M. Gomez-Romero, A. Segura-Carretero, A. Fernandez-Gutierrez, Phytochemistry 71 (2010) 1848.

[114] U. Vrhovsek, D. Masuero, M. Gasperotti, P. Franceschi, L. Caputi, R. Viola, F. Mattivi, J Agric Food Chem (2012) in press.

[115] G. Glauser, J. Boccard, S. Rudaz, J.-L. Wolfender, Phytochemical Analysis 21 (2010) 95.

[116] T. Kocher, R. Swart, K. Mechtler, Anal Chem 83 (2011) 2699.

[117] A. Staub, D. Zurlino, S. Rudaz, J.L. Veuthey, D. Guillarme, J Chromatogr A 1218 (2011) 8903.

[118] S. Sinha, L. Zhang, S. Duan, T.D. Williams, J. Vlasak, R. Ionescu, E.M. Topp, Protein Sci 18 (2009) 1573.

[119] J.P. Scarth, C. Seibert, P.R. Brown, P. Teale, G.J. Beamon, C.M. Pearce, R.A. Sams, Chromatographia 74 (2011) 593.

[120] H.Y. Li, R. Ortiz, L. Tran, M. Hall, C. Spahr, K. Walker, J. Laudernann, S. Miller, H. Salimi-Moosavi, J.W. Lee, Anal Chem 84 (2012) 1267.

[121] J. Ruta, D. Guillarme, S. Rudaz, J.L. Veuthey, Journal of Separation Science 33 (2010) 2465.

[122] C.J. Ji, N. Sadagopan, Y.Z. Zhang, C. Lepsy, Anal Chem 81 (2009) 9321.

[123] S.C. Klapoetke, J. Zhang, S. Becht, J Pharm Biomed Anal 56 (2011) 513.

[124] A.J. Percy, A.G. Chambers, J. Yang, D. Domanski, C.H. Borchers, Analytical and Bioanalytical Chemistry 404 (2012) 1089.

[125] S.A. Berkowitz, J.R. Engen, J.R. Mazzeo, G.B. Jones, Nat Rev Drug Discov 11 (2012) 527.

[126] A. Beck, S. Sanglier-Cianferani, A. Van Dorsselaer, Anal Chem 84 (2012) 4637.

[127] H. Xie, A. Chakraborty, J. Ahn, Y.Q. Yu, D.P. Dakshinamoorthy, M. Gilar, W. Chen, S.J. Skilton, J.R. Mazzeo, MAbs 2 (2010) 379

[128] J. Ahn, J. Bones, Y.Q. Yu, P.M. Rudd, M. Gilar, J Chromatogr B Analyt Technol Biomed Life Sci 878 (2010) 403.

[129] B. Chauve, D. Guillarme, P. Cleon, J.L. Veuthey, Journal of Separation Science 33 (2010) 752.

[130] L. Nováková, T. Gottvald, H. Vlčková, F. Trejtnar, J. Mandíková, P. Solich, Journal of Chromatography A (2012) in press.

[131] J. Ruta, S. Rudaz, D.V. McCalley, J.L. Veuthey, D. Guillarme, Journal of Chromatography A 1217 (2010) 8230.

[132] F. Marclay, E. Grata, L. Perrenoud, M. Saugy, Forensic Sci Int 213 (2011) 73.

[133] M. Dobrinas, E. Choong, M. Noetzli, J. Cornuz, N. Ansermot, C.B. Eap, Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences 879 (2011) 3574.

[134] I.S. Lurie, L. Li, S.G. Toske, Journal of Chromatography A 1218 (2011) 9336.

[135] M. Gilar, Y.Q. Yu, J. Ahn, H.W. Xie, H.H. Han, W.T. Ying, X.H. Qian, Anal Biochem 417 (2011) 80.

[136] Y.M. An, J.F. Cipollo, Anal Biochem 415 (2011) 67.

PART A

PRODRUG APPROACH

Cyclosporine A (CsA) is a poorly water-soluble peptide used for the treatment of a number of ocular conditions including dry eye syndrome, uveitis, conjunctivitis, postoperative ocular inflammation and corneal graft rejection. Current CsA ocular formulations suffer from tolerance and availability issues related to the use of oily vehicles necessary for CsA solubilization. Such limitations are responsible for the use of CsA as a systemic treatment requiring close patient monitoring for proper management of the ocular condition.

One solution to overcome these limitations and avoid severe CsA-related systemic side effects (such as hepatotoxicity, nephrotoxicity and hypertension) is to increase its solubility. The concept of "water-soluble CsA" based on the prodrug strategy was investigated in our group. Interestingly, CsA displays a free hydroxyl group that makes it possible to generate a panel of CsA prodrugs, which was screened according to solubility, ocular irritation potential, bioconversion and ocular distribution during a previous thesis ("Selection and evaluation of water-soluble CsA prodrugs for ophthalmic administration" by Dr. Frédéric Lallemand). With such a strategy, the "practically insoluble" CsA molecule was converted into a "freely soluble" CsA prodrug, displaying a 25'000 fold increase in water solubility.

Building on these promising results, the first part of this thesis is dedicated to the development and evaluation of prodrug formulations for ocular topical treatment of eye disease. This water-soluble CsA prodrug made it possible to develop highly concentrated CsA-based ophthalmic formulations in an aqueous vehicle, circumventing the limitations linked to conventional CsA formulations for topical ocular administration. The safety of the prodrug formulation was evaluated by assessing its *in vivo* ocular tolerance compared to a commercial CsA formulation. Then, investigations on precorneal elimination and ocular distribution of the prodrug formulation were conducted. The question of *in vitro* stability of the prodrug formulation was addressed in a preformulation study. Finally, biodistribution and ocular penetration experiments were conducted to gain a detailed understanding of the phenomena governing prodrug and CsA availability.

CHAPTER III

Cyclosporine A prodrug for improved ocular tolerance and distribution

In vivo characterisation of a novel water-soluble cyclosporine A prodrug for the treatment of dry eye disease

Marta RODRIGUEZ-ALLER[1], Béatrice KAUFMANN[1], Davy GUILLARME[1], Cinzia STELLA[1], Pascal FURRER[1], Serge RUDAZ[1], Ikram EL ZAOUI[2], Fatemeh VALAMANESH[2,3], Claudia DI TOMMASO[1], Francine BEHAR-COHEN[2,4], Jean-Luc VEUTHEY[1], Robert GURNY[1,*]

[1]School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 1211 Geneva 4, Switzerland

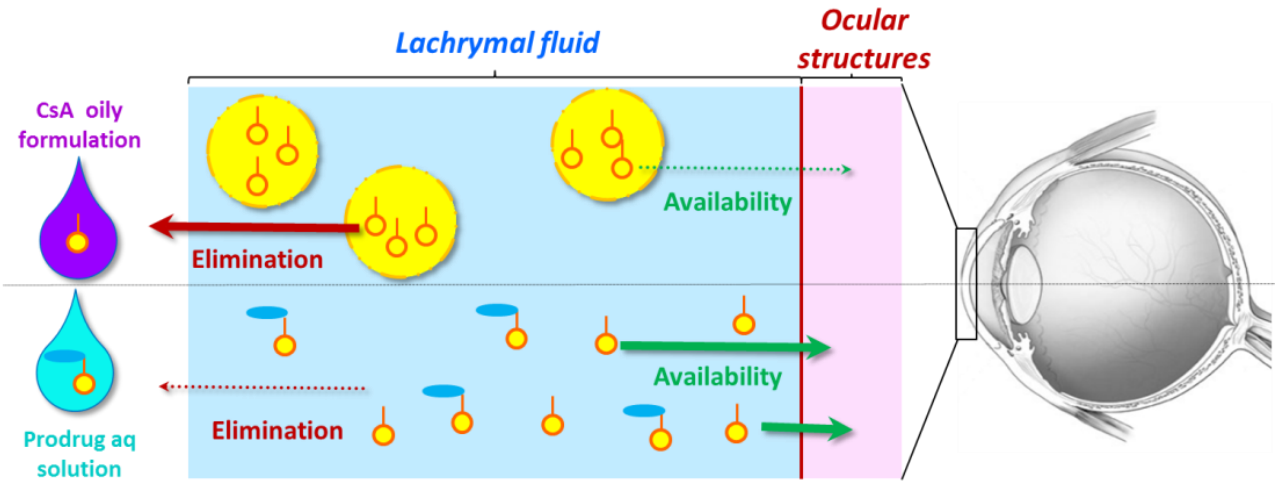
[2] INSERM, UMRS 872 Team 17, Centre de Recherche des Cordeliers, 75006 Paris, France

[3] Fondation A. De Rothschild, 25 rue Manin, 75019 Paris, France

[4] Université Paris Descartes, Faculté de médecine, Département d'ophtalmologie, Hôpital Hôtel-Dieu, APHP, Paris, France

*Correspondence: Phone: +41223793816, Fax: +41223796146, E-mail: robert.gurny@unige.ch

Published in European Journal of Pharmaceutics and Biopharmaceutics 2012; 80:544-52



ABSTRACT

Cyclosporine A (CsA) has been demonstrated to be effective for the treatment of a variety of ophthalmological conditions, including ocular surface disorders such as the dry eye disease (DED). Since CsA is characterised by its low water solubility, the development of a topical ophthalmic formulation represents an interesting pharmaceutical question. In the present study, two different strategies to address this challenge were studied and compared: i) a water-soluble CsA prodrug formulated within an aqueous solution and ii) a CsA oil-in-water emulsion (Restasis®, Allergan Inc., Irvine, CA). First, the prodrug formulation was shown to have an excellent ocular tolerance as well as no influence on the basal tear production; maintaining the ocular surface properties unchanged. Then, in order to allow *in vivo* investigations, a specific analytical method based on ultra high pressure liquid chromatography coupled with triple quadrupole mass spectrometer (UHPLC-MS/MS) was developed and optimised to quantify CsA in ocular tissues and fluids. The CsA ocular kinetics in lachrymal fluid for both formulations were found to be similar between 15 min and 48 h. The CsA ocular distribution study evidenced the ability of the prodrug formulation to penetrate into the eye, achieving therapeutically active CsA levels in tissues of both the anterior and posterior segments. In addition the detailed analysis of the *in vivo* data using a bicompartmental model pointed out a higher bioavailability and lower elimination rate for CsA when it is generated from the prodrug than after direct application as an emulsion. The interesting *in vivo* properties displayed by the prodrug solution make it a safe and suitable option for the treatment of DED.

Keywords: Cyclosporine A, prodrug, precorneal kinetic, UHPLC-MS/MS quantification, ophthalmic formulation, dry eye.

1. INTRODUCTION

The dry eye disease (DED) includes a broad spectrum of signs and symptoms that makes its definition and classification highly complex. That is one of the reasons why there are various other terms associated with this condition, such as dry eye syndrome, chronic dry eye disease, dysfunctional tear syndrome, keratoconjunctivitis sicca or keratitis sicca [1-4]. The lack of consent regarding the definition and diagnosis of the disorder contributes to the difficulty of evaluating its prevalence and impact on the patients' life. Nevertheless, a study showed that the impact of the DED on patients' quality of life could be compared with the one of a moderate angina [5], evidencing the importance of developing an appropriate treatment. As a general concept, it has been agreed that DED affects the ocular surface and has an unmistakable correlation with local inflammatory processes, in which the immune T cells and the lachrymal functional unit are highly involved. The inflammatory component of DED is assumed to play a key role in its pathology. It has been reported that some patients continue complaining about eye irritation even after adequate aqueous enhancement treatment [3]. Therefore, the underlying inflammatory process needs serious consideration for effective treatment, putting CsA in the spotlight for this application.

Cyclosporine A (CsA) is an undecapeptide produced by the microorganism *Tolypocladium inflatum Gams* that presents very useful properties for ophthalmic applications [6, 7]. CsA has very interesting mechanisms of action that can be valuable in the treatment of DED. For example, CsA binds to cyclophilins and causes an inhibition of the calcineurin pathway that results in a suppression of the expression of cytokines genes; this leads to a non activation of the T cells [8]. CsA is also known to take part in the inhibition of the c-Jun N-terminal kinases (JNK) and p38 signalling pathways involved in the activation of T cells [9]. In addition, CsA participates in the stimulation of apoptosis of the T cells that are already in an activated state [10], and it contributes to the enhancement of the release of a neurotransmitter, substance P, from the sensory nerves directing a stimulating interaction with the parasympathetic nerves [11, 12]. Once in contact with the eye, CsA leads to a decrease in inflammation and an increase of the lachrymal production; both effects being highly beneficial for the treatment of DED.

DED is an ocular surface condition; therefore it would be appropriate to treat the pathological process on a local ocular level by applying a topical ophthalmic formulation. This strategy helps to avoid some severe side effects caused by CsA when applied systemically such as nephrotoxicity, hepatotoxicity or hypertension [13].

Nevertheless, the use of CsA for ocular application represents a challenge regarding its pharmaceutical formulation. CsA is a highly hydrophobic molecule "practically insoluble" in water [14] that is difficult to be formulated in a totally hydrophilic vehicle, which makes its topical delivery into the eye a complicated matter.

Despite many attempts to address this question, only one formulation has been approved by the United States Food and Drug Administration (FDA) for the treatment of DED in humans to date; it is a CsA oil-in-water emulsion commercialised under the name of Restasis® (Allergan Inc., Irvine, CA).

In the present work, the selected procedure for the formulation of CsA into a topical ocular form was a chemical modification of the active molecule to increase its hydrophilicity. This prodrug approach is based on an ester of CsA carrying a phosphate group as a solubilising moiety, already described in previous studies [15-19]. The chemical structures of CsA and the prodrug are illustrated in Figure 1. The prodrug, OPPH 088, exhibited a solubility that was approximately 25000 times higher than CsA in isotonic phosphate buffer solution (PBS) at pH 7 [18]. In addition, OPPH 088 has the property to be quickly biotransformed into CsA once in contact with the ocular surface because of the esterase-like enzymatic activity of the tears [19].

Drug precorneal behaviour and ocular distribution are fundamental factors that strongly condition the bioavailability and efficacy of the formulations. Nevertheless, only little is known about CsA eyedrops' behaviour once in contact with the eye.

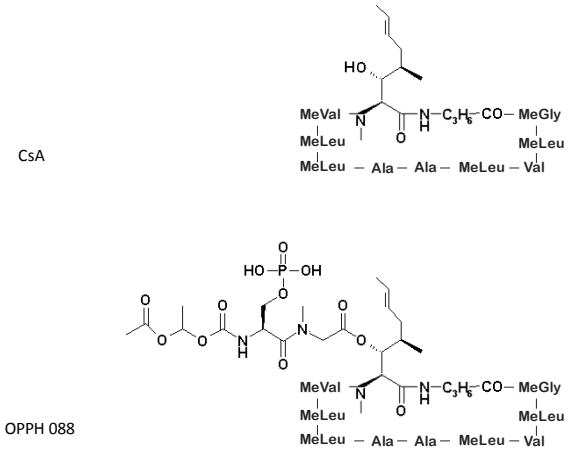


Figure 1: Chemical structures of CsA and OPPH 088.

The aim of the present study was to characterise the *in vivo* behaviour of a novel formulation based on OPPH 088, and compare it to the commercial formulation, Restasis. The influence of the formulations on ocular surface properties was evaluated by measuring the corneal damage and basal tear production after their administration. Also, a dedicated UHPLC-MS/MS (ultra high pressure liquid chromatography coupled with triple quadrupole mass spectrometer) analytical method was developed and optimised in order to quantify CsA in ocular tissues and fluids. It allowed the determination of the ocular kinetics of OPPH 088 and Restasis as well as the CsA ocular distribution after prodrug administration. This information provided a basis for a model development and comparison of the formulations.

2. MATERIALS AND METHODS

2.1. Materials

OPPH 088 was synthesized at the Institute of Chemical Sciences and Engineering of the Ecole Polytechnique Fédérale de Lausanne (Switzerland) following the procedure described by Wenger et al. [15]. CsA was kindly provided by Dr. Wenger. Deuterated CsA (d_{12}CsA), used as an internal standard (IS), was a gift from Novartis (Basel, Switzerland), and mannitol was purchased from Acros Organics (Belgium). Water was obtained from a Milli-Q Water Purification System from Millipore (Bedford, MA, USA). Acetic acid and methanol were of ULC/MS grade and were purchased from Biosolve (Valkenswaard, Netherlands). Isopropanol was provided by Sigma-Fluka (Buchs, Switzerland).

2.2. Methods

2.2.1. Preparation of the prodrug solution

A 0.066% w/v OPPH 088 aqueous formulation was prepared at a concentration equivalent to 0.050% w/v CsA using an aqueous 5% w/v mannitol solution as vehicle. The pH was measured (Metrohm, Herisau, Switzerland) and adjusted to 7 with 1N NaOH, and the isotonicity was verified (Vapor Pressure Osmometer 5500, Wescor, Logan, Utah, USA) prior to the experiment. The solution was filtered through a 0.22 µm polyvinylidene fluoride membrane (Millipore, Cork, Ireland) and kept in an appropriate eye drop container until use.

2.2.2. Analytical method

2.2.2.1. UHPLC-MS/MS instrumentation

Analyses were performed on a Waters Acquity ultra performance liquid chromatograph (UPLC) system hyphenated with a Waters TQD triple quadrupole mass spectrometer fitted with a Z-spray electrospray ionisation source (Waters, Milford, MA, USA).

The chromatographic system included a binary solvent manager with a maximum delivery flow rate of 2 mL/min, a sample manager with an injection loop volume of 2 µL (full loop injection), and a column oven set at 60°C. The chromatographic column was a Waters Acquity BEH C18 (50 x 2.1 mm I.D., 1.7 µm). Dwell volume of the UPLC-MS/MS configuration was estimated at 100 µL with a 2 µL injection loop. Chromatographic conditions for the separation were as follow: the analysis was carried out in the gradient mode at a flow rate of 600 µL/min (without splitting). The mobile phase consists of a mixture of water with 0.02% v/v acetic acid (A) and methanol with 0.02% v/v acetic acid (B). A linear gradient from 60 to 100% B was applied for 3 min, followed by a reequilibrating step of 1 min (corresponding to 5 column dead volumes). After each analysis, the injection system was washed in order to avoid any CsA adsorption onto the surface; 1000 µL of pure isopropanol were used as strong wash, and 2000 µL of pure methanol as weak wash. The sample manager temperature was maintained at 4ºC during the time of analysis to avoid transformation and degradation.

The TQD instrument possessed an upper mass limit of m/z 2000. The ESCi® ionisation source was used in the ESI positive mode, and selected reaction monitoring (SRM) was performed, using the pseudomolecular ion of each compound as the precursor ion and the most intense fragment. The use of an internal standard for the quantification of CsA in ocular tissues and fluids was highly beneficial to minimise the variability of the results. The selected molecule to be used as a reference was a 12 times deuterated CsA (d_{12}CsA) that has the same chromatographic behaviour than CsA with a higher m/z. The ratio between signals of CsA and d_{12}CsA was the basis for quantification. Collision energies and cone voltages were tuned by infusing each compound individually at 1 µg/mL using a flow rate of 600 µL/min. Optimal values were 65 V for the cone voltage and 63 eV for the collision energy. The pseudomolecular parent ion and fragment corresponding to CsA have an m/z of 1224.7 and 1112.4 respectively; concerning d_{12}CsA the parent ion has an m/z of 1236.7 and its daughter

ion of 1124.4. Nitrogen was used as the drying gas and argon as the collision gas. The capillary voltage and the source extractor voltages were set at +4 kV and +3 V, respectively. The source temperature was maintained at 140°C, the desolvation gas temperature and flow at 450°C and 600 L/h, respectively, and the cone gas flow at 50 L/h. The collision gas flow was set to 0.2 mL/min of argon and the entrance and exit potentials were adjusted to 1 and 0.5V, respectively. Finally, the inter-channel delay was set to 5 ms, and dwell time was set to 200 ms for CsA and 20 ms for d_{12}CsA to maintain enough data points across the narrow peaks produced by the UHPLC-MS/MS instrument. The high mass resolution was adjusted to 10 to improve the sensitivity of the method. Data acquisition, data handling and instrument control were performed by Masslynx v4.1 Software (Waters, Milford, MA, USA).

2.2.2.2. Solutions for calibration

Calibration standards (CSs) included samples containing known concentrations of analytes. CSs were prepared in an independent way. A series of eleven concentration levels were selected corresponding to the wide range of concentrations expected in biological samples. CSs were replicated three times and independently prepared on different days.

For the precorneal kinetic study tears from 8 rabbits were collected and pooled to obtain blank tear fluid that was subsequently spiked with 0.1 µg/mL d_{12}CsA as an internal standard and the drug concentrations was varied at: 10, 5, 2, 1, 0.5, 0.2 and 0.1 µg/mL and 5, 2, 1 and 0.5 ng/mL. For the ocular penetration study the same CsA and d_{12}CsA concentrations were used.

2.2.2.3. Analytical method for CsA quantification at kinetic time points after 24h

Two different analytical situations were defined in the determination of the kinetic profile according to the amount of CsA expected in the biological samples: during the kinetic experiment the highest levels of CsA were expected for the time points taken earlier than 3 h after administration of the drug. As a consequence, the initially developed analytical method was modified to address the specific challenges for the quantification of the active molecule in the samples collected more than 3 h after the eyedrop administration.

The modifications focus on two parameters of the method: i) dwell time for CsA was raised from 0.2 to 1 s, which allowed a higher sensitivity, and ii) calibration was performed with solutions containing 0.1 µg/mL of d_{12}CsA, and lower concentrations of CsA: 2, 1, 0.5, 0.2, 0.1 ng/mL. These results should be considered as pseudo-quantitative.

2.2.3. Animals

2.2.3.1. Rabbits for ocular tolerance evaluation, basal lachrymal production measurements and kinetic study

Female albinos New Zealand rabbits weighing approximately 4-5 kg (University Medical Center, Geneva, Switzerland) were used in this study. Animals were individually housed in stainless steel cages and maintained in a 12 h light/dark cycle at 19+/-1°C. They were allowed water and food ad libitum. All animals were healthy and free of clinically observable ocular abnormalities throughout the study. Animals were not sacrificed at the end of the experiments and remained healthy and with no observable ocular signs after the studies. All experiments were performed in accordance with the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research, and were approved by the local veterinary authority for animal experimentation.

2.2.3.2. Rats for ocular distribution study

Eight weeks old female Lewis rats weighing 150-200 g were used in this study. At the end of the study rats were sacrificed using CO_2. All experiments were performed in accordance with the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research, and were approved by the European Committee Directives for animal experimentation.

2.2.4. Ocular tolerance evaluation protocol

Twenty-five microlitres of the solution to be tested were instilled to the cornea of the right eye of a rabbit four times a day, for a period of 3 days and once on the fourth day just before observation of the cornea. After the last administration, rabbits were placed on a high-adjustable trolley in front of a camera head. A volume of 25 µL of a 0.5% w/v sodium fluorescein sterile isotonic solution was applied to allow the injured areas to be selectively marked. The eye was then rinsed for 1 min with 0.9% w/v sterile NaCl. Finally, the cornea was observed with a confocal laser scanning ophthalmoscope (CLSO® Zeiss, Oberkochen, Germany), which was modified by the addition of a set of lenses to view the cornea instead of the retina [20]. An argon ion laser operating at a 488 nm wavelength was used as the excitation light source. The fluorescence signal was detected by a photomultiplier. Images were obtained using an Epiplan-Neofluar 2.5x/0.075 NA objective lens (Zeiss, Oberkochen, Germany). Optical sectioning was performed parallel to the corneal surface at 16 equidistant focal planes; the focus shifting ranged from 0 to 470 µm covered the entire corneal thickness. The images were displayed on a digital video monitor. An image processing system (Analysis SIS, Münster, Germany) allowed the calculation of the total surface of the fluorescent zones, which indicated injured areas. Each formulation was tested on six rabbits.

Student's t test was used to compare the corneal damage. A p value of less than 0.05 was considered statistically significant.

2.2.5. Basal lachrymal production measurements

The Schirmer test was used to measure basal tear production. The evaluation was performed on 6 rabbits for each formulation; Restasis and the prodrug solution. A first sample was taken before drug administration to evaluate the tear basal production at time zero. After the administration of 25 µL of one or the other formulation, samples were collected at: 1, 2, 4, 6, 24 and 48 h. The sample collection was preceded by a topical anaesthesia with one drop of 0.4% oxybuprocaine solution; 5 min later the Schirmer test strip was inserted into the cul-the-sac and left in place for 5 min. The height of absorbed lachrymal fluid was measured in mm and reported in STT (Schirmer tear test). Student's t test was used to compare the experimental results, p values of less than 0.05 were considered statistically significant.

2.2.6. Ocular distribution study protocol

The ocular distribution of CsA was evaluated after the administration of the prodrug solution bid (twice a day) during 5 days on 6 rats. At the end of the experiment animals were sacrificed with CO_2, eyes were enucleated and the cornea, conjunctiva, aqueous humour, iris-ciliary body, vitreous humour and retina were collected and stored at -80°C in protected vial. Prior to analyses the tissue samples were thawed at room temperature, weighted, manually grinded, introduced in a vial containing 200 µL of methanol along with 0.1 µg/mL d_{12}CsA and stirred over night. The day after, samples were centrifuged and the supernatants were analysed by UHPLC-MS/MS.

2.2.7. Kinetic evaluation

Normal nasolachrymal drainage was verified three days before the experiment by applying one drop of fluorescein solution (0.5% w/v in phosphate buffered solution at pH 7). Rabbits were divided into two groups according to the tested eyedrop formulation: 6 rabbits for Restasis and others 6 rabbits for OPPH 088. The experiment began with the administration of 25 µL of the appropriate formulation in the right eye of six non-anaesthetised rabbits. Tear fluid samples were collected from the lower marginal strip using 2 µL disposable glass microcapillary tubes (Microcaps Drummond, Thomas Scientific, New Jersey). A first sample was taken before administering the drug to verify the absence of CsA before the start of the experiment. After the administration of the formulation, samples were collected at different times: 1, 3, 6, 8, 10, 12, 14, 16, 18, 20, 30, 60, 90, 120, 150, 180 min and at 24 and 48 h. After collection, the tear fluid sample was gently blown out of the capillary into a vial containing 50 µL of methanol along with 0.1 µg/mL d_{12}CsA. The vial was gently vortexed to allow the methanol to stop the conversion of the prodrug by denaturising the proteins involved in the reaction. The vials were analysed into the UHPLC-MS/MS system and CsA concentrations were determined. The statistical non-parametrical Mann-Withney U-test was chosen to compare the kinetics of the studied formulations. A bicompartment model was developed based on the experimental results.

3. RESULTS

3.1. Ocular tolerance

The percentages of corneal damage experimentally observed after the administration of Restasis and OPPH 088 formulations are shown in Figure 2 along with the value for an isotonic sterile saline solution determined during a previous study [20]. The corneal injury surface evaluated after the administration of OPPH 088 was 4.84 +/- 2.72 % of the observed area, while for Restasis it was 18.20 +/- 6.82 %; the value for a sterile saline solution was 1.15% +/- 0.35%.

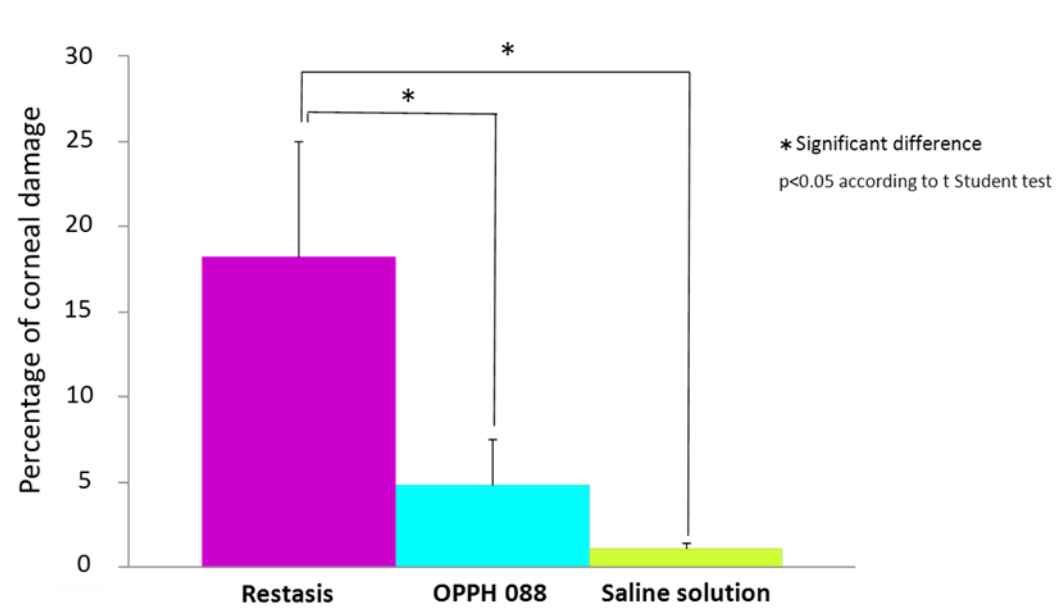


Figure 2: Percentage of corneal damage after instillation of Restasis (■), OPPH 088 (■) and saline solution (■) (the latter being already reported in a previous study performed by Furrer et al., 1999). Mean +/- SD, n=6, * significantly different according to Student t-test.

According to Student's t test the fluorescent area observed for Restasis was significantly different from that of OPPH 088 or saline solution. Restasis presented the highest corneal injury. Figure 3 clearly highlights the difference between the corneal damage caused by Restasis compared to that by OPPH 088. In addition, the statistical analysis of the values obtained with OPPH 088 compared to those of the sterile saline solution showed no significant differences between the solutions.

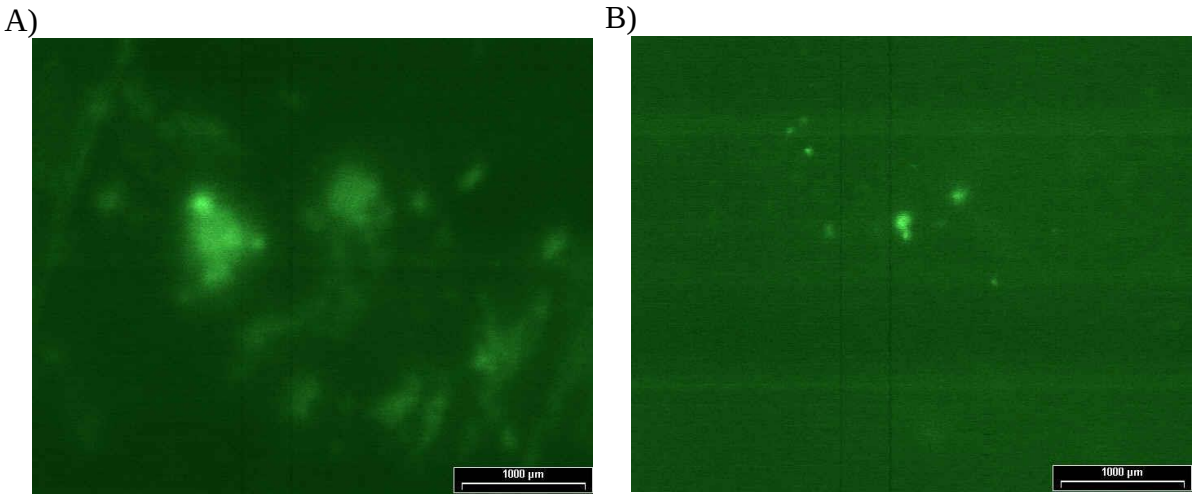


Figure 3: Representative pictures of corneal injury caused by A) Restasis and B) OPPH 088. The damaged corneal cells are permeable to the fluorescein staining and are visible as fluorescent areas.

3.2. Basal lachrymal secretion

The Schirmer test was carried out to assess the influence of the formulations on the basal tear production. Figure 4 illustrates the experimental data. The basal mean values for all animals before drug application were between 10.6 and 12.2 mm which are represented by the dashed line area in the figure. Data from the two rabbit groups do not significantly differ; values after administration of the formulations and baseline are either significantly different. Nevertheless it can be noticed that rabbits treated with the prodrug solution have values above baseline or included in the baseline area, while the Restasis group presents more fluctuations below the baseline area.

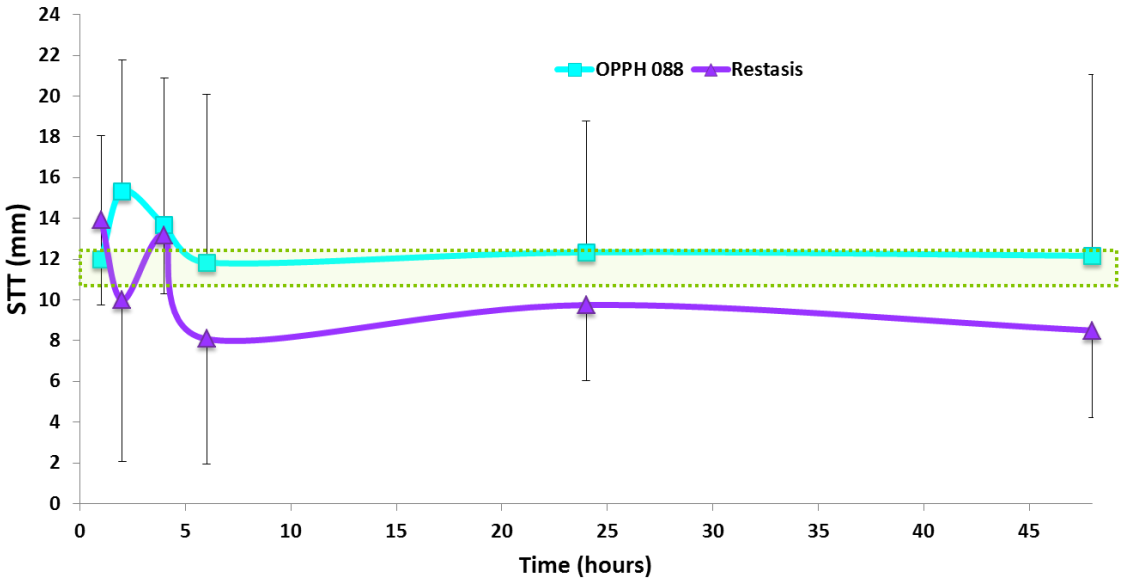


Figure 4: Basal lachrymal production after a single instillation of Restasis and the prodrug formulation (n=6) reported in STT (Schirmer tear test) in mm of wetted strip. The area with dashed line indicates the basal tear production prior to the formulation application.

3.3. Ocular distribution

The prodrug solution was administered bid during 5 days on 6 rats and CsA levels were determined in ocular tissues and fluids. The average CsA concentrations were estimated at 247 +/- 203 ng/g for the cornea, 1082 +/- 761 ng/g for the conjunctiva, 45 +/- 18 ng/g for the aqueous humour, 315 +/- 139 ng/g for the iris-ciliary body, 24 +/- 13 ng/g vitreous humour and 149 +/- 83 ng/g for the retina; as illustrated in Figure 5. The anterior and posterior structures of the eye were exposed to CsA, the highest levels being located in the anterior chamber.

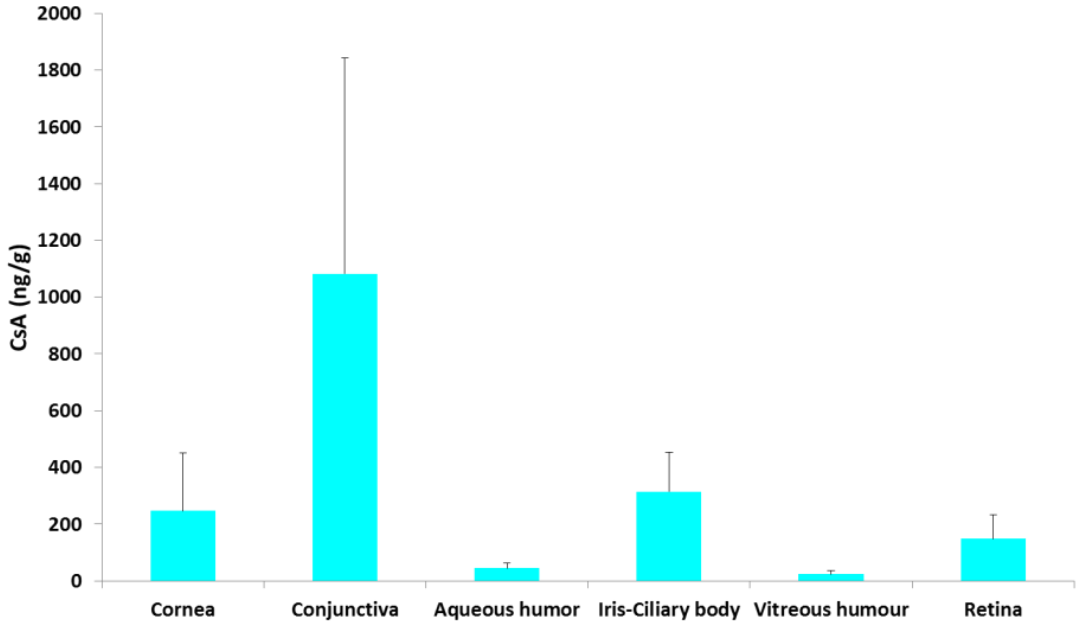


Figure 5: CsA ocular distribution after topical application of OPPH 088 formulation bid for 5 days (n=6).

3.4. *In vivo* precorneal kinetics

3.4.1. Kinetic profiles

Figure 6 illustrates the CsA tear fluid kinetic profile after administration of Restasis up to 180 min. Different kinetic phases were identified. The first phase was characterised by the rapid and marked decrease of CsA levels observed during the first 20 min. The second phase was characterised by a plateau from 20 to 180 min. Analysis of the tear samples for longer time points exhibited concentrations of CsA of 5 +/- 1 ng/mL and 2 +/- 1 ng/mL for 24 and 48 h,

Figure 7 presents the CsA tear fluid kinetic profile after administration of OPPH 088 formulation for the first 180 min. OPPH 088 appeared to have a very different general trend compared to Restasis, especially concerning the first time points and the maximum CsA concentrations.

A supplementary phase was observed in addition to the ones already identified for Restasis. In fact, the profile showed a marked increase of CsA during the first 15 min. This corresponded to the first biotransformation step required in order to allow CsA to be released into the tear film from the prodrug formulation. This first phase was linked to the formulation characteristics and explained the specificity of the profile. Later, a rapid decrease in CsA tear levels was observed and defined as the second phase. A plateau was visible up to 3 h. Furthermore, the analysis of the lachrymal fluid samples after 24 and 48 h showed levels of CsA of 5 +/- 1 ng/mL and 2 +/- 1 ng/mL, respectively. The sustained concentrations of CsA observed with Restasis were also seen for OPPH 088, constituting the third phase of its kinetic profile.

In addition, maximum CsA concentration in tears was approximately 100 times higher for Restasis than the one assessed for OPPH 088 formulation, which was expected as in the case of Restasis CsA was directly applied on the surface of the eye.

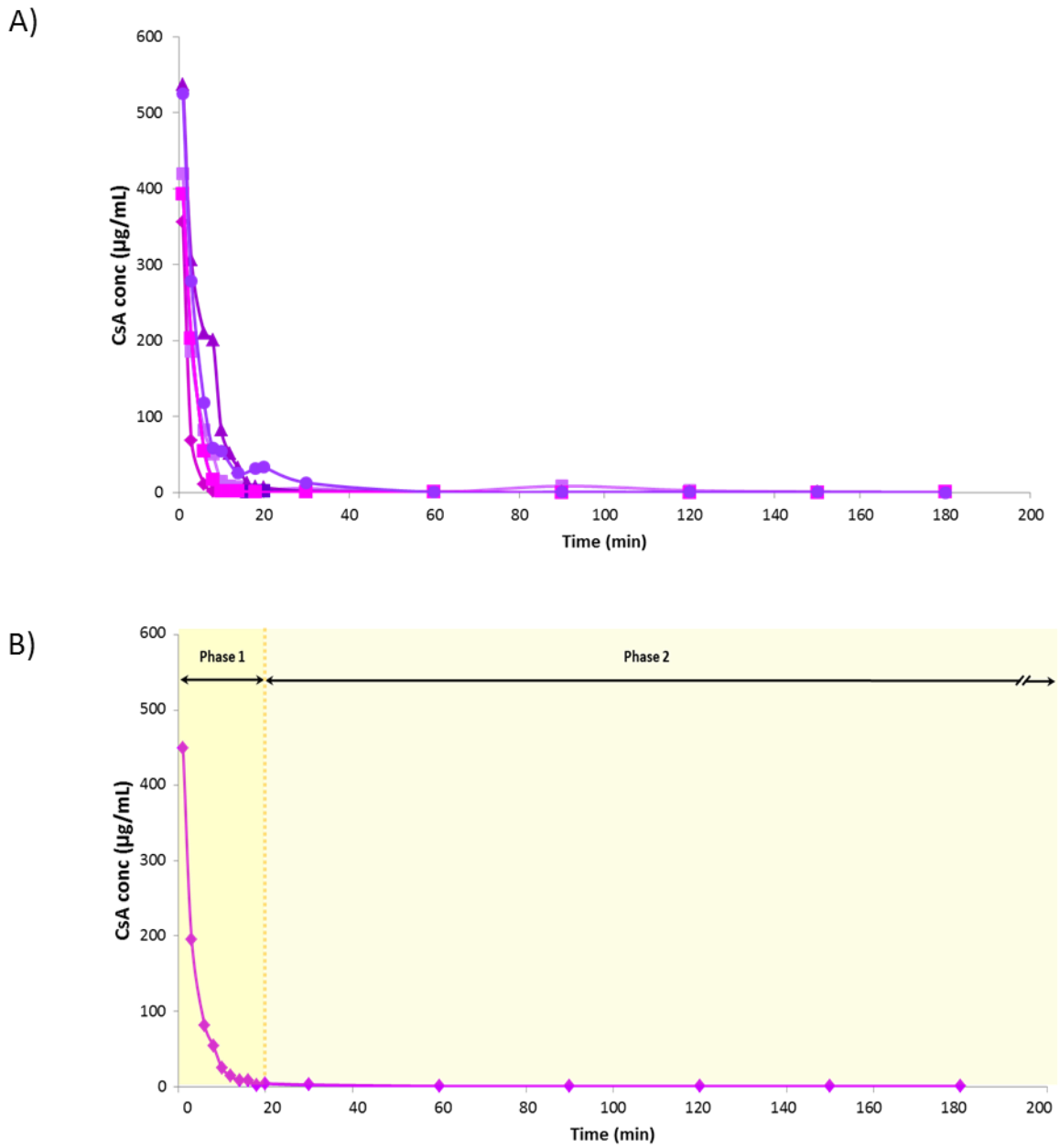


Figure 6: CsA tear fluid kinetic profile after instillation of Restasis A) for six rabbits and B) based on median values (n=6). The kinetic phases are represented in B).

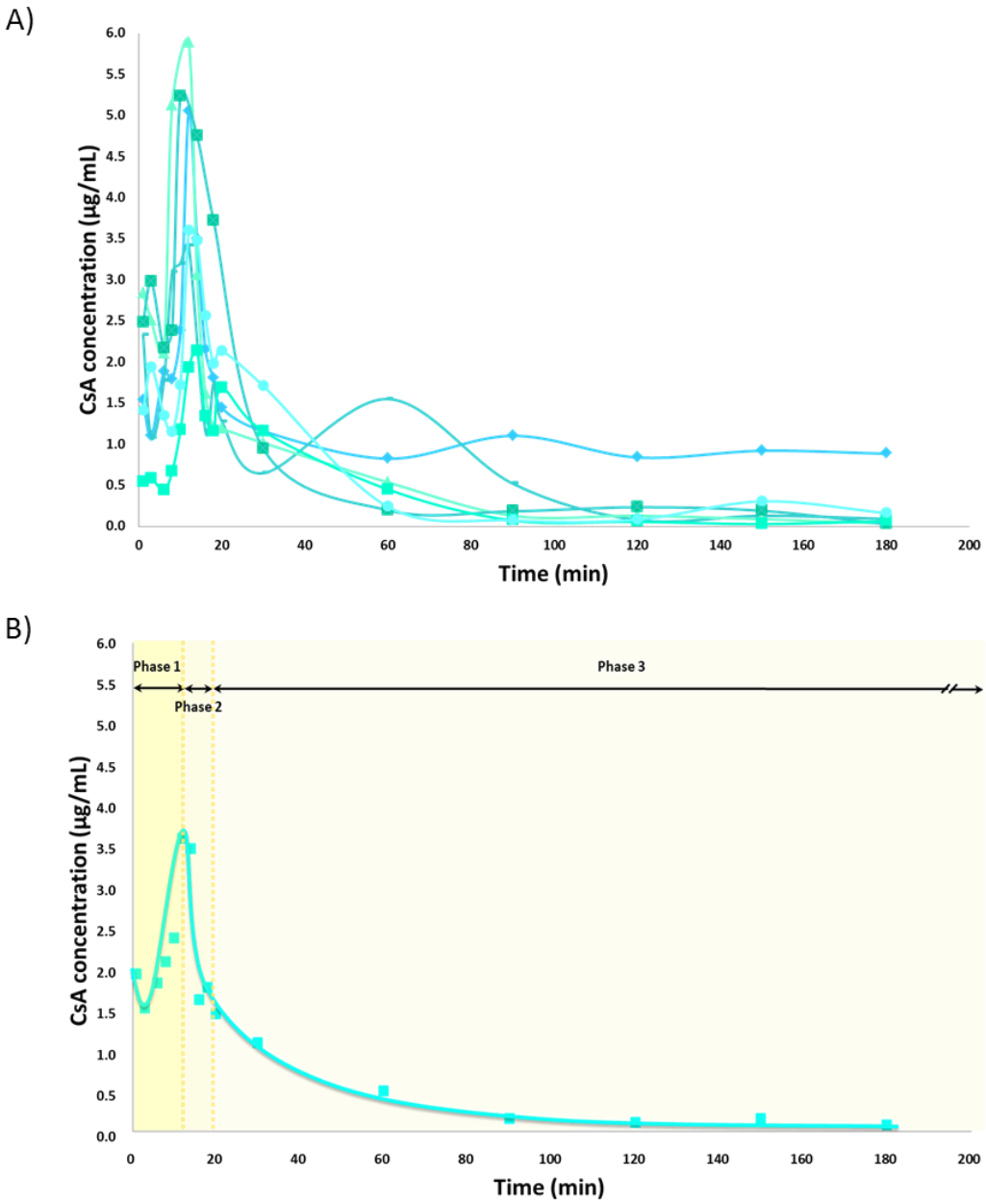


Figure 7: CsA tear fluid kinetic profile after instillation of OPPH 088 formulation A) for six rabbits and B) based on median values (n=6). The kinetic phases are represented in B).

3.4.2. Statistical analysis and comparison

A non-parametrical method was selected for this study, assuming that the distribution of *in vivo* CsA concentrations for each time point was not necessarily Gaussian. Hence, the kinetic profiles were summarised with median values for each time point, and the Mann-Withney statistical U-test was chosen to analyse the differences between the kinetics of the studied formulations. Thus, Figures 6 and 7 show two sets of data: (a) for the six rabbits, and (b) the median profile.

The major cause of disparities between the profiles was directly linked to the constitution of the formulations themselves. The observed differences during the first 15 min were linked to the nature of the formulations and other factors such as, for example, the ocular protective mechanisms or the enzymatic biotransformation specific to OPPH 088. Thus, an appropriate comparison can only be made if this variability factor is avoided. Consequently, the time points taken before 15 min were discarded from comparison, as illustrated in Figure 8.

No statistically significant differences were found for the time points between 15 min and 48 h when applying the U-test to the two rabbit groups.

Therefore, the *in vivo* behaviours of both tested formulations were considered equivalent regarding CsA levels in tears between 15 min and 48 h.

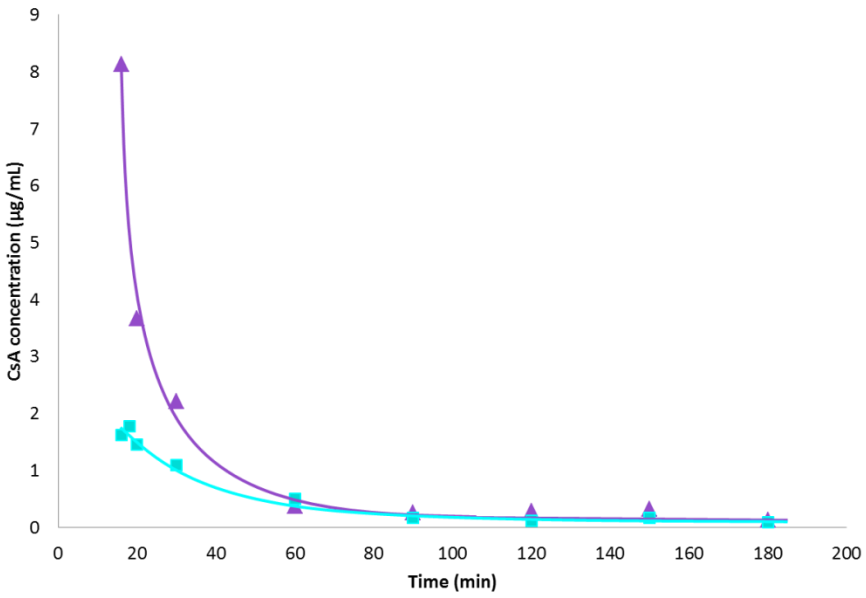


Figure 8: Restasis (▲) and OPPH 088 (■) kinetic profiles between 15 and 180 min based on median values (n=6).

3.5. Model development

The development of a model, based on the experimental data can be of great interest for a deeper understanding of the *in vivo* behaviour of each formulation, particularly of their elimination.

A two compartment model was selected based on the above presented results and the fact that the experimental kinetic curves clearly showed a profile with, at least, two phases with different linear regression constants. The central compartment was defined as the tear fluid (in which the measurements were performed during the kinetic evaluation); the peripheral one was not precisely physically delimited and included the other structures, tissues and fluids that could interact with CsA.

The bicompartmental equation took into account two main phenomena; the distribution and elimination of CsA from the tear fluid [21].

The model used for the Restasis formulation was based on the following equation:

$$C = A_0 e^{-\alpha t} + B_0 e^{-\beta t} \qquad (1)$$

where A_0, B_0, α and β were macroconstants for the hybrid disposition processes and were determined according to the method of residuals. From these macroconstants the microconstant for the pure elimination process was calculated using the following equation:

$$K_{el} = \frac{\alpha\beta(A_0 + B_0)}{A_0\beta + B_0\alpha} \qquad (2)$$

For the formulation containing OPPH 088, the additional biotransformation step had to be considered. The model equation was written as it follows:

$$C = A_0 e^{-\alpha t} + B_0 e^{-\beta t} + P_0 e^{-\gamma t} \qquad (3)$$

where A_0, B_0, P_0, α, β and γ are macroconstants for the hybrid processes that were determined according to the method of residuals. The microconstant for the pure elimination process was calculated using the following relationship:

$$K_{el} = \frac{\alpha\beta\gamma(A_0 + B_0 + P_0)}{A_0\beta\gamma + B_0\alpha\gamma + P_0\alpha\beta} \qquad (4)$$

The β elimination half life was calculated as showed in equation 5:

$$t_{1/2_\beta} = \frac{0.693}{\beta} \qquad (5)$$

The coefficients of determination, r^2, for the developed models were 0.9452 for Restasis and 0.9643 for OPPH 088.

According to the above presented equations the values of the elimination constants were 0.2193 min^{-1} for Restasis, and 0.0160 min^{-1} for OPPH 088. The Restasis elimination constant was more than ten times higher than for the prodrug regarding CsA concentration in tears. The β elimination half life values were 32 min for Restasis and 87 min for OPPH 088; approximately 3 times lower for the commercial emulsion than for the prodrug. These results suggest a higher CsA elimination process with Restasis than with OPPH 088 formulation. Model characteristics are summarised in Table 1.

Table 1: Bicompartment model characteristics for Restasis and OPPH 088. In a) are shown the bicompartment model equations where A_0, B_0, P_0, α, β and γ are macroconstants for the hybrid processes that were determined according to the method of residuals, in b) the elimination constant equation and values, in c) the β elimination half life equation and value and in d) the coefficient of determination (r^2) values.

		Restasis	**OPPH 088**
a) Bicompartment model equation		$C = A_0e^{-\alpha t} + B_0e^{-\beta t}$	$C = A_0e^{-\alpha t} + B_0e^{-\beta t} + P_0e^{-\gamma t}$
b) Elimination constant	Equation	$K_{el} = \frac{\alpha\beta(A_0 + B_0)}{A_0\beta + B_0\alpha}$	$K_{el} = \frac{\alpha\beta\gamma(A_0 + B_0 + P_0)}{A_0\beta\gamma + B_0\alpha\gamma + P_0\alpha\beta}$
	Value	0.2193 min^{-1}	0.0160 min^{-1}
c) β elimination half life	Equation	$t_{1/2_\beta} = \frac{0.693}{\beta}$	
	Value	32 min	87 min
d) Coefficient of determination (r^2) value		0.9452	0.9643

4. DISCUSSION

The possible alteration of the ocular surface was the first concern when developing a novel ocular formulation. Additionally, it was a key factor that had to be evaluated prior to the kinetic and distribution studies since it could influence the experimental results. The confocal microscopy technique seemed to be an appropriate method for the objective and sensitive evaluation of the *in vivo* ocular irritation induced by topical ophthalmic formulations, as it has been demonstrated by previous studies [20, 22]. In addition it is a simple and non-invasive procedure. As expected, the experimental results demonstrated that the two tested preparations had a good ocular tolerance (corneal damage lower than 25%) according to the classification established by Kälin [23]. Restasis has been approved by the FDA for human use; therefore, it was not surprising that no important corneal damage was determined. This result was consistent with previous *in vitro* and *in vivo* studies where no tolerability problems were evidenced [24-27]. Nevertheless, the slightly higher fluorescence observed with Restasis compared to the other formulations may be explained by the presence of surfactants, required for the oil-in-water emulsion, that act as detergents [20]. The percentage of corneal damage assessed with the saline solution is representative of the normal physiological desquamation of the surface of the rabbit eye [28]. The assessed fluorescent area for OPPH 088 did not significantly differ from the saline preparation, clearly demonstrating the excellent ocular tolerance of the OPPH 088 formulation. Moreover, the prodrug formulation is expected to exhibit an excellent ocular tolerance in humans also, as human eyes are less sensitive to ocular irritation than rabbits [29]. On the other hand the Schirmer test demonstrated that neither the prodrug formulation nor Restasis had any negative effect on basal tear production of healthy animals. Both corneal damage assessment and Schirmer test clearly demonstrated that the formulations had no negative effect on the ocular surface properties. In addition, the precorneal behaviour and ocular distribution studies were not expected to be influenced by any corneal irritation or any variation of the lachrymal fluid production.

Once the ocular tolerance of the formulation was found to be excellent, the question of the *in vivo* behaviour and efficacy of the prodrug solution could be addressed. The precorneal behaviour of both formulations as well as the ocular CsA distribution following the prodrug administration were evaluated based on the CsA quantification on ocular tissues and fluids. A specific UHPLC-MS/MS method was developed to address different analytical challenges: i) the low volume and weight of the biological fluids and tissues (2µL of tear fluid or different parts of the rat eye), ii) the low CsA levels expected in the samples to be analysed, iii) the presence of very close chemical structures in OPPH 088 samples coming from the biotransformation of the prodrug into CsA, iv) the potential interference of the biological matrices and v) the high number of samples to be analysed. The UHPLC system fulfilled the analytical requirements regarding the high throughput (analysis time of 3 min) as well as the high robustness (variation of the retention time <1%) allowing the use of an automated quantitative procedure. In addition, the inherent properties of the tandem mass spectrometric detection provided the required elevated sensitivity (LOQ of 2ng/mL) and high selectivity (ensuring no interferences from close chemical structures coming from the biotransformation of OPPH 088 into CsA or components from the matrix). Moreover the use of a last generation MS/MS instrument was highly beneficial in order to allow a sufficient number of data points across the CsA narrow peak for an accurate quantification of CsA within the short analysis time provided by the UHPLC system. Finally, the systematic addition of an internal standard, d_{12}CsA, to the samples ensured the correction of the possible variability during the ionisation process [30, 31]**.** The optimised UHPLC-MS/MS method met the analytical needs for the *in vivo* investigations and displayed interesting characteristics due to the high throughput and high sensitivity.

After the topical administration of the drug, CsA was distributed from the precorneal area between the ocular structures according to its partition coefficient [26]. Thus the quantification of CsA in the lachrymal fluid provided an overview of the precorneal ongoing processes after the administration of the drug while the determination of CsA levels in different ocular tissues allowed the evaluation of the penetration and therapeutic effect of the drug. A precorneal *in vivo* kinetic was selected primarily because the mechanism of action of the CsA in the dry eye disease is linked to the precorneal area [3, 26, 27] and because it is a

well-established, non-invasive *in vivo* procedure [32, 33]. The ocular distribution experiment was necessary in order to evaluate the therapeutic effect obtained after OPPH 088 administration since there is no established standard objective test [1, 2] neither an accepted animal model for the evaluation of DED [34]. Furthermore, the rabbit and rat animal models are well characterised and commonly used for ocular investigations [17, 26, 34-36]**.**

The ocular distribution evaluation showed the ability of the prodrug to achieve active CsA concentrations (e.i. between 50 and 300 ng/g of tissue [37]) in all the studied ocular tissues; cornea, conjunctiva, iris-ciliary body and retina. These results demonstrated the deep ocular penetration obtained with OPPH 088 and its capacity to lead to therapeutic effect in both the anterior and posterior segments of the eye. On the contrary similar ocular distribution studies performed with Restasis [38] showed lower CsA levels in all ocular tissues compared to the prodrug solution; CsA concentrations after the emulsion application being orders of magnitude below the therapeutic range. The comparison of the presented results with a former study performed with Restasis in rabbits and dogs [26] showed that the prodrug solution achieved higher concentrations in the whole eye, except for the cornea were both Restasis and the prodrug formulation lead to active CsA levels. Thus the prodrug solution clearly showed a better performance in achieving pharmacologically active CsA levels in the ocular structures compared with Restasis, highlighting its higher penetration capacity.

The target areas for the treatment of DED are located in the anterior segment of the eye and are in direct contact with the lachrymal fluid from where the CsA was redistributed depending on its affinity and bioavailability towards them. The conjunctiva is highly involved in the superficial ophthalmic inflammation reported in DED patients. The above presented ocular distribution results showed that CsA coming from the prodrug solution achieves its maximum concentration in the conjunctival tissue, being highly beneficial for the treatment of the DED. In contrast, CsA delivered from a lipophilic vehicle does not preferably penetrate into that structure [26, 38, 39]. Lachrymal glands are susceptible to an accumulation of CsA because they have specific transporters that retrieve selectively the peptide from the surrounding environment [26, 40-42]. This action could also contribute to the therapeutic effect of the drug. It has been suggested in numerous studies that the cornea plays an important role in topical ophthalmic treatments, particularly in the case of CsA formulations where the active

molecule can accumulate in the corneal epithelial cells, that can be considered a lipophilic compartment acting as a reservoir [17, 26, 43-46]. The transport of CsA into the cornea was mainly caused by its partitioning between the highly hydrophilic lachrymal fluid and the lipophilic corneal epithelial cells, therefore leading to an accumulation following the administration. The posterior redistribution and release of the drug into the tear film could be explained by the presence of specific active CsA transporters that cause a drug efflux from the cornea into the lachrymal compartment [43]. Another explanation could be that the continuous clearance of CsA from the eye surface displaced the equilibrium in favour of the tear fluid. In the case of the prodrug, the CsA located in the conjunctiva could also undergo the same redistribution processes. The redistribution phenomenon was only relevant for longer time points because during the first few minutes after the administration there was a saturation of CsA in tears that makes the passive diffusion predominant. The accumulation of CsA followed by the redistribution is consistent with the measured CsA levels in tears for both formulations until 48 h (Phase 2 for Restasis and Phase 3 for OPPH 088).

According to the postulated CsA distribution mechanism, the cornea plays a key role in the kinetics acting as a CsA reservoir. Additionally, in the case of the prodrug the conjunctiva could also take part in the phenomenon.

The kinetics could be correlated to the mentioned physiological characteristics and some hypotheses can be made to explain their profiles. In the case of Restasis the first phase of the kinetic profile can be related to the predominance of a strong and rapid precorneal drug loss linked to the interaction of CsA with the ocular structures, and to the stimulation of the specific protective mechanisms of the eye after the administration of the eyedrop. The second phase can be explained by the establishment of equilibrium in the CsA distribution which is due to the reservoir effect postulated from the corneal epithelium. The same phenomena took place for the kinetics of OPPH 088; however, it was essential for the prodrug to undergo an enzymatic biotransformation prior to the appearance of CsA in the tear fluid. The first phase represented a mixture of the precorneal loss of OPPH 088 and CsA, and the gradual biotransformation of the prodrug into CsA directly in the tear fluid. The increase of CsA levels during the first minutes strongly suggested that the predominant mechanism in that phase was the biotransformation of OPPH 088. The second phase of the kinetic of OPPH 088 was considered equivalent to the first phase of Restasis, which corresponded with a

predominant loss of drug from the surface of the eye. The third phase is characterised by sustained drug concentrations in tears suggesting a redistribution of the drug.
Despite the major disparities exposed between the general profiles, the statistical analysis based on the Mann-Whitney U-test for time points starting from the 15 min mark showed no significant difference between the kinetics of Restasis and OPPH 088. Both formulations induced equivalent CsA levels in the tear fluid between 15 min and 48 h after topical ophthalmic application.

Nevertheless, it has to be noted that the quantitative approach presented is restricted to the limitations of the technique used. A more global and qualitative view can be of interest to better understand the *in vivo* behaviour of the studied formulations. As the elimination of CsA is a continuous and subtle phenomenon, its further analysis was only possible through the presented model that allows to unveil interesting information (which would have been unnoticed with the quantitative approach). A two compartment model centred on the tear fluid was appropriate since it reflects the physiological situation [35, 46] and it is in accordance with the experimental results.
The obtained values for the elimination constant and the β half life clearly pointed out a higher ocular elimination for CsA when administered with the emulsion compared to the prodrug formulation. This result is consistent with the previously presented data for Restasis (higher corneal damage and lower CsA levels in ocular tissues). The difference observed in the elimination of the studied formulations could be linked to their nature; oil in water emulsion for Restasis versus a totally aqueous solution for OPPH 088.
It has been demonstrated that the nature of the formulation has a key influence on CsA elimination, availability and efficacy [26, 27, 47]. On one hand, the elimination could be enhanced by the stimulation of the protective mechanisms of the eye. For Restasis some reported adverse effects such as ocular burning, discharge, foreign body sensation, redness or epiphora [48] could be linked to the stimulation of the protective mechanisms of the eye that could reinforce the elimination. Previous studies showed that the ocular application of a lipophilic vehicle is not without consequences [49-51]. In the case of OPPH 088, the aqueous vehicle contributed to a lower foreign body reaction and a moderated inducement of protection mechanisms (which was consistent with the previously presented ocular tolerance results). Thus OPPH 088 formulation could lead to a lower patient's discomfort level. On the

other hand, the miscibility of the formulations with the lachrymal fluid has also to be considered. To obtain a therapeutic effect with Restasis, CsA must leave the lipophilic fraction of the emulsion to be released into the lachrymal fluid and reach the target tissues. Nevertheless, the partition coefficient of CsA between tears and its lipophilic carrier was not favourable. CsA had a higher affinity for the oily phase of the emulsion that limited its release and availability, as former studies demonstrated [47]. In addition, the lipophilic fraction of Restasis was more compatible with the superficial lipid layer of the tear film which facilitated its elimination. In the case of OPPH 088, the prodrug was easily distributed within the lachrymal fluid layer, where the enzymatic biotransformation occurred, resulting in the release of CsA directly into the tear fluid. The generated CsA is surrounded by the lachrymal hydrophilic environment from where it had a higher tendency to interact with ocular tissues. The CsA release and bioavailability are then reinforced. This postulated higher CsA availability for OPPH 088 formulation is in accordance with the previously described lower elimination and higher ocular tissues levels.

Despite the fact that the same active molecule was involved in both formulations, the implicated mechanisms were different; OPPH 088 aqueous formulation could lead to higher penetration, higher ocular tissue concentrations, lower elimination, higher availability and higher efficacy of CsA.

5. CONCLUSION

This investigation demonstrated the excellent ocular tolerance of the OPPH 088 formulation, and gives considerable insight on the *in vivo* behaviour of the studied ophthalmic formulations. On one hand, it demonstrated the ability of OPPH 088 formulation to achieve therapeutically active CsA levels in both the anterior and posterior segments of the eye, highlighting higher CsA tissue levels and penetration capacity compared to Restasis. On the other hand it evidenced a lower elimination and higher availability for CsA when generated from the prodrug than after direct application with the emulsion. Therefore, it can be suggested that the use of OPPH 088 represents a safe and suitable option for the treatment of DED. In addition OPPH 088 has some interesting advantages such as an increased patient comfort and higher compliance, which are of major importance since DED requires long periods of treatment.

Ongoing *in vivo* investigations will allow a deeper understanding of the behaviour of CsA-based topical ophthalmic formulations.

ACKNOWLEDGMENTS

The authors would like to acknowledge the financial support from Ophthalmopharma (Sarnen, Switzerland). The animal experiments have been made with the help of Catherine Siegfried. The authors would also like to thank Dr. Raul Nicoli for his help with analytical questions.

REFERENCES

[1] M.A. Lemp, Advances in understanding and managing dry eye disease, Am J Ophthalmol, 146 (2008) 350-356.

[2] H.D. Perry, Dry eye disease: pathophysiology, classification, and diagnosis, Am J Manag Care, 14 (2008) S79-87.

[3] K. Gumus, D.H. Cavanagh, The role of inflammation and antiinflammation therapies in keratoconjunctivitis sicca, Clin Ophthalmol, 3 (2009) 57-67.

[4] M.E. Johnson, P.J. Murphy, Changes in the tear film and ocular surface from dry eye syndrome, Prog Retin Eye Res, 23 (2004) 449-474.

[5] R.M. Schiffman, J.G. Walt, G. Jacobsen, J.J. Doyle, G. Lebovics, W. Sumner, Utility assessment among patients with dry eye disease, Ophthalmology, 110 (2003) 1412-1419.

[6] F. Lallemand, O. Felt-Baeyens, K. Besseghir, F. Behar-Cohen, R. Gurny, Cyclosporine A delivery to the eye: a pharmaceutical challenge, Eur J Pharm Biopharm, 56 (2003) 307-318.

[7] S. Kashani, A.A. Mearza, Uses and safety profile of ciclosporin in ophtalmology, Expert Opin. Grug Saf., 7 (2008) 79-89.

[8] S.M. Stepkowski, Molecular targets for existing and novel immunosuppressive drugs, Expert Rev Mol Med, 2 (2000) 1-23.

[9] S. Matsuda, S. Koyasu, Mechanisms of action of cyclosporine, Immunopharmacology, 47 (2000) 119-125.

[10] N. Andre, B. Roquelaure, J. Conrath, Molecular effects of cyclosporine and oncogenesis: a new model, Med Hypotheses, 63 (2004) 647-652.

[11] A. Yoshida, T. Fujihara, K. Nakata, Cyclosporin A increases tear fluid secretion via release of sensory neurotransmitters and muscarinic pathway in mice, Exp Eye Res, 68 (1999) 541-546.

[12] H. Toshida, K. Nakayasu, A. Kanai, Effect of cyclosporin A eyedrops on tear secretion in rabbit, Jpn J Ophthalmol, 42 (1998) 168-173.

[13] A.G. Palestine, R.B. Nussenblatt, C.-C. Chan, Side effects of systemic cyclosporine in patients not undergoing transplantation, The American Journal of Medicine, 77 (1984) 654-656.

[14] E. Clarke, Clarke's isolation and identification of drugs, 2nd ed., Pharmaceutical Press, London, 1986.

[15] A.R. Hamel, F. Hubler, A. Carrupt, R.M. Wenger, M. Mutter, Cyclosporin A prodrugs: design, synthesis and biophysical properties, J Pept Res, 63 (2004) 147-154.

[16] F. Lallemand, O. Felt-Baeyens, S. Rudaz, A.R. Hamel, F. Hubler, R. Wenger, M. Mutter, K. Besseghir, R. Gurny, Conversion of cyclosporine A prodrugs in human tears vs rabbits tears, Eur J Pharm Biopharm, 59 (2005) 51-56.

[17] F. Lallemand, P. Furrer, O. Felt-Baeyens, M. Gex-Fabry, J.M. Dumont, K. Besseghir, R. Gurny, A novel water-soluble cyclosporine A prodrug: ocular tolerance and *in vivo* kinetics, Int J Pharm, 295 (2005) 7-14.

[18] F. Lallemand, P. Perottet, O. Felt-Baeyens, W. Kloeti, F. Philippoz, J. Marfurt, K. Besseghir, R. Gurny, A water-soluble prodrug of cyclosporine A for ocular application: a stability study, Eur J Pharm Sci, 26 (2005) 124-129.

[19] F. Lallemand, E. Varesio, O. Felt-Baeyens, L. Bossy, G. Hopfgartner, R. Gurny, Biological conversion of a water-soluble prodrug of cyclosporine A, Eur J Pharm Biopharm, 67 (2007) 555-561.

[20] P. Furrer, B. Plazonnet, J.M. Mayer, R. Gurny, Application of *in vivo* confocal microscopy to the objective evaluation of ocular irritation induced by surfactants, Int J Pharm, 207 (2000) 89-98.

[21] L. Shargel, S. Wu-Pong, A.B.C. Yu, Applied biopharmaceutics & pharmacokinetics, 5th ed., Appleton & Lange Reviews/McGraw-Hill, Medical Pub. Division, New York, 2005.

[22] P. Furrer, R. Gurny, Recent advances in confocal microscopy for studying drug delivery to the eye: concepts and pharmaceutical applications, Eur J Pharm Biopharm, 74 (2010) 33-40.

[23] P. Kälin, Contribution à la validation d'un test de tolérance oculaire sur la souris., in, University of Lausanne, Lausanne, 1994.

[24] G. Singh, R.L. Lindstrom, D.J. Doughman, Cyclosporin A on human corneal endothelium, Cornea, 3 (1984) 272-277.

[25] L. Gan, Y. Gan, C. Zhu, X. Zhang, J. Zhu, Novel microemulsion in situ electrolyte-triggered gelling system for ophthalmic delivery of lipophilic cyclosporine A: *in vitro* and *in vivo* results, Int J Pharm, 365 (2009) 143-149.

[26] D.D. Tang-Liu, A. Acheampong, Ocular pharmacokinetics and safety of ciclosporin, a novel topical treatment for dry eye, Clin Pharmacokinet, 44 (2005) 247-261.

[27] D. Stevenson, J. Tauber, B.L. Reis, Efficacy and safety of cyclosporin A ophthalmic emulsion in the treatment of moderate-to-severe dry eye disease: a dose-ranging, randomized trial. The Cyclosporin A Phase 2 Study Group, Ophthalmology, 107 (2000) 967-974.

[28] P. Furrer, J.M. Mayer, B. Plazonnet, R. Gurny, Ocular tolerance of absorption enhancers in ophthalmic preparations, AAPS PharmSci, 4 (2002) E2.

[29] C.M. Hutak, R.B. Jacaruso, Evaluation of primary ocular irritation: Alternative to the Draize test., in: I.K. Reddy (Ed.) Ocular Therapeutics and Drug Delivery: A Multidisciplinary Approach, Technomic Publishing Co., Inc., Lancaster, Pennsylvania, USA, 1996, pp. 489-525.

[30] L. Novakova, H. Vlckova, A review of current trends and advances in modern bio-analytical methods: chromatography and sample preparation, Anal Chim Acta, 656 (2009) 8-35.

[31] D. Guillarme, J. Schappler, S. Rudaz, J.L. Veuthey, Coupling ultra-high-pressure liquid chromatography with mass spectrometry, Trac-Trend Anal Chem, 29 (2010) 15-27.

[32] G. Dumortier, J.C. Chaumeil, Lachrymal determinations: methods and updates on biopharmaceutical and clinical applications, Ophthalmic Res, 36 (2004) 183-194.

[33] Y. Ohashi, M. Dogru, K. Tsubota, Laboratory findings in tear fluid analysis, Clin Chim Acta, 369 (2006) 17-28.

[34] S. Barabino, M.R. Dana, Animal models of dry eye: a critical assessment of opportunities and limitations, Invest Ophthalmol Vis Sci, 45 (2004) 1641-1646.

[35] N. Worakul, J.R. Robinson, Ocular pharmacokinetics/pharmacodynamics, European Journal of Pharmaceutics and Biopharmaceutics, 44 (1997) 71-83.

[36] D. BenEzra, G. Maftzir, Ocular penetration of cyclosporine A in the rat eye, Arch Ophthalmol, 108 (1990) 584-587.

[37] R.L. Kaswan, Intraocular penetration of topically applied cyclosporine, Transplant Proc, 20 (1988) 650-655.

[38] C. Di Tommaso, F. Valamenesh, F. Miller, P. Furrer, M. Rodriguez-Aller, F. Behar-Cohen, R. Gurny, M. Moller, A novel cyclosporin A aqueous formulation for dry eye treatment: *in vitro* and *in vivo* evaluation, Submitted, (2011).

[39] A. Urtti, Challenges and obstacles of ocular pharmacokinetics and drug delivery, Adv Drug Deliv Rev, 58 (2006) 1131-1135.

[40] D. BenEzra, G. Maftzir, C. de Courten, P. Timonen, Ocular penetration of cyclosporin A. III: The human eye, Br J Ophthalmol, 74 (1990) 350-352.
[41] A.A. Acheampong, M. Shackleton, D.D. Tang-Liu, S. Ding, M.E. Stern, R. Decker, Distribution of cyclosporin A in ocular tissues after topical administration to albino rabbits and beagle dogs, Curr Eye Res, 18 (1999) 91-103.
[42] A. Poon, M. Constantinou, E. Lamoureux, H.R. Taylor, Topical Cyclosporin A in the treatment of acute graft rejection: a randomized controlled trial, Clin Experiment Ophthalmol, 36 (2008) 415-421.
[43] E. Mannermaa, K.S. Vellonen, A. Urtti, Drug transport in corneal epithelium and blood-retina barrier: emerging role of transporters in ocular pharmacokinetics, Adv Drug Deliv Rev, 58 (2006) 1136-1163.
[44] D. BenEzra, G. Maftzir, Ocular penetration of cyclosporin A. The rabbit eye, Invest Ophthalmol Vis Sci, 31 (1990) 1362-1366.
[45] J. Theng, L. Zhou, D. Tan, K.W. La, Distribution of cyclosporin A in the cornea after topical or oral administration, J Ocul Pharmacol Ther, 18 (2002) 83-88.
[46] M. Wiederholt, D. Kossendrup, W. Schulz, F. Hoffmann, Pharmacokinetic of topical cyclosporin A in the rabbit eye, Invest Ophthalmol Vis Sci, 27 (1986) 519-524.
[47] M. Kuwano, H. Ibuki, N. Morikawa, A. Ota, Y. Kawashima, Cyclosporine A formulation affects its ocular distribution in rabbits, Pharm Res, 19 (2002) 108-111.
[48] K. Sall, O.D. Stevenson, T.K. Mundorf, B.L. Reis, Two multicenter, randomized studies of the efficacy and safety of cyclosporine ophthalmic emulsion in moderate to severe dry eye disease. CsA Phase 3 Study Group, Ophthalmology, 107 (2000) 631-639.
[49] R.A. Laibovitz, S. Solch, K. Andriano, M. O'Connell, M.H. Silverman, Pilot trial of cyclosporine 1% ophthalmic ointment in the treatment of keratoconjunctivitis sicca, Cornea, 12 (1993) 315-323.
[50] A.G. Secchi, M.S. Tognon, A. Leonardi, Topical use of cyclosporine in the treatment of vernal keratoconjunctivitis, Am J Ophthalmol, 110 (1990) 641-645.
[51] M.W. Belin, C.S. Bouchard, S. Frantz, J. Chmielinska, Topical cyclosporine in high-risk corneal transplants, Ophthalmology, 96 (1989) 1144-1150.

CHAPTER IV

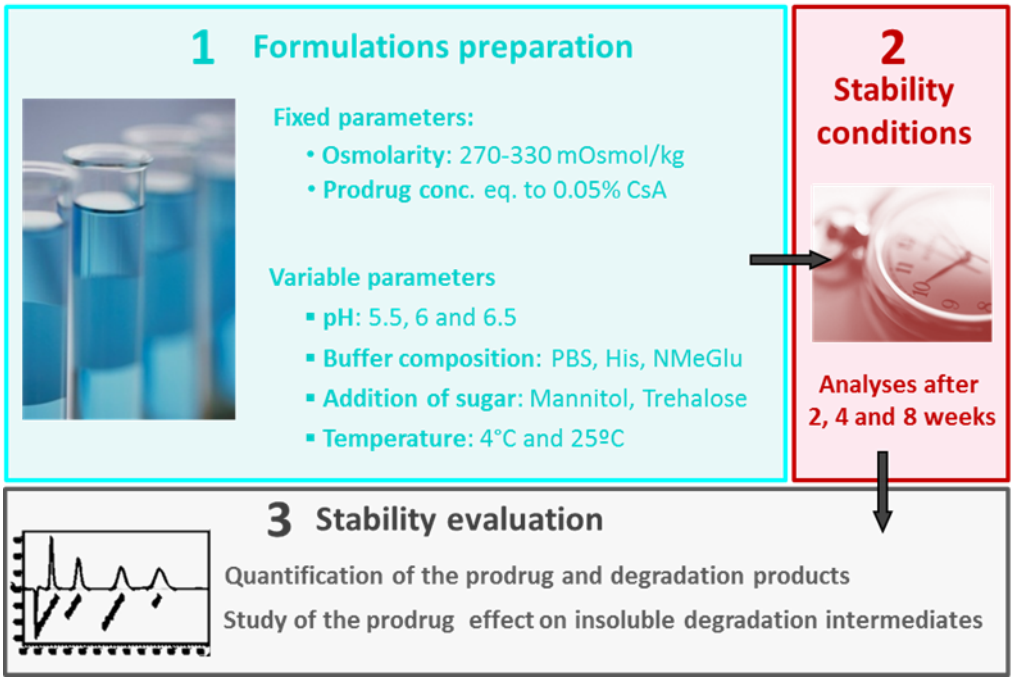
Preformulation study of cyclosporine A prodrug for preclinical investigations

Preformulation study of a water-soluble cyclosporine A prodrug for preclinical investigations

Marta RODRIGUEZ-ALLER, Béatrice KAUFMANN, Davy GUILLARME, Jean-Luc VEUTHEY, Robert GURNY*

School of Pharmaceutical Sciences, University of Geneva, University of Lausanne,
30, Quai Ernest Ansermet, 1211 Geneva 4, Switzerland
*Correspondence: Phone: +41223793816, Fax: +41223796146, E-mail: robert.gurny@unige.ch

To be submitted.



ABSTRACT

A water-soluble cyclosporine A prodrug has been developed to address the challenges related to the poor water solubility of cyclosporine A (CsA). This prodrug was demonstrated to efficiently deliver CsA to the eye with minimal local side effects. The development of a formulation containing an ester prodrug is known to be challenging due to potential instability issues. Prodrugs are designed to display a high reactivity once in contact with the biological medium, but they need to be stable during storage. The question of prodrug stability therefore needed to be addressed to guarantee the quality of the formulation over a reasonable period of time allowing further preclinical investigations. This study presents the stability evaluations of different types of aqueous prodrug formulations for the determination of optimal composition and storage conditions ensuring stability. The stability was assessed through the quantification of the prodrug and its degradation products based on an UHPLC-UV analytical method. The variables tested were pH, temperature, nature of the buffering system and addition of sugars. The experimental results showed that the formulation based on PBS at pH 5.5 and stored at 4°C maintained approximately 99% of the initial prodrug after three months. Interestingly, besides stability, these investigations pointed out a potential solubilizing effect of the prodrug on insoluble degradation intermediates and CsA.

Keywords: water soluble cyclosporine A prodrug, stability, chemical degradation

1. INTRODUCTION

During early stage preclinical investigations, adequate formulation of the active compound is of major importance. An inappropriate formulation could be responsible for suboptimal performances of a compound, not reflecting its real potential. Therefore, even if the formulation used for the investigation does not have the final composition of the drug product, it is important to conduct preformulation studies to maximize the chances of properly evaluating the drug [1].

Former experiments have demonstrated that the water-soluble CsA prodrug is a promising candidate for the management of ocular conditions [2-4]. Prodrugs need to find equilibrium between the high reactivity required for a rapid biotransformation and the stability necessary for a long shelf life. The stability profile of the water-soluble CsA prodrug is of particular importance since it belongs to the group of ester prodrugs, which are known to be challenging in regards to their *in vitro* stability [5, 6]. In addition, temperature and pH were found to have a strong influence on prodrug stability [2]. Thus, the question of prodrug stability in the formulation needed to be addressed prior to any further *in vivo* investigations. The stability study was based on four variables: i) pH, ranging from 5.5 to 6.5, ii) buffering system, including phosphate buffer solution (PBS), histidine and N-methyl-glucamine (NMeGlu), iii) presence of sugars, including mannitol or trehalose and iv) temperature, that was set at 4º and 25ºC. The osmolarity was maintained in the iso-osmolar range (270-330 mOsm/L) and the prodrug concentration was set at 0.066% w/v (equivalent to the commercial 0.050% CsA formulation). The prodrug chemical degradation pathways are illustrated in Figure 1. This information was used for the development of the stability indicating assay based on an UHPLC-UV analysis.

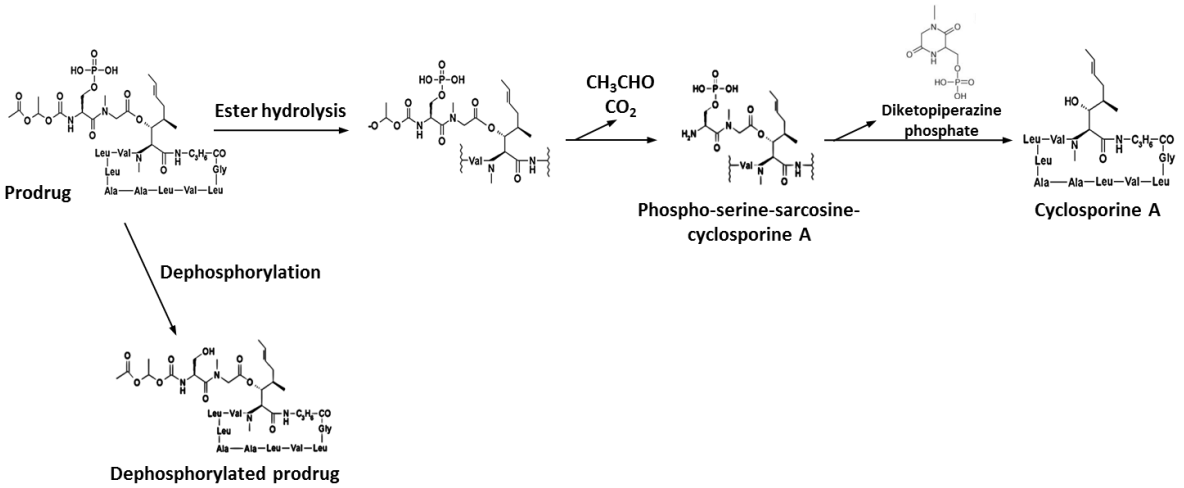


Figure 1: Pathways of *in vitro* chemical degradation of the cyclosporine A prodrug (adapted from [2]).

The aim of this work was to develop an aqueous prodrug formulation that guarantees its quality over a reasonable period of time, allowing further preclinical investigations. The experiments were designed to evaluate the stability of different prodrug formulations leading to the selection of the most stable one, with a minimum of 95% of the initial prodrug content after eight weeks. In addition, these experiments would gather important information about the behaviour of the prodrug when in solution with its degradation intermediates.

2. MATERIALS AND METHODS

2.1. Materials

The CsA prodrug, phospho-serine-sarcosine-CsA (pSer-Sar-CsA) and dephosphorylated prodrug (deP-prodrug) were synthesized at the Institute of Chemical Sciences and Engineering of the Swiss Federal Institute of Technology of Lausanne (EPFL, Switzerland). CsA and sodium chloride were purchased from Fluka (Buchs, Switzerland). Mannitol was purchased from Acros Organics (Geel, Belgium). Water was obtained from a Milli-Q Water Purification System from Millipore (Bedford, MA, USA). Acetic acid and methanol were of ULC/MS grade and purchased from Biosolve (Valkenswaard, Netherlands). N-methyl-glucamine (NMeGlu), histidine, trehalose, phosphate monopotassium, phosphate monosodium, phosphate disodium, chlorhydric acid, phosphoric acid and sodium hydroxide were provided by Sigma-Fluka (Buchs, Switzerland). Acetonitrile and trifluoroacetic acid were purchased from Biosolve (Valkenswaard, Netherlands). The 0.22 µm filters were from Millipore (Cork, Ireland).

The pH and osmolarity values were adjusted using a SevenMulti pH-meter (Mettler-Toledo, Schwerzenbach, Switzerland) and a Vapor Pressure Osmometer 5500 (Wescor, Logan, USA).

2.2. Methods

2.2.1. Manufacturing of the prodrug formulations

Aqueous formulations of the prodrug were prepared by dissolving the appropriate amount of solid prodrug in water and using PBS, histidine or NMeGlu as buffering systems for the adjustment of the pH at pH 5.5, 6.0 or 6.5 (fine pH adjustment was achieved if necessary with sodium hydroxide, hydrochloric acid and phosphoric acid respectively). The buffer systems also contained 5% w/v mannitol or trehalose when indicated. The final prodrug concentration was equivalent to 0.050% w/v CsA (i.e. 0.066% w/v prodrug). The isotonicity was adjusted with sodium chloride. Each formulation was 0.22-µm filtered and dispatched into ten vials for stability testing. Half of each set of vials was kept at 4°C and the other half at 25°C. After two, four and eight weeks, prodrug, CsA, pSer-Sar-CsA and deP-prodrug were quantified.

2.2.2. UHPLC-UV analytical method

Analyses were performed on a Waters Acquity ultra performance liquid chromatographic (UPLC) system coupled with a UV detector (Waters, Milford, MA, USA). The sample manager was kept at 25ºC. A volume of 10 µL (partial loop with needle overfill injection) was injected into the chromatographic column, a Waters Acquity BEH C18 (50 x 2.1 mm I.D., 1.7 µm) heated at 80°C. The mobile phase consisted of a mixture of water and acetonitrile both with 0.1% trifluoroacetic acid. An isocratic elution was used with 60% acetonitrile applied for 2.5 min at a flow rate of 800 µL/min. After each analysis, the injection system was washed with 200 µL of a water/acetonitrile 10:90 solution and 600 µL of a water/acetonitrile 50:50 mixture. The detection was performed at 210 nm. Data acquisition, data handling and instrument control were performed by Empower v2.0 Software (Waters, Milford, MA, USA).

Calibration standards for the prodrug contained 10, 20, 50, 100 and 200 µg/mL. For the calibration of CsA, deP-prodrug and pSer-Sar-CsA the concentrations used were: 0.5, 1, 2, 5, 10, 20, 50, and 100 µg/mL.

3. RESULTS

3.1. Influence of pH and temperature on stability

Figure 2A illustrates the stability profiles at 4ºC of the PBS, histidine and NMeGlu buffered formulations at pH 5.5, 6.0 and 6.5. The graphical representation clearly shows that the PBS formulations had the highest stability, with prodrug levels above 90% of the initial amount after eight weeks. For each buffering system, pH 5.5 formulations showed a lower prodrug degradation compared to pH 6.0 and 6.5. A general classification from most stable to less stable would be: i) PBS-based formulations, ii) histidine formulations and iii) NMeGlu formulations.

Figure 2B illustrates the stability of the formulations at 25ºC. The PBS and NMeGlu formulations displayed similar high stability profiles, which can be considered as equivalent. Surprisingly, with the histidine formulation, the prodrug levels were under 10% after two weeks at 25ºC, and this for all tested pH levels. It is worth mentioning that all histidine-buffered formulations presented a fine precipitate after four weeks at 25ºC. From this data, the formulations can be classified from most stable to less stable as follows: i) PBS formulations and NMeGlu formulations and ii) histidine formulations.

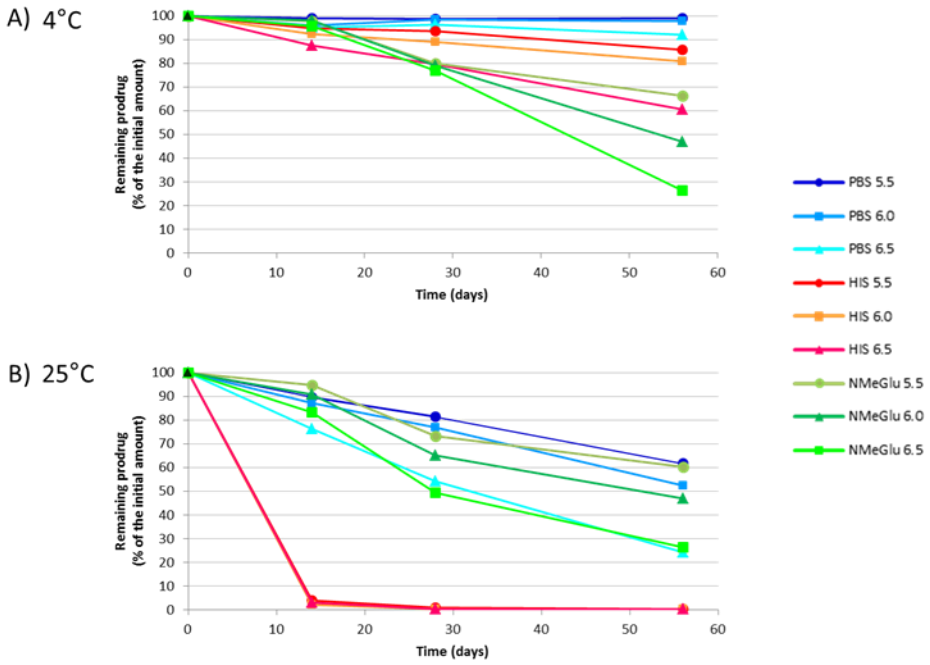


Figure 2: Prodrug content in PBS, histidine and N-methyl-glucamine formulations stored at A) 4°C and B) 25°C. Formulations presented pH values of 5.5, 6.0 and 6.5.

3.2. Influence of sugars on stability

Figure 3 illustrates how sugars affect the stability of the prodrug and the generation of its degradation products in PBS pH 5.5 formulations stored at 4ºC. This data shows that the different formulations can be considered as equivalent in terms of prodrug stability, regardless of the presence of sugars. The three formulations conserved over 98% of their initial prodrug content after eight weeks at 4°C. Similarly, no significant difference was observed in the generation of CsA, pSer-Sar-CsA or deP-prodrug.

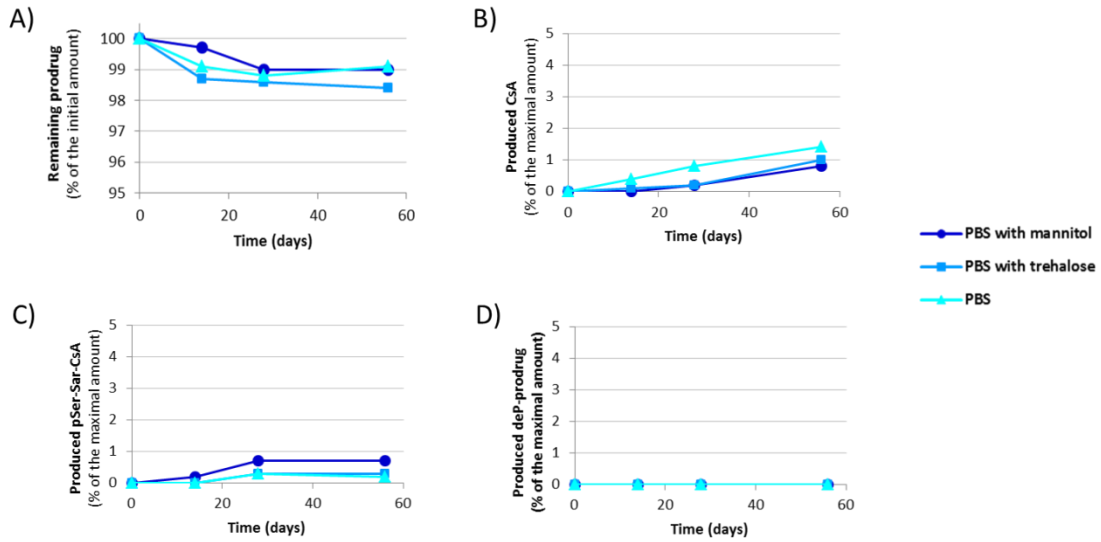


Figure 3: Stability of the prodrug in PBS formulations at pH 5.5 stored at 4°C with the addition of mannitol, trehalose or no sugar. The content in prodrug as well as its degradation products was measured over time: A) remaining prodrug, B) generated CsA, C) generated pSer-Sar-CsA and D) generated deP-prodrug.

4. DISCUSSION

The stability of the prodrug was tested in three buffering systems. The first buffer was PBS, which is frequently used in ophthalmic formulations. The second buffer was based on histidine, known for its physiological buffering capacities and its excellent *in vivo* tolerance [7]. Its buffering capacity relies on the imidazole group with a pK_a of 6.8 and it has an effective buffering range from 5.5 to 7.4. The third buffer was NMeGlu, which was selected because it was already present in the formulation as the counterion of the prodrug salt and it displays buffering capacities [8]. The use of NMeGlu could be an advantage for upscaling, as it would make the manufacturing process easier. In addition, the use of mannitol and trehalose was based on preliminary experimental observations suggesting that the formulations containing sugars could present a slightly enhanced stability (data not shown). Two different sugars were added separately to determine if they had a potential stabilizing effect.

As expected, the environment in which the prodrug was formulated highly influenced its stability. The pH was confirmed to be the most important factor conditioning prodrug degradation. The lowest tested pH value, pH 5.5, led to the highest stability. The other pH values, pH 6.0 and 6.5, were not investigated further. It is important to note that a pH of 5.5 is not an issue for *in vivo* ocular administration. Acidic pH formulations were demonstrated to be safe for ocular administration [9]. As a matter of fact, Propine® (Allergan, Irvine, US) is a well-tolerated pH 3 dipivefrin formulation administered every twelve hours for the long-term treatment of glaucoma [10].

Temperature was the second factor identified as determinant for prodrug stability. As expected, the formulations displayed a higher degradation at 25ºC than at 4°C. This marked temperature-related instability was the reason for the selection of 4ºC as the best option for formulation storage. It is interesting to note that the histidine buffered formulations were particularly unstable, showing a near complete degradation of the prodrug after two weeks. This low stability could be linked to a potential reactivity between the deprotonated carboxylic acid of the histidine (displaying a pK_a of 1.70) and the ester, carbamate and phosphate groups of the prodrug moiety, as illustrated in Figure 4. The chemical reactions between histidine and the prodrug could lead to the production of CsA and the dephosphorylated prodrug by the pathways already presented in Figure 1. These hypotheses

were confirmed by the presence of elevated amounts of CsA and dephosphorylated prodrug in the histidine buffered formulations (data not shown).

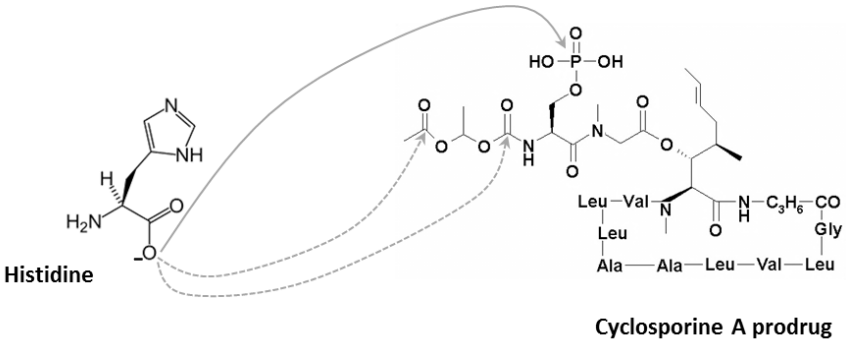


Figure 4: Possible chemical reactions between histidine and the cyclosporine A prodrug leading to the production of dephosphorylated prodrug (— solid line) or CsA (---- dashed line).

Interestingly, the highly degraded histidine formulations showed atypical CsA and deP-prodrug profiles, particularly at pH 6.5. CsA reached a peak concentration after two weeks and then decreased. This phenomenon could be linked to the absence of prodrug and the appearance of precipitates in the histidine formulations, which were not observed in the other formulations. These findings suggest that the absence of prodrug could lead to CsA precipitation, illustrating possible surfactant-like properties of the prodrug. The prodrug is an amphiphilic molecule with two clearly separated polar and apolar sections (the polar one being the prodrug moiety and the apolar one being the CsA backbone). Thus, the prodrug could potentially play a role in the solubilization of CsA, deP-prodrug or other insoluble intermediates. The surfactant-like properties were also supported by the fact that the prodrug formulations present a persisting foam after vortexing. The ability of amphiphilic drugs and prodrugs to act as surfactants has already been reported in former studies [11, 12].

According to these results, the best conditions for prodrug formulation were pH 5.5 and a storage temperature of 4ºC. When comparing the stability of the formulations under these conditions, the PBS formulations seem to be the best option, displaying the highest stability. Neither histidine, nor NMeGlu formulations were found to be superior to the commonly used PBS, and no additional benefits in stability could be achieved with their use. Among the PBS-based formulations, the addition of sugars did not affect stability. Therefore, the PBS pH 5.5 formulation stored at 4ºC was selected as the lead formulation to perform further preclinical investigations.

5. CONCLUSION

This study led to the development of a stable prodrug formulation guaranteeing the quality of the formulation for up to eight weeks and allowing further preclinical investigations. Among the tested excipients and conditions, the most stable formulation was obtained with PBS as a buffering system, a pH of 5.5 and a storage temperature of 4ºC. With this formulation, less than 1% of the prodrug was degraded after eight weeks. None of the other buffer systems or sugar additives tested managed to provide additional stabilizing effects. The histidine buffering system resulted in a high instability, probably due to its chemical reactivity with the prodrug.

Besides stability, these experiments brought interesting information about the potential solubilizing effect of the surfactant-like prodrug on CsA and other insoluble intermediates, and this phenomenon is currently under investigation. If future preclinical experiments confirm that the prodrug is an effective option for the management of ocular conditions in humans, further fine-tuning of the composition, packaging and storage would be required to reach a fully optimized clinical formulation.

REFERENCES

[1] Y. Kawabata, K. Wada, M. Nakatani, S. Yamada, S. Onoue, Formulation design for poorly water-soluble drugs based on biopharmaceutics classification system: basic approaches and practical applications, International journal of pharmaceutics, 420 (2011) 1-10.

[2] F. Lallemand, P. Perottet, O. Felt-Baeyens, W. Kloeti, F. Philippoz, J. Marfurt, K. Besseghir, R. Gurny, A water-soluble prodrug of cyclosporine A for ocular application: a stability study, European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences, 26 (2005) 124-129.

[3] J.L. Bourges, F. Lallemand, E. Agla, K. Besseghir, J.M. Dumont, D. BenEzra, R. Gurny, F. Behar-Cohen, Evaluation of a topical cyclosporine A prodrug on corneal graft rejection in rats, Molecular vision, 12 (2006) 1461-1466.

[4] M. Rodriguez-Aller, B. Kaufmann, D. Guillarme, C. Stella, P. Furrer, S. Rudaz, I. El Zaoui, F. Valamanesh, C. Di Tommaso, F. Behar-Cohen, J.L. Veuthey, R. Gurny, *In vivo* characterisation of a novel water-soluble Cyclosporine A prodrug for the treatment of dry eye disease, European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V, 80 (2012) 544-552.

[5] B.D. Anderson, R.A. Conradi, K.E. Knuth, Strategies in the design of solution-stable, water-soluble prodrugs I: a physical-organic approach to pro-moiety selection for 21-esters of corticosteroids, Journal of pharmaceutical sciences, 74 (1985) 365-374.

[6] R. Liu, Water-insoluble drug formulation, 2nd ed., CRC Press, Boca Raton, FL, 2008.

[7] A.R. Karow, S. Bahrenburg, P. Garidel, Buffer capacity of biologics--from buffer salts to buffering by antibodies, Biotechnology progress, 29 (2013) 480-492.

[8] V. Chromy, L. Zahradnicek, J. Voznicek, Use of N-methyl-D-glucamine as buffer in the determination of serum alkaline phosphatase activity, Clin Chem, 27 (1981) 1729-1732.

[9] J.P. Remington, Remington, the science and practice of pharmacy, in, Mack Pub. Co. Pharmaceutical Press, Easton, Pa.
London, UK, 1995, pp. v.

[10] Physicians' Desk Reference, 65th ed., Medical Economics Company, Inc., Montvale, New Jersey, 2010.

[11] Y. Surakitbanharn, R. McCandless, J.F. Krzyzaniak, R.M. Dannenfelser, S.H. Yalkowsky, Self-association of dexverapamil in aqueous solution, Journal of pharmaceutical sciences, 84 (1995) 720-723.

[12] B.D. Anderson, R.A. Conradi, K. Johnson, Influence of premicellar and micellar association on the reactivity of methylprednisolone 21-hemiesters in aqueous solution, Journal of pharmaceutical sciences, 72 (1983) 448-454.

CHAPTER V

Cyclosporine A prodrug for improved topical delivery to the eye

In vivo distribution and *ex vivo* permeation of cyclosporine A prodrug aqueous formulations for ocular application

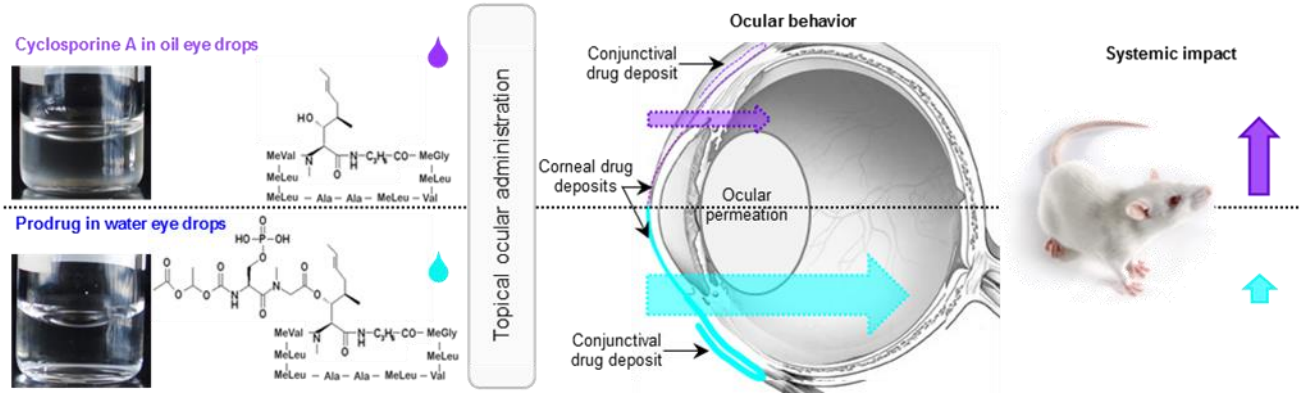
Marta RODRIGUEZ-ALLER[1], Davy GUILLARME[1], Mohamed El SANHARAWI[2], Francine BEHAR-COHEN[2], Jean-Luc VEUTHEY[1], Robert GURNY[1,*]

[1] School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 30, Quai Ernest Ansermet, 1211 Geneva 4, Switzerland

[2] INSERM, UMRS 872 Team 17, Centre de Recherche des Cordeliers, 75006 Paris, France

*Correspondence: Phone: +41223793816, Fax: +41223796146, E-mail: robert.gurny@unige.ch

Published in Journal of Controlled Release 2013; 170:153-9.



ABSTRACT

Cyclosporine A is a poorly water-soluble, immunosuppressive drug used to treat a variety of ocular diseases. Its limited solubility makes challenging the development of a cyclosporine A-based eye drop for ocular topical application. Based on the prodrug strategy, the practically insoluble cyclosporine A was converted into a freely soluble prodrug. Such a water-soluble prodrug made it possible to develop water-based concentrated eye drops. The prodrug formulations were tested for their *ex vivo* permeation and *in vivo* distribution at three concentrations (equivalent to 0.05%, 0.50% and 2.00% w/v cyclosporine A). The *ex vivo* permeation experiments were performed on corneal and conjunctival epithelia. The *in vivo* distribution evaluated the total cyclosporine A present in the ocular structures as well as in serum, spleen and cervical lymphatic ganglions. Each prodrug formulation was compared to conventionally used cyclosporine A eye drops at an equivalent concentration. The experimental results showed that the tested eye drops behaved differently. The prodrug formulation was characterized by the following: i) preferential conjunctival penetration, ii) an interesting capacity to create large tissue deposits and iii) a lower risk of systemic complications and immunosuppression. The prodrug aqueous eye drop was demonstrated to be a patient-friendly option for the treatment of ocular diseases requiring high ocular levels of cyclosporine A, pushing the boundaries of the current therapeutic arsenal.

Keywords: cyclosporine A, water formulation, prodrug, *in vivo* distribution, *ex vivo* permeation, eye.

1. INTRODUCTION

The poor water solubility of active molecules is a major pharmaceutical challenge, as 90% of newly discovered active compounds and 40% of marketed drugs are poorly soluble, according to the biopharmaceutical classification system [1]. Cyclosporine A (CyA) belongs to this group [2]. It is an immunosuppressive peptide that has a variety of applications in ophthalmology, including dry eye syndrome, prevention of corneal graft rejection, blepharitis, rosacea, different types of keratoconjunctivitis or conjunctival graft vs. host disease [3, 4]. Nevertheless, the formulation of such a molecule as an eye drop remains challenging. As a consequence, the highest concentration of eye drop conventionally used is 2.00% w/v CyA in oil vehicles [3, 4]. When high CyA concentrations are required, ophthalmologists have no option but systemic administration. Unfortunately, systemic treatments of CyA demand intensive patient monitoring to prevent and manage potential systemic side effects, which can be as severe as nephrotoxicity, hepatotoxicity or hypertension [5, 6]. The patient's quality of life is drastically changed.

The limitations of ocular formulations of CyA are linked to its poor solubilization. CyA requires the use of oily vehicles, which are linked with tolerance issues [7-11] and availability limitations [12-14]. One way to increase the water solubility of CyA was to develop a water-soluble CyA prodrug [2, 15-19]. With such a strategy, the "practically insoluble" CyA molecule was converted into a "freely soluble" CyA prodrug, according to Clarke's classification of drugs [20]. This ester prodrug makes it possible to develop highly concentrated CyA-based ophthalmic formulations within an aqueous vehicle. In addition, this water-based prodrug eye drop avoids tolerance issues linked to the oil vehicles.

In the present study, the *ex vivo* and *in vivo* permeation and distribution of the newly developed aqueous prodrug formulation were evaluated at different concentrations (0.05% w/v, 0.50% w/v and 2.00% w/v equivalent CyA). Each prodrug formulation was compared to conventional CyA eye drops at the equivalent concentration. In this way, the behaviors of both formulation types could be characterized and compared. Together, *ex vivo* and *in vivo* experiments provided essential information on CyA and prodrug formulations, allowing a deep understanding of their interactions with the ocular structures and with the rest of the organism.

2. MATERIALS AND METHODS

2.1. Materials

The CyA prodrug N-methyl-glucamine salt, was synthesized at the Institute of Chemical Sciences and Engineering of the Ecole Polytechnique Fédérale de Lausanne (Switzerland) following the procedure previously described [15]. The prodrug has a molecular weight of 1570 g/mol and its solubility in isotonic PBS at pH 7 was evaluated at 128.28 +/- 0.07 mg/mL [2, 15-19], which represents an increase in solubility of more than 25,000 times compared to CyA itself. CyA and sodium chloride were purchased from Fluka (Buchs, Switzerland). Deuterated CyA was a gift from Novartis (Basel, Switzerland). Mannitol was purchased from Acros Organics (Belgium). Hepes was purchased from AppliChem (Darmstadt, Germany). Water was obtained from a Milli-Q Water Purification System from Millipore (Bedford, MA, USA). Acetic acid and methanol were of ULC/MS grade and purchased from Biosolve (Valkenswaard, Netherlands). Isopropanol and sodium hydroxide were provided by Sigma-Fluka (Buchs, Switzerland).

2.2. Methods

2.2.1. Preparation of the formulations

Prodrug aqueous formulations were prepared at concentrations equivalent to 0.05, 0.50 and 2.00% w/v CyA using PBS pH 5.5 (Metrohm, Herisau, Switzerland) as the vehicle. Isotonicity was adjusted with NaCl (Vapor Pressure Osmometer 5500, Wescor, Logan, Utah, USA). The solution was filtered through a 0.22-µm polyvinylidene fluoride membrane (Millipore, Cork, Ireland) and kept in sterile conditions until use. Each prodrug formulation was compared to a reference CyA formulation at the same concentration: i) 0.05% w/v: commercial oil-in-water emulsion Restasis® (Allergan Inc., Irvine, CA), ii) 0.50% w/v: oily solution made at the pharmacy of the hospital "Hotel Dieu" in Paris and iii) 2.00% w/v: oily solution based on protocol described elsewhere [21]. The 0.05% w/v CyA formulation contained 40 mg of CyA, 40 mg of ethanol, 7.6 mL of castor oil; corn oil was used to adjust the final volume to 8 mL. The 2.00% w/v CyA formulation was produced by evaporating 2 mL of Sandimmun® (Novartis International SA, Basel, Switzerland) containing 100mg of CyA along with 65% w/v of castor oil and 26% w/v of ethanol. After evaporation castor oil was added to adjust the final volume to 5 mL.

2.2.2. Analytical method

The analytical method was published previously [19]. Minor modifications were made to adapt it to the present investigation. Briefly, the mobile phase consisted of a mixture of water with 0.1% v/v acetic acid (A) and methanol with 0.1% v/v acetic acid (B). A linear gradient from 60 to 100% B was applied for 3 min at a flow rate of 600 µL/min followed by a re-equilibrating step of 1 min. Twelve-times deuterated CyA (d_{12}CyA) was used as internal standard. The ratio between the signals of the analytes and d_{12}CyA was the basis for quantification. The pseudo-molecular parent ion and fragment had an m/z of 1224.7 and 1112.4, respectively, for CyA; 1594.0 and 1185.0, respectively, for the prodrug molecule and 1236.7 and 1124.4, respectively, for d_{12}CyA. The inter-channel delay was set to 5 ms, and dwell time was set to 200 ms for CyA and the prodrug and 20 ms for d_{12}CyA. Data acquisition, data handling and instrument control were performed by Masslynx v4.1 Software (Waters, Milford, MA, USA).

Calibration standards contained 0.1 µg/mL d_{12}CyA. CyA calibrators included the following concentrations: 1, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01, 0.005 and 0.002 µg/mL. Prodrug calibrators were prepared at 10, 5, 2, 1, 0.5 and 0.2 µg/mL.

The relative standard deviation on retention times was 0.2% for CyA and 0.0% for the prodrug. When considering calibration standards, the relative standard deviation on peak areas for 3 independent injections for CyA was 3.0% at 0.002 µg/mL, 1.6% at 0.02 µg/mL and 6.7% at 0.2 µg/mL; while for the prodrug it was 9.4% at 0.5 µg/mL and 9.2% at 2 µg/mL.

2.2.3. *Ex vivo* permeation

Ex vivo experiments were performed using pig eyes obtained from a local slaughterhouse, where eyes were collected directly after sacrificing the animals. Eyes were kept in a physiological buffer solution on ice during transportation.

Corneas and conjunctivas with no signs of epithelial damage were dissected and carefully placed on a vertical diffusion cell (Verrerie de Carouge, Geneva, Switzerland). The aqueous prodrug solution and its equivalent CyA oily formulation were tested at a concentration of 2.00% w/v. The 5-mL receptor compartment presented one single arm for sampling and was filled with 25 mM HEPES buffer pH 7.4 preheated to 37°C. A volume of 200 µL of the appropriate formulation was added to the donor compartment. The area of tissue available for diffusion corresponded to 0.64 cm^2. Diffusion cells were kept at 37°C, and the receptor compartment was magnetically stirred during the experiment. Samples of 500 µL were taken every hour and diluted 1:2 with methanol containing 0.2 µg/mL internal standard. The same volume of fresh buffer was subsequently added to the receptor compartment. After 5 h, tissues were removed from the diffusion cells, and the 0.9 cm diameter disc that was in contact with the formulation was dissected. The tissue sections were weighed, ground, put into vials containing 1 mL methanol and 0.1 µg/mL d_{12}CyA and stirred overnight. The next day, samples were centrifuged, and CyA and prodrug were quantified in the supernatants. Six corneas and six conjunctivas were used, results are expressed in mean total CyA +/- standard deviation. Experimental results were compared according to Mann-Whitney U-test.

2.2.4. *In vivo* distribution

Seven-week-old female albinos Lewis rats weighing approximately 200 g received the formulations. Rats were sacrificed on the 6^{th} day using CO_2 after a wash-out period of 18 h after the last administration. All experiments were performed in accordance with the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research, and were approved by the European Committee Directives for animal experimentation.

The distribution was evaluated after the administration of 25 µL of the selected formulations 5 times a day for 5 days on 6 rats. The tested formulations were the prodrug aqueous solutions and the conventional CyA oily formulations at different concentrations (0.05%, 0.50% and 2.00% w/v). At the end of the experiment, eyes, blood, ocular draining lymphatic ganglions and spleen were collected. Each eye was dissected; the cornea, conjunctiva, aqueous humor, iris-ciliary body, vitreous humor and retina were collected. All samples were stored at -80°C in protected vials. Prior to analyses, samples were thawed at room temperature, weighed, manually ground, put into a vial containing methanol and 0.1 µg/mL d_{12}CyA and stirred overnight. The volume of methanol used was 200 µL for the ocular samples and 400 µL for the other samples. The next day, samples were centrifuged, and CyA and prodrug were quantified in the supernatants. Each formulation was tested on six rats and results are expressed in mean CyA +/- standard deviation. Experimental results were analyzed according to Mann-Whitney U-test.

3. RESULTS

The biotransformation of the prodrug into CyA starts with the cleavage of the prodrug ester bound by esterases (enzymes with a high activity in the ocular globe). This bioconversion leads to the release of CyA into the biological medium, making possible its pharmacological activity. The *in vivo* esterase activity being the driving force of the biotransformation, it is important to note that the esterase activity can suffer from variations [22, 23] which can impact on the experimental observations. Therefore, to limit the external variability factors, results are expressed as "total CyA" which represents the sum of the converted and unconverted CyA.

3.1. *Ex vivo* permeation and accumulation

The corneal permeation data showed a similar behavior of prodrug in water and CyA in oil formulations towards the cornea, as shown in Figure 1A. Their profiles were not significantly different. Only CyA was found in the receptor compartment. The CyA corneal permeability coefficient [24] was 6.33 10^{-11} cm/s for the CyA in oil formulation and 6.99 10^{-11} cm/s for the prodrug formulation.

The conjunctival permeation profiles showed a significant difference, illustrated in Figure 1B. The involved concentrations for the prodrug eye drop were 200 to 500 times higher than for CyA in oil. The prodrug formulation demonstrated its capability to release both molecules, the converted and unconverted CyA, into the receptor chamber. The conjunctival permeability coefficients were 6.46 10^{-10} cm/s for the CyA in oil while 3.73 10^{-7} cm/s for the prodrug formulation. The percentage of prodrug molecule was approximately 80% of the permeated amount (see Figure 1B1), highlighting its ability to cross this physiological barrier in its native state. When only the amounts of permeated CyA were compared, the prodrug formulation still presented higher concentrations than CyA in oil (20 to 140 times higher).

When the corneal and conjunctival permeations were compared, it was found that both formulations had a higher tendency to permeate through the conjunctiva than through the cornea. For CyA in oil, conjunctival permeation was higher than that of the cornea. However, experimental values became significantly different only for time points starting from 3 h. For the prodrug formulation, the conjunctival concentrations were 200 to 1900 times higher than

the corneal concentrations. The amount of total CyA in the tissue sections in contact with formulations for 5 h is shown in Figure 2. The prodrug formulation led to a significantly higher amount of CyA in the conjunctiva compared to the cornea. The prodrug molecule represented 95% of the total cumulated CyA in the cornea and 52% for the conjunctiva. In contrast, for the oil formulation, the difference between the amounts of CyA in the conjunctiva and the cornea was not significant.

When both formulations were compared, a marked difference was observed. The prodrug aqueous formulation led to total CyA amounts 130 times higher than the CyA in oil formulation.

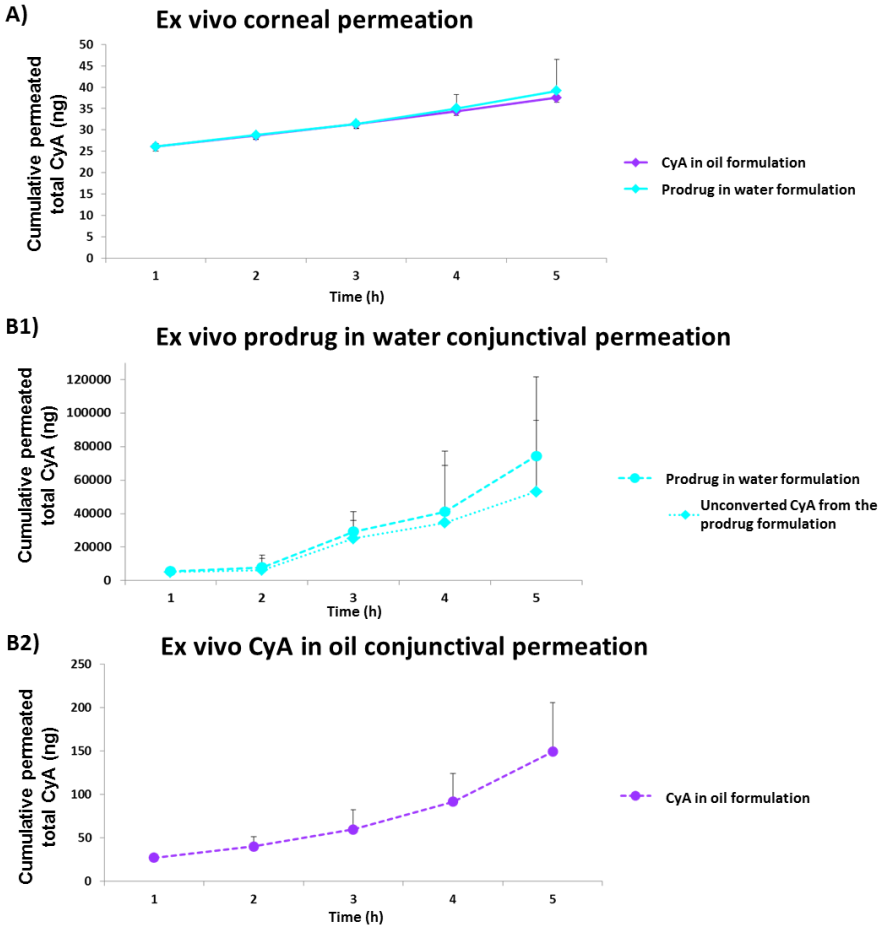


Figure 1: Cumulative amount of total CyA permeated through A) the cornea (n=6) and B) the conjunctiva (n=6) for both formulations. The conjunctival permeation of the prodrug formulation was illustrated in B1) with the additional information of the unconverted CyA levels permeated. The conjunctival CyA in oil formulation illustrated alone in B2) with a smaller scale. The error bars correspond to the standard deviation. The *ex vivo* profile was based on sample collection from the receptor compartment of the diffusion cell every hour.

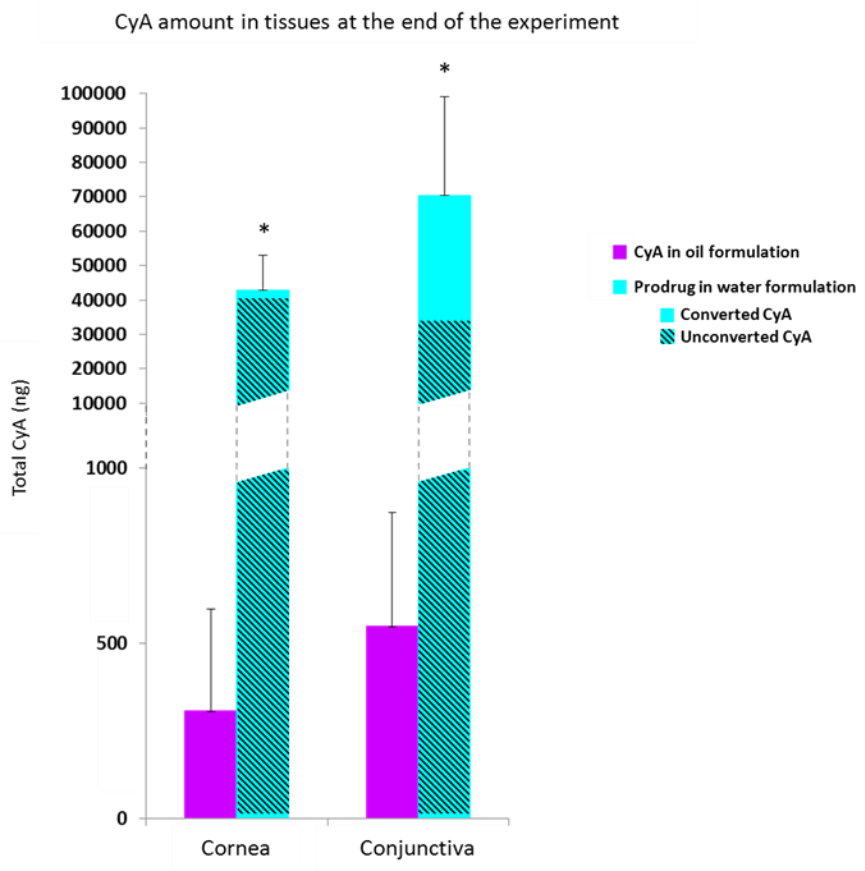


Figure 2: Total CyA in ng in the tissue section in contact with the formulation and the receptor compartment during 5 h (n=6) for CyA in oil (■) and prodrug in water (■) formulations. The fraction of unconverted CyA detected for the prodrug formulations is illustrated with oblique lines (▨). The error bars correspond to the standard deviation. (★$p<0.05$ according to Mann-Whitney U-test).

3.2. *In vivo* distribution

The ocular distribution data are shown in Figure 3. The CyA concentrations in serum, spleen and cervical lymphatic ganglions are shown in Figure 4.

The ocular distribution data of the formulations at 0.05% w/v confirmed the higher CyA levels for the prodrug formulation in the conjunctiva, aqueous humor, iris-ciliary body, vitreous and retina than for the CyA formulation. The concentrations found in the cornea were not significantly different. For the prodrug formulation, the highest concentration was found in the conjunctiva, with 2114.8 +/- 875.1 ng/g, followed by the cornea. For the CyA formulation, the cornea presented the highest tissue level, with 126.3 +/- 103.6 ng/g (approximately 70 times lower than levels obtained with the prodrug formulation), as shown in Figure 3. Unconverted CyA levels were detectable and quantifiable only in the conjunctiva, as shown in Figure 3A. When comparing both formulations based on converted CyA levels conclusions remain unchanged. The systemic distribution showed serum values slightly higher for the prodrug formulation, whereas the concentrations found in the spleen and cervical ganglions were significantly lower when compared to the CyA formulation, as shown in Figure 4.

The ocular distribution data of the formulations at 0.50% w/v showed that the prodrug formulation was able to achieve significantly higher concentrations than the CyA formulation in the cornea and in the conjunctiva. These tissues presented the highest CyA ocular levels for both formulations. The CyA amounts from the prodrug formulation were approximately 40 times higher than those obtained with the CyA formulation (4110.7 +/- 429.4 ng/g vs. 113.1 +/- 30.5 ng/g for the cornea and 4704.9 +/- 2511.5 ng/g vs. 313 +/- 214 ng/g for the conjunctiva). For the other ocular structures, the CyA formulation achieved concentrations between 1 and 5 times higher than those obtained with the prodrug formulation. Unconverted CyA levels were quantifiable in the cornea and conjunctiva, as shown in Figure 3B. When comparing only converted CyA levels for both formulations the nature of the conclusions do not change. Concerning the systemic levels, serum, spleen and cervical ganglions, the concentrations were 3 to 7 times higher for the CyA formulation, as shown in Figure 4.

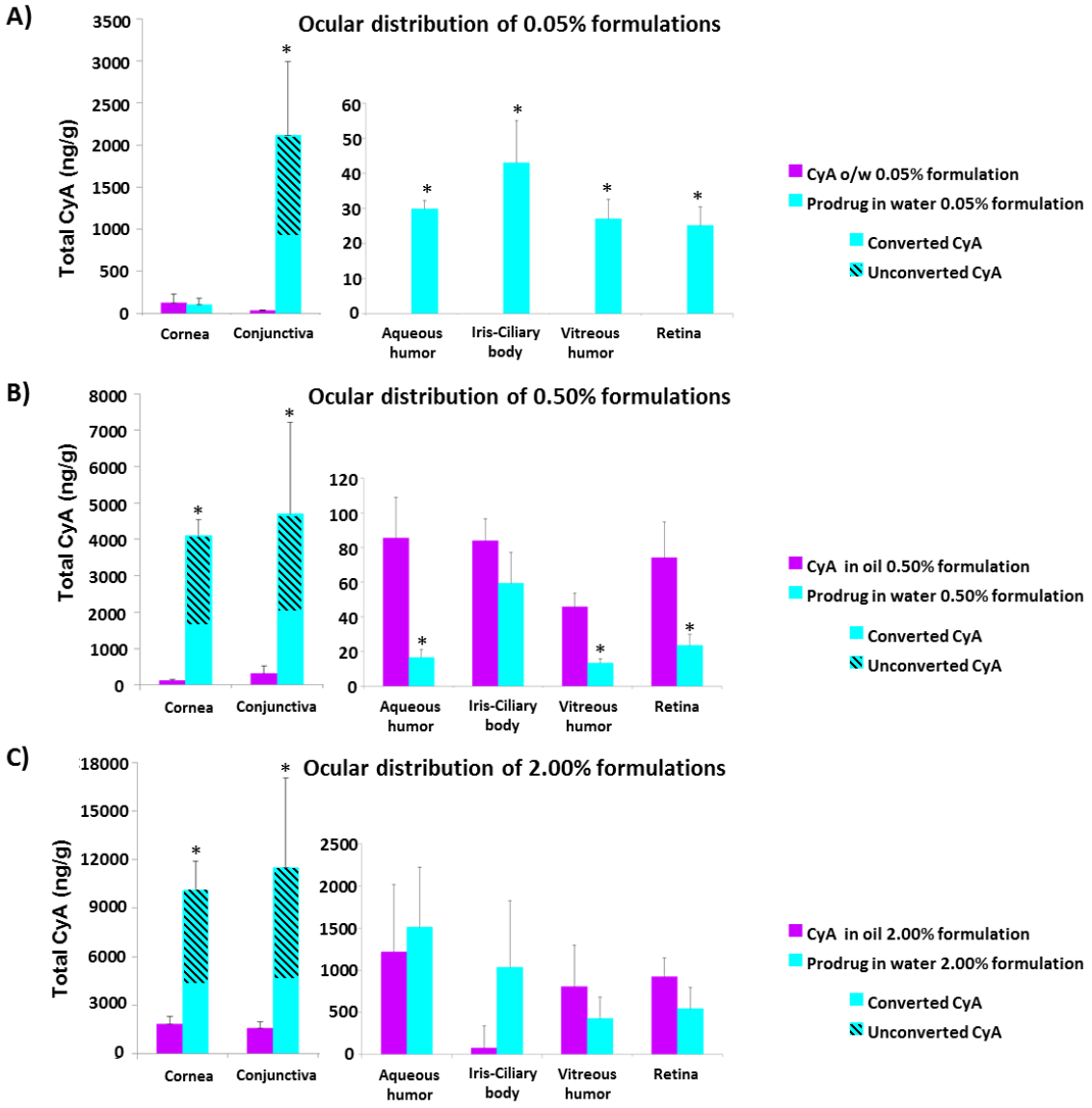


Figure 3: Ocular distribution of total CyA in ng/g of cornea, conjunctiva, aqueous humor, iris-ciliary body, vitreous humor or retina after the application of CyA and prodrug formulations 5 times a day during 5 days (n=6). The error bars correspond to the standard deviation. Each histogram illustrates the ocular distribution for the CyA oily formulation (■) and its equivalent prodrug in water formulation (■) at different concentration levels: A) 0.05% w/v, B) 0.50% w/v and C) 2.00% w/v. The fraction of unconverted CyA detected for the prodrug formulations is illustrated with oblique lines (▨) (★$p<0.05$ according to Mann-Whitney U-test).

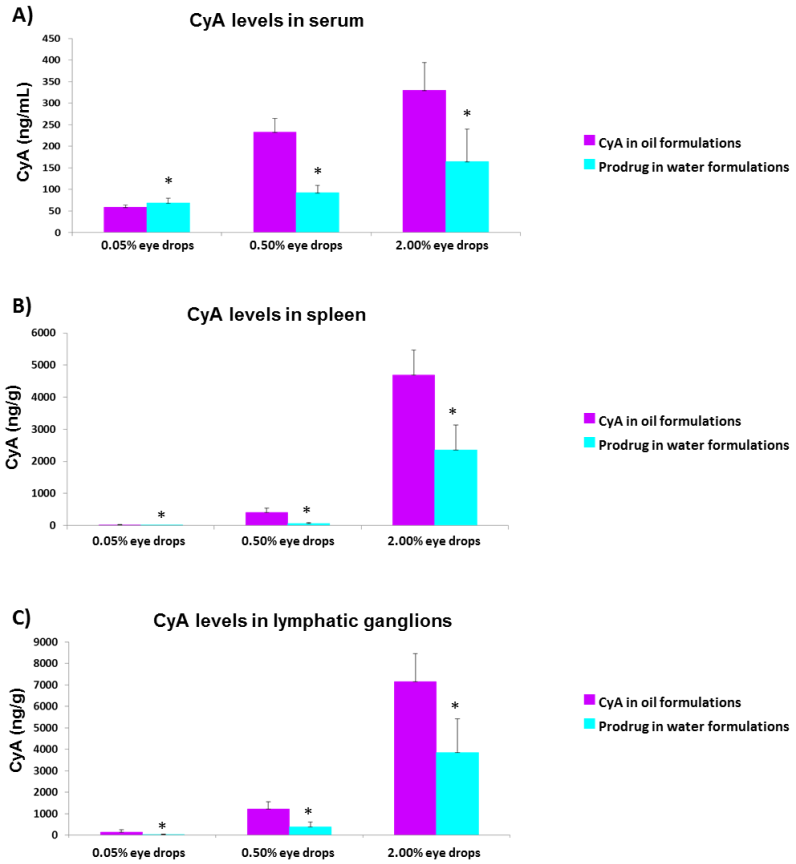


Figure 4: CyA levels in A) serum, B) spleen and C) lymphatic ganglions after the application of CyA or prodrug formulations 5 times a day during 5 days (n=6). The histograms illustrates the distribution for the CyA oily formulations (■) and their equivalent prodrug in water formulations (■). No unconverted CyA was detectable. The error bars correspond to the standard deviation. (★$p<0.05$ according to Mann-Whitney U-test).

Finally, CyA and prodrug formulations at 2.00% w/v gave significantly different corneal and conjunctival concentrations. The prodrug formulation achieved higher concentrations than the CyA formulation in the mentioned tissues (10091.0 +/- 1808.5 ng/g vs. 1842.3 +/- 448.4 ng/g for the cornea and 11495.7 +/- 5535.5 ng/g vs. 1568 +/- 397.8 ng/g for the conjunctiva). Again, the cornea and conjunctiva had the highest CyA levels. The rest of ocular structures did not differ in their concentrations, as shown in Figure 3. Unconverted CyA levels were quantifiable in the cornea and conjunctiva, as shown in Figure 3C. The comparison between the 2.00% formulations based on converted CyA levels do not change the nature of the

conclusions already mentioned. Regarding the systemic concentrations, the CyA formulation was demonstrated to lead to approximately 2 times higher concentrations than the prodrug formulation, as shown in Figure 4.

For the prodrug formulations, the unconverted CyA was quantifiable only in the cornea and the conjunctiva. It represented 59 +/- 23% of the total CyA amount found in these tissues for the 0.50% and 2.00% concentrations as well as for the corneal amount measured for the 0.05% formulation, as shown in Figure 3.

The relationship between the concentration of the formulation and the achieved corneal and conjunctival levels is illustrated in Figure 5. This dependency profile clearly shows the capacity of the prodrug formulation to lead to higher corneal and conjunctival levels than the CyA formulations.

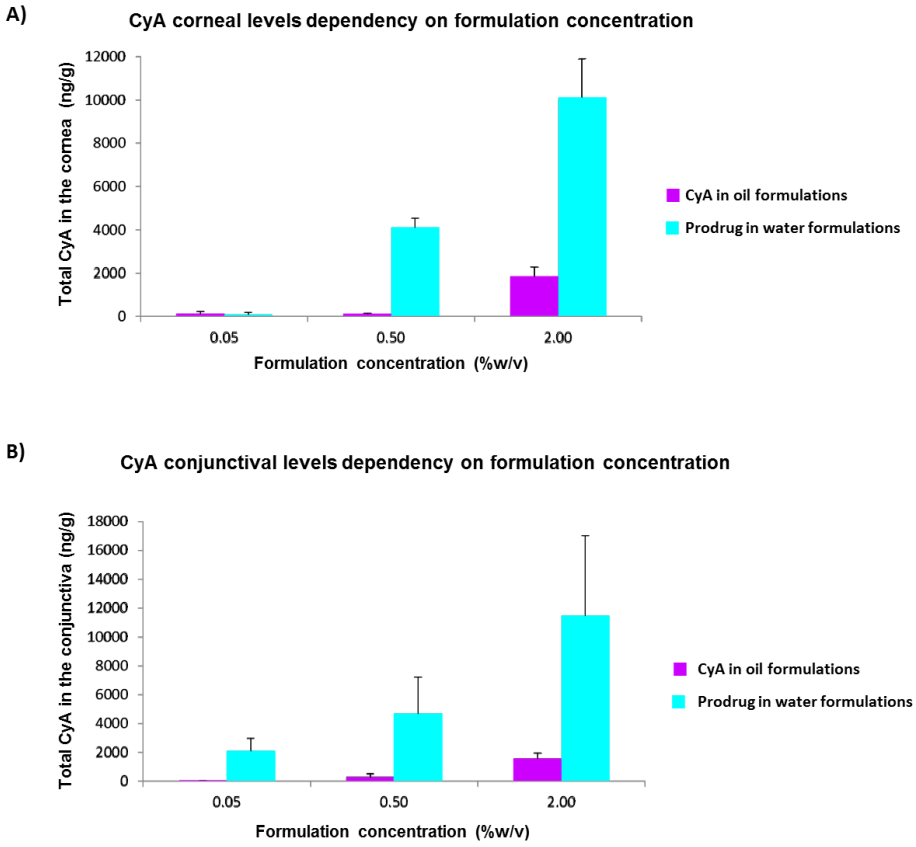


Figure 5: Dependency of the CyA tissues accumulation on the concentration of the applied formulation; in A) the cornea and B) the conjunctiva.

4. DISCUSSION

The *ex vivo* and *in vivo* experiments performed in this study provided complementary information. The *ex vivo* experiments were particularly interesting as they allowed important information to be gathered about pure processes (i.e., permeation, tissue accumulation) that were difficult to be individually evaluated *in vivo*. The major *in vivo* constraint was related to the fact that many other parameters were in equilibrium with permeation and accumulation (i.e., elimination, biotransformation in the lachrymal fluid). In addition, the *ex vivo* set up made it possible to differentiate between corneal and conjunctival processes. Nonetheless, it is important to note that the *ex vivo* conditions were different than the *in vivo* conditions (i.e., longer residence time, no lachrymal fluid, no elimination); thus, extrapolations have to be made with caution, as mentioned elsewhere [25]. In addition, *in vivo* experiments gave paramount information about the ocular and systemic behavior of the drug after its administration. The analysis was based on the concentrations encountered in the ocular structures and in other important organs, tissues and fluids. The ocular distribution allowed the prediction of the potentially achieved therapeutic effect. Particularly interesting was the information gathered from the serum, spleen and cervical lymphatic ganglions. Serum levels gave information about the potential systemic impact, and spleen and lymphatic ganglion concentrations helped to evaluate the potential undesired systemic immunosuppression. Together, the *ex vivo* and the *in vivo* experiments provided detailed information on how CyA and prodrug formulations behave towards their target organ and the rest of the organism. Additionally, these investigations allowed a deep understanding of some of the mechanisms governing such interactions.

It is worth reminding that the *in vivo* model presents some limitations. One of the major differences between the selected rat model and humans is the size of the studied structures. The rat eye is approximately 4 times smaller than the human one and presents anatomical differences [26]. In addition, large variations can be observed in serum concentrations measured after ocular topical application of CyA in small experimental animals [11]. This disparity could also be linked to the differences in the applied volumes and the nasolachrymal overflow of experimental animals. In addition, the present *in vivo* study was performed on non-pigmented rats, implying the absence of melanin. It has been reported that melanin can

bind lipophilic drugs [27] and impact the ocular CyA distribution. Thus, higher CyA levels can be expected in the uveal tract, iris-ciliary body, choroid and retinal pigmented epithelium of pigmented animals. Nevertheless, the mentioned higher drug levels do not necessarily involve higher pharmacological activity as the fraction of CyA that would be bound to melanin would not be available for interaction with its target [28].

The experimental results clearly pointed out different *ex vivo* and *in vivo* behaviors (i.e., distribution, permeation and accumulation profiles) when comparing CyA and prodrug formulations.

The prodrug formulations showed a clear preference for the conjunctiva in their permeation and accumulation. *In vivo,* the highest total CyA concentration was reached in the conjunctiva for the three tested concentrations (see Figure 3). This repeated pattern highlighted the high interaction of the formulation with this particular epithelium, as already seen in previous *in vivo* studies [19]. *Ex vivo,* the prodrug eye drop appeared to permeate preferentially through the conjunctiva (see Figure 1) where it also accumulated at higher levels than in the cornea (see Figure 2). These findings are in line with former publications showing that large hydrophilic molecules, such as hydrophilic peptides, preferentially enter the eye through the conjunctival route [25, 29-31]. Interestingly, conjunctival permeation involves a number of appealing features. First, the conjunctival area represents approximately 94% of the ocular surface primarily in contact with the eye-drop formulation [31-33]; thus, the probability of interaction and availability are greatly enhanced. Second, the conjunctival route allows active compounds to directly enter the posterior segment [25]. Finally, the conjunctiva is known to have a high esterase activity that ensures the prodrug bioconversion [22, 23, 34-36].

CyA formulations followed a different pattern. *In vivo,* CyA formulations preferentially interacted with the cornea and the conjunctiva without any apparent preference between these two epithelia. *Ex vivo* experiments showed no significant difference in tissue accumulation after 5 h (see Figure 2); nevertheless, a higher conjunctival permeation profile was observed after 3 h of permeation compared to the corneal profile. Such a difference in permeation was not as apparent *in vivo*. This observation was directly related to the experimental conditions. The conjunctiva and the cornea are two physiological barriers with different properties. The cornea is characterized by a squamous epithelium with a high number of tight junctions, whereas the conjunctiva is columnar and has fewer tight junctions and higher

porosity [31, 37]. Indeed, the conjunctival permeability is between 15 and 25 times higher than that of the cornea [31]. *In vivo* conditions highly limit the observation of such a difference, mainly due to short residence times and important precorneal losses. *In vivo*, the molecules contained in the eye-drop formulations have the opportunity to interact with the superficial tissues for only short periods of time, making the difference in permeability between the cornea and the conjunctiva less evident.

Despite the mentioned disparities, both formulations shared a common feature: they both had a preference for the superficial tissues of the eye (i.e., cornea and conjunctiva) over the other studied structures. For both types of formulations, the cornea and conjunctiva had the highest CyA levels, which was in line with former studies [38-43]. It is important to note that the 18-h washout period ensured that only the amounts of CyA stored for at least 18 h were visible. The experimental results strongly suggest that the cornea and conjunctiva were able to accumulate and store both converted and unconverted CyA, creating a drug deposit. A depletion of such deposits could be expected over time (as already seen for CyA in previous studies [41-44]). This depletion would lead to a potential prolonged release. From a quantitative point of view, the amounts of total CyA that accumulated in the cornea and conjunctiva were higher for the prodrug aqueous formulation than for the CyA oily formulation. Thus, the prodrug eye drop showed the interesting ability to create important drug deposits in the superficial tissues of the eye. This drug deposit was particularly interesting for two main reasons. Firstly, it consisted of 59 +/- 23% prodrug molecule that, due to its high hydrophilicity, is expected to have a lower clearance rate compared to CyA [30]. Secondly, the prodrug formulation made available an enormous amount of CyA and prodrug compared to the oil formulation. This idea is strongly reinforced by the fact that the area of the conjunctiva represents approximately 20 times that of the cornea [32].

This ability of the prodrug formulation to lead to extensive corneal and conjunctival accumulation is particularly evident from the data presented in Figure 5. For example, to achieve a conjunctival concentration of 1500 ng/g, a 2.00% CyA formulation must be applied, whereas a 0.05% concentration of the prodrug formulation (40 times less concentrated) is enough.

The question of the biotransformation of the prodrug once it had entered the ocular structures was also a matter of concern. Extensive studies regarding the esterase activity in the eye have been performed [22, 23, 34-36]. According to these investigations, tissues and fluids can be classified based on their esterase activity. The classification is the following, starting from the highest activity: i) conjunctiva, ii) iris - ciliary body, iii) corneal endothelium, iv) corneal epithelium and v) serum. It is worth mentioning that the conjunctiva has by far the highest esterase activity, ensuring between 60 and 75% of the biotransformation [22]. In light of this information, the fact that the prodrug molecule preferentially interacted with the conjunctiva represented an interesting advantage.

One of the main advantages of the local route vs. oral or systemic administration is the prevention of systemic secondary effects. In this study the potential systemic consequences of the administration of the formulations were studied through the analysis of three types of samples: serum, spleen and ocular draining cervical lymphatic ganglions (see Figure 4). Concerning serum levels, conventional CyA oral dosage involved systemic concentrations orders of magnitude higher than those obtained after eye drop administration [40, 45]. The prodrug formulations led to lower serum levels than conventional CyA formulations for the two higher concentrations (0.50% and 2.00%). However, serum levels did not directly inform about potential immunosuppression, as previously observed [46]. Consequently, potential general immunosuppression was studied based on CyA levels in tissues related to the immune system, such as spleen and lymphatic ganglions. *In vitro* investigations demonstrated that immunosuppression appeared at CyA concentrations ranging from 100 to 1000 ng/g [47]. For prodrug formulations, spleen CyA levels were above this limit only at the higher concentration (2.00% w/v), whereas the two higher concentrations of CyA formulations (2.00 and 0.50% w/v) led to spleen immunosuppression. Regarding lymphatic ganglion levels, the three CyA formulations led to significant immunosuppression, whereas only the two higher prodrug concentrations did so. It can be concluded that the prodrug formulation led to lower systemic toxicity and immunosuppression compared to the CyA formulation. This finding represents an important benefit of the use of the prodrug.

The potential pharmacological effect achieved in the eye deserves special mention. Former studies demonstrated that CyA achieved immunosuppression in ocular structures starting from 50 ng/g [48].The prodrug formulation attained pharmacologically active CyA concentrations in the anterior and posterior chambers of the eye, as shown in Table 1. These results highlight the efficiency of the prodrug eye drops for the treatment of ocular diseases affecting both the anterior and posterior chambers. The prodrug concentration to be used can be selected according to the ocular pathology to be treated.

Table 1: Overview the ocular levels and pharmacological effect achieved by the prodrug aqueous formulation at different concentrations. The squares are filled in green (■) when therapeutically active CyA concentrations are reached [48].

Prodrug concentration (equivalent CyA)	Cornea	Conjunctiva	Aqueous humor	Iris-Ciliary body	Vitreous humor	Retina
0.05%	101.4 ng/g +/-77.1 ng/g	2114.8 ng/g +/-875.1 ng/g	29.9 ng/g +/-2.3ng/g	43.0 ng/g +/-12.0 ng/g	27.1 ng/g +/-5.5 ng/g	25.2 ng/g +/-5.3 ng/g
0.50%	4110.7 ng/g +/-429.4 ng/g	4704.9 ng/g +/-2511.5 ng/g	16.5 ng/g +/-4.5ng/g	59.3 ng/g +/-17.8 ng/g	13.3 ng/g +/-2.6 ng/g	23.5 ng/g +/-6.3 ng/g
2.00%	10091.0 ng/g +/-1808.5 ng/g	11495.7 ng/g +/-5535.5 ng/g	1514.2 ng/g +/-709.8 ng/g	1037.1 ng/g +/-788.7 ng/g	426.8 ng/g +/-254.5 ng/g	542.6 ng/g +/-254.4 ng/g

During these investigations, major differences were observed between the prodrug and CyA formulations. Three features that governed such differences could be identified. First, the prodrug formulation was an aqueous solution. A water vehicle gives a major advantage avoiding tolerance issues [7-11] and availability limitations [12-14] linked to CyA oily vehicles. Interestingly, the prodrug in water was demonstrated to have excellent ocular tolerance and a low elimination rate [19]. Second, the prodrug molecule could interact in its native state with ocular structures. Such an interaction could occur in two different manners: i) permeation through the ocular structures to enter the eye or ii) tissue accumulation and subsequent biotransformation and release (or vice versa). Third, the prodrug formulation generated large drug deposits in the ocular superficial tissues. Interestingly, the clearance of the prodrug deposits was potentially lower than for CyA [30].

5. CONCLUSIONS

This study presented a possible strategy to address the problem of CyA solubility and availability based on a prodrug approach. The *ex vivo* and *in vivo* evaluation of the prodrug and CyA formulations gives important insight on the differences and similarities between their behaviors . This information explains in part the mechanisms behind the modulation of the distribution and therapeutic effect that can be achieved through the use of such formulations. The prodrug eye drops appeared to involve a number of advantages over conventional CyA formulations: i) higher permeation and accumulation capacities, ii) entrance into the eye preferentially through the conjunctival route, iii) the ability to create large tissue deposits and iv) a lower risk of systemic complications and general immunosuppression.

Interestingly, the prodrug makes it possible to develop ophthalmic formulations at concentrations higher than 2.00% w/v, pushing the boundaries of the current therapeutic arsenal for ocular diseases. Such highly concentrated formulations are currently under investigation.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Dr. Wenger for the synthesis of the prodrug molecule and Ikram El Zaoui for her assistance in the *in vivo* experiments conditions. The authors would also like to thank Dr. Ingo Alberti for making available the equipment for *ex vivo* experiments and Dr. Tais Gratieri for her training on *ex vivo* ocular permeation.

REFERENCES

[1] T. Loftsson, M.E. Brewster, Pharmaceutical applications of cyclodextrins: effects on drug permeation through biological membranes, J Pharm Pharmacol, 63 (2011) 1119-1135.

[2] F. Lallemand, P. Perottet, O. Felt-Baeyens, W. Kloeti, F. Philippoz, J. Marfurt, K. Besseghir, R. Gurny, A water-soluble prodrug of cyclosporine A for ocular application: a stability study, European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences, 26 (2005) 124-129.

[3] C.A. Utine, M. Stern, E.K. Akpek, Clinical review: topical ophthalmic use of cyclosporin A, Ocular immunology and inflammation, 18 (2010) 352-361.

[4] E. Donnenfeld, S.C. Pflugfelder, Topical ophthalmic cyclosporine: pharmacology and clinical uses, Survey of ophthalmology, 54 (2009) 321-338.

[5] W. Vine, L.D. Bowers, Cyclosporine: structure, pharmacokinetics, and therapeutic drug monitoring, Critical reviews in clinical laboratory sciences, 25 (1987) 275-311.

[6] A.G. Palestine, R.B. Nussenblatt, C.C. Chan, Side effects of systemic cyclosporine in patients not undergoing transplantation, The American journal of medicine, 77 (1984) 652-656.

[7] C.A. Le Bourlais, F. Chevanne, B. Turlin, L. Acar, H. Zia, P.A. Sado, T.E. Needham, R. Leverge, Effect of cyclosporine A formulations on bovine corneal absorption: ex-vivo study, Journal of microencapsulation, 14 (1997) 457-467.

[8] P. Versura, M. Cellini, P.G. Zucchini, R. Caramazza, R. Laschi, Ultrastructural and immunohistochemical study on the effect of topical cyclosporin A in the rabbit eye, Cornea, 8 (1989) 81-89.

[9] J.M. Benitez del Castillo, C. del Aguila, S. Duran, J. Hernandez, J. Garcia Sanchez, Influence of topically applied cyclosporine A in olive oil on corneal epithelium permeability, Cornea, 13 (1994) 136-140.

[10] L. Goichot-Bonnat, P. Chemla, Y. Pouliquen, [Cyclosporin A eyedrops in the prevention of high-risk corneal graft rejection. II. Postoperative clinical results], Journal francais d'ophtalmologie, 10 (1987) 213-217.

[11] D.L. Williams, A comparative approach to topical cyclosporine therapy, Eye, 11 (Pt 4) (1997) 453-464.

[12] M. Kuwano, H. Ibuki, N. Morikawa, A. Ota, Y. Kawashima, Cyclosporine A formulation affects its ocular distribution in rabbits, Pharmaceutical research, 19 (2002) 108-111.
[13] M. Diaz-Llopis, J.L. Menezo, Penetration of 2% cyclosporin eyedrops into human aqueous humour, The British journal of ophthalmology, 73 (1989) 600-603.
[14] L. Cheeks, R.L. Kaswan, K. Green, Influence of vehicle and anterior chamber protein concentration on cyclosporine penetration through the isolated rabbit cornea, Current eye research, 11 (1992) 641-649.
[15] A.R. Hamel, F. Hubler, M. Mutter, Water-soluble prodrugs of cyclosporine A with tailored conversion rates, The journal of peptide research : official journal of the American Peptide Society, 65 (2005) 364-374.
[16] F. Lallemand, O. Felt-Baeyens, S. Rudaz, A.R. Hamel, F. Hubler, R. Wenger, M. Mutter, K. Besseghir, R. Gurny, Conversion of cyclosporine A prodrugs in human tears vs rabbits tears, European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V, 59 (2005) 51-56.
[17] F. Lallemand, P. Furrer, O. Felt-Baeyens, M. Gex-Fabry, J.M. Dumont, K. Besseghir, R. Gurny, A novel water-soluble cyclosporine A prodrug: ocular tolerance and *in vivo* kinetics, International journal of pharmaceutics, 295 (2005) 7-14.
[18] F. Lallemand, E. Varesio, O. Felt-Baeyens, L. Bossy, G. Hopfgartner, R. Gurny, Biological conversion of a water-soluble prodrug of cyclosporine A, European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V, 67 (2007) 555-561.
[19] M. Rodriguez-Aller, B. Kaufmann, D. Guillarme, C. Stella, P. Furrer, S. Rudaz, I. El Zaoui, F. Valamanesh, C. Di Tommaso, F. Behar-Cohen, J.L. Veuthey, R. Gurny, *In vivo* characterisation of a novel water-soluble Cyclosporine A prodrug for the treatment of dry eye disease, European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V, 80 (2012) 544-552.
[20] E. Clarke, Clarke's isolation and identification of drugs, 2nd ed., Pharmaceutical Press, London, 1986.
[21] H. Nourry, S. Perrot, C. Martin, C. Chaumeil, C. Cambourieu, P. Rat, J.M. Warnet, [Cytotoxicity evaluation of different eyes drops with cyclosporine oral solution (Sandimmun)], Journal francais d'ophtalmologie, 29 (2006) 251-257.

[22] M.A. Redell, D.C. Yang, V.H.L. Lee, The Role of Esterase-Activity in the Ocular Disposition of Dipivalyl Epinephrine in Rabbits, International journal of pharmaceutics, 17 (1983) 299-312.

[23] V.H. Lee, K.W. Morimoto, R.E. Stratford, Jr., Esterase distribution in the rabbit cornea and its implications in ocular drug bioavailability, Biopharmaceutics & drug disposition, 3 (1982) 291-300.

[24] R.D. Schoenwald, H.S. Huang, Corneal penetration behavior of beta-blocking agents I: Physiochemical factors, Journal of pharmaceutical sciences, 72 (1983) 1266-1272.

[25] K. Jarvinen, T. Jarvinen, A. Urtti, Ocular Absorption Following Topical Delivery, Advanced drug delivery reviews, 16 (1995) 3-19.

[26] R.W. Massof, F.W. Chang, A revision of the rat schematic eye, Vision research, 12 (1972) 793-796.

[27] B. Leblanc, S. Jezequel, T. Davies, G. Hanton, C. Taradach, Binding of drugs to eye melanin is not predictive of ocular toxicity, Regulatory toxicology and pharmacology : RTP, 28 (1998) 124-132.

[28] R. Gaudana, H.K. Ananthula, A. Parenky, A.K. Mitra, Ocular drug delivery, The AAPS journal, 12 (2010) 348-360.

[29] D.H. Geroski, H.F. Edelhauser, Transscleral drug delivery for posterior segment disease, Advanced drug delivery reviews, 52 (2001) 37-48.

[30] A. Urtti, Challenges and obstacles of ocular pharmacokinetics and drug delivery, Advanced drug delivery reviews, 58 (2006) 1131-1135.

[31] K.M. Hamalainen, K. Kananen, S. Auriola, K. Kontturi, A. Urtti, Characterization of paracellular and aqueous penetration routes in cornea, conjunctiva, and sclera, Investigative ophthalmology & visual science, 38 (1997) 627-634.

[32] M.A. Watsky, M.M. Jablonski, H.F. Edelhauser, Comparison of conjunctival and corneal surface areas in rabbit and human, Current eye research, 7 (1988) 483-486.

[33] M.R. Prausnitz, J.S. Noonan, Permeability of cornea, sclera, and conjunctiva: a literature analysis for drug delivery to the eye, Journal of pharmaceutical sciences, 87 (1998) 1479-1488.

[34] A. Tsuji, I. Tamai, K. Sasaki, Hydrolysis of Prednisolone Succinate by Esterase in Rabbit Ocular Tissue, Ophthalmic Res, 19 (1987) 322-329.

[35] V.H. Lee, D.S. Iimoto, K.A. Takemoto, Subcellular distribution of esterases in the bovine eye, Current eye research, 2 (1982) 869-876.
[36] V.H. Lee, S.C. Chang, C.M. Oshiro, R.E. Smith, Ocular esterase composition in albino and pigmented rabbits: possible implications in ocular prodrug design and evaluation, Current eye research, 4 (1985) 1117-1125.
[37] B. Nichols, C.R. Dawson, B. Togni, Surface features of the conjunctiva and cornea, Investigative ophthalmology & visual science, 24 (1983) 570-576.
[38] D. BenEzra, G. Maftzir, Ocular penetration of cyclosporine A in the rat eye, Archives of ophthalmology, 108 (1990) 584-587.
[39] D. BenEzra, G. Maftzir, Ocular penetration of cyclosporin A. The rabbit eye, Investigative ophthalmology & visual science, 31 (1990) 1362-1366.
[40] D. BenEzra, G. Maftzir, C. de Courten, P. Timonen, Ocular penetration of cyclosporin A. III: The human eye, The British journal of ophthalmology, 74 (1990) 350-352.
[41] A.A. Acheampong, M. Shackleton, D.D. Tang-Liu, S. Ding, M.E. Stern, R. Decker, Distribution of cyclosporin A in ocular tissues after topical administration to albino rabbits and beagle dogs, Current eye research, 18 (1999) 91-103.
[42] L. Gan, Y. Gan, C. Zhu, X. Zhang, J. Zhu, Novel microemulsion in situ electrolyte-triggered gelling system for ophthalmic delivery of lipophilic cyclosporine A: *in vitro* and *in vivo* results, International journal of pharmaceutics, 365 (2009) 143-149.
[43] J. Shen, Y. Deng, X. Jin, Q. Ping, Z. Su, L. Li, Thiolated nanostructured lipid carriers as a potential ocular drug delivery system for cyclosporine A: Improving *in vivo* ocular distribution, International journal of pharmaceutics, 402 (2010) 248-253.
[44] M. Wiederholt, D. Kossendrup, W. Schulz, F. Hoffmann, Pharmacokinetic of topical cyclosporin A in the rabbit eye, Investigative ophthalmology & visual science, 27 (1986) 519-524.
[45] N. Maziers, P. Bulpa, J. Jamart, L. Delaunois, P. Eucher, P. Evrard, Correlations between cyclosporine concentrations at 2 hours post-dose and trough levels with functional outcomes in de novo lung transplant recipients, Transplant Proc, 44 (2012) 2880-2884.
[46] P. Cugini, M. Cavallini, P. Pozzilli, C. Letizia, M. Sepe, L. Di Palma, P. Battisti, Circadian rhythm of T-lymphocyte subsets, cortisol and cyclosporin in kidney-transplanted subjects, Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association, 6 (1991) 512-517.

[47] K.J. Lafferty, J.F. Borel, P. Hodgkin, Cyclosporine-a (Csa) - Models for the Mechanism of Action, Transplant P, 15 (1983) 2242-2247.
[48] R.L. Kaswan, Intraocular penetration of topically applied cyclosporine, Transplant Proc, 20 (1988) 650-655.

PART B

COMPLEXATION WITH CYCLODEXTRINS

Latanoprost is a poorly water-soluble prostaglandin $F_{2\alpha}$ analog used for the management of glaucoma, which requires lifelong treatment. Latanoprost is an ester prodrug of the active latanoprost acid. The latanoprost ester group has two opposite roles: *in vitro*, it needs to have a low reactivity to ensure drug stability, while *in vivo* it needs to be rapidly cleaved to release the active latanoprost acid. Latanoprost acid acts by stimulating intraocular prostanoic receptors type F (FP receptors), leading to a decrease in intraocular pressure. In contrast, it can also stimulate the precorneal prostanoic receptors type E (EP receptors) leading to nociception, vasodilatation, increased vascular permeability and hyperemia. A large number of studies identified ocular irritation as the major issue related to topical ocular administration of prostaglandin analogs.

Latanoprost is a challenging molecule to formulate as an eyedrop due to its poor water solubility, chemical instability and potential for ocular irritation. One approach to simultaneously address these limitations is to use cyclodextrins (CDs). CDs and poorly water-soluble drugs are known to form water-soluble complexes, which can solubilize, stabilize, carry and protect the drug. Interestingly, the use of latanoprost-CD complexes illustrates the combination of two approaches, the prodrug design and the complexation with CDs, for the delivery of the active latanoprost acid to the eye.

The investigations presented in the following part evaluated the use of a complexation of latanoprost with CDs for a topical ocular delivery. The first step was to screen a panel of CDs to determine the CD with the best tradeoff between latanoprost stability and availability, since not all the CDs can be expected to have a positive impact on latanoprost properties. The selected combination was then further investigated: the complexation mechanisms and the complex structure were characterized to better understand the influence of the CD on latanoprost. Finally, *in vivo* ocular tolerance experiments were performed to determine if the complexation of latanoprost with the selected CD decreased the ocular irritation linked to topical ocular administration of latanoprost.

CHAPTER VI

Complexation of latanoprost with cyclodextrins for improved stability, solubility and ocular tolerance

New prostaglandin analog formulation for glaucoma treatment containing cyclodextrins for improved stability, solubility and ocular tolerance

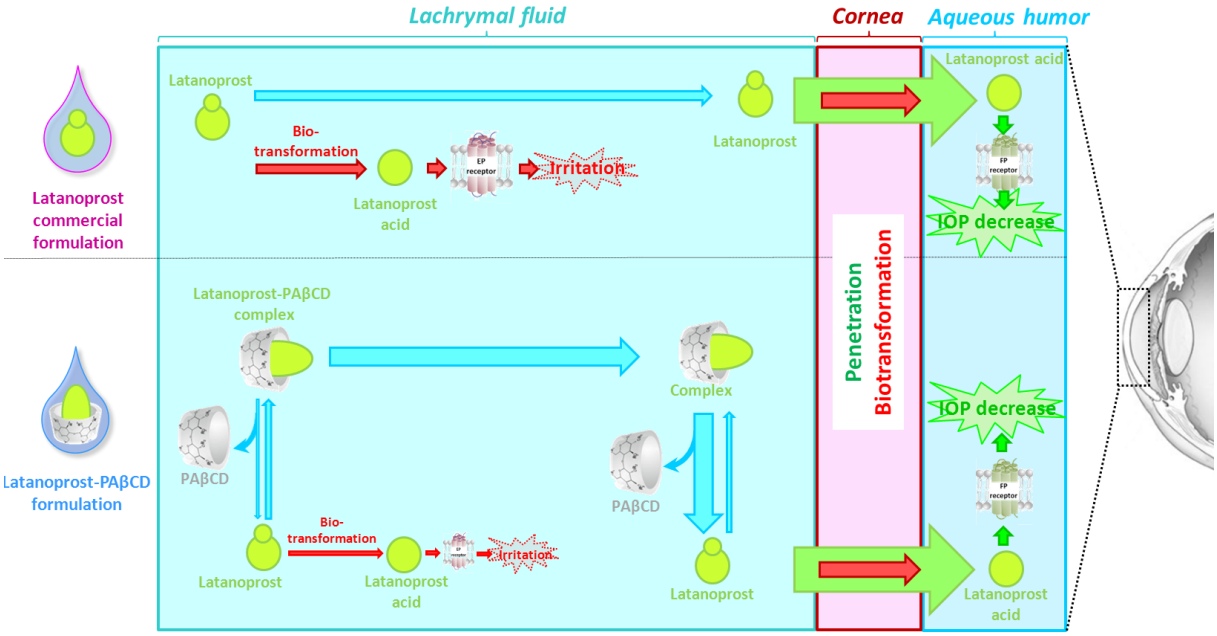
Marta RODRIGUEZ-ALLER[1], Sylvie GUINCHARD[1], Davy GUILLARME[1], Marion PUPIER[2], Damien JEANNERAT[2], Elisabeth RIVARA-MINTEN[1], Jean-Luc VEUTHEY[1], Robert GURNY[1]*

[1]School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 30, Quai Ernest Ansermet, 1211 Geneva 4, Switzerland

[2]Department of Organic Chemistry, University of Geneva, 30, Quai Ernest Ansermet, 1211 Geneva 4, Switzerland

*Correspondence: Phone: +41223793816, Fax: +41223796146, E-mail: robert.gurny@unige.ch

To be submitted.



ABSTRACT

Latanoprost is a practically insoluble prostaglandin F2α analog considered a first-line agent for glaucoma treatment. From a pharmaceutical point of view, latanoprost is challenging to formulate as an eye drop due to its poor water solubility and the presence of an ester bond which needs to be cleaved *in vivo* but maintained unchanged during storage. Cyclodextrins (CDs) are known to form complexes with hydrophobic drugs, influencing their stability, availability, solubility and tolerance in a non-predictable manner. A variety of CDs including native α, β and γCDs as well as substituted hydroxypropylβCD, hydroxypropylγCD, dimethylβCD, sulphatedβCD and propylaminoβCD were screened and the most appropriate CD for the formulation of latanoprost for an ocular topical application was selected. Among the tested CDs, propylaminoβCD had the best tradeoff between latanoprost stability and availability, which was confirmed by its complex constant value of 3129 M^{-1}. Phase-solubility and NMR investigations demonstrated that the propylaminoβCD effectively formed a complex involving the ester group of latanoprost providing protection to its ester bond, while ensuring proper latanoprost solubilization. Furthermore, *in vivo* experiments demonstrated that the latanoprost-propylaminoβCD formulation led to lower ocular irritation than the commercial latanoprost formulation used as a reference. The latanoprost-propylaminoβCD formulation was demonstrated to successfully address the main stability, solubility and tolerance limitations of topical ocular latanoprost therapy for glaucoma.

Keywords: latanoprost, cyclodextrins, propylaminoβcyclodextrin, stability, *ex vivo* availability, complex structure, complexation, ocular irritation

1. INTRODUCTION

Glaucoma can be defined as a group of sight threatening conditions that involve damage to the optic nerve. This affects more than sixty million people worldwide and is the second leading cause of blindness [1-3]. Maintaining intraocular pressure (IOP) within physiological range prevents the development and progression of the disease. Chronic elevated IOP can easily go unnoticed until the first signs of vision loss evidence the optic neuropathy. After a glaucoma diagnosis, a lifelong treatment needs to be initiated as soon as possible to minimize further vision impairment.

Latanoprost is a prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) analog, considered a first-line agent for glaucoma treatment and has drastically changed the management of glaucoma from a surgical approach to a local pharmacological treatment. Latanoprost was demonstrated to decrease the IOP in a highly efficient manner by enhancing the uveoscleral outflow, while inducing low systemic side effects [4, 5]. Latanoprost design was based on the chemical structure of $PGF_{2\alpha}$, and is both a prodrug and an analog of $PGF_{2\alpha}$, as illustrated in Figure 1. The naturally occurring $PGF_{2\alpha}$ was demonstrated to efficiently decrease the IOP after a topical ocular administration, but also induces unacceptable levels of ocular irritation and hyperemia [6]. Compared to $PGF_{2\alpha}$, latanoprost displays a higher ocular penetration and lower ocular irritation while achieving effective IOP reductions [5, 7]. Once topically administered to the eye, latanoprost efficiently permeates the cornea, where it is enzymatically biotransformed; this leads to the release of the active latanoprost free acid to the anterior chamber allowing the active to reach the iris-ciliary body where it achieves the therapeutic effect, as illustrated in Figure 1B [8]. In contrast, the direct topical administration of latanoprost acid could be related to poor ocular penetration reported with PG analogs presenting low lipophilicity [7]. The mechanisms behind the ocular effects of latanoprost acid were elucidated in previous studies, reporting that the therapeutic IOP reducing effect was linked to the stimulation of prostanoic receptors type F (FP receptors) present in the ciliary body and iris, while the ocular irritation was related to the stimulation of prostanoic receptors type E (EP receptors) present in the cornea and conjunctiva [5-7, 9-14].

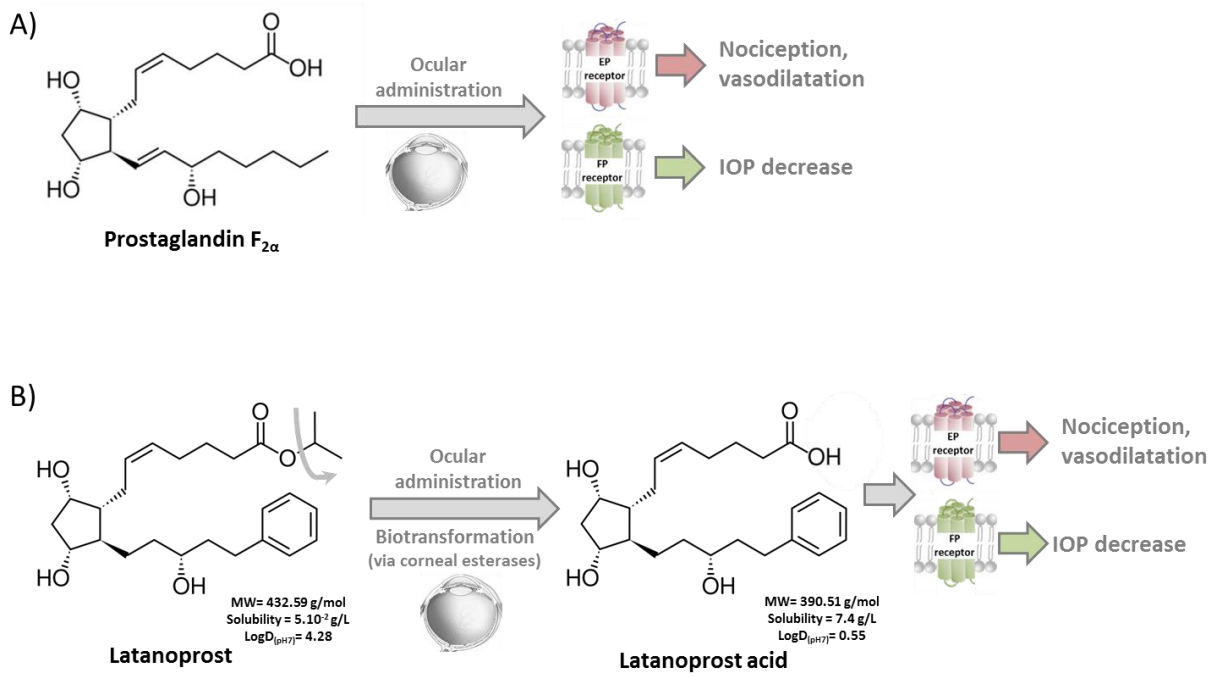


Figure 1: Prostanoic receptor-mediated ocular effects after direct topical application of A) prostaglandin $F_{2\alpha}$ and B) latanoprost. The latanoprost biotransformation process and the physico-chemical characteristics of latanoprost and latanoprost acid are illustrated in B). (IOP: intraocular pressure)

From a pharmaceutical point of view, the formulation of latanoprost for ocular topical administration with an appropriate stability, solubility and tolerance can be challenging. First of all, *in vitro* stability of the formulation can be difficult to achieve since the ester bond of the latanoprost needs to be stable *in vitro*, but rapidly cleaved *in vivo* to produce the active latanoprost free acid. An adequate equilibrium between *in vitro* stability and *in vivo* reactivity is difficult to reach. As a matter of fact, latanoprost was reported to be unstable under UV and thermal stresses [15-19]. Secondly, the solubility of latanoprost is challenging since it has a low solubility in water (water solubility of 50 mg/L) and a high lipophilicity ($LogD_{(pH7)}$ of 4.28) leading to a proven tendency to adsorb onto the surfaces of laboratory equipment and formulation containers [20-23]. These characteristics, together with the fact that latanoprost has a high potency and is formulated at a low concentration (0.005% w/v), can lead to a significant drug loss and result in a potential decrease in effective dosage and treatment efficacy. Finally, the tolerance of latanoprost is also a challenge since ocular irritation was

reported after the topical ocular administration of PG analogs [5, 7, 9, 24-27]. This ocular irritation can lead to compliance issues, which are known to be particularly problematic in life-long glaucoma management. The importance of the ocular irritation issue is supported by the fact that new formulations with lower PG analogs concentrations are being developed to decrease the associated ocular irritation. This is the case for Lumigan® (Allergan, Irvine, USA), a bimatoprost eyedrop formulation, which has recently been relaunched at 0.01% instead of the initial 0.03% formulation.

The use of cyclodextrins (CDs) in combination with latanoprost represents an interesting option to simultaneously address the stability, solubility and tolerance issues related to latanoprost formulation for ocular therapies. Cyclodextrins are cylindrical oligosaccharides presenting a hydrophilic surface and a lipophilic cavity known to form complexes with poorly water-soluble drugs [28]. Such complexes present a number of interesting features for the formulation of latanoprost for ocular topical treatments since they can act as i) stabilizers by preventing drug hydrolysis [29], ii) solubilizers by creating water-soluble complexes with the drug [28, 30-34], iii) anti-irritants by shielding irritating drugs [35] and iv) drug carriers by improving drug delivery to the eye [36]. In addition, CDs are considered as non-toxic due to their very low penetration through biological barriers [29, 35, 37]. However, the effect of CDs on drug properties is not predictable and not all CDs can be expected to have the desired effects on drug properties.

The aim of this study was to develop a new latanoprost formulation containing CDs to address current stability, availability, solubility and tolerance issues related to the formulation of latanoprost for topical ocular administration. In a first step, a panel of CDs was screened to evaluate their impact on drug stability *in vitro* and on drug availability *ex vivo*. The latanoprost-CD combination displaying the best tradeoff between stability and availability was then selected for further characterization and investigation regarding the complex. Finally, the *in vivo* ocular tolerance of the latanoprost-CD formulation was investigated to evaluate the potential anti-irritating effect of the CD. The commercially available latanoprost 0.005% formulation (Xalatan®, Pfizer Inc., NY, USA) was used as reference for all the experiments.

2. MATERIALS AND METHODS

2.1. Materials

Latanoprost, deuterated latanoprost, latanoprost free acid and deuterated latanoprost free acid were purchased from Cayman chemicals (Michigan, USA). Xalatan was purchased from Pfizer (Zurich, Switzerland). Pharmaceutical grade native α, β and γ cyclodextrins (αCD, βCD, γCD), hydroxypropyl-β-cyclodextrin (HPβCD) and hydroxypropyl-γ-cyclodextrin (HPγCD) were a kind gift from ISP Global Technologies Deutschland GmbH (Cologne, Germany). Dimetyl-β-cyclodextrin (DMβCD), highly sulphated-β-cyclodextrin (HSβCD) and disodium-monohydrogenophosphate were obtained from Sigma-Aldrich (Buchs, Switzerland). The 6-monodeoxy-6-N-mono(3-hydroxy)propylamino-β-cyclodextrin (PAβCD) was obtained from Cyclolab (Budapest, Hungary). Monosodium-dihydrogenophosphate and benzalkonium chloride were from Hanseler (Herisau, Switzerland). Sodium chloride was from Reactolab (Servion, Switzerland).

Methanol, acetonitrile and formic acid of ULC/MS grade were purchased from Biosolve (Valkenswaard, Netherlands). Water for formulations was purchased from Dr. G. Bichsel AG (Interlaken, Switzerland). The water used for experimentation was obtained from a Milli-Q Water Purification System from Millipore (Bedford, MA, USA). Phosphate buffered saline solution for *ex vivo* experiments was purchased from Gibco Life Technologies (Zug, Switzerland).

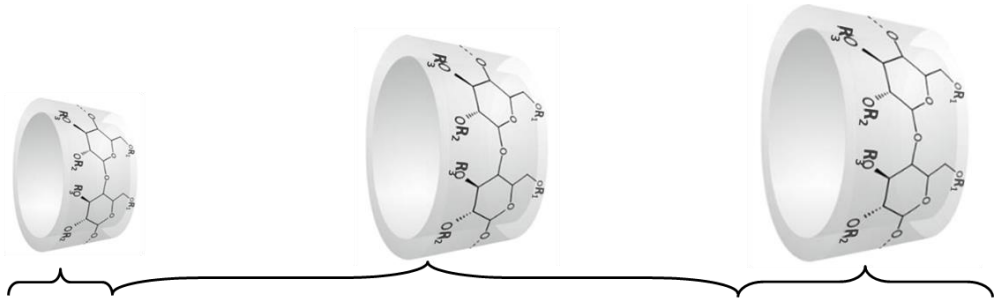
2.2. Methods

2.2.1. Preparation of the formulations

Latanoprost 0.005% w/v aqueous formulations were prepared with pH 6.8 buffered aqueous solutions containing the CDs to be tested. Eight CDs (αCD, βCD, HPβCD, PAβCD, DMβCD, HSβCD, γCD, HPγCD) were selected to obtain a panel of different ring sizes, substituents and polarities, which are summarized in Table 1. For stability and availability tests, CD levels were set at molar concentrations 10 and 50 times higher than for latanoprost, and will be referred to as latanoprost-CD 1:10 and latanoprost-CD 1:50 formulations, respectively.

Briefly, 50 µL of latanoprost stock solution was evaporated under vacuum (centrifuge evaporator RC 10.22 and cold trap RCT90, Jouan Ltd, Herts, UK) and the appropriate buffered aqueous CD solutions containing 0.02% w/v benzalkonium chloride were added to the vial containing pure latanoprost. The vials were sonicated, vortexed and then magnetically stirred for 96 hours. The osmolarity was then adjusted to 290 mOsm/L with NaCl (Vapor Pressure Osmometer 5500, Wescor, Logan, Utah, USA). Latanoprost formulations were finally filtered through 0.2 µm membranes (Exapure, Bussigny-près-Lausanne, Switzerland) and kept in appropriate containers and conditions until use.

Table 1: Cyclodextrins evaluated for the formulation of latanoprost for topical ocular administration.



Name	αCD	βCD	HPβCD	PAβCD	DMβCD	HSβCD	γCD	HPγCD
Glucopyranose units	6	7	7	7	7	7	8	8
Substituents (R_1, R_2, R_3)	H	H	H and hydroxypropyl	H and 6-monodeoxy-6-N-mono (3-hydroxy) propylamino	H and methyl	H and sulfo	H	H and hydroxypropyl

2.2.2. Analytical methods

The analyses were performed on a Waters Acquity ultra performance liquid chromatography (UPLC) system hyphenated with a DAD UV detection system or a triple quadrupole mass spectrometer (Waters, Milford, USA).

2.2.2.1. In vitro analyses

For the *in vitro* stability and phase solubility assessments, the sample manager of the UPLC was maintained at 25°C. A volume of 5 µL (partial loop with needle overfill injection) was injected into the chromatographic column, a Waters Acquity BEH C18 (50 x 2.1 mm I.D., 1.7 µm) heated at 40°C. The mobile phase consisted of a mixture of ammonium acetate 20mM pH 5.0 buffer and acetonitrile. A linear gradient from 20 to 80% acetonitrile was applied for 4 min at a flow rate of 500 µL/min, followed by a re-equilibrating step of 3 min. After each analysis, the injection system was washed with 200 µL of acetonitrile and 600 µL of a mix water/acetonitrile 80:20. The detection was performed at 210 nm. Data acquisition, data handling and instrument control were performed by the Empower v2.0 Software (Waters, Milford, USA).

For the stability study, latanoprost and latanoprost free acid calibrators were prepared at 2, 10 and 30 µg/mL. For the phase-solubility assessment, the latanoprost concentrations used for calibration were 2, 10, 30, 50, 100 and 150 µg/mL.

2.2.2.2. Ex vivo analyses

MS/MS detection was used for the analyses of *ex vivo* samples. The method was based on the injection of 5 µL of each sample kept at 4ºC in the sample manager. The chromatographic column was a Waters Acquity BEH C18 (50 x 2.1 mm I.D., 1.7 µm) connected to a C18 VanGuard precolumn (5 x 2.1 mm I.D., 1.7 µm) both heated at 40°C. The mobile phase was a mixture of water with 0.1% v/v formic acid (A) and methanol with 0.1% v/v formic acid (B). A linear gradient from 50 to 90% B was applied for 3 min at a flow rate of 500 µL/min, followed by a re-equilibrating step of 1 min. After each analysis, the injection system was washed with 300 µL of methanol and 500 µL of water. The detection was performed in ESI positive mode and selected reaction monitoring (SRM). A 4 times deuterated latanoprost

($d_4$latanoprost) and 4 times deuterated latanoprost free acid ($d_4$latanoprost acid) were used as internal standards. The quantifications were based on the ratios between the signals of the analytes and the internal standards. Collision energies and cone voltages were tuned by infusing each compound individually. Optimal values were 12 V for the cone voltage and 10 eV for the collision energy. The pseudomolecular parent ion and the fragment mass-to-charge ratios (m/z) for the analytes of interest were 433.0 and 397.0, respectively, for latanoprost; 391.0 and 355.0, respectively, for latanoprost free acid; 437.0 and 401.0, respectively for $d_4$latanoprost; and 395.0 and 359.0, respectively for $d_4$latanoprost free acid. The capillary voltage and the source extractor voltage were set at +2 kV and +3 V, respectively. The source temperature was maintained at 140°C and the desolvation nitrogen temperature and flow at 400°C and 800 L/h, respectively. The cone gas flow was at 20 L/h. The collision gas flow was set to 0.2 mL/min of argon and the entrance and exit potentials were adjusted to 1 and 0.5 V, respectively. The inter-channel delay was set to 5 ms and dwell time was set to 100 ms for all compounds. The low and high mass resolutions were adjusted to 15 and 13, respectively. Data acquisition, data handling and instrument control were performed with the Masslynx v4.1 Software (Waters, Milford, USA).

Calibration standards contained 0.1 µg/mL $d_4$latanoprost and 0.5 µg/mL $d_4$latanoprost free acid along with the following concentrations of latanoprost and latanoprost free acid: 0.01, 0.05, 0.1, 0.5, and 1 µg/mL. Freshly prepared calibrators were used for each analytical run.

2.2.3. *In vitro* stability investigations

Two sets of conditions were selected to evaluate the stability of latanoprost-based formulations. One set was exposed to direct sunlight and the other was kept at 25°C in the dark. The degradation profiles from samples exposed to sunlight allowed to identify the CDs that provide protection against both photodegradation and thermal degradation. The formulations kept under usual storage conditions (room temperature, no light) allowed the evaluation of the potential shelf life of the new formulations compared to the commercial reference. For each time point, 100 µL of each tested formulation was diluted in the same volume of acetonitrile. Samples were analyzed with the UHPLC-UV method for latanoprost and latanoprost free acid quantification. Formulations stability was initially evaluated over six months and prolonged for six additional months for the most promising formulations.

2.2.4. *Ex vivo* corneal permeation evaluation

Ex vivo experiments were performed on bovine eyes obtained from a local slaughterhouse where eyes were collected directly after animal sacrifice. The eyes were collected with the eyelids, were carefully closed to maintain the quality and integrity of the corneal epithelium and were kept on ice during transportation.

The corneas were carefully dissected and placed on one arm diffusion cells (GlassTechnology, Meyrin, Switzerland). The area of tissue available for diffusion was 0.64 cm^2. The receptor compartment was filled with 5 mL of PBS at 25 mM, pH 7.4, preheated at 37°C. A volume of 100 µL of latanoprost-HPβCD, latanoprost-PAβCD, latanoprost-DMβCD, latanoprost-HSβCD formulations or the commercial formulation Xalatan was added to the donor compartment. The experiment was conducted on six corneas for each formulation. The diffusion cells were kept at 37°C and the receptor compartments were magnetically stirred during the experiment. The experiments were conducted during 4 hours, 500 µL samples were taken each hour and replaced with fresh buffer. The samples were diluted 1:2 with methanol containing 0.2 µg/mL $d_4$latanoprost and 1.0 µg/mL $d_4$latanoprost free acid for latanoprost and latanoprost free acid quantification by UHPLC-UV.

After 4 hours, tissues were removed from the diffusion cells and the 0.9 cm diameter disc that was in contact with the formulation was dissected. The tissue sections were weighed, grinded, placed in vials containing methanol along with 0.1 µg/mL $d_4$latanoprost and 0.5 µg/mL $d_4$latanoprost free acid and stirred overnight. The following day, samples were centrifuged and supernatants were analyzed for latanoprost and latanoprost free acid content. Experimental results were compared with the Mann-Whitney U-test.

2.2.5. Complex characterization

2.2.5.1. Phase-solubility investigations of complexation

The effect of the cyclodextrin PAβCD on latanoprost solubility in water was evaluated according to the protocol described by Loftsson et al. [38]. Briefly, a latanoprost stock solution at 10 mg/mL was added to a vial and evaporated under vacuum (Centrifuge evaporator RC 10.22 and cold trap RCT90, Jouan Ltd, Herts, UK). A volume of 700 µL of aqueous cyclodextrin solutions at different concentrations were added to the pure latanoprost. Vials were sealed and sonicated for 1h at 70°C. They were then allowed to reach room temperature (RT) over night while agitated. The following day, an additional excess of drug was added to each vial and the vials were maintained under agitation for 120 h at 25°C. The latanoprost-CD solutions were filtered through a 0.45 µm membrane and the first third of the filtrate was discarded. The amount of latanoprost in solution was evaluated with the UHPLC-UV method described above. The phase-solubility diagram was constructed by representing the concentration of dissolved latanoprost as a function of PAβCD concentration. The type of diagram was determined following to Higuchi and Connors classification including A_P, A_L, A_N, B_S and B_I diagram types [39]. Complex stoichiometry, complex stability constant, complexation efficacy and optimal latanoprost-PAβCD ratio for formulation were calculated according to the equations described elsewhere [29, 40, 41].

Briefly, the stoichiometry of the complex was derived from the slope of the linear regression.

The complex stability constant was based on the following equation:

$$Complex\ stability\ constant = \frac{\text{Slope}}{\text{S}\ (1 - \text{Slope})}$$

where S is the intrinsic latanoprost solubility, which is 0.116 mM, and Slope is the slope of the linear regression of the phase-solubility diagram.

The complexation efficacy determination was based on the following equation:

$$Complexation\ efficacy = \frac{\text{Slope}}{(1 - \text{Slope})}$$

The optimal latanoprost-PAβCD ratio for latanoprost formulation was determined according to the following equation:

$$Optimal\ latanoprost - PA\beta CD\ ratio = 1/(\ 1 + \frac{1}{Complexation\ efficacy})$$

2.2.5.2. NMR investigation of complex structure

Solutions of PAβCD and of the latanoprost-PAβCD complex were prepared in D_2O for analysis of the complex structure by NMR. For the PAβCD sample, 14 mg of PAβCD were dissolved in 0.7 mL of D_2O. To make the latanoprost-PAβCD complex sample, 500 µL of latanoprost stock solution at 10 mg/mL were evaporated under vacuum (centrifuge evaporator RC 10.22 and cold trap RCT90, Jouan Ltd, Herts, UK) to retrieve 5 mg of pure latanoprost. The latanoprost was then mixed with 0.7 mL of D_2O containing 14 mg of PAβCD (corresponding to a 1:1 molar ratio) for the formation of the complex.

The NMR experiments were performed on a Fourier Transform Bruker NMR Spectrometer (Bruker, Fällanden, Switzerland) operating at a ^{1}H Larmor frequency of 500 MHz and equipped with a cryogenic detection probe. All experiments were recorded at 298°K. The PAβCD and latanoprost-PAβCD complex were studied though one-dimensional ^{1}H and ^{13}C NMR experiments as well as two-dimensional ^{1}H-^{1}H correlation spectroscopy (COSY) and one-bound ^{1}H-^{13}C heteronuclear single-quantum coherence spectroscopy (HSQC). The ^{1}H-NMR spectra were referenced to the resonance line of HOD at 4.70. Further two-dimensional rotating frame nuclear Overhauser effect spectroscopy (ROESY) experiments were carried out with a mixing time of 300 ms, a recovery delay of 1.9 s and 256 time increments with 16 scans per increment.

2.2.6. *In vivo* ocular tolerance evaluation

Three formulations were tested for their ocular tolerance on New Zealand rabbits. The tested formulations were: i) the commercial latanoprost formulation Xalatan containing 0.005% w/v latanoprost and 0.02% w/v benzalkonium chloride in isotonic PBS at pH 6.8, ii) a latanoprost-PAβCD formulation containing 0.005% w/v latanoprost, 0.02% w/v benzalkonium chloride and 0.142% w/v PAβCD in isotonic PBS at pH 6.8 and iii) the vehicle of the commercial formulation containing benzalkonium chloride 0.02% w/v in PBS at pH 6.8. Three groups of rabbits were created, one for each formulation to be tested. Each group contained six rabbits, except the group receiving the vehicle of the commercial formulation (control group) which contained three rabbits. Prior to the experiment, the basal ocular irritation of each rabbit was evaluated and animals were randomly assigned to each group. Thirty microliters of the appropriate formulation were topically administered in both eyes of the rabbits, six times per day over fourteen days. Ocular hyperemia was evaluated twice a day during the fourteen days of administration (after the third and sixth daily doses). Clinical observations were performed by trained clinicians who assessed the hyperemia of rabbit eyes. Clinicians were blinded to the animal groups to minimize bias. Over the fourteen days of administration, one clinical observation of ocular irritation was made each day for each animal based on the two evaluations made during the same day. A clinical observation of ocular irritation was reported when bilateral hyperemia was observed at the two evaluation points of the same day. The incidence of ocular irritation for each formulation was calculated as the percentage of total clinical observations reporting ocular irritations over the fourteen days of administration.

At the end of the experiment animals were sacrificed. Eyes were collected, embedded in paraffin, sectioned, mounted on glass slides, and hematoxylin-eosin stained for microscopic histopathology evaluation. The histology study was performed in a blind manner to minimize bias.

3. RESULTS

3.1. *In vitro* stability

The stability profiles for the formulations exposed to direct sunlight for six and twelve months are presented in Figure 2A and 2B, respectively. Under direct sunlight, native αCD and γCD formulations led to marked latanoprost degradation compared to the commercial formulation and precipitates became visible in these formulations after 2 weeks. On the other hand, βCD formulations showed an improved stability profile compared to the reference formulation. After 12 months under direct sunlight, the commercial formulation contained 33.5 +/- 1.8% of the initial latanoprost dose, while the latanoprost-DMβCD 1:50 and latanoprost-PAβCD 1:50 still contained 81.9 +/- 1.3% and 79.6 +/- 0.9% of the initial latanoprost amount, respectively. The formulations with DMβCD, PAβCD, HPβCD and HSβCD displayed the best stability profiles among the tested CDs. In regards to the CD concentration, the formulations containing βCDs at 1:50 were more stable than their equivalent 1:10 formulations. On the contrary, for αCD and γCDs, the 1:10 formulations were more stable than the 1:50 ones.

The stability profiles under usual storage conditions (darkness at room temperature) for six months of latanoprost-DMβCD, -PAβCD, -HPβCD, -HSβCD formulations and of the reference formulation are illustrated in Figure 2C. Among the tested formulations, only the latanoprost-HSβCD 1:50 displayed a significantly lower stability profile than the commercial reference. The latanoprost-DMβCD, latanoprost-PAβCD and latanoprost-HPβCD formulations were not significantly different from the commercial reference, and after 6 months at 25°C these formulations still contained more than 95% of the initial latanoprost content.

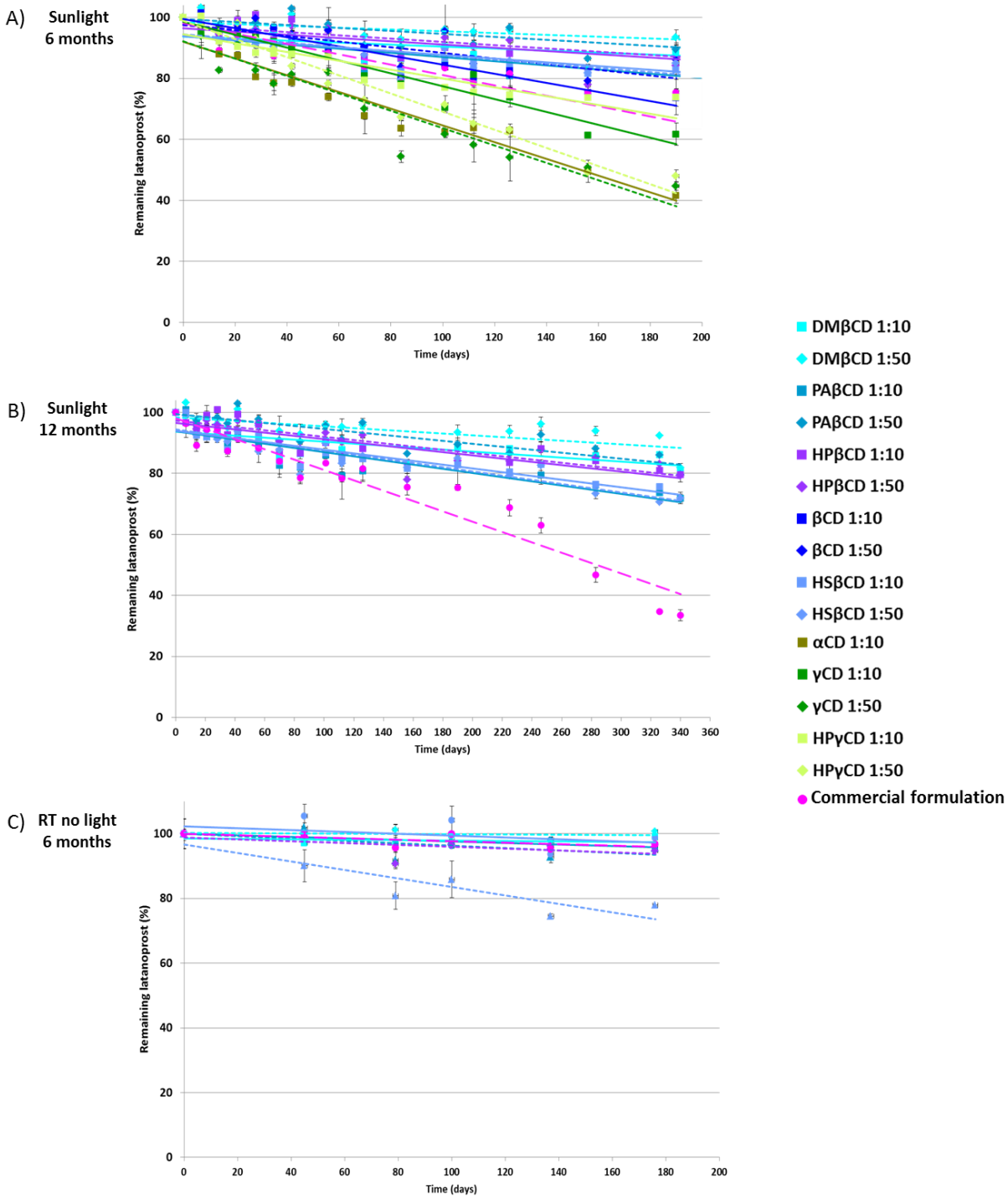


Figure 2: Stability profile of latanoprost formulations over A) 6 months under direct sunlight exposure, B) 12 months under direct sunlight exposure and C) 6 months at room temperature in the dark. Solid lines represent latanoprost-CD formulations with a latanoprost-CD ratio of 1:10 and dotted lines represent formulations at 1:50 ratios. The commercial latanoprost formulation Xalatan was used as a reference.

3.2. *Ex vivo* availability

Figure 3 illustrates the *ex vivo* corneal permeation profiles of the latanoprost-HPβCD, latanoprost-PAβCD, latanoprost-DMβCD, latanoprost-HSβCD formulations and the commercial formulation using a diffusion cell experimental setup. The comparison of the permeation profiles of the latanoprost-CD formulations and the reference CD-free latanoprost formulation allowed evaluating the potential effect of CDs on latanoprost availability. Latanoprost permeation profiles were determined according to the percentage of latanoprost that was found in the receptor compartment as latanoprost acid, since biotransformation occurs inside the cornea during permeation and no latanoprost was detectable in the receptor compartment. The permeation profiles of latanoprost-HPβCD 1:10 and 1:50, latanoprost-PAβCD 1:10 and 1:50 and latanoprost-DMβCD 1:10 did not significantly differ from the one of the commercial formulation. In contrast, latanoprost-DMβCD 1:50 and latanoprost-HSβCD 1:10 showed a lower permeation profile compared to the commercial formulation.

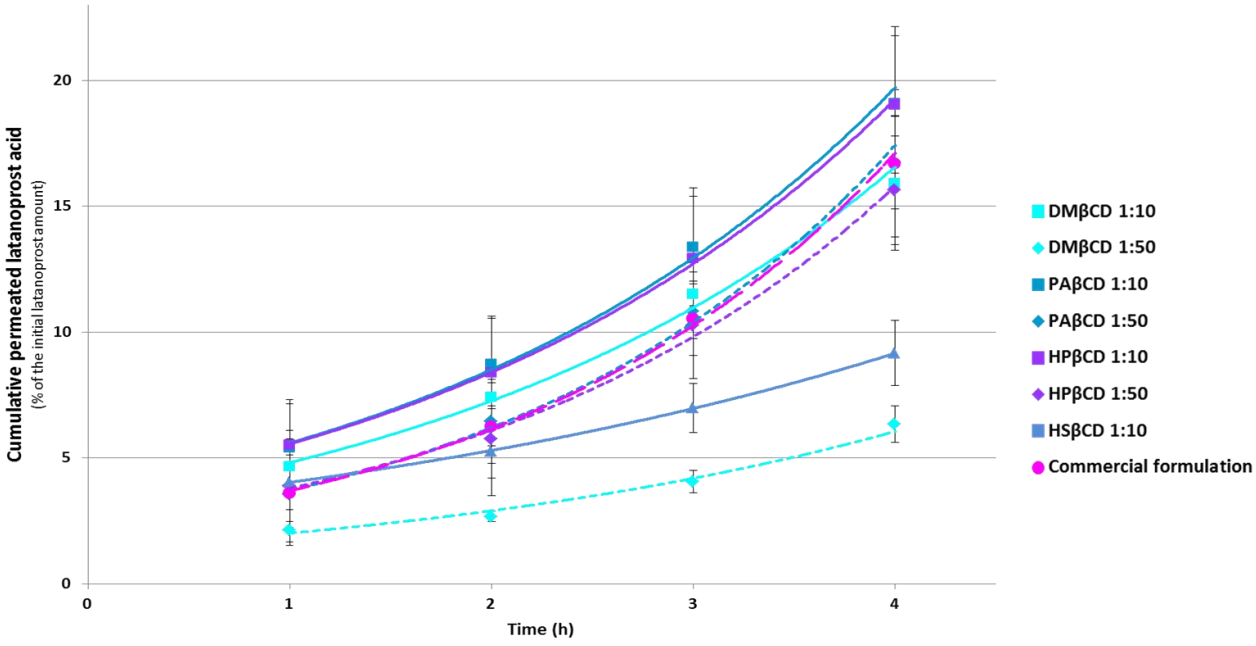


Figure 3: *Ex vivo* corneal permeation profiles of latanoprost formulations based on the percentage of latanoprost added to the donor compartment that was found in the receptor compartment of the diffusion cell as latanoprost acid. No latanoprost was found in the receptor compartment since biotransformation took place during corneal permeation. The experiments were performed on six cells for each formulation. Solid lines are used for latanoprost-CD formulations with a latanoprost-CD ratio of 1:10 and dotted lines are used for latanoprost-CD formulations at a 1:50 ratio. The commercial formulation Xalatan was used as a reference.

After 4 hours, the corneal tissues were disintegrated and the latanoprost and latanoprost free acid contents were quantified (data not shown). No significant difference was observed in latanoprost and latanoprost acid content in the cornea between latanoprost-PAβCD 1:10, latanoprost-PAβCD 1:50 formulations and the commercial formulation.

3.3. Latanoprost-PAβCD complex characterization

3.3.1. Complexation process

The PAβCD was demonstrated to stabilize latanoprost while ensuring adequate ocular availability so the latanoprost-PAβCD formulation was selected for further study. Figure 4 illustrates the phase-solubility diagram of latanoprost in presence of PAβCD. The plot shows a linear increase in latanoprost solubility as a function of PAβCD concentration, demonstrating the solubilizing effect of the PAβCD on latanoprost.

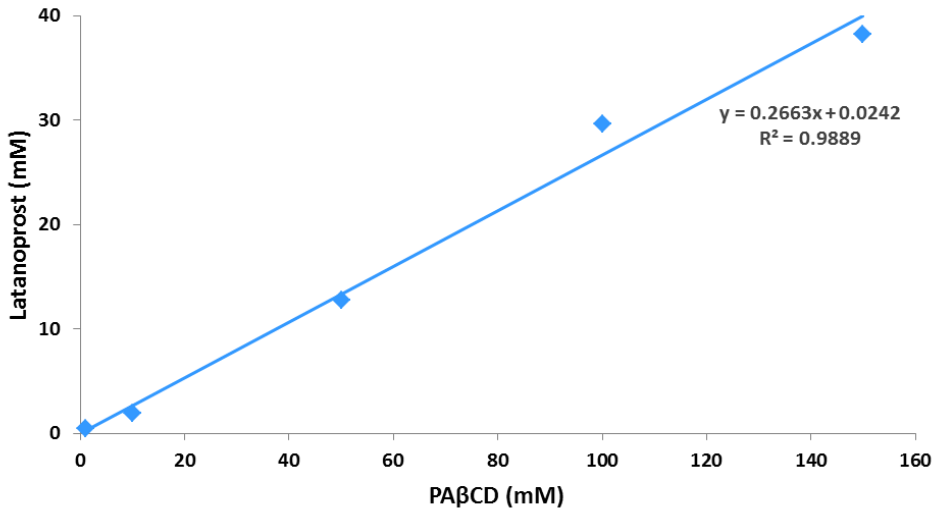


Figure 4: Phase-solubility diagram of latanoprost in presence of propylaminoβCD (PAβCD) represented as latanoprost solubilization as a function of PAβCD concentration.

According to the described methodology, the diagram was classified as "A_L-type", the complex stoichiometry was 1:1, the complex stability constant was determined to be 3129 M^{-1}, the complexation efficacy was 0.36 and the optimal latanoprost-PAβCD ratio was 1:4.

3.3.2. Complex structure

One-dimensional ^{1}H and ^{13}C and two-dimensional ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC NMR experiments were performed to determine the signals of PAβCD in its free form and its complexated form with latanoprost. The signals of the non-substituted sugar units of PAβCD were overlapping and assigned as a group and the other signals were individually assigned. The comparison of the signals of PAβCD in its free and complexated forms allowed the identification of the protons interacting with latanoprost, which show ^{1}H chemical shift displacements. The largest chemical shifts were reported for the internal H3 and H5 of the PAβCD, which displayed chemical displacements of 0.024 ppm and 0.055 ppm, respectively. On the contrary, external H1, H2 and H4 displayed negligible chemical shifts (lower than 0.006 ppm). These results indicate a modification of the chemical environment of the hydrophobic cavity of the PAβCD in presence of latanoprost, evidencing the formation of a latanoprost-PAβCD complex.

The two dimensions ROESY experiments investigated dipolar coupling interactions taking place when nuclei are in close proximity. The ROESY spectrum illustrated in Figure 5 shows clear intermolecular interactions between the methyl groups of the latanoprost isopropyl ester and the PAβCD internal cavity, demonstrating the efficient formation of a latanoprost-PAβCD complex where the ester group of latanoprost was located inside the cyclodextrin cavity.

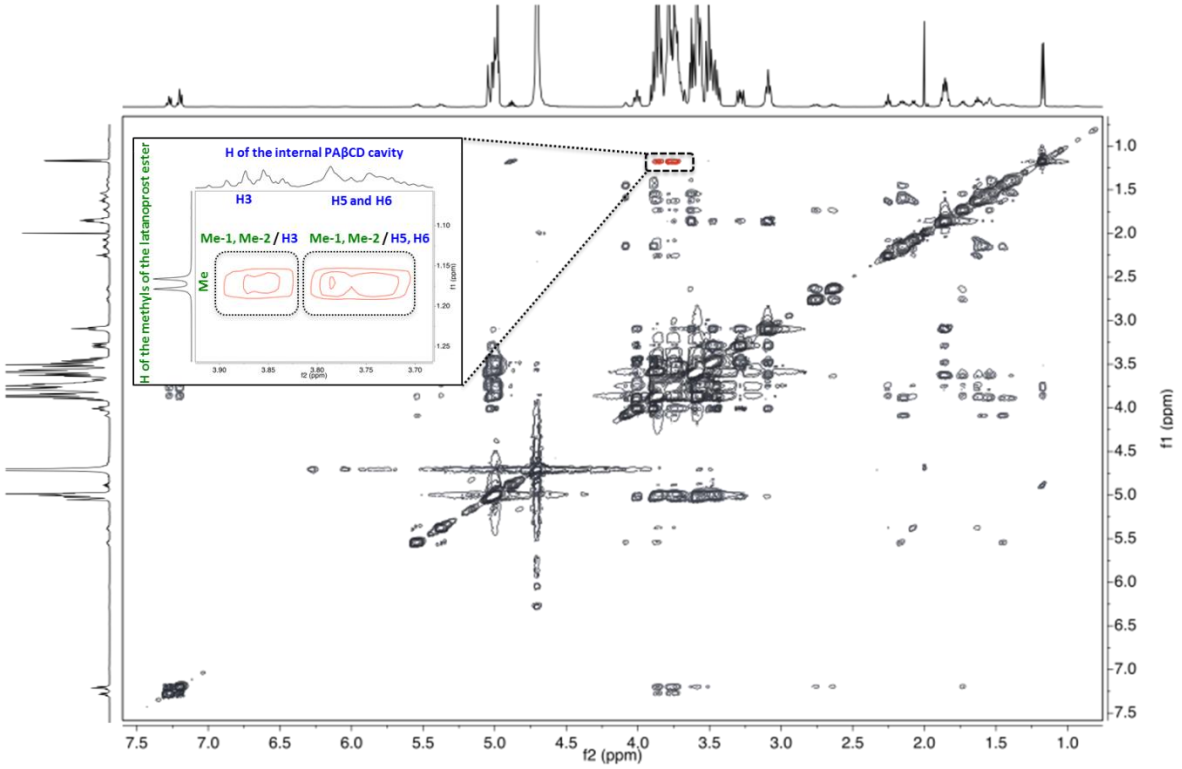


Figure 5: NMR rotating frame nuclear Overhauser effect spectroscopy (ROESY) spectrum of the latanoprost-PAβCD complex in D_2O. The most significant intermolecular interactions between latanoprost and PAβCD are highlighted in the insert. The signals correlate the protons of the two methyl groups of the latanoprost isopropyl ester (Me-1 and Me-2) with the protons located in the cavity of PAβCD (H3, H5 and H6).

3.4. *In vivo* ocular irritation

The latanoprost-PAβCD formulation, the latanoprost commercial formulation Xalatan, as well as the vehicle of the commercial formulation were evaluated for *in vivo* ocular irritation in the rabbit model over 14 days. The ocular irritation was evaluated for each formulation through the percentage of ocular irritation reported as clinical observations during the fourteen days of administration considering that each day one clinical observation was made for each animal. The ocular irritation measured for each group was 15.5% with the latanoprost commercial formulation, 9.5% with the latanoprost-PAβCD formulation and 7.1% for the vehicle of the formulations, as illustrated in Figure 6. The latanoprost-PAβCD formulation presented a lower percentage of ocular irritations than the commercial formulation and the vehicle of the formulation induced the lowest irritation. Microscopic evaluation of ocular tissues showed that Xalatan induced higher inflammatory mixed cell infiltrates than the latanoprost-PAβCD formulation and the vehicle, as illustrated in Figure 6.

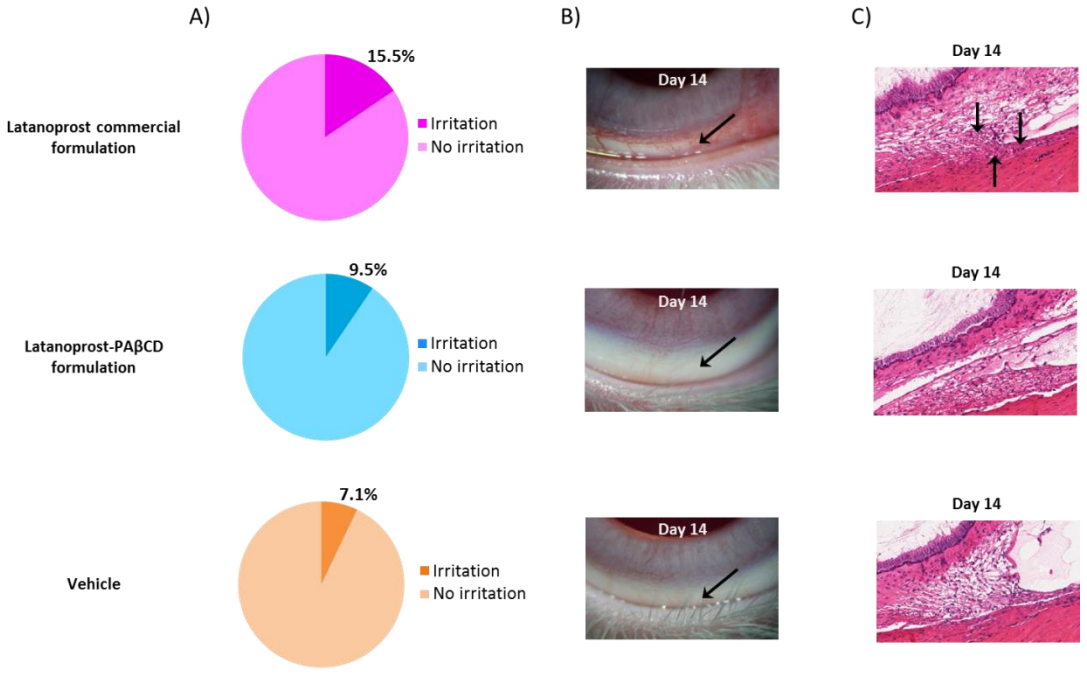


Figure 6: A) Percentage of total clinical observations reporting ocular irritation made during the fourteen days of administration for the latanoprost commercial formulation Xalatan, the latanoprost-PAβCD formulation and the vehicle (with no latanoprost and no PAβCD). B) Representative images of ocular irritation observed for each group on the last day of administration. Conjunctivas are indicated by arrows. C) Representative histological images of the lower ocular drainage angle for each group after fourteen days of administration. Inflammatory cellular infiltrates are indicated by arrows.

4. DISCUSSION

The complexation with CDs is an interesting approach since it offers the possibility to simultaneously address stability, solubility and tolerance issues related to the formulation of latanoprost for ocular topical administration. The effect of latanoprost complexation with a CD cannot be predicted, and not all CDs can be expected to have a positive impact on latanoprost formulation properties [35, 42]. Thus, the screening of different CDs was necessary to identify a CD that would improve the stability, availability, solubility and tolerance of latanoprost.

The CDs selected for investigation presented a range of different ring sizes, substituents and polarities. The ring size determines the diameter of the internal cavity, while the substituents and charges finely modulate the complexation capacity by allowing additional interactions or modifying the shape of the cavity [43-45]. It is worth mentioning that monographs of α, β, γCDs and HPβCD are included in the US Pharmacopeia [21]. The βCD ring was chosen as the base for the evaluation of various substituents: hydroxypropyl, 6-monodeoxy-6-N-mono(3-hydroxy)propylamino, methyl and sulfo groups, which are found in the HPβCD, PAβCD, DMβCD and HSβCD, respectively. In addition, these substituted CDs provided a range of polarities. The βCDs could be classified from positive to negative as follows: PAβCD, DMβCD, βCD, HPβCD and HSβCD.

The stabilities of the latanoprost-CD formulations were the first criterion for CD selection. The complexation of latanoprost could either stabilize or destabilize latanoprost in a non-predictable manner [35, 42]. In addition, since latanoprost is an ester, a potential chemical degradation could occur if the complex involved close proximity between the ester group of latanoprost and nucleophilic hydroxyl groups of the CDs, as previously reported [35, 42, 46, 47]. In a first step, the formulations were exposed to sunlight (which includes IR, visible and UV radiation as well as heat), mimicking an in-life use. The formulations were also kept at room temperature in the dark, simulating usual storage conditions. The commercial latanoprost formulation was included in all stability studies as a reference. The degradation profiles for the commercial latanoprost formulation were in line with former studies [17, 18, 48, 49] and confirmed the reported latanoprost instability after UV radiation [15, 16].

The solar stress stability profiles highlighted major differences between the commercial reference and the latanoprost-CD formulations. A clear distinction could be made between α, β and γCDs, the size of the CD ring therefore being a key parameter. Latanoprost seemed to be unstable in presence of α and γCDs. Both physical and chemical instabilities were hypothesized to be responsible for this behavior. The physical instability of the latanoprost-αCD and latanoprost-γCD complexes was revealed by the presence of precipitates in the vials and was related to the reported tendency of CDs and complexes to self-associate creating aggregates [50, 51]. The chemical instability of the α and γCD complexes was seen through the degradation of latanoprost and could be related to the previously mentioned reactivity between the latanoprost ester and the CD nucleophilic hydroxyl groups. In contrast to the α and γCDs, all the βCD formulations had a higher stability than the commercial product, suggesting that the β glucopyranose ring had particularly favorable characteristics for latanoprost stabilization. Pomponio and coworkers studied the effect of CDs on nicardipine photodegradation and reported a similar stabilizing effect of βCD and destabilizing effect of αCD [52]. The stability evaluation of the βCD-formulations under usual storage conditions demonstrated that latanoprost-DMβCD, latanoprost-PAβCD and latanoprost-HPβCD formulations could be expected to have similar shelf-lives than the commercial formulation. According to this stability study, the best CD-based formulations of latanoprost were with DMβCD and PAβCD.

The second criterion for CD screening was their influence on the latanoprost ocular availability, which is essential for formulation efficacy. The availability evaluation was based on an *ex vivo* corneal permeation experiment that was designed to specifically evaluate the influence of CDs on latanoprost ocular availability, regardless of the potential carrier-like activity that could be displayed by CDs as previously reported [36]. This *ex vivo* availability evaluation was of particular importance since CDs could lead to drug availability issues if the drug-CD interactions were too strong and hinders complex dissociation [42]. According to this statement, in this experiment the HSβCD formulation was used as a negative control since it is known to display a high complexation capacity responsible for a limited release of latanoprost from the complex as it was evidenced in the obtained permeation profile [53]. Interestingly, the two formulations providing the highest latanoprost stabilization showed

opposite effects on latanoprost availability. On one hand, the latanoprost-DMβCD 1:50 formulation displayed the lowest latanoprost permeation among the tested formulations, suggesting that the interaction between latanoprost and the DMβCD was too high to be used for a latanoprost ocular formulation. These findings were in line with previous studies showing that DMβCD was inappropriate for *in vivo* administration due to its high complexation capacity [32, 54, 55]. On the other hand, the latanoprost-PAβCD formulation, which was also selected for its excellent stabilizing effect, resulted in a latanoprost permeation profile equivalent to that of the CD-free commercial formulation, showing that latanoprost availability was not impaired when complexated with PAβCD. Consequently, among the tested CDs, PAβCD was the best candidate for latanoprost formulation for topical ocular application.

The fact that PAβCD resulted in the best tradeoff between latanoprost stability and availability was consistent with the latanoprost-PAβCD complex stability constant of 3129 M^{-1}. This value fell within the optimal range of 100-10'000 M^{-1}, which was reported to guarantee not only drug complexation *in vitro* but also drug availability *in vivo* [29, 41, 45, 56-58]. Stability constants above this range could lead to a high stabilization, but a low availability, which could be the case for the latanoprost-DMβCD formulation. In addition, the NMR experiments gave important insight on the structure of the latanoprost-PAβCD complex. On one hand, the NMR spectra confirmed efficient complexation between latanoprost and PAβCD, which was responsible for the observed stabilizing and solubilizing effects. On the other hand, the NMR 2D experiments unequivocally demonstrated that the ester group of latanoprost was included inside the PAβCD. This finding proved that the stabilizing effect was related to the inclusion of the labile part of latanoprost inside the cavity of the PAβCD, excluding superficial interaction-based hypotheses.

Furthermore, the efficient complexation of latanoprost with the PAβCD was demonstrated to drastically increase its solubility in the phase-solubility investigations. By maintaining latanoprost solubilized, the reported risk of drug loss by adsorption onto the eyedropper surface could be minimized, avoiding potential decrease in effective dosage and therapeutic efficacy [23, 59]. In addition, from a pharmaceutical perspective, the latanoprost-PAβCD complexation efficacy of 0.36 and optimal latanoprost-PAβCD ratio of 1:4 demonstrated that

the use of the PAβCD for latanoprost formulation represents a limited increase in the weight of the components of the formulation, making of it a suitable option for pharmaceutical production.

Besides *in vitro* stability and solubility improvements, the complexation of latanoprost with the PAβCD resulted in an improved ocular tolerance, which represents an important advantage for glaucoma treatment.

Ocular irritation was identified as the major issue related to the topical ocular administration of PG analogs, as reported by a number of studies [5, 7, 9, 24-27]. Conjunctival hyperemia (red eyes) was reported in 45% of the patients treated with PG analogs and was responsible for the noncompliance of 37% of these patients (27% discontinued the treatment and 10% skipped medication because of hyperemia) [26]. Thus, the improved ocular tolerance displayed by the latanoprost-PAβCD formulation represents a major advantage over conventional formulations since it can be expected to have a positive impact on patient compliance which is of particular importance in the life-long glaucoma treatment [27].

From a mechanistic point of view, these superficial ocular irritation issues have been related to the stimulation of corneal and conjunctival EP receptors that lead to nociception, vasodilatation, increased vascular permeability and hyperemia [5-7, 9-14]. Latanoprost ester is not active on these receptors and its presence in the lachrymal fluid should not lead to ocular irritation. Nevertheless, tears are known to display esterase-like activity, which could catalyze to the biotransformation of latanoprost into latanoprost acid [60]. Latanoprost acid can stimulate the EP receptors so its presence in the lachrymal fluid could lead to ocular irritation, as illustrated in Figure 7. In addition, latanoprost acid has a low lipophilicity, which could limit its corneal penetration and enhance its residence in the lachrymal fluid, further contributing to undesirable precorneal irritation. To reduce ocular irritation, the amount of active latanoprost acid in the lachrymal fluid should therefore be minimized. From these observations, it can be hypothesized that the anti-irritating effect of the latanoprost-PAβCD

formulation results from the protection of latanoprost from enzymatic biotransformation in the lachrymal fluid, as illustrated in Figure 7.

For this study, the commercial latanoprost formulation Xalatan was selected as reference product since it has a large history of clinical use and it has been extensively studied in the eighteen years it has been on the market (Xalatan being the second PG analog eyedrop commercialized for the treatment of glaucoma). Since Xalatan contains 0.02% w/v of benzalkonium chloride, the new latanoprost-PAβCD and the vehicle used as negative control for these experiments were formulated with the same concentration of benzalkonium chloride. Therefore, the experimental results correctly portrayed the difference in the ocular irritation caused by latanoprost and successfully demonstrated that PAβCD improves the ocular tolerance of latanoprost.

Nevertheless, the presence of benzalkonium chloride in ocular formulations is controversial due to contradictory observations. On one hand, latanoprost formulations containing 0.02% w/v of benzalkonium chloride were related to higher ocular irritation than preservative-free latanoprost formulations in acute toxicological stress tests in rabbits [61]. On the other hand, no direct correlation was found between benzalkonium chloride concentration and ocular irritation in studies involving glaucoma patients [26, 62]. Furthermore, Jessen and coworkers evidenced a direct correlation between latanoprost dosage and ocular irritation using preservative-free latanoprost subconjunctival implants, demonstrating that the ocular irritation is related to the active molecule itself [63]. Also, the ocular irritation effect, reported for PG analog formulations, was related to the structure of the PG analog and latanoprost was demonstrated to lead to the lowest irritation among commercialized PG analogs [10, 64, 65]. Consequently, it could be stated that the main irritating effect is related to latanoprost, but it cannot be denied that benzalkonium chloride could have a slight irritating effect, as measured in this study. It would therefore be interesting to develop a latanoprost-PAβCD formulation without benzalkonium chloride and investigate if ocular irritation could be further reduced.

The latanoprost-PAβCD formulation presents a promising solution for the long-term management of glaucoma as it successfully addresses the main challenges of stability, solubility and tolerance related to latanoprost formulations. Additional advantages could also be associated with such a formulation: i) enhanced ocular spreading and retention due to its positive charge [66] and ii) carrier-like effect, allowing latanoprost to reach the corneal surface more easily [36]. Further studies on this promising formulation should therefore be conducted to investigate different formulation vehicles for a further reduced ocular irritation, as well the effect of PAβCD on ocular retention time and carrier-like properties.

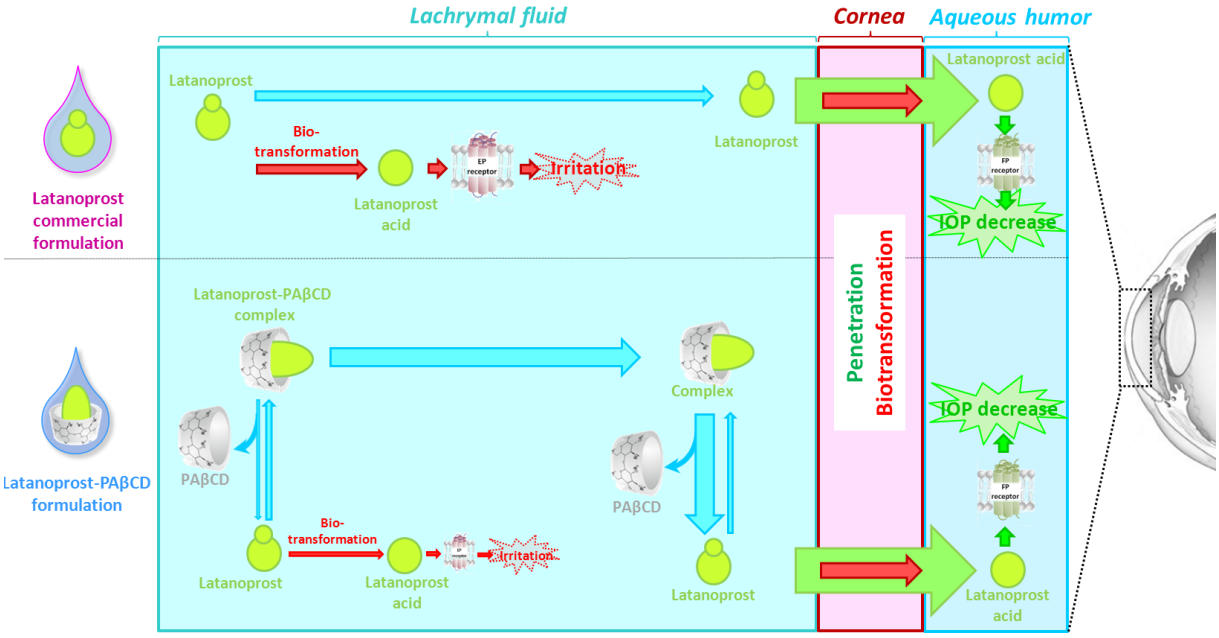


Figure 7: Schematic representation of the hypothesized *in vivo* behavior of both the commercial latanoprost formulation and the latanoprost-PAβCD formulation in the lachrymal fluid and anterior chamber of the eye, explaining the intraocular pressure lowering effect and the potential difference in ocular irritation.

5. CONCLUSIONS

This study presented the development of a latanoprost-CD formulation for the ocular topical treatment of glaucoma. A first screening step was mandatory since it cannot be assumed that all CDs will positively influence latanoprost properties. A panel of CDs constituted of αCD, βCD, HPβCD, PAβCD, DMβCD, HSβCD, γCD and HPγCD was selected to evaluate the influence of different ring sizes, substituents and charges. The stability and availability experiments led to the selection of PAβCD as the best CD candidate for latanoprost formulation for ocular topical administration. The PAβCD was demonstrated to shield the ester group of latanoprost inside its cavity, offering an effective protection from *in vitro* degradation and *in vivo* biotransformation. Moreover, PAβCD was demonstrated to resolve the stability, solubility and tolerance issues currently related to the formulation of latanoprost for the topical treatment of glaucoma. On one hand, PAβCD was demonstrated to stabilize latanoprost while ensuring its adequate availability, successfully addressing latanoprost-related stability issues without affecting its ocular availability. On the other hand, the complexation of latanoprost with PAβCD was proven to drastically increase latanoprost solubility avoiding potential drug loss related to its adsorption onto the surface of formulation containers. Finally, the latanoprost-PAβCD formulation led to a decrease in ocular irritation compared to the commercial latanoprost formulation, successfully addressing the main current challenge of latanoprost formulations. This improved ocular tolerance could be expected to have a positive impact on patient compliance, which is of major importance in the life-long glaucoma treatment. In addition, the PAβCD showed interesting characteristics from a pharmaceutical point of view since it led to only a limited increase in formulation bulk and because it can potentially improve drug delivery by increasing its retention time and acting as a drug carrier.

The latanoprost-PAβCD formulation represents an attractive solution for the long-term management of glaucoma addressing the main challenges related to latanoprost formulation. The mechanisms behind its anti-irritating effect, its potential to increase ocular retention time and its carrier-like properties should be investigated in future experiments.

ACKNOLEDGMENTS

The authors would like to thank Dr. Firas El Saleh for kindly providing us with pharmaceutical grade αCD, βCD, γCD, HPβCD and HPγCD. Dr. Laurence Marcourt is acknowledged for her help with preliminary NMR investigations. The authors would also like to thank Dr. Julien Boccard for his help with *in vivo* data analyses.

REFERENCES

[1] C. Cook, P. Foster, Epidemiology of glaucoma: what's new?, Canadian journal of ophthalmology. Journal canadien d'ophtalmologie, 47 (2012) 223-226.

[2] S. Resnikoff, D. Pascolini, D. Etya'ale, I. Kocur, R. Pararajasegaram, G.P. Pokharel, S.P. Mariotti, Global data on visual impairment in the year 2002, Bulletin of the World Health Organization, 82 (2004) 844-851.

[3] E.M. Quigley, Commentary: synbiotics and gut microbiota in older people--a microbial guide to healthy ageing, Alimentary pharmacology & therapeutics, 38 (2013) 1141-1142.

[4] M. Digiuni, P. Fogagnolo, L. Rossetti, A review of the use of latanoprost for glaucoma since its launch, Expert opinion on pharmacotherapy, 13 (2012) 723-745.

[5] I. Dams, J. Wasyluk, M. Prost, A. Kutner, Therapeutic uses of prostaglandin F(2alpha) analogues in ocular disease and novel synthetic strategies, Prostaglandins & other lipid mediators, 104-105 (2013) 109-121.

[6] G. Giuffre, The effects of prostaglandin F2 alpha in the human eye, Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie, 222 (1985) 139-141.

[7] J. Stjernschantz, Studies on ocular inflammation and development of a prostaglandin analogue for glaucoma treatment, Experimental eye research, 78 (2004) 759-766.

[8] S. Basu, B. Sjoquist, J. Stjernschantz, B. Resul, Corneal permeability to and ocular metabolism of phenyl substituted prostaglandin esters *in vitro*, Prostaglandins, leukotrienes, and essential fatty acids, 50 (1994) 161-168.

[9] A. Alm, J. Stjernschantz, Effects on intraocular pressure and side effects of 0.005% latanoprost applied once daily, evening or morning. A comparison with timolol. Scandinavian Latanoprost Study Group, Ophthalmology, 102 (1995) 1743-1752.

[10] J.W. Stjernschantz, From PGF(2alpha)-isopropyl ester to latanoprost: a review of the development of xalatan: the Proctor Lecture, Investigative ophthalmology & visual science, 42 (2001) 1134-1145.

[11] C.B. Camras, E.C. Siebold, J.S. Lustgarten, J.B. Serle, S.C. Frisch, S.M. Podos, L.Z. Bito, Maintained reduction of intraocular pressure by prostaglandin F2 alpha-1-isopropyl ester applied in multiple doses in ocular hypertensive and glaucoma patients, Ophthalmology, 96 (1989) 1329-1336; discussion 1336-1327.

[12] J. Villumsen, A. Alm, Prostaglandin-F-2-Alpha-Isopropylester Eye Drops - Effects in Normal Human Eyes, Brit J Ophthalmol, 73 (1989) 419-426.
[13] J. Villumsen, A. Alm, Ocular Effects of 2 Different Prostaglandin-F2-Alpha Esters - a Double-Masked Cross-over Study on Normotensive Eyes, Acta ophthalmologica, 68 (1990) 341-343.
[14] J.G. Crowston, J.D. Lindsey, C.A. Morris, L. Wheeler, F.A. Medeiros, R.N. Weinreb, Effect of bimatoprost on intraocular pressure in prostaglandin FP receptor knockout mice, Investigative ophthalmology & visual science, 46 (2005) 4571-4577.
[15] P.V. Morgan, S. Proniuk, J. Blanchard, R.J. Noecker, Effect of temperature and light on the stability of latanoprost and its clinical relevance, Journal of glaucoma, 10 (2001) 401-405.
[16] T.M. McGrath, S. Lam, J. Walt, J. Page, Evaluation of the Stability of Xalatan and Lumigan After Dispensing and Simulated Patient Use, Invest. Ophthalmol. Vis. Sci., 44 (2003) 4448-.
[17] T.V. Johnson, P.K. Gupta, D.K. Vudathala, I.A. Blair, A.P. Tanna, Thermal stability of bimatoprost, latanoprost, and travoprost under simulated daily use, Journal of ocular pharmacology and therapeutics : the official journal of the Association for Ocular Pharmacology and Therapeutics, 27 (2011) 51-59.
[18] Y. Sakai, S. Yasueda, A. Ohtori, Stability of latanoprost in an ophthalmic lipid emulsion using polyvinyl alcohol, International journal of pharmaceutics, 305 (2005) 176-179.
[19] M.Y. Kahook, R.D. Fechtner, L.J. Katz, R.J. Noecker, D.A. Ammar, A comparison of active ingredients and preservatives between brand name and generic topical glaucoma medications using liquid chromatography-tandem mass spectrometry, Current eye research, 37 (2012) 101-108.
[20] European Pharmacopeia, 8th edition ed., Strasbourg, 2014.
[21] United States Pharmacopeia and National Formulary, Rockville,, 2014.
[22] J.P. Remington, Remington, the science and practice of pharmacy, in, Mack Pub. Co. Pharmaceutical Press, Easton, Pa.
London, UK, 1995, pp. v.
[23] A. Ochiai, K. Danjo, The stabilization mechanism of latanoprost, International journal of pharmaceutics, 410 (2011) 23-30.
[24] R.K. Parrish, P. Palmberg, W.P. Sheu, X.L.T.S. Group, A comparison of latanoprost, bimatoprost, and travoprost in patients with elevated intraocular pressure: a 12-week,

randomized, masked-evaluator multicenter study, American journal of ophthalmology, 135 (2003) 688-703.

[25] R.S. Noecker, M.S. Dirks, N.T. Choplin, P. Bernstein, A.L. Batoosingh, S.M. Whitcup, G. Bimatoprost/Latanoprost Study, A six-month randomized clinical trial comparing the intraocular pressure-lowering efficacy of bimatoprost and latanoprost in patients with ocular hypertension or glaucoma, American journal of ophthalmology, 135 (2003) 55-63.

[26] T.J. Zimmerman, S.R. Hahn, L. Gelb, H. Tan, E.E. Kim, The impact of ocular adverse effects in patients treated with topical prostaglandin analogs: changes in prescription patterns and patient persistence, Journal of ocular pharmacology and therapeutics : the official journal of the Association for Ocular Pharmacology and Therapeutics, 25 (2009) 145-152.

[27] J.P. Nordmann, N. Auzanneau, S. Ricard, G. Berdeaux, Vision related quality of life and topical glaucoma treatment side effects, Health and quality of life outcomes, 1 (2003) 75.

[28] M.E. Davis, M.E. Brewster, Cyclodextrin-based pharmaceutics: past, present and future, Nature reviews. Drug discovery, 3 (2004) 1023-1035.

[29] T. Loftsson, M.E. Brewster, Pharmaceutical applications of cyclodextrins: basic science and product development, J Pharm Pharmacol, 62 (2010) 1607-1621.

[30] T. Loftsson, M.E. Brewster, Pharmaceutical applications of cyclodextrins .1. Drug solubilization and stabilization, Journal of pharmaceutical sciences, 85 (1996) 1017-1025.

[31] R.A. Rajewski, V.J. Stella, Pharmaceutical applications of cyclodextrins .2. *In vivo* drug delivery, Journal of pharmaceutical sciences, 85 (1996) 1142-1169.

[32] V.J. Stella, R.A. Rajewski, Cyclodextrins: their future in drug formulation and delivery, Pharmaceutical research, 14 (1997) 556-567.

[33] K. Uekama, F. Hirayama, T. Irie, Cyclodextrin Drug Carrier Systems, Chemical reviews, 98 (1998) 2045-2076.

[34] M.E. Brewster, T. Loftsson, Cyclodextrins as pharmaceutical solubilizers, Advanced drug delivery reviews, 59 (2007) 645-666.

[35] T. Loftssona, T. Jarvinen, Cyclodextrins in ophthalmic drug delivery, Advanced drug delivery reviews, 36 (1999) 59-79.

[36] T. Loftsson, E. Stefansson, Cyclodextrins in eye drop formulations: enhanced topical delivery of corticosteroids to the eye, Acta ophthalmologica Scandinavica, 80 (2002) 144-150.

[37] T. Irie, K. Uekama, Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation, Journal of pharmaceutical sciences, 86 (1997) 147-162.
[38] T. Loftsson, D. Hreinsdottir, M. Masson, Evaluation of cyclodextrin solubilization of drugs, International journal of pharmaceutics, 302 (2005) 18-28.
[39] T. Higuchi, K.A. Connors, Phase-solubility techniques, Advan. Anal. Chem. Instr., 4 (1965) 117-212.
[40] E.M. Martin Del Valle, Cyclodextrins and their uses: a review, Process Biochem, 39 (2004) 1033-1046.
[41] H.D. Williams, N.L. Trevaskis, S.A. Charman, R.M. Shanker, W.N. Charman, C.W. Pouton, C.J. Porter, Strategies to address low drug solubility in discovery and development, Pharmacological reviews, 65 (2013) 315-499.
[42] R. Liu, Water-insoluble drug formulation, 2nd ed., CRC Press, Boca Raton, FL, 2008.
[43] M. Másson, T. Loftsson, S.d. Jónsdóttir, H. Fridriksdóttir, D.S. Petersen, Stabilisation of ionic drugs through complexation with non-ionic and ionic cyclodextrins, International journal of pharmaceutics, 164 (1998) 45-55.
[44] V. Zia, R.A. Rajewski, V.J. Stella, Effect of cyclodextrin charge on complexation of neutral and charged substrates: Comparison of (SBE)(7M)-beta-CD to HP-beta-CD, Pharmaceutical research, 18 (2001) 667-673.
[45] Y. Zheng, I.S. Haworth, Z. Zuo, M.S. Chow, A.H. Chow, Physicochemical and structural characterization of quercetin-beta-cyclodextrin complexes, Journal of pharmaceutical sciences, 94 (2005) 1079-1089.
[46] E. Albers, B.W. Muller, Cyclodextrin derivatives in pharmaceutics, Critical reviews in therapeutic drug carrier systems, 12 (1995) 311-337.
[47] K.-H. Frömming, J.z. Szejtli, Cyclodextrins in pharmacy, Kluwer Academic Publishers, Dordrecht ; Boston, 1994.
[48] R. Varma, J. Winarko, T. Kiat-Winarko, B. Winarko, Concentration of latanoprost ophthalmic solution after 4 to 6 weeks' use in an eye clinic setting, Investigative ophthalmology & visual science, 47 (2006) 222-225.
[49] G.D. Novack, R. Evans, Commercially Available Ocular Hypotensive Products: Preservative Concentration, Stability, Storage, and In-life Utilization, Journal of glaucoma, 10 (2001) 483-486.

[50] T. Loftsson, A. Magnusdottir, M. Masson, J.F. Sigurjonsdottir, Self-association and cyclodextrin solubilization of drugs, Journal of pharmaceutical sciences, 91 (2002) 2307-2316.

[51] Y. He, P. Fu, X. Shen, H. Gao, Cyclodextrin-based aggregates and characterization by microscopy, Micron, 39 (2008) 495-516.

[52] R. Pomponio, R. Gotti, J. Fiori, V. Cavrini, P. Mura, M. Cirri, F. Maestrelli, Photostability studies on nicardipine-cyclodextrin complexes by capillary electrophoresis, Journal of pharmaceutical and biomedical analysis, 35 (2004) 267-275.

[53] C.E. Evans, A.M. Stalcup, Comprehensive strategy for chiral separations using sulfated cyclodextrins in capillary electrophoresis, Chirality, 15 (2003) 709-723.

[54] T. Jansen, B. Xhonneux, J. Mesens, M. Borgers, Beta-cyclodextrins as vehicles in eye-drop formulations: an evaluation of their effects on rabbit corneal epithelium, Lens and eye toxicity research, 7 (1990) 459-468.

[55] K. Jarvinen, T. Jarvinen, A. Urtti, Ocular Absorption Following Topical Delivery, Advanced drug delivery reviews, 16 (1995) 3-19.

[56] V.J. Stella, Q. He, Cyclodextrins, Toxicologic pathology, 36 (2008) 30-42.

[57] A. Ascenso, R. Guedes, R. Bernardino, H. Diogo, F.A. Carvalho, N.C. Santos, A.M. Silva, H.C. Marques, Complexation and full characterization of the tretinoin and dimethyl-beta-cyclodextrin complex, AAPS PharmSciTech, 12 (2011) 553-563.

[58] B.R. Schipper, T. Ramstad, Determination of the binding constant between alprostadil and alpha-cyclodextrin by capillary electrophoresis: implications for a freeze-dried formulation, Journal of pharmaceutical sciences, 94 (2005) 1528-1537.

[59] A. Ochiai, M. Ohkuma, K. Danjo, Investigation of surfactants suitable for stabilizing of latanoprost, International journal of pharmaceutics, 436 (2012) 732-737.

[60] F. Lallemand, E. Varesio, O. Felt-Baeyens, L. Bossy, G. Hopfgartner, R. Gurny, Biological conversion of a water-soluble prodrug of cyclosporine A, European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik e.V, 67 (2007) 555-561.

[61] H. Liang, C. Baudouin, M.O. Faure, G. Lambert, F. Brignole-Baudouin, Comparison of the ocular tolerability of a latanoprost cationic emulsion versus conventional formulations of prostaglandins: an *in vivo* toxicity assay, Molecular vision, 15 (2009) 1690-1699.

[62] J.T. Whitson, W.B. Trattler, C. Matossian, J. Williams, D.A. Hollander, Ocular surface tolerability of prostaglandin analogs in patients with glaucoma or ocular hypertension, Journal of ocular pharmacology and therapeutics : the official journal of the Association for Ocular Pharmacology and Therapeutics, 26 (2010) 287-292.

[63] B.A. Jessen, M.H. Shiue, H. Kaur, P. Miller, R. Leedle, H. Guo, M. Evans, Safety assessment of subconjunctivally implanted devices containing latanoprost in Dutch-belted rabbits, Journal of ocular pharmacology and therapeutics : the official journal of the Association for Ocular Pharmacology and Therapeutics, 29 (2013) 574-585.

[64] O. Eyawo, J. Nachega, P. Lefebvre, D. Meyer, B. Rachlis, C.W. Lee, S. Kelly, E. Mills, Efficacy and safety of prostaglandin analogues in patients with predominantly primary open-angle glaucoma or ocular hypertension: a meta-analysis, Clinical ophthalmology, 3 (2009) 447-456.

[65] W.C. Stewart, A.E. Kolker, J.A. Stewart, J. Leech, A.L. Jackson, Conjunctival hyperemia in healthy subjects after short-term dosing with latanoprost, bimatoprost, and travoprost, American journal of ophthalmology, 135 (2003) 314-320.

[66] S. Klang, M. Abdulrazik, S. Benita, Influence of emulsion droplet surface charge on indomethacin ocular tissue distribution, Pharmaceutical development and technology, 5 (2000) 521-532.

PART C

INTRAOCULAR ADMINISTRATION

As previously mentioned, cyclosporine A (CsA) is frequently used in ophthalmology for the treatment of ocular inflammatory conditions. However, being poorly water-soluble, it requires oily formulation vehicles, which lead to ocular irritation and a poor CsA availability. Therefore, ocular pathologies that need to be treated with high CsA concentrations in the ocular globe require a systemic administration of CsA and subsequent patient monitoring. Intraocular administration presents an interesting alternative to overcome the poor topical ocular availability of CsA while avoiding the severe side effects linked to systemic administration (such as nephrotoxicity, hepatotoxicity and hypertension).

To administer CsA intraocularly, a new formulation needed to be developed to ensure adequate delivery of the drug according to clinical requirements. Our strategy was to formulate the poorly water-soluble CsA in a solid dispersion using poly-lactic-glycolic acid 50:50 (PLGA) as a matrix. A novel manufacturing process was developed combining the solvent evaporation method with a multilayer approach for the production of a highly reproducible and versatile multilayer drug delivery system (DDS). Interestingly, this section illustrates the combination of two strategies to overcome the poor water solubility of CsA: a solid dispersion formulation and the use of intraocular administration. For its intraocular implantation, the DDS was associated with an intraocular lens based on the “all-in-one” concept previously developed by our group.

The investigations presented in the following part evaluated the effectiveness of intraocular CsA administration for the long-term management of complicated ocular inflammations. This approach was based on the intraocular implantation of a CsA loaded DDS in an animal model presenting an ocular inflammation and undergoing a cataract surgery. This model mimicked concomitant uveitis and postoperative inflammation, which is a complicated situation frequently faced by ophthalmologists. The development and characterization of the CsA loaded DDS was described before presenting an *in vivo* study for the evaluation of CsA release from the implant and its efficacy for the treatment of complicated ocular inflammation.

CHAPTER VII

Intraocular administration of cyclosporine A for prolonged delivery to the eye

A new drug delivery system inhibits uveitis in an animal model after cataract surgery

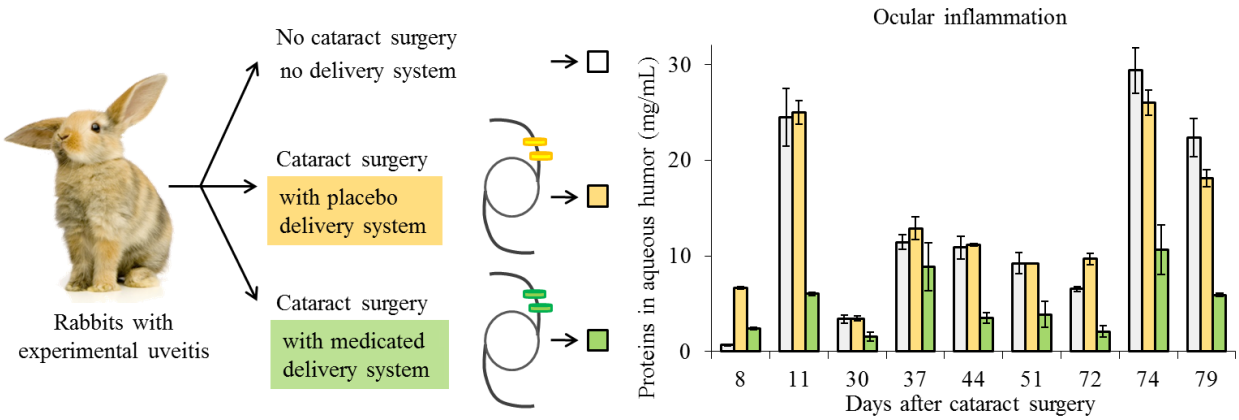
Simone EPERON[a, *, 1], Marta RODRIGUEZ-ALLER[b, 1], Konstantinos BALASKAS[a], Robert GURNY[b], Yan GUEX-CROSIER[a]

[1] The first two authors have equally contributed to this study

[a] Jules Gonin Eye Hospital, Lausanne University, 15, Av. de France, 1000 Lausanne 7, Switzerland, [b] School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, 30, Quai E.- Ansermet, 1211 Geneva 4, Switzerland

* Correspondence: Tel+41216268259; Fax+41216268122; E-mail: simone.eperon@fa2.ch

Published in International Journal of Pharmaceutics 2013; 443: 254-261.



ABSTRACT

Cataract surgery is a common ocular surgical procedure consisting in the implantation of an artificial intraocular lens (IOL) to replace the aging, dystrophic or damaged natural one. The management of postoperative ocular inflammation is a major challenge especially in the context of pre-existing uveitis. The association of the implanted IOL with a drug delivery system (DDS) allows the prolonged intraocular release of anti-inflammatory agents after surgery. Thus IOL-DDS represents an "all in one" strategy that simultaneously addresses both cataract and inflammation issues. Polymeric DDS loaded with two model anti-inflammatory drugs (triamcinolone acetonide (TA) and cyclosporine A (CsA)) were manufactured in a novel way and tested regarding their efficiency for the management of intraocular inflammation during the three months following surgery. The study involved an experimentally induced uveitis in rabbits. Experimental results showed that medicated DDS efficiently reduced ocular inflammation (decrease of protein concentration in aqueous humor, inflammatory cells in aqueous humor and clinical score). Additionally, more than 60% of the loading dose remained in the DDS at the end of the experiment, suggesting that the system could potentially cover longer inflammatory episodes. Thus, IOL-DDS were demonstrated to inhibit intraocular inflammation for at least three months after cataract surgery, representing a potential novel approach to cataract surgery in eyes with pre-existing uveitis.

Keywords: Cyclosporine A; Triamcinolone acetonide; Polymeric drug delivery system; Ocular inflammation; Rabbit; Uveitis.

1. INTRODUCTION

Cataract is a common ocular condition linked to the damage of the crystalline lens. Its treatment always requires surgery. Cataract surgery is the most frequently performed ocular procedure and the number of patients with cataract is continuously increasing. In the United States, the number of patients with cataract is expected to be around 30 million by 2020 [1, 2]. Cataract surgery raises significant additional management concerns in people suffering from concomitant ocular pathologies such as uveitis. Pre-existing pathologies may be exacerbated after surgery and could even lead to permanent visual loss [3].

Corticosteroids and nonsteroidal anti-inflammatory drugs are the golden standard for the treatment of severe ocular inflammation following cataract surgery [4, 5]. Nevertheless, it has been reported that the administration of steroids by periocular or intravitreal injections may cause severe ocular side effects and complications such as increased intraocular pressure, vitreous haemorrhage, retinal detachment or endophthalmitis [6, 7]. Immunosuppressive agents represent the second-line therapy in case of steroids side effects or steroid-resistant patients [8]. Nevertheless it has to be noted that systemic immunosuppressive therapy could lead to severe side effects that could be prevented by local ocular administration.

Biodegradable drug delivery systems (DDS) overcome systemic side effects of therapeutic agents and increase drug bioavailability and efficacy. It has been demonstrated that DDS prolongs drug release in comparison to other modes of administration [6, 9, 10]. Thus, many types of DDS have been developed during the last decade. Ozurdex® is a biodegradable intravitreal DDS delivering dexamethasone approved by the FDA to treat macular oedema secondary to non-infectious intermediate or posterior uveitis [8, 11, 12]. Scleral plugs loaded with immunosuppressive agents, such as tacrolimus or cyclosporine A (CsA) were tested on rabbits to treat experimental uveitis [13, 14]. Pellet DDS, similar to Surodex®, were loaded with CsA and freely implanted in the anterior chamber of rabbit eye [15].

An “all in one” system was developed in our group based on biodegradable DDS made out of poly(D,L-lactide-co-glycolide) associated with the intraocular lens (IOL) to be inserted during cataract surgery [16-18]. The IOL-DDS concept is illustrated in Figure 1A. This IOL-DDS loaded with triamcinolone acetonide (TA) was demonstrated to be able to significantly inhibit post-surgery inflammation [16]. Interestingly, a novel and versatile manufacturing process for

the DDS was developed and optimized for the present investigations, allowing the administration of TA and CsA based on a single IOL-DDS device.

The aim of the present study was to test the efficacy of IOL-DDS loaded with TA and CsA as a generic treatment strategy for the management of pre-existing concomitant intraocular inflammation during the months following cataract surgery. The association of TA and CsA was found to be particularly interesting for the treatment of uveitis of various aetiologies. The combination of steroids and immunosuppressants had been successfully used in clinics for the management of various inflammatory conditions [19-21]. The association of TA and CsA strategy allows a more comprehensive approach to the treatment of intraocular inflammation during the postoperative period.

Thus, the IOL was associated with two DDS, loaded with TA and CsA, respectively, and implanted during cataract surgery on rabbits already presenting uveitis at the time of the surgery. For this, rabbits were preimmunized with inactivated *Mycobacterium tuberculosis* H37RA and experimental uveitis was induced by intravitreal injections of the antigen (e.g. “challenges”). Uveitis was maintained over 79 days.

2. MATERIALS AND METHODS

2.1. Materials

The polymer, poly(D,L-lactide-co-glycolide) or PLGA, was a 50:50 mixture of lactide and glycolide with a molecular weight of 34,000 (Boehringer Ingelheim, Ingelheim, Germany). Triamcinolone acetonide was purchased from Sigma-Aldrich (Seelze, Germany) and CsA from Dynapharm (Dynapharm Distribution, Meyrin, Switzerland). All reagents were of analytical grade.

2.2. Novel manufacturing process of the DDS

A novel manufacturing process was developed and optimized for this application. An amount of 667 mg of PLGA was dissolved in 2 ml of acetonitrile and 333 mg of CsA or 400 mg of TA were added to the solution; the mixtures were vortexed and sonicated in order to obtain homogeneous preparations. A monolayer film and a bilayer film were needed to prepare DDS loaded with TA and CsA, respectively.

The mixture was poured on the liner of the Laboratory Coating Unit Type SV (Mathis, Zurich, Switzerland) after knife setting at the desired height (1mm for CsA and 1.5 mm for TA). The viscous suspension was spread with a constant thickness by translating the knife above each preparation. The liner supporting the preparations was put on an oven at 40°C for 12h to allow the solvent to evaporate. This procedure was repeated for each layer. The final film was taken from the liner, 2-mm diameter discs were cut and a hole was made in the centre of each disc. After sterilization by gamma-radiation, the DDS (one loaded with TA and the other loaded with CsA) were threaded on one haptic of IOL (AcrySof® MA30BA, Alcon Switzerland, Hünenberg) under sterile conditions and kept into appropriate packaging until their *in vivo* implantation.

2.3. Physico-chemical characterization of the DDS

Scanning Electron Microscopy (SEM) was used in order to investigate the internal structure of the DDS.

The thickness, diameter and weight of the DDS were assessed for each type of DDS to analyse the reproducibility of the manufacturing process. For the evaluation of the drug loading, the DDS were dissolved in 1.5 ml of Water/Acetonitrile 20:80. The amount of CsA was assessed by ultra high pressure liquid chromatography coupled with a UV detection (UHPLC-UV; Waters, Milford, MA, USA) using a Waters Acquity BEH C18 column (50 x 2.1 mm I.D., 1.7 µm) heated at 80°C. The mobile phase was a mixture of water with 0.1% v/v trifluoro-acetic acid and acetonitrile with 0.1% v/v trifluoro-acetic acid at a flow rate of 0.8 ml/min. The absorbance was assessed at 210 nm for CsA quantification.

The amount of TA was evaluated using the same system, mobile phase, flow rate and column. The analysis was carried out at 60°C and the detection was made at 236 nm.

The residual solvent amount was measured by GC Headspace (6850 series, Agilent Technologies, Valbronn, Germany).

2.4. Animals

This study was performed in accordance with the Swiss laws and institutional guidelines for animal experimentation and approved by the local and federal authorities. It followed EU directive 2010/63/EU for animal experiments and adhered to the tenets of the NIH statement for the use of Animals in Research. Female adult pigmented rabbits (4 – 5 kg) were provided by an authorized farmer. They were housed at a temperature of 20^{o}C in a day/night cycle of 12h/12h and had food and drink ad libitum. All eyes were checked with a slit lamp after anaesthesia (Rodenstock Instrumente, München, Germany) and only animals with no sign of ocular pathology were included in the study.

All rabbits went through the presensitization and uveitis induction procedures according to a method [22], which was modified for the present study. Rabbits were divided into three experimental groups. The first one, the control group, had no cataract surgery (control, n=6). The second and the third groups had the cataract surgery and different IOL-DDS were inserted: IOL with unloaded DDS (n=4) for the placebo group and IOL with DDS loaded with TA and CsA (n=4) for the medicated group.

2.5. Presensitization

Desiccated *M. tuberculosis* H37RA (Axonlab, Le Mont-sur-Lausanne, Switzerland) was resuspended in 150 mM sterile PBS. The antigen was mixed with TiterMax® Gold Adjuvant (Sigma, Saint Louis, MO, USA) to produce a water-in-oil emulsion. Rabbits were preimmunized with a multisite s.c. injection of a total of 400 µl of the emulsion containing 200 µg *M. tuberculosis* H37RA. Rabbits were checked 5 days a week at the sites of injection and regarding both their general comportment and state until the end of the experiment.

2.6. Uveitis induction

Experimental uveitis was induced by three intravitreal injections of the *M. tuberculosis* antigen (challenges) at days 29, 51 and 93 after presensitization, which corresponded to days 8, 30 and 72 after cataract surgery as illustrated in Figure 2. Rabbits were anesthetized with an i.m. injection of a new mix of 20 mg Ketamine /kg body weight (Ketalar® 50 mg/ml, Pfizer, Parke-Davis, Zürich, Switzerland) and 300 µg Medetomidine / kg body weight (Dorbene® 1 mg/ml, Graeub AG, Bern, Switzerland). After topical anaesthesia with Oxybuprocaïne 0.4% SDU Faure (Théa Pharma SA, Schaffhausen, Switzerland), the eye was disinfected with aqueous Betadine® (Mundipharma, Hamilton, Bermuda). We intravitreally injected 100 µl of a suspension of *M. tuberculosis* H37RA (15 µg) in Balanced Salt Solution (BSS®, Alcon Laboratories, Inc., Fort Worth, TX, USA). One drop of Ofloxacin (Floxal®, Bausch & Lomb, Steinhausen, Switzerland) was instilled at the end of injection and during the 3 following days.

2.7. Cataract surgery

We performed cataract surgery at day 21 after presensitization on rabbits of both placebo and medicated groups. After general anaesthesia of the rabbit, the eye was disinfected and anesthetized. Mydriasis was induced by one drop of Tropicamide 0.5% SDU Faure (CIBA Vision, Hettlingen, Switzerland), Atropine 1% SDU Faure (Novartis Ophthalmics, Hettlingen, Switzerland) and Néosynéphrine 5% Faure (Europhta, Monaco). During surgery, the eye was constantly moistened with drops of BSS®. Surgery was performed with the Series 20000™ Legacy® (Alcon Surgical, Fort Worth, TX, USA) and the pulverized lens aspirated. The IOL-DDS was inserted. The wounds were sutured with Dafilon 10/0 (B/Braub, Aesculap AG, Tuttlingen, Germany). Topical lomefloxacine (Okacin®, Novartis, Switzerland) was instilled in the eye at the end of surgery and the 3 following days.

2.8. Ocular inflammation assessment

Rabbits were systemically and topically anesthetized at each time point for clinical assessment of both eyes. Intraocular pressure was measured by a TonoPen™ XL Tonometer for animals (Mediconsult AG, Roggwil, Switzerland) at 19 time points. An ophthalmologist scored with a slit lamp, in a blind manner: i) the presence of fibrin and/or synechiae (score = 1 if presence of fibrin and/or synechiae; score = 0 if absence of both fibrin and synechiae), ii) anterior chamber aqueous cell density on a semi-quantitative scale from 0 to 3 and iii) anterior chamber flare grade from 0 to 3. The mean of these 3 parameters was called microscopical score because its assessment needed a slit lamp. Mydriasis was induced if necessary.

Aqueous humor (AH) was aspirated under systemic and local anaesthesia. Quadruplicate samples of AH were dried on slides and stained by trypan blue for inflammatory cell counting. Remaining AHs were spun and acellular AH frozen. Protein concentration was measured in duplicate or quadruplicate samples with the Coomassie® Plus Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) (Bradford, 1976). Results were given in means ± SEM. Data were statistically compared with the Mann-Whitney and Wilcoxon-Kruskall Wallis test.

At day 79 after cataract surgery, final ocular and systemic samples were collected prior to i.v. injection of pentobarbital. Eyes were removed and dissected into conjunctiva, cornea, iris, lens or IOL- DDS, vitreous humor, retina, choroid and sclera. All tissues were stored in protected vial at -80^{0}C for subsequent evaluation of active agent.

2.9. CsA quantification in explanted DDS and in ocular tissues / fluids

Explanted DDS were dissolved in 1.5 ml of Water/Acetonitrile 20:80 and analysed by UHPLC-UV for CsA quantification.

Prior to analyses, tissues and fluids were thawed, weighted, manually grinded, introduced in a vial containing 200 µl of methanol along with 0.1 µg/ml d_{12}CsA and stirred overnight. The day after, samples were centrifuged and supernatants were collected and analysed by UHPLC-MS/MS for CsA quantification [23]. Analyses were performed on a UHPLC system coupled with a triple quadrupole mass spectrometer (Waters Acquity ultra performance liquid chromatograph and TQD, Milford, MA, USA). The sample manager temperature was set at 4ºC. For each sample, three injections of 2 µl were performed in the Waters Acquity BEH C18 column (50 x 2.1 mm I.D., 1.7 µm) heated at 60°C. The mobile phase consisted on a mixture of water with 0.02% v/v acetic acid (A) and methanol with 0.02% v/v acetic acid (B). A linear gradient from 60 to 100% B was applied for 3 min at a flow rate of 600 µl/min. The ratio between signals of CsA and d_{12}CsA (used as an internal standard) was the basis for quantification. The pseudomolecular parent ion and fragment corresponding to CsA have a mass to charge ratio of 1224.7 and 1112.4 respectively; concerning d_{12}CsA the parent ion has a mass to charge ratio of 1236.7 and its daughter ion of 1124.4. Data acquisition, data handling and instrument control were performed by Masslynx v4.1 Software (Waters, Milford, MA, USA).

3. RESULTS

3.1. DDS characterization

The manufacturing process for TA loaded DDS was based on a monolayer system. While for CsA, a bilayer system was necessary to ensure the appropriate drug loading of the DDS (because of the limited CsA solubility in PLGA solvents) together with reproducible thickness, diameter and weight. The internal structure of the bilayer DDS was studied based on SEM in order to verify that the delivery system presents a homogeneous internal structure. As shown in Figure 1B, SEM pictures showed a homogeneous structure and porosity inside the two layers of the DDS. The junction area between the different layers was also visible.

Thickness, diameter and weight of DDS were measured. Table 1 shows the average values +/- standard deviation of each parameter, along with the drug loading for each type of DDS. Results show a good reproducibility.

The residual solvent was determined to be below 2%.

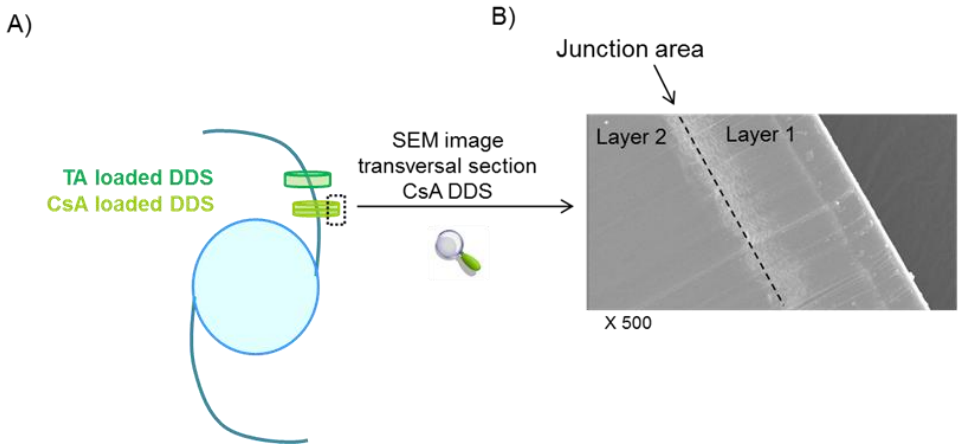


Figure 1: A) Schematic of the IOL-DDS system inserted into the rabbits eye during cataract surgery. The IOL-DDS consists in an IOL associated with a monolayer triamcinolone acetonide DDS and a bilayer Cyclosporine A DDS. B) Scanning electron microscopy image of a transversal section of a bilayer drug delivery system loaded with Cyclosporine A.

Table 1: Physical characterization and drug loading of the drug delivery systems.

Active molecule	Thickness (mm)	Diameter (mm)	Weight (mg)	Drug loading (mg)
Cyclosporine A	0.65 +/- 0.05	2.05 +/- 0.04	2.43 +/- 0.15	0.78 +/- 0.02
Triamcinolone acetonide	0.61 +/- 0.04	2.1 +/- 0.03	2.69 +/- 0.12	0.99 +/- 0.04

3.2. Protein concentration into the aqueous humor (AH)

AH was aspirated at 9 time points and protein concentration was evaluated to assess the severity of ocular inflammation (Figure 2). Each intravitreal injection of *M. tuberculosis* H37RA provoked an increase in protein concentration in AH as shown in Figure 3. It can be noted that ocular inflammation tended to diminish with time in a more pronounced manner after the 1st challenge than after the other two challenges. In addition, the 3rd challenge was found to be the most efficient in inducing uveitis, since > 25 mg protein/ml AH was measured. The comparison between the three experimental groups showed that medicated DDS significantly decreased protein concentration in AH, highlighting a decrease in ocular inflammation. The inhibition of uveitis by medicated DDS was found to be statistically significant for 8 out of the 9 time points. The comparison between the control and placebo groups showed significant difference only for two time points (8 and 72 days). At 8 days, control eyes did not show any inflammation (0.68 mg protein /ml AH), whereas eyes implanted with IOL-placebo DDS presented 6.7 mg/ml protein.

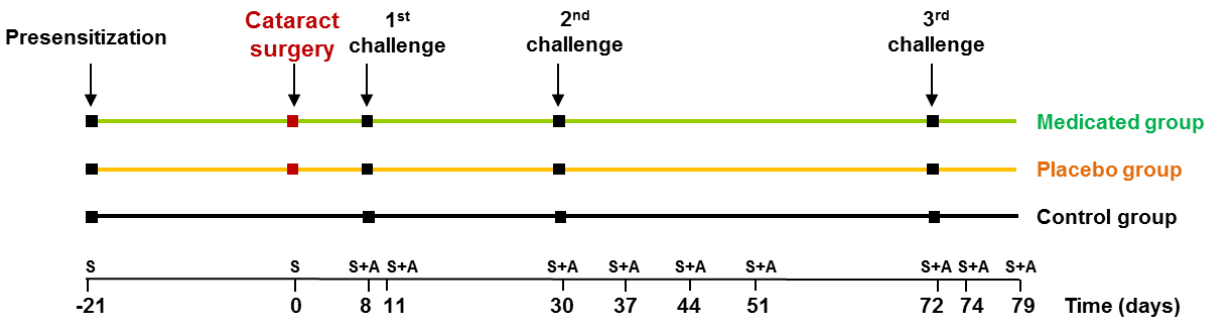


Figure 2: Schematic of the study design. Rabbits were presensitized with a s.c. injection of *Mycobacterium tuberculosis* H37RA prior to cataract surgery. The surgery was performed only in the placebo group and the medicated group, leaving the control group without any surgical intervention. Uveitis was induced and maintained in all rabbits by intravitreal injections of *M. tuberculosis* H37RA at days 8, 30 and 72 after the day of surgery. Clinical and microscopical scoring (S) as well as aqueous humor aspiration (A) were performed at indicated time points.

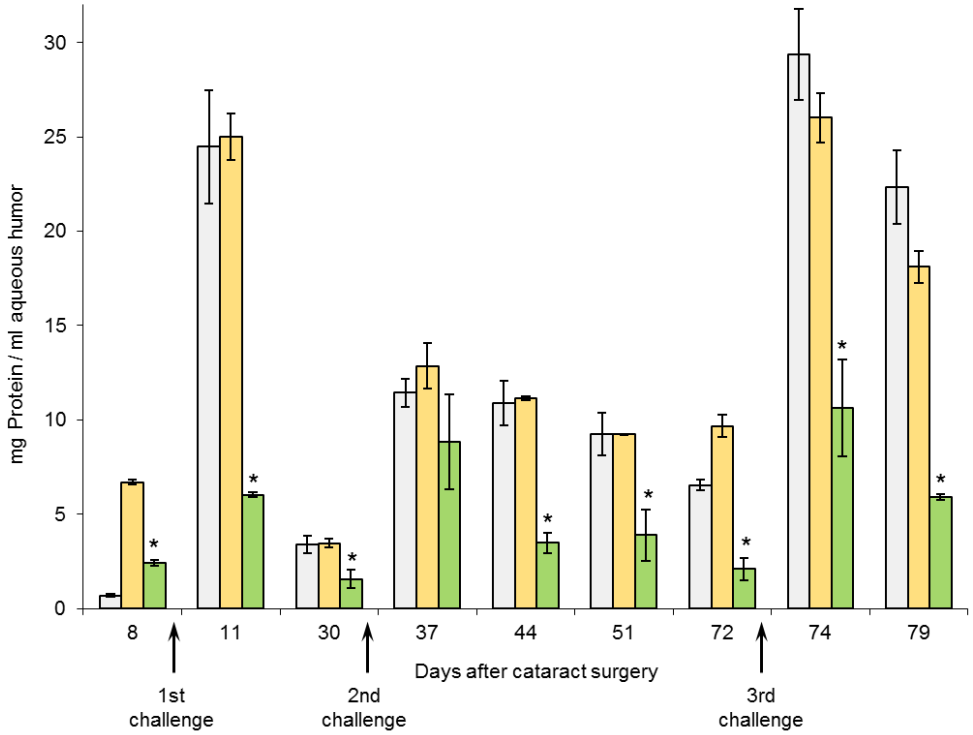


Figure 3: Evolution of the protein concentration in mg/ml in the aqueous humor of rabbits presenting uveitis vs time (in days). Control group did not have surgery, placebo and medicated groups had surgery and received whether a IOL-unloaded DDS or a IOL-medicated DDS, respectively. Uveitis was maintained through intravitreal injections of *M. tuberculosis* designated as "challenges" in the figure.

* indicates a statistically significant difference ($P<0.05$) according to Wilcoxon statistical test.

3.3. Number of inflammatory cells in AH

The number of inflammatory cells in the AH was an essential parameter for evaluating the degree of ocular inflammation. As expected, intravitreal injections of *M. tuberculosis* H37RA challenged invasion of AH by inflammatory cells, as illustrated by Figure 4. Experimental results showed that the presence of medicated DDS into the eye significantly inhibited cell invasion at every studied time point. The most pronounced inhibitory effect was visible after the 3rd challenge where the cell invasion remained below 22 ± 3 cells/µl for the medicated group, while it reached a maximum for both control and placebo groups (1342 ± 29 cells/µl and 1210 ± 136 cells/µl, respectively).

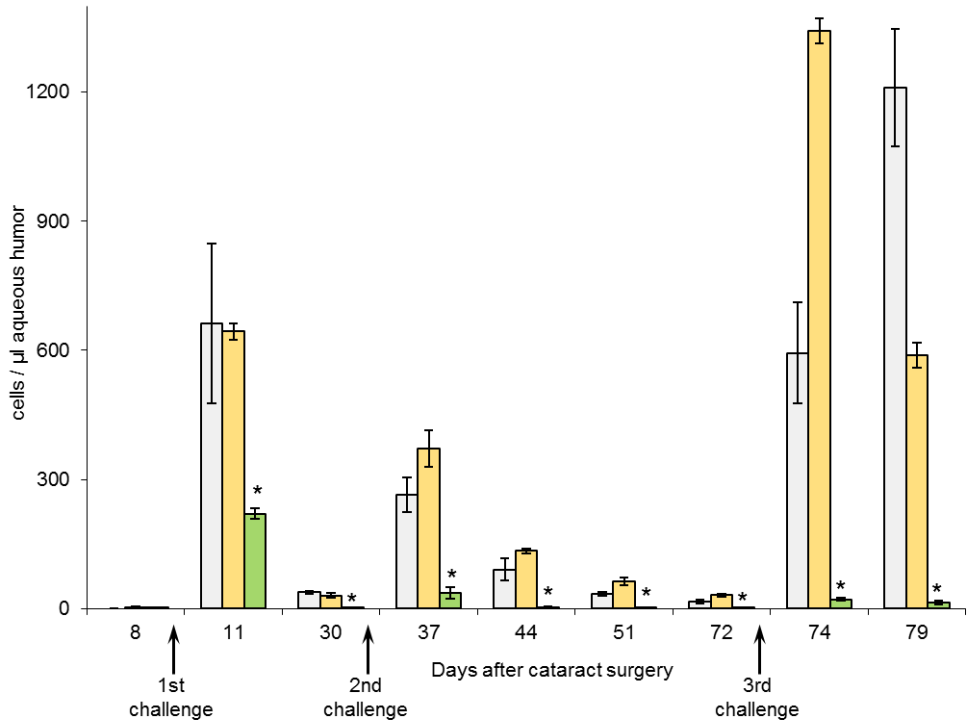


Figure 4: Evolution of the number of inflammatory cells counted in 1 ml of aqueous humor of rabbits presenting uveitis vs time (in days). Control group did not have surgery, placebo and medicated groups had surgery and received whether a IOL-unloaded DDS or a IOL-medicated DDS, respectively. Uveitis was maintained through intravitreal injections of *M. tuberculosis* designated as "challenges" in the figure.

* indicates a statistically significant difference ($P<0.05$) according to Wilcoxon statistical test.

3.4. Microscopical score (slit lamp)

Medicated DDS inserted with the IOL reduced the microscopical score assessed by slit lamp, as compared to unloaded DDS associated to IOL (Figure 5). Reduction of the microscopical score was statistically significant at 30, 49, 51 and 79 days after cataract surgery.

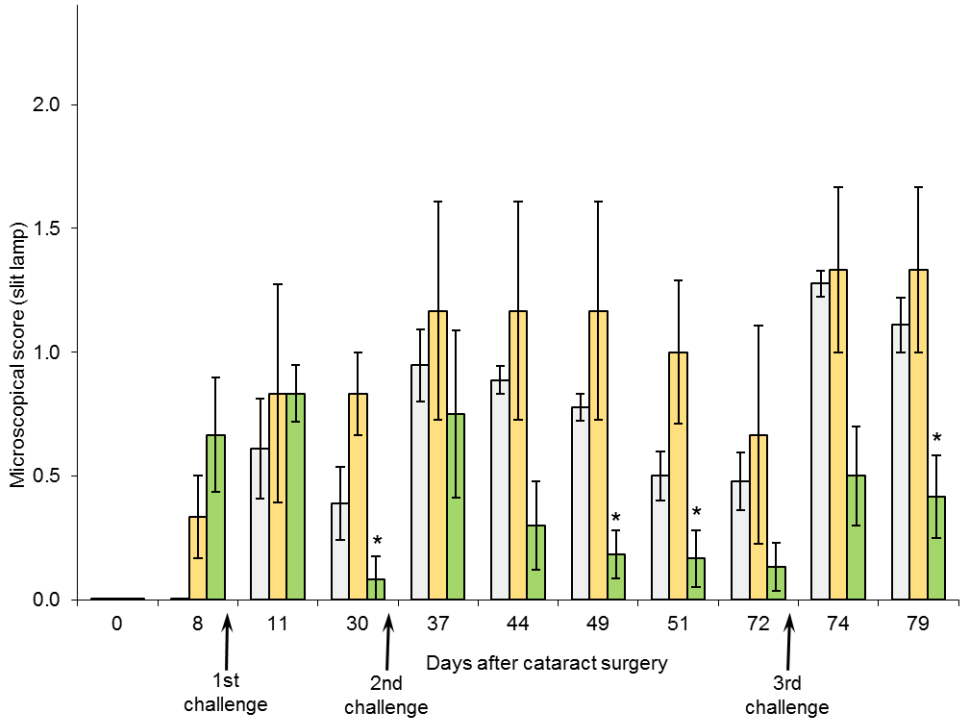


Figure 5: Evolution of the microscopical score estimated by slit lamp examination of rabbits presenting uveitis vs time (in days). Control group did not have surgery, placebo and medicated groups had surgery and received whether a IOL-unloaded DDS or a IOL-medicated DDS, respectively. Uveitis was maintained through intravitreal injections of *M. tuberculosis* designated as "challenges" in the figure.

* indicates a statistically significant difference ($P<0.05$) according to Wilcoxon statistical test.

3.5. Intraocular pressure (IOP)

Mean values of IOP varied between 7 and 17 mm Hg during the time of the experiments (Figure 6), with no significant difference between the control and placebo groups. Intraocular pressure of the medicated eyes showed a tendency to increase after the 1st antigen challenge (reaching a maximum of 21.5 ± 4.5 mm Hg at day 30 after cataract surgery). However, IOP in these medicated eyes showed no significant difference compared to IOP in control eyes: P values were 0.1386 at 14 days, 0.2361 at 21 days, 0.2361 at 30 days, 0.0756 at 31 days and 0.0833 at 74 days after cataract surgery.

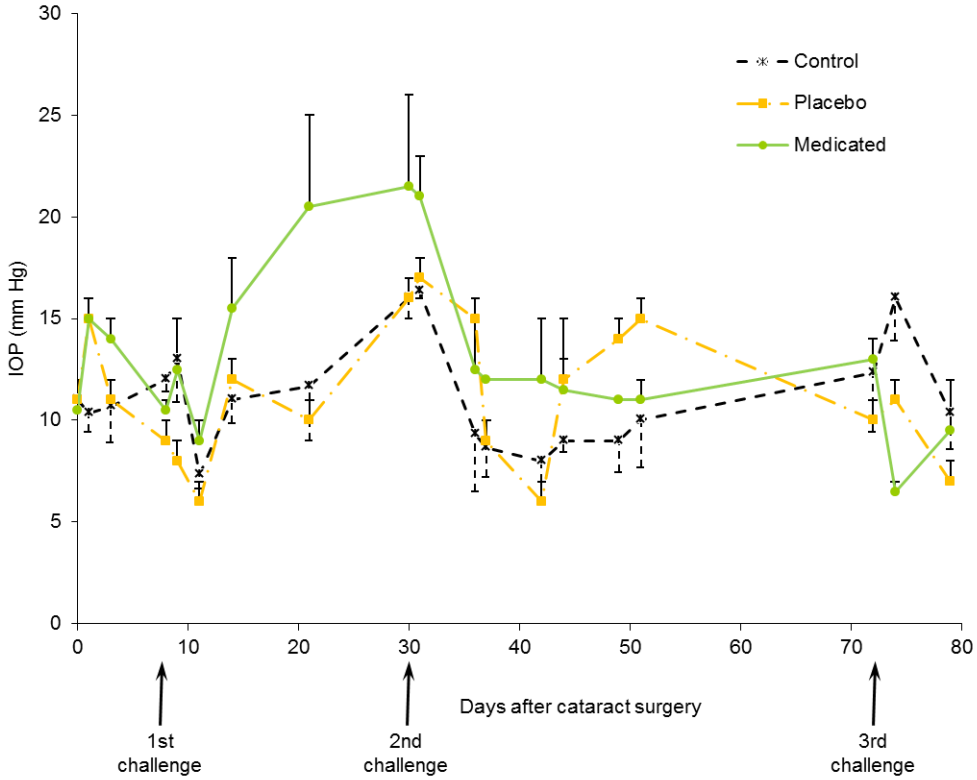


Figure 6: Intraocular pressure (IOP) of rabbits presenting experimental uveitis vs. time (in days). Control group (– –) did not have surgery; placebo (— · —) and medicated groups (—) had surgery and received either a IOL-unloaded DDS or a IOL-medicated DDS, respectively. Experimental uveitis was maintained through intravitreal injections (challenges) of *M. tuberculosis* H37RA. The IOP values in the medicated group were not statiscally different from IOP values in the control group.

3.6. Ocular distribution of CsA

At day 79 after cataract surgery, the amount of CsA was evaluated and expressed in µg of CsA per g of ocular tissue or fluid; 22.72 µg/g for the cornea, 4.64 µg/g for the conjunctiva, 0.77 µg/g for the sclera, 0.32 µg/g for the AH, 4.71 µg/g for the iris, 0.18 µg/g for the vitreous humour, 0.89 µg/g for the retina and 0.20 µg/g for the choroid, as illustrated in Figure 7. Systemic CsA levels were found to be 0.002 µg/ml.

At day 79 after cataract surgery, TA was below the limit of quantification in the collected ocular tissues and fluids.

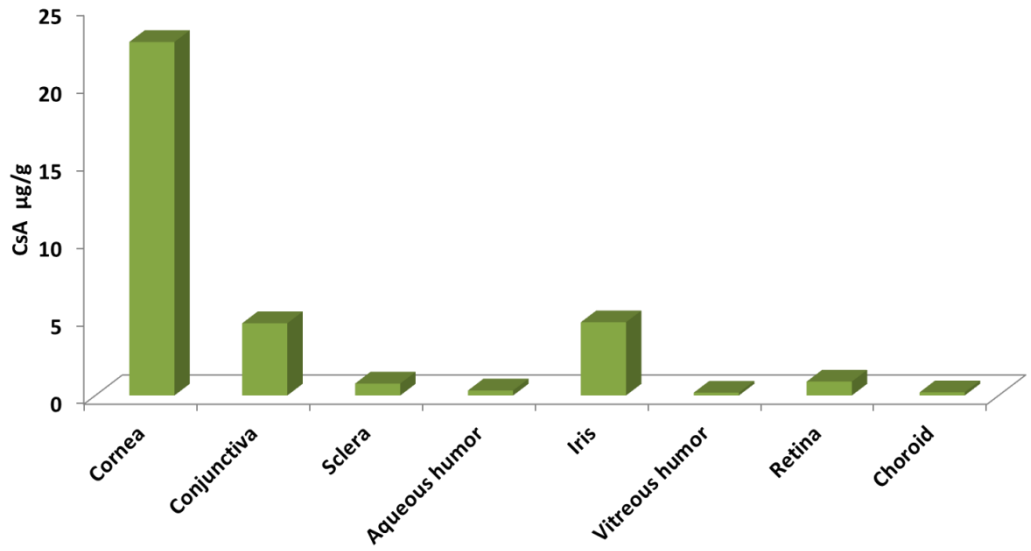


Figure 7: Ocular levels of Cyclosporine A at day 79 after cataract surgery in µg/g of tissue.

3.7. Remaining active molecule in DDS

At day 79 after cataract surgery, the medicated DDS still contained 64% and 73% of the initial dose of CsA and TA, respectively. These results highlighted that elevated amounts of active molecules remained available to be released into the eye even after 79 days.

4. DISCUSSION

Uveitis and cataract are often connected; one condition could lead directly or indirectly to the other and *vice versa*. Indeed, it has been reported that cataract represents a common complication in uveitic patients that occurs in up to 50% of the anterior or intermediate uveitic cases [24]. On the other hand, cataract surgery presents an obvious risk of uveitic complications. Thus, the ocular inflammatory condition needs serious consideration before and after cataract surgery. The recovery from surgery partially lies on the ability to manage uveitis during the postoperative period.

In the light of this information, the selected animal model presenting uveitis at the time of surgery seems to be particularly adapted for the efficiency evaluation, reflecting a commonplace situation for ophthalmologists.

The experimental results demonstrated that TA and CsA, delivered together into the eye, were able to decrease protein concentration and the number of inflammatory cells in the AH as well as the slit lamp microscopical score. Thus, it is clear that the presented IOL-DDS efficiently inhibit experimentally induced uveitis. The association of an immunosuppressive agent and a steroid agent had already been reported in ophthalmology to solve particularly complicated clinical cases [19-21]. Particularly interesting is the use of systemic CsA and a steroid agent for the treatment of sight threatening uveitis in Behçet's disease patients resulting in stabilization or even an improvement of visual acuity in 75.7% of the cases [21]. Nevertheless, the main drawback of this therapy is that the active agents have to be systemically administered and require the management of the systemic side effects. The IOL-DDS technology allows the administration of both therapeutic agents locally, considerably reducing systemic side effects, which is an important advantage of this strategy. Thus, the combination of both active molecules for the first time within the same system allows the treatment of severe uveitis with minimal systemic complications.

Ocular side effects remain limited by the fact that the TA amount is kept as low as possible to ensure the therapeutic effect while minimizing the rise of the IOP, which is one of the major concerns after TA administration [25-27]. In our study, rabbit IOP was fluctuating within the normal range. Rabbits implanted with IOL-medicated DDS showed values which were not significantly different from those of the control rabbits. Moreover, such values were comparable to previously measured IOP [15].

Thus, the local administration of low doses of TA and CsA enlarges the application scope of the IOL-DDS by combining in a single device the first and second-line treatment options for the management of ocular inflammation, while minimizing potential side effects [28]. Consequently, this strategy opens new possibilities for a more comprehensive approach to treating postoperative inflammatory complications and inflammatory concomitant pathologies after cataract surgery.

In addition, the implantation of IOL-DDS during cataract surgery would alleviate post-operative treatments and drastically increase patients' compliance. Former studies demonstrated that the relapse of pre-existing uveitis is minimized by perioperative treatments [27, 29]. One potential application would be the use of IOL-DDS in patients with Behçet's disease undergoing cataract surgery. These patients present a particularly poor visual prognosis [30] and require minimal surgical interventions to avoid potential complications.

Moreover, the elevated amount of active compound remaining in the DDS after 79 days of implantation strongly suggests that the therapeutic effect could be prolonged for more than 3 months. These findings open new possibilities in long-term treatment of inflammatory conditions after cataract surgery.

The *in vivo* results, regarding CsA ocular levels, showed that IOL-medicated DDS loaded with less than 1 mg of CsA was capable of delivering clinically relevant amounts of CsA [31] in the anterior and posterior segments of the eye suggesting that the system could prevent inflammation in the entire ocular structure for at least three months. Furthermore, systemic CsA levels measured in our rabbits were orders of magnitude below the active concentration, suggesting that no systemic side effects such as malaise, nephrotoxicity and hypertension could be expected [6, 32].

The IOL-DDS technology presents various advantages compared to other DDS. First, it delivers the active compounds in an efficient manner into the eye. Compared to another DDS system loaded with 0.5 mg of CsA freely inserted in the inferior angle of the anterior chamber for 12 weeks [15], the IOL-DDS achieves approximately 10 times higher CsA concentrations in the iris, conjunctiva, AH and vitreous humor. Secondly, its biodegradability avoids additional procedures to remove the implant, which is the case for other implants anchored to the sclera by a suture [13, 33]. Third, its biocompatibility ensures no ocular additional damage or inflammation linked to the DDS. In the present study, the PLGA DDS were demonstrated to cause no additional inflammation when comparing the control group (no DDS) with the

placebo group (unloaded DDS). Indeed, the inflammation observed at day 8 after cataract surgery in the placebo group corresponds to the expected post-surgery inflammation [29]. Besides, previous studies have demonstrated good tolerance of PLGA [14, 16]. Finally, the implantation of the DDS is performed during an otherwise necessary surgery and the DDS is immobilized within the capsule with the IOL, being an advantage compared to freely inserted DDS [10, 34, 35].

The novel manufacturing procedure allowed obtaining reproducible DDS regarding their thickness, diameter and weight, as well as their drug loading. In addition, such a manufacturing process was found to be particularly versatile. It allows the production of a variety of DDS with different layers and thicknesses in a reproducible and simple manner. Interestingly, the adaptation of the technique for the inclusion of new active molecules or polymers requires only limited effort and time, which is particularly promising, as discussed by Pillai *et al.* in their review on polymers [36].

Standard commercially available foldable IOLs were used in this experimental study and were implanted through a 3.2 mm clear corneal incision. In both placebo and medicated IOLs, the DDS was adjusted to one of the haptics without detrimental effect on the ease of IOL insertion nor need for extension of the corneal wound. Sutureless surgery was performed in all but one case where a 10/0 Dafilon suture was placed to seal off the wound. No major technical adaptations or manufacturing improvements would be required for expanding our model for use in human subjects. It is nevertheless evident that a well-designed phase I safety study would be a pre-requisite to any further efficacy assessment on human subjects.

The presented IOL-DDS displayed an interesting versatility and could be used for the delivery of other active molecules such as steroids, antivirals, antibiotics, antimetabolites or biological agents [6, 27, 37, 38]. A similar device based on norfloxacin-loaded hydrogel attached onto IOL haptics was developed to counteract postoperative intraocular infections [39]. Other pathologies such as cystoid macular oedema, various retinopathies or age-related macular degeneration could also benefit from this technology because such a strategy minimizes their relapse after cataract surgery, as previously demonstrated [29].

5. CONCLUSIONS

Through the present study, the new type of biodegradable DDS loaded with TA and CsA were demonstrated to efficiently reduce the ocular inflammation signs for more than 3 months. Particularly interesting features are: i) the novel manufacturing process, ii) the concept of implanting a DDS during cataract surgery to ensure the postoperative pharmacological treatment and iii) the local administration of a steroid and an immunosuppressant together for the coverage of a larger variety of inflammatory processes.

The possibility of combining cataract surgery and postoperative pharmacological treatment in a single procedure represents an advantageous possibility brought by the IOL-DDS technology. This "all in one" system is an efficient option for the management of uveitis in patients needing cataract surgery, ensuring paramount factors for patients' recovery such as proper therapeutic treatment and compliance for long periods of time. It is worth mentioning that besides improving patients' quality of life, IOL-DDS could considerably reduce health costs, which is not without relevance.

ACKNOWLEDGEMENTS

Our warm thanks go to Catherine Guex-Crosier and Jean Vaudaux for their help in surgery, and to the animal keeper Nathalie Terrier. This work has been supported by the Swiss National Foundation (# 3200B0-117951). The authors would also like to thank Dr. Beatrice Kaufmann for her help with analytical questions.

REFERENCES

[1] J.C. Erie, K.H. Baratz, D.O. Hodge, C.D. Schleck, J.P. Burke, Incidence of cataract surgery from 1980 through 2004: 25-year population-based study, J. Cataract Refract. Surg., 33 (2007) 1273-1277.

[2] S.-E. Hankinson, Epidemiology of Age-Related Cataract, in: D.M. Albert, F.A. Jakobiec, D.T. Azar, E.S. Gragoudas (Eds.) Principles and Practice of Ophthalmology, Vol 1, W. B. Saunders Company, Philadelphia, 2000, pp. 511-521.

[3] A.P. Adamis, D.T. Shima, The role of vascular endothelial growth factor in ocular health and disease, Retina, 25 (2005) 111-118.

[4] M. Nagpal, K. Nagpal, P.N. Nagpal, Postcataract cystoid macular edema, Ophthalmol. Clin. North Am., 14 (2001) 651-659.

[5] J.N. Simone, M.M. Whitacre, Effects of anti-inflammatory drugs following cataract extraction, Curr. Opin. Ophthalmol., 12 (2001) 63-67.

[6] M.T. Cahill, G.J. Jaffe, Intraocular sustained-release drug delivery in uveitis, in: G.J. Jaffe, P. Ashton, P.A. Pearson (Eds.) Intraocular Drug Delivery, Taylor & Francis Group, New York, 2006, pp. 265-278.

[7] K.G. Janoria, S. Gunda, S.H. Boddu, A.K. Mitra, Novel approaches to retinal drug delivery, Expert. Opin. Drug Deliv., 4 (2007) 371-388.

[8] S.S. Siddique, R. Shah, A.M. Suelves, C.S. Foster, Road to remission: a comprehensive review of therapy in uveitis, Expert Opin. Investig. Drugs, 20 (2011) 1497-1515.

[9] Y.E. Choonara, V. Pillay, M.P. Danckwerts, T.R. Carmichael, L.C. du Toit, A review of implantable intravitreal drug delivery technologies for the treatment of posterior segment eye diseases, J. Pharm. Sci., 99 (2010) 2219-2239.

[10] D.T. Tan, S.P. Chee, L. Lim, J. Theng, M. Van Ede, Randomized clinical trial of Surodex steroid drug delivery system for cataract surgery: anterior versus posterior placement of two Surodex in the eye, Ophthalmology, 108 (2001) 2172-2181.

[11] C.R. Ghosn, Y. Li, W.C. Orilla, T. Lin, L. Wheeler, J.A. Burke, M.R. Robinson, S.M. Whitcup, Treatment of experimental anterior and intermediate uveitis by a dexamethasone intravitreal implant, Invest. Ophthalmol. Vis. Sci., 52 (2011) 2917-2923.

[12] C. Lowder, R. Belfort, Jr., S. Lightman, C.S. Foster, M.R. Robinson, R.M. Schiffman, X.Y. Li, H. Cui, S.M. Whitcup, Dexamethasone intravitreal implant for noninfectious intermediate or posterior uveitis, Arch. Ophthalmol., 129 (2011) 545-553.

[13] G.J. Jaffe, C.S. Yang, X.C. Wang, S.W. Cousins, R.P. Gallemore, P. Ashton, Intravitreal sustained-release cyclosporine in the treatment of experimental uveitis, Ophthalmology, 105 (1998) 46-56.

[14] E. Sakurai, M. Nozaki, K. Okabe, N. Kunou, H. Kimura, Y. Ogura, Scleral plug of biodegradable polymers containing tacrolimus (FK506) for experimental uveitis, Invest. Ophthalmol. Vis. Sci., 44 (2003) 4845-4852.

[15] J.T. Theng, S.E. Ti, L. Zhou, K.W. Lam, S.P. Chee, D. Tan, Pharmacokinetic and toxicity study of an intraocular cyclosporine DDS in the anterior segment of rabbit eyes, Invest. Ophthalmol. Vis. Sci., 44 (2003) 4895-4899.

[16] S. Eperon, L. Bossy-Nobs, I.K. Petropoulos, R. Gurny, Y. Guex-Crosier, A biodegradable drug delivery systems for the treatment of post-operative inflammation, Int. J. Pharm., 352 (2008) 240-247.

[17] O. Felt-Baeyens, S. Eperon, P. Mora, Y. Guex-Crosier, S. Sagodira, P. Breton, R. Gurny, Scleral implants - A potential route of administration?, in: Monduzzi, Editore (Eds.) Proceedings of the 5th Int Symp of Ocul Pharmacol and Therapeutics, Monaco, France, 11-14 march 2004, Medimond S.r.l., Pianoro (Bologna), Italy, 2004, pp. 41-46.

[18] O. Felt-Baeyens, S. Eperon, P. Mora, D. Limal, S. Sagodira, P. Breton, B. Simonazzi, L. Bossy-Nobs, Y. Guex-Crosier, R. Gurny, Biodegradable scleral implants as new triamcinolone acetonide delivery systems, Int. J. Pharm., 322 (2006) 6-12.

[19] R.B. Nussenblatt, A.G. Palestine, C.C. Chan, Cyclosporine therapy for uveitis: long-term followup, J. Ocul. Pharmacol., 1 (1985) 369-382.

[20] H.M. Towler, P.H. Whiting, J.V. Forrester, Combination low dose cyclosporin A and steroid therapy in chronic intraocular inflammation, Eye, 4 (Pt 3) (1990) 514-520.

[21] S.M. Whitcup, E.C. Salvo, Jr., R.B. Nussenblatt, Combined cyclosporine and corticosteroid therapy for sight-threatening uveitis in Behcet's disease, Am. J. Ophthalmol., 118 (1994) 39-45.

[22] S. Eperon, K. Balaskas, J.D. Vaudaux, Y. Guex-Crosier, Experimental uveitis can be maintained in rabbits for a period of six weeks after a safe sensitization method, Curr.Eye Res., accepted for publ. (2013).

[23] M. Rodriguez-Aller, B. Kaufmann, D. Guillarme, C. Stella, P. Furrer, S. Rudaz, Z. El, I, F. Valamanesh, T.C. Di, F. Behar-Cohen, J.L. Veuthey, R. Gurny, *In vivo* characterisation of a novel water-soluble Cyclosporine A prodrug for the treatment of dry eye disease, Eur. J. Pharm. Biopharm., 80 (2012) 544-552.

[24] P.L. Hooper, N.A. Rao, R.E. Smith, Cataract extraction in uveitis patients, Surv. Ophthalmol., 35 (1990) 120-144.

[25] T.A. Ciulla, J.D. Walker, D.S. Fong, M.H. Criswell, Corticosteroids in posterior segment disease: an update on new delivery systems and new indications, Curr. Opin. Ophthalmol., 15 (2004) 211-220.

[26] J.B. Jonas, R.F. Degenring, I. Kreissig, I. Akkoyun, B.A. Kamppeter, Intraocular pressure elevation after intravitreal triamcinolone acetonide injection, Ophthalmology, 112 (2005) 593-598.

[27] A. Sallam, S.R. Taylor, S. Lightman, Review and update of intraocular therapy in noninfectious uveitis, Curr. Opin. Ophthalmol., 22 (2011) 517-522.

[28] R.B. Nussenblatt, A.G. Palestine, C.C. Chan, Cyclosporin A therapy in the treatment of intraocular inflammatory disease resistant to systemic corticosteroids and cytotoxic agents, Am. J. Ophthalmol., 96 (1983) 275-282.

[29] P. Taravati, D.L. Lam, T. Leveque, R.N. Van Gelder, Postcataract surgical inflammation, Curr. Opin. Ophthalmol., 23 (2012) 12-18.

[30] M. Takeuchi, T. Iwasaki, T. Kezuka, Y. Usui, Y. Okunuki, J. Sakai, H. Goto, Functional and morphological changes in the eyes of Behcet's patients with uveitis, Acta Ophthalmol., 88 (2010) 257-262.

[31] R.L. Kaswan, Intraocular penetration of topically applied cyclosporine, Transplantation proceedings, 20 (1988) 650-655.

[32] G. Deray, M. Benhmida, H.P. Le, P. Maksud, B. Aupetit, A. Baumelou, C. Jacobs, Renal function and blood pressure in patients receiving long-term, low-dose cyclosporine therapy for idiopathic autoimmune uveitis, Ann. Intern. Med., 117 (1992) 578-583.

[33] B.C. Gilger, E. Malok, T. Stewart, D. Horohov, P. Ashton, T. Smith, G.J. Jaffe, J.B. Allen, Effect of an intravitreal cyclosporine implant on experimental uveitis in horses, Vet. Immunol. Immunopathol., 76 (2000) 239-255.

[34] S.Y. Lee, S.P. Chee, V. Balakrishnan, S. Farzavandi, D.T. Tan, Surodex in paediatric cataract surgery, Br. J. Ophthalmol., 87 (2003) 1424-1426.

[35] A.C. Wadood, A.M. Armbrecht, P.A. Aspinall, B. Dhillon, Safety and efficacy of a dexamethasone anterior segment drug delivery system in patients after phacoemulsification, J. Cataract Refract. Surg., 30 (2004) 761-768.
[36] O. Pillai, R. Panchagnula, Polymers in drug delivery, Curr. Opin. Chem. Biol., 5 (2001) 447-451.
[37] N. Kuno, S. Fujii, Biodegradable intraocular therapies for retinal disorders: progress to date, Drugs Aging, 27 (2010) 117-134.
[38] T. Yasukawa, Y. Ogura, E. Sakurai, Y. Tabata, H. Kimura, Intraocular sustained drug delivery using implantable polymeric devices, Adv. Drug Deliv. Rev., 57 (2005) 2033-2046.
[39] S. Garty, R. Shirakawa, A. Warsen, E.M. Anderson, M.L. Noble, J.D. Bryers, B.D. Ratner, T.T. Shen, Sustained antibiotic release from an intraocular lens-hydrogel assembly for cataract surgery, Invest. Ophthalmol. Vis. Sci., 52 (2011) 6109-6116.

CONCLUSIONS

AND

PERSPECTIVES

Conclusions and Perspectives

This thesis has explored three strategies for the delivery of poorly water soluble cyclosporine A (CsA) and latanoprost to the eye based on: i) a chemical modification, ii) a physical modification and iii) the use of an alternative administration route. CsA and latanoprost are particularly interesting model drugs for two main reasons. First, because they are "practically insoluble" drugs (displaying a water solubility of 0.005 mg/mL and 0.050 mg/mL respectively) whose topical ophthalmic formulations need to be improved in regards to their stability, safety or availability. The second reason is that they are currently being used for the management of ocular conditions as common as the dry eye syndrome, uveitis, conjunctivitis, postoperative ocular inflammation and glaucoma.

The chemical modification approach based on the use of a water soluble prodrug of CsA was demonstrated to be a new and optimized drug delivery system of its own. The water soluble prodrug transformed the "practically insoluble" parent CsA into a "very soluble" CsA prodrug, making it possible to develop transparent and highly concentrated aqueous eyedrops. The *in vitro*, *ex vivo* and *in vivo* experiments revealed that the covalent linking of the prodrug moiety to the CsA molecule had an impact on many more parameters than just solubility. When compared to conventional CsA formulations, the aqueous prodrug solution was demonstrated to have: i) an excellent ocular tolerance, ii) a reduced precorneal elimination, iii) a preferential conjunctival penetration, iv) the ability to create drug deposits in the superficial ocular tissues and v) a lower systemic impact. Therefore, the prodrug strategy not only allowed the formulation of CsA in an aqueous environment, but also allowed to deliver it in an efficient and patient-friendly manner.

The fate of the CsA deposits in the corneal and conjunctival tissues and their potential for prolonged CsA release would deserve further investigation. It would also be interesting to conduct additional studies to further optimize the stability of the formulation.

The physical modification based on the complexation of latanoprost with cyclodextrins (CDs) was demonstrated to efficiently address the issues of solubility, stability and tolerability related to latanoprost eyedrops. The screening of a panel of CDs led to the selection of propylaminoβCD (PAβCD) as it presented the best tradeoff between latanoprost stability and availability. Latanoprost was shown to lodge inside the PAβCD cavity forming a 1:1 complex allowing for solubilization and impeding the cleavage of its ester bond. Interestingly, *in vivo* experiments demonstrated the ability of the PAβCD to prevent the ocular irritation reported with the use of the latanoprost commercial formulation. This sophisticated CD approach was demonstrated to allow latanoprost solubilization and stabilization, while preventing ocular irritation after administration.

The therapeutic effects of the latanoprost-PAβCD formulation would deserve further preclinical investigations using an animal model with an elevated intraocular pressure.

The modified administration approach based on the intraocular implantation of a CsA loaded drug delivery system (DDS) was demonstrated to allow efficient delivery of CsA into the eye, successfully overcoming ocular barriers. The novel DDS was produced with a new multilayer process to obtain a CsA solid dispersion inside a PLGA (poly-lactic-glycolic acid 50:50) matrix. This DDS was associated with an intraocular lens (IOL) and was implanted during cataract surgery. This approach was demonstrated to efficiently manage pre-existing uveitis and postoperative inflammation in animals during three months after the surgical intervention as a result of the prolonged release of CsA inside the eye.

The novel manufacturing process developed for the multilayer DDS could potentially allow the production of DDS with tailored release profiles for a variety of poorly water soluble drugs and should be the subject of further investigations.

The three approaches explored in this thesis were successful in overcoming the challenges with ocular delivery of poorly water soluble drugs. Three new formulations were successfully developed based on the use of a CsA prodrug, the complexation of latanoprost with a CD and the intraocular implantation of CsA loaded DDS. The three developed formulations were demonstrated to improve drug stability, safety and availability, addressing the current challenges of CsA and latanoprost topical ophthalmic formulations. These new formulations represent promising new options for the management of highly prevalent ocular conditions as the dry eye syndrome, uveitis, conjunctivitis, postoperative ocular inflammation or glaucoma. The developed formulations allowed for the successful treatment of the targeted diseased areas while having a low impact on healthy tissues, which is indeed our ultimate goal as formulators.

Summary

Summary

The water solubility of a drug is a key parameter in its formulation and delivery. A drug needs to be in solution to permeate biological barriers and interact with its pharmacologic target. On the other hand, the number of pharmacologically active molecules with low water solubility has been constantly increasing in the last decades due to the surge of new drug discovery strategies, the identification of new therapeutical targets and the demand for high drug potency. This has led to compounds with low solubility and poor properties for easy development as final drug products. Current pharmaceutical technologies present powerful tools to solve poor water solubility issues and include three types of approaches: i) chemical modifications including pH adjustment and prodrug design, ii) physical modifications with alteration of the solid state, use of small particles, cosolvents, surfactants, lipids and cyclodextrins and iii) the use of alternative routes of administration. Besides solubility, drug delivery to the eye presents an additional challenge. The eye has highly efficient physiological barriers (i.e. cornea and conjunctiva) and strong protective mechanisms (eyelid movement, tears enzymatic activity, lachrymation and naso-lachrymal drainage) that need to be overcome for the efficient ocular delivery of a drug. Topical drug delivery is a non-invasive ocular route, but is subject to a rapid drug precorneal loss, a limited retention time and an efficient ocular barrier function. The intraocular administration route can overcome ocular barriers, but can potentially lead to complications such as infections, inflammation or pain.

This thesis has explored three strategies for the delivery of poorly water soluble cyclosporine A (CsA) and latanoprost to the eye based on: i) a chemical modification, ii) a physical modification and iii) the use of an alternative administration route. CsA and latanoprost are particularly interesting model drugs for two main reasons. First, because they are "practically insoluble" drugs (displaying a water solubility of 0.005 mg/mL and 0.050 mg/mL respectively) whose topical ophthalmic formulations need to be improved in regards to their stability, safety or availability. The second reason is that they are currently being used for the management of ocular conditions as common as the dry eye syndrome, uveitis, conjunctivitis, postoperative ocular inflammation and glaucoma.

The chemical modification approach based on the use of a water soluble prodrug of CsA constituted a new and optimized drug delivery system of its own. The water soluble prodrug allowed the conversion of the "practically insoluble" parent CsA into a "very soluble" CsA prodrug, making it possible to develop transparent and highly concentrated aqueous eyedrops. The *in vitro*, *ex vivo* and *in vivo* experiments revealed that the covalent link between the prodrug moiety and the CsA had a strong influence on many more factors than just solubility. When compared to conventional CsA formulations, the prodrug aqueous solution was demonstrated to have: i) an excellent ocular tolerance, ii) a lower precorneal elimination, iii) a preferential conjunctival penetration, iv) the ability to create drug deposits in the superficial ocular tissues and v) a lower systemic impact. The prodrug strategy therefore not only allowed the formulation of CsA in an aqueous environment, but also its delivery in an efficient and patient-friendly manner.

The physical modification based on the complexation of latanoprost with cyclodextrins (CDs) was demonstrated to efficiently address the solubility, stability and tolerability issues related to latanoprost eyedrops. The screening of a panel of CDs allowed for the selection of the propylaminoβCD (PAβCD) since it presented the best tradeoff between latanoprost stability and availability, which was confirmed by further investigations of the latanoprost-PAβCD complex (i.e. complexation constant and structure). Latanoprost was demonstrated to lodge inside the cyclodextrin cavity forming a 1:1 complex, thus allowing its solubilization and impeding the cleavage of its ester bond. Interestingly, *in vivo* experiments demonstrated the ability of the CD to avoid ocular irritation that reportedly occurs with the use of latanoprost commercial formulations. This sophisticated CD approach was demonstrated to allow latanoprost solubilization and stabilization, while preventing ocular irritation after administration.

The modified administration approach based on the intraocular implantation of a CsA loaded drug delivery system (DDS) was demonstrated to allow efficient delivery of CsA into the eye, bypassing the ocular barriers. The novel DDS was produced with a new multilayer process to obtain a CsA solid dispersion inside a PLGA (poly-lactic-glycolic acid 50:50) matrix. This DDS was associated with an intraocular lens (IOL) and implanted during cataract surgery. This product was demonstrated to efficiently manage pre-existing uveitis and postoperative inflammation in animals during three months following the surgical intervention as a result of the prolonged release of CsA from the solid dispersion directly inside the eye.

The three approaches explored in this thesis were successful in overcoming the challenges with ocular delivery of poorly water soluble drugs. Three new formulations were successfully developed based on the use of a CsA prodrug, the complexation of latanoprost with a CD and the intraocular implantation of CsA loaded DDS. The three developed formulations were demonstrated to improve drug stability, safety and availability, addressing the current challenges of CsA and latanoprost topical ophthalmic formulations. These new formulations represent promising new options for the management of highly prevalent ocular conditions as the dry eye syndrome, uveitis, conjunctivitis, postoperative ocular inflammation or glaucoma. The developed formulations allowed for the successful treatment of the targeted diseased areas while having a low impact on healthy tissues, which is indeed our ultimate goal as formulators.

Summary in French

(Résumé)

Résumé

La solubilité dans l'eau des principes actifs est un paramètre clef pour leur formulation et leur libération. En effet, le principe actif doit se trouver en solution pour pouvoir traverser les barrières biologiques et interagir avec sa cible thérapeutique. Cependant, le nombre de molécules pharmacologiquement actives qui présentent une faible solubilité dans l'eau n'a fait qu'augmenter ces dernières décennies. Cela est dû à des facteurs tels que l'apparition de nouvelles stratégies pour la découverte de principes actifs, l'identification de nouvelles cibles thérapeutiques et le désir d'avoir des principes actifs présentant des activités élevées. Ainsi, les nouvelles molécules pharmacologiquement actives sont souvent peu solubles dans l'eau et peu aptes à être transformées en médicaments. La technologie pharmaceutique représente un puissant outil permettant la résolution des problèmes liés aux molécules peu solubles dans l'eau par le biais de trois types de stratégies; i) les modifications chimiques comme l'ajustement du pH ou le développement de prodrogues, ii) les modifications physiques, comme la modification de l'état solide, l'utilisation de petites particules, cosolvents, surfactants, lipides ou cyclodextrines et enfin iii) les modifications de la stratégie d'administration en faisant appel à d'autres voies d'administration.

Mis à part la solubilité, l'administration oculaire représente aussi un défi de taille. L'œil présentant d'efficaces barrières physiologiques (comme la cornée et la conjonctive) ainsi que d'importants mécanismes de protection (comme le clignement, l'activité enzymatique des larmes, la lacrymation ou le drainage nasolacrymal) qui doivent être surmontés pour que le principe actif soit libéré sur son site d'action. L'application topique oculaire est la voie oculaire de prédilection, celle-ci étant non invasive ; cependant elle présente l'inconvénient majeur de la rapide élimination précornéene. D'autre part, l'administration intraoculaire permet au principe actif d'atteindre plus facilement sa cible thérapeutique, mais présente des risques de complications telles que les infections, l'inflammation ou la douleur post intervention. Les investigations liées au développement et la caractérisation de formulations à usage oculaire requièrent des techniques analytiques permettant l'analyse d'un nombre important d'échantillons de volume très réduit en peu de temps, ainsi qu'une bonne résolution, sensibilité et sélectivité. L'UHPC-MS (chromatographie liquide à très haute

pression couplée à une détection de type spectrométrie de masse) répondant à tous ces critères, elle a été la technique de choix pour ces recherches.

Cette thèse explore trois différentes stratégies pour la formulation et l'administration oculaire de principes actifs peu solubles dans l'eau tels que la cyclosporine A (CsA) et le latanoprost en se basant sur trois stratégies différentes. La CsA et le latanoprost étant connus pour leur faible solubilité dans l'eau (0.005 mg/mL pour la CsA et 0.050 mg/mL pour le latanoprost) leurs formulations oculaires à usage topique actuellement disponibles sur le marché souffrent de limitations liées à leur stabilité, toxicité ou biodisponibilité. Cependant elles sont utilisées dans le contexte de maladies oculaires aussi fréquentes que l'œil sec, l'uvéite, la conjonctivite, l'inflammation postopératoire ou le glaucome.

Une partie introductive présente les stratégies pharmaceutiques permettant de résoudre les problèmes liés aux principes actifs peu solubles dans l'eau et présente l'UHPLC-MS comme un outil permettant de faire face aux défis analytiques liés aux investigations basées sur des formulations ophtalmiques.

L'utilisation d'une prodrogue de CsA soluble dans l'eau pour le développement d'une formulation à usage topique ophtalmique est présentée dans une seconde partie. Cette modification chimique de la CsA représente à elle seule une nouvelle formulation permettant une meilleure libération de la CsA au niveau de l'œil pour le traitement de l'œil sec, le rejet de greffes cornéennes, la conjonctivite ou l'uvéite. Au travers de cette stratégie, l'insoluble CsA est transformée en la très soluble prodrogue (dont la solubilité 25000 fois supérieure à celle de la CsA). Cette prodrogue permet ainsi l'obtention de formulations aqueuses concentrées. Les expériences *in vitro, ex vivo* et *in vivo* révèlent que l'ajout covalent du groupement prodrogue à la structure de la CsA a des conséquences bien au-delà de la solubilité. Ainsi, la comparaison des nouvelles formulations à base de prodrogue avec les formulations conventionnelles de CsA ont mis en valeur des propriétés propres à la prodrogue comme: 1) son excellente tolérance oculaire, 2) sa plus faible élimination précornéene, 3) sa pénétration majoritairement conjonctivale, 4) sa capacité à créer des dépôts au niveau de la conjonctive et la cornée ainsi que 5) son plus faible effet systémique comparé aux

formulations conventionnelles à base de CsA actuellement sur le marché. Par conséquent, la stratégie basée sur l'utilisation d'une prodrogue de CsA soluble dans l'eau a non seulement permis la formulation de la CsA dans un environnement aqueux, mais aussi une libération oculaire plus efficace et une administration plus confortable pour le patient.

Une troisième partie présente une modification physique basée sur l'utilisation des cyclodextrines (CDs) pour la formulation du latanoprost pour le traitement du glaucome. Une sélection de CDs a été testée en vue d'identifier celle qui permet une stabilisation du latanoprost face à la dégradation liée au rayonnement solaire et à la température. De plus, la capacité des CDs à libérer le principe actif a aussi été évaluée au travers d'une étude *ex vivo*. Les résultats de stabilité et de disponibilité montrent que la formulation comportant le latanoprost et la propylaminoβCD (PAβCD) serait la plus prometteuse. Ainsi, la formulation latanoprost- PAβCD a été retenue pour des investigations plus poussées sur la formation du complexe (constante de stabilité, stœchiométrie) et sa structure (résonnance magnétique nucléaire). De plus, les études *in vivo* démontrent que la PAβCD est capable d'empêcher l'irritation oculaire observée après l'application des formulations commerciales contenant du latanoprost. L'utilisation de CDs s'est avérée efficace pour la solubilisation et stabilisation du latanoprost tant *in vitro* que *in vivo*, résolvant ainsi les problèmes liés à son application oculaire.

Une troisième partie présente une modification de la stratégie d'administration, avec l'administration intraoculaire de CsA grâce à des implants à base de PLGA (acide poly-lactique-glycolique 50 :50). Un nouveau processus de fabrication a d'abord été mis en place pour l'obtention d'implants multicouche avec une grande reproductibilité. Ces implants ont été associés à des lentilles intraoculaires (IOL) remplaçant le cristallin après une opération de cataracte, se servant de cet acte chirurgical pour leur implantation. Les études *in vivo* ont permis d'évaluer l'efficacité du système IOL-DDS (implant intraoculaire associé au IOL) sur un modèle animal présentant une inflammation oculaire concomitante au moment de l'intervention chirurgicale, une telle situation se présentant fréquemment en clinique. Dans ce contexte, les IOL-DDS ont démontré leur efficacité pour le traitement de l'uvéite préexistante, ainsi que de l'inflammation postopératoire grâce à la libération prolongée de la CsA directement dans l'organe cible.

Les trois approches explorées au travers de cette thèse ont permis de faire face aux défis liés à l'administration oculaire de principes actifs peu solubles dans l'eau. Trois nouvelles formulations ont ainsi été développées avec succès, basées sur : l'utilisation d'une prodrogue de CsA, la complexation du latanoprost avec une CD et l'administration intraoculaire d'implants à base de CsA. Ces trois nouvelles formulations ont été capables de résoudre les problèmes de stabilité, toxicité et biodisponibilité dont souffrent les formulations de CsA et latanoprost pour application topique oculaire qui sont actuellement sur le marché. Ces nouvelles formulations se sont avérées particulièrement prometteuses pour le traitement de maladies aussi fréquentes que l'œil sec, l'uvéite, la conjonctivite, l'inflammation postopératoire ou le glaucome. Les formulations développées ont permis de traiter efficacement et de façon ciblée les zones pathologiques tout en ayant un impact minimal sur les tissus sains ; ce qui représente notre but ultime en tant que formulateurs.

Abbreviations

Abbreviations

5-FU	5-Fluoro-Uracil
API	Active Pharmaceutical Ingredients
AUC	Area under the curve
Bid	*bis in die* or two times per day
CD	Cyclodextrin
CLSO	Confocal laser scanning ophthalmoscope
Cmax	Maximal concentration
CMC	Critical micellar concentration
COSY	Correlation spectroscopy
CS	Calibration standard
CsA	Cyclosporine A
CyA	Cyclosporine A
$d_4$latanoprost	Four times deuterated latanoprost
$d_4$latanoprost acid	Four times deuterated latanoprost acid
d_{12}CsA	Twelve times deuterated cyclosporine A
DDS	Drug delivery system
DED	Dry eye disease
deP-prodrug	Dephosphorylated cyclosporine A prodrug
DMA	Dimethylacetamide
DMSO	Dimethylsolfoxide
DMβCD	Dimetyl-β-cyclodextrin
DSC	Differencial Scanning Calorimetry
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FTIR	Fourrier transformed infra-red spectroscopy
HPLC	High pressure liquid chromatography
HPMA	Hydroxypropylmetacrylamide
HPβCD	Hydroxypropyl-β-cyclodextrin
HPγCD	Hydroxypropyl-γ-cyclodextrin

HSQC	Heteronuclear single-quantum coherence
HSβCD	Highly sulphated-β-cyclodextrin
i.m.	Intramuscular
i.v.	Intravenous
IOL-DDS	Intraocular lens associated with a drug delivery system
IOP	Intraocular pressure
IS	Internal standard
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NCE	New chemical entity
NMeGlu	N-methyl-glucamine
NMP	N-methyl-2-pyrrolidone
NMR	Nuclear magnetic resonnance
o/w	Oil-in-water
OPPH 088	Cyclosporine A prodrug
PAβCD	6-monodeoxy-6-N-mono(3-hydroxy)propylamino-β-cyclodextrin
PBS	Phosphate buffer solution
PEG	Polyethylene glycol
PG	Propylene glycol
$PGF_{2\alpha}$	Prostaglandin F 2α
pKa	Ionization constant
PLGA	Poly(D,L-lactide-co-glycolide)
pSer-Sar-CsA	Phospho-serine-sarcosine-CsA
ROESY	Rotating frame nuclear overhauser effect spectroscopy
RT	Room temperature
s.c.	Subcutaneous
SEM	Scanning electron microscopy.
SRM	Single reaction monitoring
STT	Schirmer tear test
TA	Triamcinolone acetonide
UHPLC	Ultra-high pressure liquid chromatography

UHPLC-MS/MS	Ultra-high pressure liquid chromatography coupled with a tandem mass spectrometric detection
w/o	Water-in-oil