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TESIS DOCTORAL

"Design and development of novel cyclosporine A lipid nanoparticle formulations appropriate for oral delivery"

Trabajo presentado por Melissa Guada Ramírez para obtener el Grado de Doctor

Fdo. Melissa Guada Ramírez
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A mi familia

–El tiempo de Dios es perfecto–

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ABBREVIATIONS

ASCs	adipose-derived stem cells
AUC	area under the concentration-time curve
BUN	blood urea nitrogen
Caco-2	human colon adenocarcinoma cell line
Capmul MCM	medium chain mono- and diglycerides
C_{max}	maximum blood concentration
Con A	concanavalin A
CREA	creatinine
CsA	cyclosporine A
Cys-PEG-SA	thiolated polyethylene glycol monostearate
DCM	dichloromethane
DCs	dendritic cells
DLS	dynamic light scattering
DMAB	N,N-didodecyl-N,N-dimethylammonium
DMSO	dimethyl sulfoxide
DSC	differential scanning calorimetry
DSS	dextran sodium sulfate
EE	entrapment efficiency
EL14	carboxylated multi-block copolymer of lactic acid and ethylene glycol
FBS	heat-inactivated fetal bovine serum
F_{rel}	relative oral bioavailability
GCPQ	quaternary ammonium palmitoyl glycol chitosan
GMO	glyceryl monooleate
GRAS	Generally Recognized as Safe
HCV	hepatitis C virus
hexPLA	hexyl-substituted poly(lactides)
HPMC K100M	hydroxypropylmethylcellulose
IBD	inflammatory bowel disease
IC50	50% inhibitory concentration
Lec	L-α-phosphatidylcholine, phosphatidilcholine
Lipo 320	trilaurin
LN	lipid nanoparticles
log P	partition coefficient value

LPS	lipopolyssacharide
LTP	liver-targeting peptide
MP	microparticles
MPO	myeloperoxidase
mPEG	methoxypolyethyleneglycol
MRT	mean residence time
MS	microspheres
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFAT	nuclear factor of activated T-cells
NLC	nanostructured lipid carriers
NMP	N-methylpyrrolidone
NP	nanoparticles
P_{app}	apparent permeability coefficient
PCL	poly-ε-caprolactone
PDI	polydispersity index
PEG	polyethylene glycol
PEG-SA	polyethylene glycol monostearate
PEO N80	polyethylene oxide with a molecular weight of 400 kDa
PL	Pluronic® F127
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic) acid
PVA	polyvinyl alcohol
PVP K30	polyvinylpyrrolidone K30
RT	room temperature
SDC	sodium deoxycholate
SLM	solid lipid microparticles
SLN	solid lipid nanoparticles
SNEDDS	self-nanoemulsifying drug delivery systems
SPC	soybean phosphatidylcholine
TBI	traumatic brain injury
TC	taurocholic acid sodium salt hydrate, taurocholate
TEER	trans-epithelial electrical resistance
TEM	transmission electron microscopy
T_{max}	time to reach maximum blood concentration
TNF	tumor necrosis factor

TPGS	polyethylene glycol 1000 succinate
Tw	Tween® 80
UC	ulcerative colitis
UHPLC–MS/MS	ultra-high-performance liquid chromatography tandem mass spectrometry
w/v	weight/volume
w/w	weight/weight
wt	weight
XPS	X-ray photoelectron spectroscopy
XRD	X-ray powder diffraction

INTRODUCTION

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REFORMULATING CYCLOSPORINE A (CsA): MORE THAN JUST A LIFE CYCLE MANAGEMENT STRATEGY

Melissa Guada[a,b†], Ana Beloqui[c†], M. N. V. Ravi Kumar[d], Veronique Preat[c], Maria del Carmen Dios-Viéitez[a,b], Maria J. Blanco-Prieto[a,b*]

[a] *Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Navarra, C/Irunlarrea 1, E-31008 Pamplona, Spain.*

[b] *Instituto de Investigación Sanitaria de Navarra, IdiSNA, C/Irunlarrea 3, E-31008 Pamplona, Spain.*

[c] *Université catholique de Louvain, Louvain Drug Research Institute, Advanced Drug Delivery and Biomaterials, Brussels, Belgium.*

[d] *Department of Pharmaceutical Sciences, Texas A&M Health Science Center, College Station, Texas 77845, USA*

† Both authors contributed equally to this work.

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***Corresponding author:**

Maria J. Blanco-Prieto, PhD

Department of Pharmacy and Pharmaceutical Technology

School of Pharmacy, University of Navarra, C/Irunlarrea 1, E-31008 Pamplona, Spain

Office phone: + 34 948 425 600 ext. 6519

Fax: + 34 948 425 649

e-mail: mjblanco@unav.es

ABSTRACT

Cyclosporine A (CsA) is a well-known immunosuppressive agent that gained considerable importance in transplant medicine in the late 1970s due to its selective and reversible inhibition of T-lymphocytes. While CsA has been widely used to prevent graft rejection in patients undergoing organ transplant it was also used to treat several systemic and local autoimmune disorders. Currently, the neuro- and cardio-protective effects of CsA (CiCloMulsion®; NeuroSTAT®) are being tested in phase II and III trials respectively and NeuroSTAT® received orphan drug status from US FDA and Europe in 2010. The reformulation strategies focused on developing Cremophor® EL free formulations and address variable bioavailability and toxicity issues of CsA. This review is an attempt to highlight the progress made so far and the room available for further improvements to realize the maximum benefits of CsA.

Keywords: cyclosporine A, drug delivery system, immunosuppressant, microparticle, nanoparticle

1. INTRODUCTION

Cyclosporine A (CsA) is a well-known immunosuppressive agent that has played a very important role in transplant medicine since the late 1970s. At that time, the fact that it was found to produce selective and reversible inhibition of T-lymphocytes while causing low cytotoxicity won worldwide recognition of CsA as a promising agent in immune therapy. This compound was first isolated from the fungal extract of *Tolypocladium inflatum* in 1973 but its immunosuppressive activity was discovered later by Borel in 1976. After promising outcomes regarding graft survival after renal transplantation, CsA obtained the US FDA's clinical approval in 1983 for use in prevention of allograft rejection in transplantation. In 1987, the immunosuppressant was registered for the treatment of several autoimmune disorders, and it was in 2003 that the agency approved its use for dry eye disease [1]. Over the years, animal studies and clinical trials have revealed the effectiveness of CsA in other pathologies, such as T-cell large granular lymphocyte leukemia [2], traumatic brain injury (TBI) [3] or ischemic heart disease [4], among others. However, FDA approval has not yet been given for these diseases.

Different CsA formulations are currently available on the market, but there is still a need for improvement. Nowadays, the use of CsA has been limited owing to the related side effects, not only caused by the agent itself but also by the excipients present in the formulations (e.g. high quantities of organic solvents and surfactants). It is also worth mentioning that its unpredictable pharmacokinetics and its narrow therapeutic window are still concerns. In order to overcome these limitations, many promising drug delivery system alternatives based on particulate carriers are now being investigated [5-8]. The scientific efforts devoted to reformulating CsA have been oriented to improve the drug absorption and to modify its tissue distribution. The final goal is to achieve a better pharmacokinetic profile and controlled drug release, thus increasing its therapeutic range, while avoiding the use of Cremophor® as a vehicle, thereby diminishing the number of related side effects.

In this review, innovative CsA delivery systems developed during recent years are summarized, specifically focusing on those consisting on nano- and micro-carriers. The different sections cover (i) the drug background, (ii) the pharmaceutical and clinical aspects that make CsA a challenging drug to formulate, (iii) the critical points to consider for suitable delivery systems depending on the routes of administration, (iv) and current experimental findings and their contribution to the pharmaceutical field.

1.1. Chemical structure and physical properties

CsA ($C_{62}H_{111}N_{11}O_{12}$) occurs as a white power with a melting point of 148-151ºC, which is barely soluble in water and n-hexane, but highly soluble in other organic solvents and lipids [9]. It has a partition coefficient value (log P) of 2.92 [10]. This lipophilic compound is a neutral cyclic polypeptide consisting of 11 aminoacid residues with a molecular weight of 1202.61 Da (Figure 1).

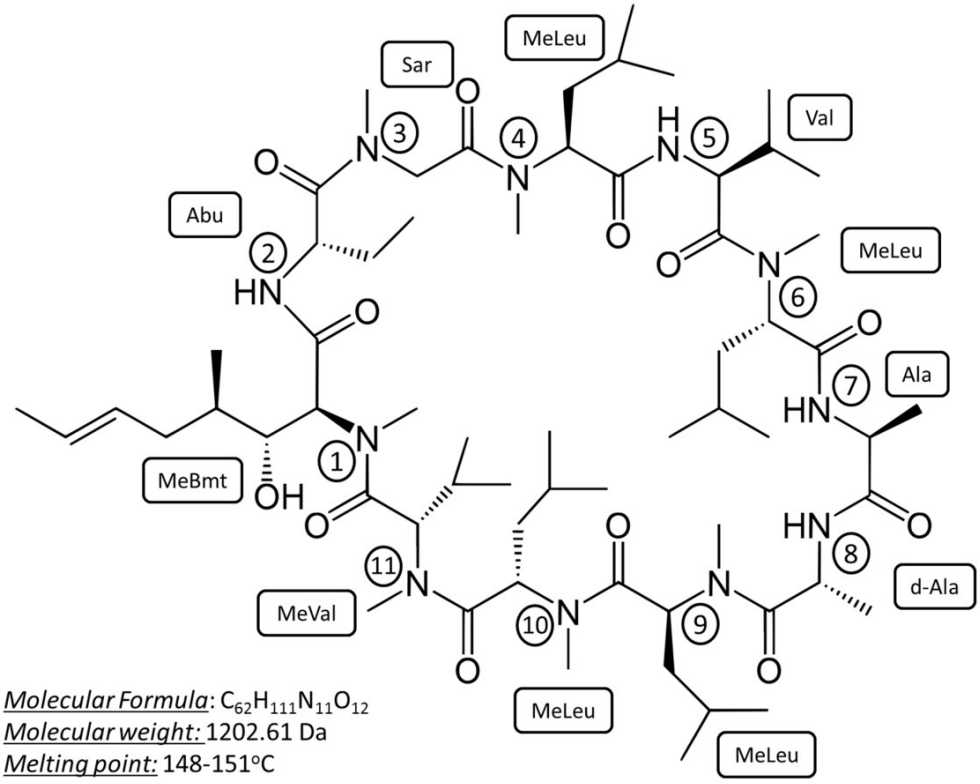

Figure 1. Molecular structure of CsA.

The aminoacids present in the molecule are: (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine (MeBmt) at position 1, unknown until the isolation of CsA, L-aminobutyric acid (Abu) at position 2, sarcosine (Sar) at position 3, methyl-leucine (MeLeu) at positions 4, 6, 9 and 10, L-valine (Val) at position 5, L-alanine (Ala) at position 7, D-alanine (d-Ala) at position 8 and methylvaline (MeVal) at position 11. The aminoacids at positions 1, 3, 4, 6, 9, 10 and 11 are N-methylated at the amide nitrogens and are responsible for the highly lipophilic nature of the molecule. The methylamide between residues 9 and 10 is located in the *cis* configuration and the other remaining fractions are in the *trans* form. On the other hand, the amide groups at positions 2, 5, 7 and 8 produce four intramolecular hydrogen bonds with the carbonyl groups of residues 5, 2, 11 and 6, respectively, ensuring high rigidity in the structure. Finally, the unsaturated chain at position 1 and the aminoacids at position 2, 3 and 11 are responsible for immunosuppressive activity [11].

1.2. Mechanism of action

The immunosuppressive activity of CsA is attributed to the formation of a complex resulting from the high affinity of the drug with immunophilins, mainly one called cyclophilin A (a cytoplasmic receptor protein of the targeted cells). The CsA-cyclophilin complex formed binds to calcineurin causing the inhibition of its phosphatase activity. Calcineurin is the protein responsible for regulating the nuclear translocation and activation of the nuclear factor of activated T-cells (NFAT) transcription factors. The prevention of the dephosphorylation of NFAT stimulated by the cytosolic calcium hinders their penetration to the core. As a consequence, the transcription of important cytokine genes, including those of IL-2, IL-4, TNF-α and INF-γ, is blocked. Therefore, the proliferation and activation of T-lymphocytes (T-helper and T-cytotoxic cells) are inhibited, the cells do not respond to specific antigen stimulation and thus, the immune system is weakened [12,13].

Furthermore, CsA also binds to cyclophilin D, a protein located in the mitochondria, leading to the blockage of the mitochondrial permeability transition pore (mPTP) and the prevention of mitochondrial mega-pore formation. This mechanism may be involved in the cardio- and neuroprotective effects attributed to CsA [3,14].

1.3. Pharmacokinetics

CsA is considered a highly variable drug and its efficacy depends greatly on the patient population. Several factors strongly influence CsA disposition through the body and lead to a high intra- and inter-individual variability in the pharmacokinetic parameters. These factors include age, gender, genetics, pathology, diet, dosing time after transplantation, and concomitant administration with other drugs, among others [15].

There are two main routes for CsA administration, intravenous and oral. Although oral administration is preferred, the bioavailability of this lipophilic substance is low and highly variable, ranging from 8 to 60%, with the maximum drug concentration achieved 1 to 8 hours after the administration [15-17].

Once in the bloodstream, CsA is widely distributed throughout the body as a result of its lipophilic nature. Its apparent volume of distribution ranges from 2.9 to 4.7 L/kg in humans. From the dose absorbed found in whole blood, CsA is distributed as follows: erythrocytes (58%), plasma (33%), granulocytes (4%) and lymphocytes (5%). In plasma, most of the drug is bound to proteins, mainly to lipoproteins. CsA reaches higher concentrations in lymphoid

tissues, such as thymus, spleen, lymph nodes, and bone marrow, rather than in blood. Also, the drug accumulates in lipid-containing tissues, like liver, pancreas, adrenal glands, and adipose tissue, while it barely penetrates into the central nervous system [15].

CsA is largely metabolized in the liver by the oxidation produced by the cytochrome P450 system, specifically by the CYP3A4. Also, the gut wall and the kidney are involved in the drug biotransformation, but to a lesser extent. The cyclic structure of this molecule makes it resistant to metabolization, nevertheless oxidation and demethylation of the side chains lead to the formation of at least 30 metabolites in bile, feces, blood and urine of different species. Some of these metabolites boost the immunosuppressant activity of CsA while others induce toxic effects [17].

Biliary excretion is the main pathway of CsA elimination, which is mostly excreted as metabolites, and only 1% as intact drug. Less important, but also implicated in the drug elimination, is the renal route; approximately 6% of the dose is eliminated in urine. The clearance is approximately 0.35 L/h per kg and the elimination half-life of the drug can vary significantly among patients from mean values of 6.4 hours in heart transplantation patients to 20.4 hours in patients with hepatic dysfunctions [16].

1.4. Clinical applications

The most important clinical indication of CsA is the prophylaxis of rejection of several transplanted organs, such as kidneys, liver, heart, lung, small bowel, cornea or skin. Moreover, it has been indicated in bone marrow transplantation and graft-versus-host disease. The success of CsA in the transplantation field arises from its selective immunosuppressive effect that allowed it to significantly decrease the rejection rate in the 1980s, and to prolong patient and allograft survival [18]. Due to its successful outcomes in transplantation, the therapeutic application of CsA was extended to the treatment of various autoimmune disorders (Figure 2). These include severe rheumatoid arthritis, psoriasis, nephrotic syndrome, severe atopic dermatitis, and uveitis, when patients do not respond adequately to conventional therapy [19]. CsA is also used for the treatment of various ocular disorders with evidence of inflammation, like dry eye disease, posterior blepharitis, venral and atopic keratoconjunctivitis, among others [1]. CsA's therapeutic activity in treating ulcerative colitis has also been reported [20]. For some physicians, this is the preferred immunosuppressant used as rescue therapy in patients with acute colitis that do not respond to the intravenous steroid treatment, the main reason being that therapeutic levels of CsA can

be rapidly reached [21]. Moreover, CsA therapy has been effective in T-cell large granular lymphocyte leukemia and well tolerated regardless of the patient population [2,22]. In the last decade, CsA has attracted special attention as a cardio- and neuroprotective agent. Preliminary data from preclinical studies and early stage clinical trials have demonstrated the beneficial properties of CsA in TBI, stroke and other neuronal conditions [3,4]. Its ability to protect neuronal cells and the mitochondria in the cardiac tissue damaged during a heart attack makes CsA a potential candidate for addressing neurological and cardiovascular disorders (Figure 2). Phase II/III clinical trials are in progress to test CsA's efficacy in the treatment of these disorders and thus, contribute to the limited existing regimens for these purposes. However, there is some concern about the effective dose-toxicity relation since high doses and chronic administration are needed to evoke the cardio- and neuroprotective effect.

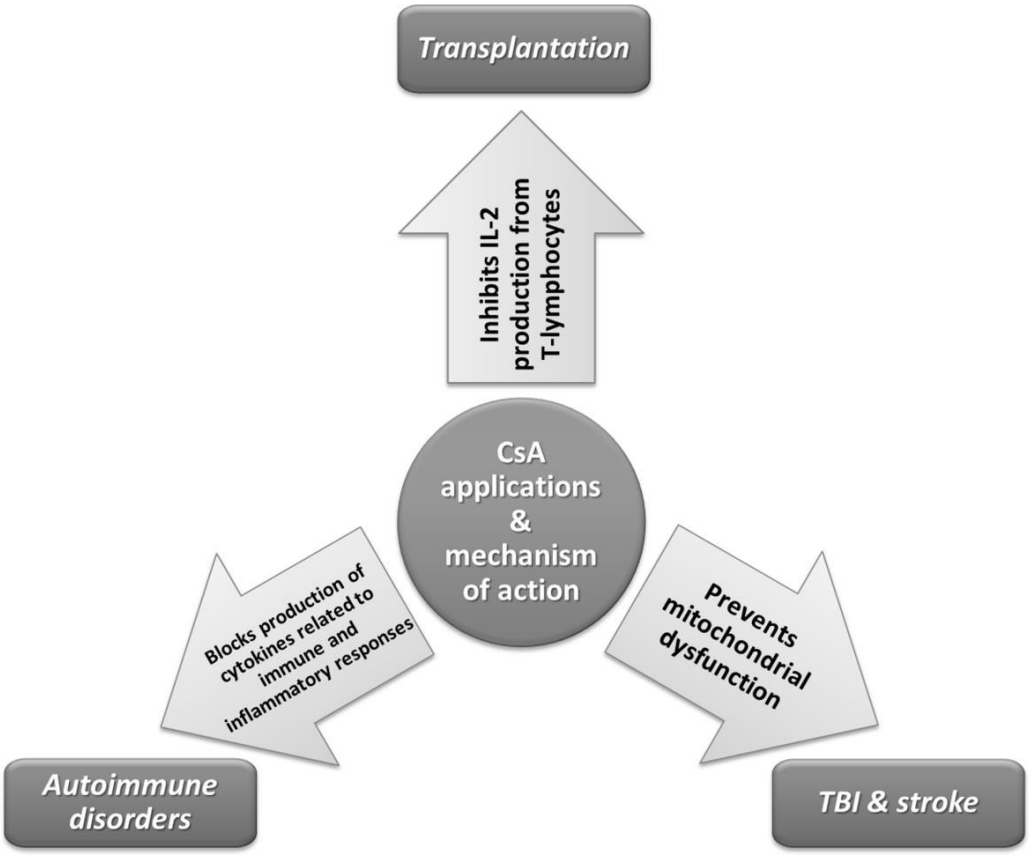

Figure 2. CsA clinical application and mechanism of action for the different indications.

Additionally, CsA has exhibited promising results in the treatment of pathologies such as asthma, primary biliary cirrhosis, myasthenia gravis, and insulin-dependent diabetes mellitus, among others [1]. However, more scientific studies are needed for CsA to become part of the established regimens in clinical practice.

1.5. Dosage

The dosing regimen and duration of CsA therapy greatly depends on the patient's individual condition. The treatment period may last months or years, or may become a lifelong therapy. The therapy is conditioned by the clinical response of the patient and his/her tolerability.

For transplantation, the common dose used is 10-15 mg/kg/day of CsA orally within the 12 hours prior to the surgery, and is maintained for the first 2 weeks post-transplantation. After this period, this dose is gradually reduced to a maintenance dose of 2-6 mg/kg/day. When the intravenous route is required, the dose is reduced to the third part of the oral dose [23]. Generally, the blood drug concentration is monitored at two hours post dosing (C-2) and the dose is adjusted during the treatment to achieve the desired therapeutic range for an individual patient. The therapeutic CsA C-2 levels can vary from 1000 to 1700 ng/mL during the three first months, depending on the transplanted organ, and followed by a progressive reduction to 600-800 ng/mL [24].

For the treatment of autoimmune diseases, the doses usually employed are lower, starting from 2.5 mg/kg/day of CsA and increasing gradually up to 5 mg/kg/day, if significant clinical enhancement is not observed and the therapy has been well-tolerated. In some cases, discontinuation of the CsA treatment leads to relapse of the pathology [25].

1.6. Adverse effects

Nephrotoxicity is the major concern in patients exposed to CsA therapy. The acute nephrotoxicity is characterized by a reduction of the glomerular filtration rate along with an increase in serum biochemical parameters, such as urea and creatinine. Nevertheless, if the levels of these parameters are carefully monitored in the initial stage of the treatment, the impairment of the renal function can be avoided, since they usually respond to a dose reduction. Inadequate dose adjustment can lead to chronic nephrotoxicity, also related to long-term CsA treatment. In this case, structural damage of the kidney arises and becomes progressive and irreversible, occurring as an interstitial fibrosis, tubular atrophy, arteriolar hyalinosis, and glomerulosclerosis [26]. The renal tubular injury is associated with metabolic disorders, including wasting of magnesium, calcium and phosphate as well as distal tubular acidosis, and impaired renal potassium excretion [27]. In turn, the magnesium loss may cause muscle cramps, weakness, paresthesia and sometimes convulsions. Additionally, hypertension is one of the most common pathologies that appear at the initial stage of CsA

treatment and is also related to electrolyte imbalance. Presumably, CsA's mechanism of action is also related to its side effects since the inhibition of the calcineurin-NFAT pathway produced by this molecule is not specific to immune cells. However, other factors have been studied as responsible for renal CsA susceptibility such as the variability in P-glycoprotein and CYP3A4/5 expression or activity, aged kidneys, salt depletion, concomitant medication, and genetic polymorphisms in genes like TGF-β and angiotensin converting enzyme [28].

Other adverse effects that have been reported for CsA therapy include hepatotoxicity, hirsutism, gingival hyperplasia, lymphoproliferative malignancy, etc [29].

1.7. Commercially available formulations

So far, CsA is available for oral, intravenous and ophthalmic administration. The first CsA formulation on the market was Sandimmune®, supplied as an oral solution or soft gelatin capsules and also as a concentrate solution for intravenous infusion.

Sandimmune® (oral dosage forms) consists of a conventional oil-based formulation containing corn oil, a large amount of ethanol, and inter-esterified corn oil. From this emulsion, CsA absorption is dependent on the presence of bile salts in gastrointestinal environment and its digestion by pancreatic enzymes. As a consequence, the bioavailability of CsA from this formulation has been reported to be low and very variable [30], leading to an erratic relationship between oral dose and total exposure of the compound. Years later Sandimmune Neoral® (hereafter referred as Neoral®) was introduced to the market in order to reach a better pharmacokinetic profile. This is a reformulated product consisting of a preconcentrate microemulsion containing DL-α-tocopherol, ethanol in high proportion, propylene glycol, corn glycerides and Cremophor® RH 40. Unlike the conventional Sandimmune® that forms oil droplets in the micrometric size, the more recent formulation can form homogeneous emulsion droplets of approximately 30 nm immediately after its contact with gastrointestinal fluids, promoting CsA absorption. In this regard, Neoral® has been shown to be less bile-dependent and provide superior and more reproducible bioavailability of CsA, which has been attributed to the micellar solubilization effect and the reduced particle size [31,32]. Despite the better performance in pharmacokinetics for the microemulsion, there is no evidence that Neoral® reduces the risk of side effects arising from Sandimmune® therapy. In addition, achieving sustained constant levels of the drug in blood within the therapeutic window is still a concern, and therefore costly and unpleasant drug monitoring is required [33]. There are other CsA formulations in the market for oral

administration, Gengraf®, Deximune® and Panimun Bioral™ as well as several generic formulations; however, they are not bioequivalent [34]. Switching to a different CsA formulation requires supervision of the physicians, and the drug levels must be carefully monitored during the first weeks.

Sandimmune® concentrate for the intravenous route consists of Cremophor® EL and ethanol. It should be diluted in saline solution or 5% glucose before administration. Due to the risk of anaphylactic reactions caused by Cremophor® EL, its use is limited to those cases in which the oral route is not well-tolerated or there are gastrointestinal disorders that threaten drug absorption. Recently, two intravenous CsA formulations have been developed, named CicloMulsion® and NeuroSTAT®, the first one for the treatment of heart reperfusion injury following stenting in patients with myocardial infarction, and the second one for the treatment of severe TBI. Both of them consist of Cremophor® free formulations, ready-to-use, which contain physiological fats and phospholipids, characteristics that make them advantageous over the existing marketed formulations. Hence, clinical trials are ongoing in order to obtain the marketing authorization. NeuroSTAT® received orphan drug status from US FDA and Europe in 2010 [35,36].

CsA is also available as an ophthalmic emulsion (Restasis®) containing castor oil, glycerin, polysorbate 80 and carbomer copolymer type A.

Furthermore, another two CsA formulations, specifically for veterinary use, are currently commercialized. One is Atopica®, an oral formulation indicated in atopic dermatitis; and the other one is Optimmune®, which consists of an ophthalmic ointment based on white petrolatum, used in dogs for the management of keratoconjunctivitis sicca or chronic superficial keratitis.

2. Limitations of CsA

Although CsA is available in the market in different dosage forms for different applications and administration routes, its use has been limited owing to certain side effects, which are not only associated with the drug but also with the components used for their preparation. Figure 3 summarizes some of the pharmaceutical and clinical problems related to CsA, which are explained in more detail in the following sections.

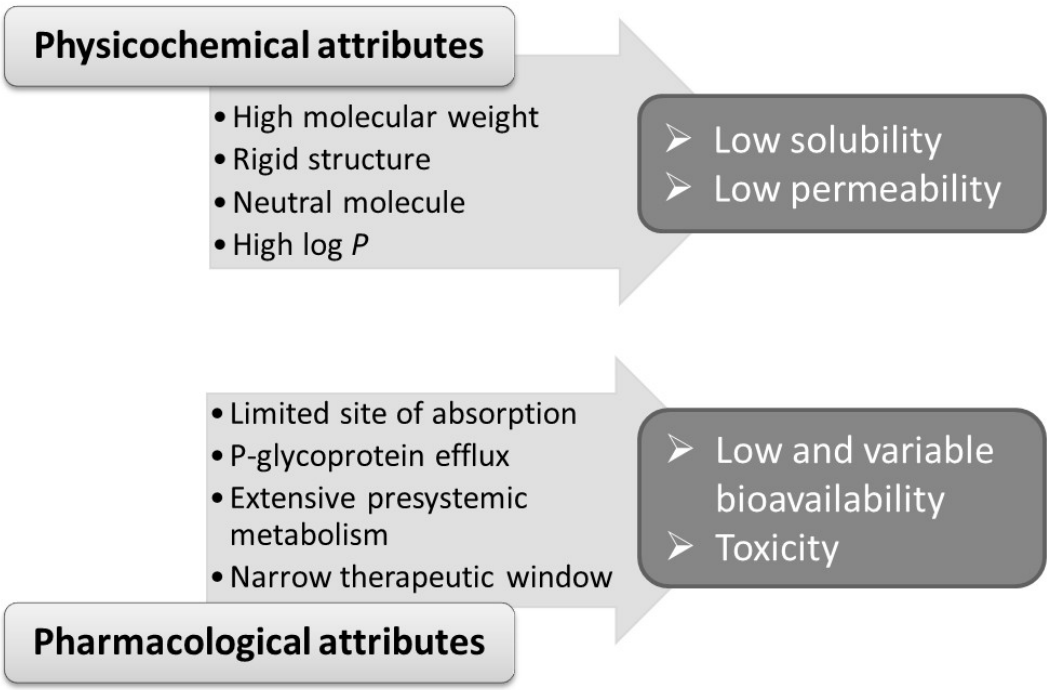

Figure 3. The impact of physicochemical and pharmacological attributes of CsA on its clinical outcomes.

2.2. Physicochemical attributes

Due to its poor biopharmaceutical properties, CsA is a challenging drug to formulate as a suitable delivery system able to ensure not only the efficacy of the drug but also its safety, regardless of the route of administration. Problems associated with CsA include high molecular weight, a rigid structure and a lipophilic nature, which are characteristics that lead to the low solubility of the compound. Consequently, CsA is poorly absorbed across several biological barriers such as the gastrointestinal tract, the stratum corneum and the corneal epithelium, causing an erratic relationship between the administered dose and total exposure, so that the drug concentration achieved in the site of action may be ineffective. Besides, the neutral characteristics of the molecule and the absence of ionizable functional groups make it impossible to obtain a more soluble form of the compound, which is one of the strategies usually employed to achieve improved solubility. Owing to its low solubility and low permeability through the physiological barriers, CsA is classified as Class IV according to the Biopharmaceutics Classification System [23,37]. Nonetheless, this compound has also been classified as Class II according to the same system when surfactants are implicated in its formulation [31]. In the search for alternatives to increase CsA solubility, special excipients have been used to formulate the currently marketed formulations.

However, they also contribute to the shortcomings of CsA therapy. Ethanol is one of the organic solvents used in both oral and intravenous forms, but it may be harmful for certain patient populations, such as pregnant or breastfeeding women, in patients with hepatic dysfunction or epilepsy, in alcoholic patients or pediatric patients, which restricts its use. Along with this, organic solvents may interact with the shell of the soft gelatin capsules causing the precipitation of some compounds and storage instability [8]. Moreover, one of the solubilizers employed for the microemulsion preparation, Cremophor® RH 40, might cause gastrointestinal disorders that, as mentioned above, significantly alter drug absorption. Similarly, the concentrate for intravenous infusion contains Cremophor® EL as carrier medium. This solubilizer is known to produce serious side effects, including anaphylactic reactions, hyperlipidemia, abnormal lipoprotein patterns, aggregation of erythrocytes and peripheral neuropathy [9].

2.3. Pharmacological attributes

The main drawbacks of CsA administration not only involve its limited and variable absorption through the biological barriers, but also its low safety - efficacy correlation. Several factors can explain the deficient CsA performance characterized by the low and unpredictable bioavailability. First, the site of absorption of the compound is limited to a part of the small intestine. Moreover, the P-glycoprotein efflux and the extensive presystemic metabolism in enterocytes and liver can influence the drug levels in the general circulation. The intra- and inter-individual variability in bioavailability has been associated with genetic aspects as well as the liver function. Among patients, the polymorphism of the cytochrome P450 system in the liver and enterocytes can differ, so that the drug metabolism and thus, drug concentration would be different between individuals. The production of bile salts and its flow can also affect the drug absorption, this variable being dependent on the patient's condition. Metabolic state, diarrhea and motility of the gastrointestinal tract are also inherent factors that can alter the permeability of CsA [15]. In transplantation, low levels of CsA can lead to organ rejection, whereas high levels of the drug can result in acute or chronic toxicity. Minimal change in dose might alter the clinical outcome of the patient. This means that CsA has a narrow therapeutic window and therefore drug blood levels with this immunosuppressant therapy must be carefully monitored [33]. The CsA dose adjustment also may be accompanied by monitoring of renal and hepatic functions to diminish the risk of toxicity leading to unpleasant and costly health care measures. In addition, given that CsA is a P-glycoprotein substrate, its concomitant administration with certain active agents can

either increase or decrease CsA blood levels by inhibition or induction of CYP3A4 and P-glycoprotein transporter [15], and hence drug adjustment is mandatory.

3. Suitable CsA delivery systems: pharmaceutical and clinical considerations

Several strategies have been investigated to reduce CsA-related side effects. Among these, the co-administration of antioxidants that might induce protective effects against renal injury [38], or the combination with other immunosuppressants in order to minimize CsA dose [19] are the most promising. However, no reliable evidence ensuring patient safety has been demonstrated. Besides, these patients are usually polymedicated so the inclusion of more actives that can interact with the standard treatment is not recommended. In this regard, the best strategy to overcome some of the above-mentioned limitations and enhance the therapeutic efficacy of CsA is to design a suitable CsA delivery system considering some key aspects such as the route of administration, the dosage and the intended indication (Figure 4).

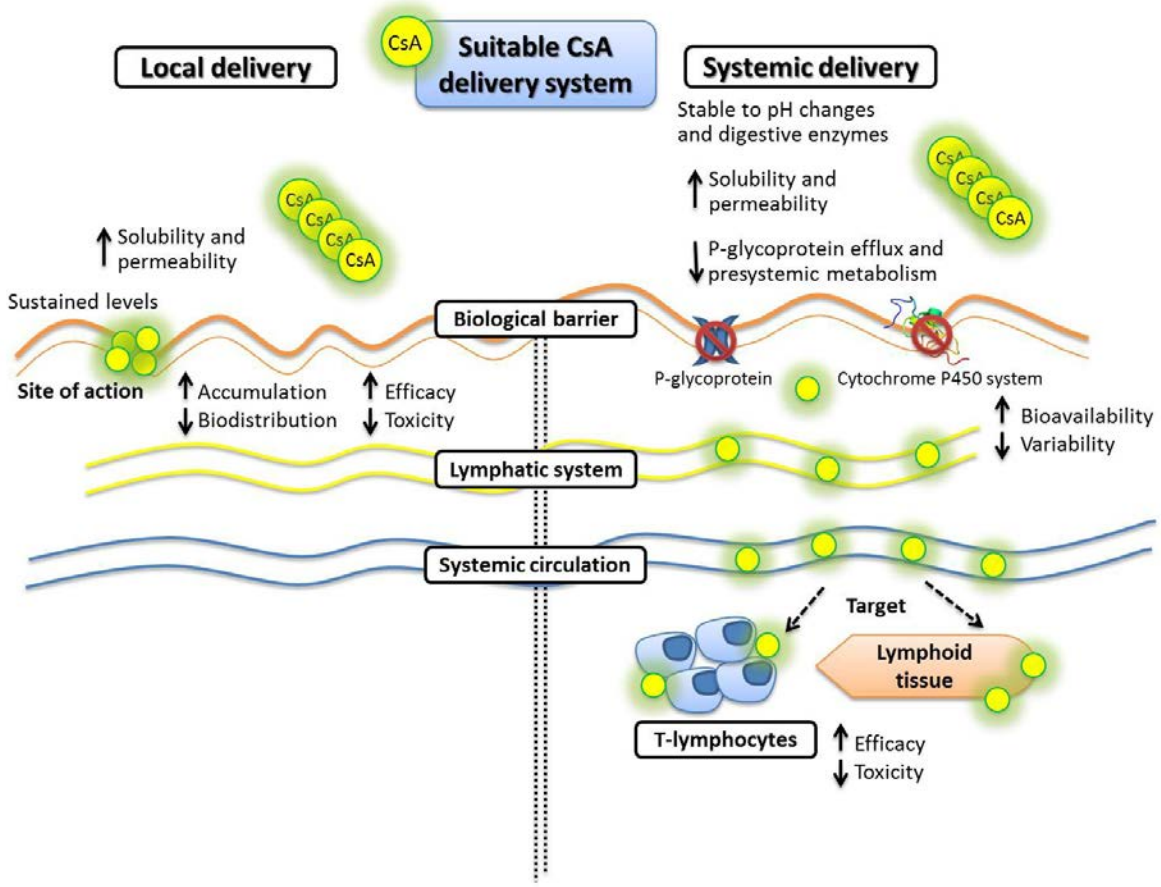

Figure 4. Benefits offered by a suitable CsA delivery system after its systemic and local delivery.

Moreover, the stability of the final product in the different storage conditions is an important aspect to be considered. These delivery systems must ensure efficacy and safety of CsA administration and enable patient comfort and compliance. The following sections focus on the specific considerations required for an optimal CsA performance for the different administration routes.

3.1. Systemic delivery

In transplant and systemic autoimmune disorders CsA delivery by the oral route is preferred. For an optimal CsA systemic delivery, the active should be efficiently and reproducibly absorbed and, once in the bloodstream, target the site of action at therapeutic concentration without compromising safety (Figure 4). In order to enhance drug oral absorption, it is important to increase the solubility of CsA in the vehicle and keep it dissolved in the gastrointestinal fluid, attempting to prevent drug precipitation in the biological environment. The vehicle has to exhibit high drug loading capacity using a minimum amount of excipients and be as safe as possible. The oral delivery system should be stable in the physiological environment, including pH changes and digestive enzymes, as well as capable of modulating the P-glycoprotein efflux and avoiding the presystemic metabolism, in order to decrease variability in CsA oral absorption and thus, decrease the risk of acute graft rejection or nephrotoxicity. Rapid release of the drug might be desirable for shortening the time to reach the steady-state concentration and therefore better immunosuppression. Moreover, targeting lymphoid tissue after systemic administration, orally or intravenously, may be advantageous for improving CsA activity on T-lymphocytes. Along with this, sustaining blood levels of CsA within the therapeutic window with a controlled release system can increase dosing intervals and thus enhance patient compliance. For the therapy of neurological disorders, a high concentration of CsA is required to achieve a therapeutic effect. Therefore, in this particular case, it may be advantageous to have a CsA delivery system capable of penetrating the blood brain barrier or/and delivering sustained and localized drug concentrations reaching the desired levels, and also limiting the organ distribution. For the parenteral route, it is important to highlight the use of safe excipients, which avoid the need for Cremophor® EL, which prevents side effects and improves therapeutic efficacy.

3.2. Local delivery

The development of a CsA delivery system for local administration is mainly focused on the skin, cornea and lung, according to the CsA indications. The strategy should consist of achieving the maximal therapeutic effect without compromising the complete immune system of the body. In this way, the adverse effects associated with systemic delivery would be reduced (Figure 4). For that reason, the delivery system should be able to accumulate high concentrations of CsA in the specific site of action and prevent its distribution to other organs. The vehicle for CsA ophthalmic administration has to be resistant to ocular fluids, increase corneal uptake, be well-tolerated by the corneal epithelium and reduce the precorneal clearance of the drug in order to achieve sustained therapeutic levels in the intraocular tissue for prolonged periods of time. For percutaneous delivery, the vehicle has to facilitate the permeation across the skin, avoiding its irritation and improving drug delivery into the damage tissue. In the development of a pulmonary delivery system, it is expected to target the entire lung tissue providing efficient CsA deposition and retention after inhalation, using an appropriate vehicle for aerosolization able to solubilize CsA but which is harmless to the lungs.

4. Current trends toward the development of novel CsA delivery systems

The present section aims to give an overview of the current state of the art of drug delivery systems for CsA delivery through novel lipid and polymeric drug delivery systems, providing examples of successful outcomes.

4.1. Lipid-based nano/microcarriers

Newly developed lipid-based formulations encapsulating CsA have been mainly exploited *via* the ocular and oral route (Table 1). The challenge when delivering CsA to the eye is to deliver a CsA therapeutic dose at the targeted ocular tissue with a low toxicity. However, currently available oils to deliver CsA topically to the eye are poorly tolerated and provide a low bioavailability [39]. Here we present examples of different lipid-based drug delivery system which overcome the aforesaid limitations.

Table 1. Lipid-based formulations encapsulating CsA *via* different routes of administration

Route of administration	Type of carrier	Ligad grafting/coating	Composition	Outcomes
Ocular	NLC	PEG-SA or Cys-PEG-SA	70% Precifac ATO®5 30% Mygliol®840 20 wt% CsA 2 wt% Tween® 80 2 wt% PEG-SA Coating: 2% Cys-PEG-SA or 2% PEG-SA	• Good ocular tolerance *in vivo* in New Zealand male rabbits • AUC_{0-24} and MRT_{0-24} of Cys-PEG-SA NLC in the eye were significantly higher than an oily solution and NLC or PEG-SA-NLC [40]
	SLN	-	6% Dinasan®116 or Compritol®888 ATO 0.10% CsA 1.50% Octadecylamine 0.01% Benzalkonium chloride 4% Tween® 80	• Prolonged *in vivo* released of CsA in sheep from Dinasan-SLN • CsA concentration in aqueous and vitreous humor below the limit concentrations for ocular immune system suppression [41]
	SLN	Chitosan	Compritol®888 ATO or Precirol ATO®5 (100 mg) Pluronic® F68 (50 mg) Tween® 80 (50 mg) Chitosan 0.2 or 2% (w/w) CsA (10 mg)	• Increased *ex vivo* permeation of CsA loaded Compritol versus Precirol SLN, whereas no CsA in solution did penetrate across excised corneas [42,43]
	SLM	-	8-20% (w/w) Precirol ATO®5 or 8-20% (w/w) Compritol®888 ATO 2-4% Mygliol®812 2% Witepsol® H15 1-3% Tween® 80 0.5% Cremophor® EL 1% Span 80	• CsA concentration 2% higher than commercial ocular emulsion • *In vitro* prolonged release for 48 h [44]
Oral	SLN	-	Precirol ATO®5 (200 mg) 2.5% (w/w) CsA Tween® 80 or Pluronic® F127 or Lec or TC	• *In vitro* IL-2 secretion inhibition in Con A activated Jurkat cells • Same effects compared to Neoral® • Similar or enhanced bioavailability and comparable biodistribution profile to that of Neoral® in Balb/c mice [45,46]
	SNEDDS	-	9:14:7 (w/w) Labrafil M® 1944 CS, Cremophor® EL and Transcutol P® 8% CsA 10% PVP K30	• Equivalent oral bioavailability of liquid SNEDDS to that of Neoral® in dogs, while decreased when being solidified into pellets [6]
		-	30-70% (w/w) oil (vitamin E TPGS) 10-70% (w/w) surfactant (Tween® 20 or 40 or 60 or 80 or Gelucire® or Cremophor® EL or Cremophor® RH) 0.30% (w/w) co-surfactant (Labrafil M® 1944 CS, ethylene glycol, Transcutol, PEG, ethanol, prurol oleique)	• Increased oral bioavailability and reduced CsA induced nephrotoxicity in mice compared to marketed Bioral® [47]
	SNEDDS from osmotic pump tablets	-	CsA (3.3 mg/tablet) Labrafil M® 1944 CS (7.6 mg/tablet) Transcutol P® (10.1 mg/tablet) Cremophor® EL (20.2 mg/tablet) Sucrose (61.6 mg/tablet) Lactose monohydrate (61.6 mg/tablet) PEO N80 (123.3 mg/tablet) Pregelatinized starch (12.3 mg/tablet)	• Prolonged CsA T_{max} and MRT, reduced C_{max} compared to Neoral® in dogs [48]
	Lipospheres	-	8% (w/w) CsA 14% (w/w) Tween® 20 14% (w/w) Span® 80 14% (w/w) Cremophor® RH 7% (w/w) Epikuron 200 28% ethyl lactate 14% (w/w) Dynasan® 110 or 114 or116 or 118 or lipo 320	• Bioequivalent with Neoral® in humans • Stable at room temperature for over 24 months [49,50]
	Liposomes	-	5% (w/v) SPC/SDC or SPC/cholesterol CsA (2 mg/mL)	• Improved absorption of CsA in SPC/SDC liposomes compared to SPC/cholesterol liposomes or Neoral® in rats [51]
Buccal	SLN containing gel	-	Compritol®888 ATO Poloxamer 188 Tween® 80, CsA 1 µg 1-2.5% HPMC K100M or 1-2.5% Carbopol 974 P NF	• Rapid decrease in ulcer size and increased mucosal repair in an oral ulcer model compared to the untreated group in rabbits [52]
Intracoronary	NP	-	Lipoid E 80 Poloxamer 188 Lipoid MCT CsA	• Enhanced therapeutic efficacy of ASCs (NP+ASCs) in a myocardial infarction in pigs compared to NP-treated or ASC-treated groups: left ventricular ejection fraction increased, decreased infarct size and neovascularization [19,53]

4.1.1. Ocular route

All the reported studies encompassing CsA *via* the ocular route in the last few years include solid lipid based-formulations [40-44]. Lipid-based nanocarriers have been reported to enhance the bioavailability of ophthalmic formulations [54], particularly in the case of anti-inflammatory drugs [55]. Solid lipid nanoparticles (SLN) are made of biocompatible lipids and present the advantage of avoiding an organic solvent during the preparation method, while presenting a high stability *in vivo* as they remained solid at body temperature [56], thus representing an alternative also to previous lipid-based formulations (e.g. liposomes). Başaran *et al*. [41] incorporated CsA (0.1% w/w) into cationic SLN containing Dynasan® or Compritol® as solid lipid and obtained positively charged nanoparticles presenting a mean particle size ~180 nm. The authors chose Dynasan-SLN over Compritol-SLN for *in vivo* studies as the latter presented a wider distribution size and higher zeta potential. *In vivo*, Dynasan-SLN were applied topically to sheep and samples from the aqueous and vitreous humor were withdrawn at 2, 16, 24 and 48 h, respectively. The ophthalmic amounts of CsA *in vivo* in both the aqueous and the vitreous humor (21.30 and 15 ng/mL, respectively) were found to be below the immunosuppressive concentration of CsA, which has been reported to be 0.05-0.30 μg/mL in blood and 0.10 μg/mL in vitreous humor. However, the increased CsA concentrations 48 h upon administration highlights the prolonged CsA released *in vivo* from SLN compared to previously reported nanoparticles in which CsA concentrations were found to decrease after 8 h [39]. Battaglia *et al*. [43] evaluated the toxicity of neutral, cationic and anionic SLN *ex vivo* in rabbit corneas using the bovine corneal opacity and permeability test (BCOP). Regarding SLN toxicity, the authors reported no irritation measured in terms of opacity and permeability. Regarding SLN permeability, higher permeation of fluorescently labeled CsA was reported for SLN compared to CsA emulsion or the drug in suspension. Cationic nanoparticles, obtained by coating SLN with chitosan, exhibited higher permeability values compared to bare nanoparticles (anionic and neutral). Sandri *et al*. [42] further confirmed these results. Indeed, chitosan-based nanocarriers have been described as a promising platform for ocular therapeutics [57], including CsA administration [58]. Wolska *et al*. [44] reported that CsA concentration could be increased at least 2% within solid lipid microspheres (SLM) (1-10μm) compared to the commercial ocular emulsion, while prolonging CsA released for at least 48 h. These findings are in agreement with the data reported by Başaran *et al*. [41] on CsA release from SLN.

Nanostructured lipid carriers (NLC), a second generation of SLN comprising both liquid and solid lipids, have been also exploited *via* the ocular route toward CsA delivery. Compared to

SLN, these nanoparticles favor increased drug loading due to their unstructured matrix [59]. Shen *et al*. [40] cross-linked the conjugate of cysteine-polyethylene glycol monostearate (Cys-NLC) into NLC to prepare thiolated NLC (Cys-NLC). Upon topical ocular administration to rabbits, the $AUC_{0\text{-}24h}$ and the $MRT_{0\text{-}24h}$ of Cys-NLC in aqueous humor, tear and eye tissues were significantly higher compared to those obtained for non-thiolated NLC and an oil solution. The authors attributed these increased concentrations to the ability of thiolated-NLC to prolong the pre-corneal residence time, thus improving CsA distribution in the conjunctiva.

Compared to Restasis® (marketed CsA ophtalmic emulsion), these formulations offer (i) prolonged CsA release that might allow us to lower the daily dose of CsA, (ii) increased CsA encapsulation rates and (iii) good tolerability even at high concentrations. Cationic over neutral or anionic lipid nanoparticles might be more appropriate to obtain increased adhesion into the ocular surface.

4.1.2. Oral route

Most of the studies based on lipid-based formulations aimed at increased CsA bioavailability have been carried out in self-emulsifying drug delivery systems (SEDDS), concretely in self-nanoemulsifying drug delivery systems (SNEDDS) [6,47,48]. SNEDDS are clear isotropic mixtures of oils, water-soluble surfactants and, optionally, hydrophilic co-solvents and are thus spontaneously generating oil-in-water colloidal nanoemulsions in gastrointestinal fluids. This formulation has been commonly used to improve the solubility of poorly water-soluble drugs and has been demonstrated to prevent the enzymatic and/or chemical hydrolysis of encapsulated drugs [60]. Lei *et al*. [6] studied the pharmacokinetics of CsA-loaded SNEDDS pellets compared to liquid SNEDDS and the commercial Neoral® in beagle dogs. Compared to Neoral®, liquid SNEDDS exhibited equivalent CsA absorption but with higher C_{max}. However, solid SNEDDS exhibited a lower absorption compared to liquid SNEDDS and Neoral®. Interestingly, the solidification of SNEDDS led to decreased CsA absorption. The authors attributed the differences in CsA absorption between liquid and solid SNEDDS to the particle size (21 nm and 54 nm, respectively) and the redispersing velocity (10 min and 20 min in water, respectively). However, this statement is somehow controversial since the oral bioavailability of CsA containing delivery systems (average particle size of 150 μm, x1000 bigger than Neoral®) has been found to be equivalent to Neoral® in healthy volunteers, thus discarding particle size-bioavailability correlation [32]. Jain *et al*. [47] evaluated the bioavailability and nephrotoxicity of CsA-TPGS-loaded SNEDDS *in vivo* in Sprague-Dawley rats and Swiss mice, respectively, and compared to (i) the marketed formulation Bioral™ and

(ii, iii and iv) CsA and TPGS alone or in combination, respectively. An increased bioavailability was observed only for CsA-TPGS-loaded SNEDDS compared to Bioral™, which was attributed by the authors to the increased CsA solubilization within TPGS-SNEDDS, the P-glycoprotein inhibition ability of TPGS and the increased encapsulation of CsA within the SNEDDS. Regarding nephrotoxicity, CsA-TPGS-SNEDDS exhibited a significant reduction in nephrotoxicity biochemical markers (creatinine and urea) compared to Bioral™, thus highlighting the safety of CsA-TPGS-SNEDDS over the marketed Bioral™. Zhang *et al*. [48] formulated CsA-SNEDDS into osmotic pump tablets (SNEOPT) and evaluated CsA bioavailability in dogs. Compared to Neoral®, SNEOPT presented a prolonged T_{max} and MRT, and significantly reduced C_{max}. However, similar CsA bioavailability values were obtained.

Avramoff *et al*. [50] evaluated lipospheres as CsA-loaded lipid-based delivery systems and proved equivalent bioavailability compared to marketed Neoral®. More recently, the authors have improved the formulation, preparing a CsA-loaded liposphere oral pro-dispersion stable at room temperature for over 24 months [49]. Guada *et al*. [45] evaluated *in vitro* the immunosuppressive effect of different SLN encapsulating CsA and observed a significant IL-2 secretion decreased in activated Jurkat compared to untreated cells, although an equivalent effect was observed for Neoral®. Likewise, a relative bioavailability of approximately 100% was observed when Precirol LN stabilized with a mixture of L-α-phosphatidylcholine (Lec)/taurocholic acid sodium salt hydrate (TC) or Pluronic® F127/TC were administered to Balb/c mice using Neoral® as reference formulation. Interestingly, an improved bioavailability was observed for LN containing Tween® 80, attributed to the more resistant properties of Tween® 80 against the gastrointestinal environment. A similar biodistribution profile 24 h-post dosing was obtained for these lipid nanosystems compared to the marketed microemulsion. The authors highlighted the advantages of the novel CsA lipid carriers regarding long-term stability and the safety of the excipients used compared to the commercial formulations [46].

Liposomes have also been exploited as lipid nanocarriers for CsA delivery. Guan *et al*. [51] evaluated liposomes containing a bile salt, sodium deoxycholate (SDC) as oral drug delivery system for CsA. They compared the widely used soybean phosphatidylcholine (SPC)/cholesterol liposomes with SDC/SPC liposomes and observed that both formulations released less than 5% CsA *in vitro* after 12 h. However, *in vivo* SPC/SDC liposomes exhibited increased absorption when compared to conventional liposomes or Neoral® in rats (120% versus 98%, respectively, with Neoral® as reference).

In general terms, CsA-loaded lipid-based nanocarriers exhibited (i) equivalent bioavailability compared to Neoral® (except few exceptions), (ii) decreased toxicity and (iii) long-term stability at room temperature.

4.1.3. Other routes of administration

Recently, a bioadhesive gel formulation containing CsA SLN for the treatment of recurrent aphthous stomatitis has been described [52]. The suitability of the formulation intended for the buccal route was carried out in rabbits in terms of distribution on the buccal mucosa and efficacy in wound healing. After 12 days, the gel containing CsA-loaded SLN showed a statistically significant increased rate of mucosal repair compared to the untreated and the unloaded gel, exhibiting 68% of the formulation retained on the buccal mucosa 6 h after application.

A newly and innovative application of CsA-loaded lipid-based formulations was reported by Yin *et al*. [53]. In this study, a combination of adipose-derived stem cells (ASCs) with a CsA nanoparticle emulsion (CsA NP) in a swine myocardial infarction model in pigs *via* the intracoronary route and compared the effect with untreated, CsA NP-treated and ASCs-treated groups. The cardiac function was evaluated 8 weeks later, revealing a significantly increased left ventricular ejection fraction and a significantly decreased infarct size in the ASCs + CsA SLN-treated group compared to CsA NP- and ASCs-treated groups ($p<0.05$). Moreover, the ASCs + CsA SLN treatment promoted neovasculatization and cardiomyocyte apoptosis ($p< 0.05$).

4.2. Polymeric-based nano/microcarriers

Polymeric formulations encapsulating CsA have been mainly exploited *via* the oral, intravenous and ocular route (Table 2). The main matter of discussion regarding especially these routes of administration of CsA is the safety of the formulation. The main aim of these formulations is to increase the absorption of CsA and obtain higher blood concentrations. However, high CsA concentrations in blood lead to nephrotoxicity, among other things. In other words, there is a need for a balance in CsA formulations: on the one hand, adequate CsA concentrations for inducing the desired effect, on the other hand, reduced CsA blood levels so that they are innocuous.

Table 2 summarizes the latest polymer-based drug delivery systems tested *in vivo*. In addition to these examples, several authors have provided interesting data on CsA-loaded polymeric carriers, providing new insights on CsA encapsulation within different types of carriers (e.g. effect of different polymers on CsA encapsulation, different preparation techniques, stability of the formulations, etc). However, these have not been tested *in vivo* and thus, have not being included within the following table [61-72].

4.2.1. al route

As with lipid-based formulations, many efforts have been made in the formulation of CsA within polymeric nano- or microparticles, micelles, microspheres, etc, toward an increased bioavailability. Ankola *et al*. [73] compared conventional PLGA NP (~100 nm) with EL14 (a carboxylated multi-block copolymer of lactic acid and ethylene glycol) NP (~135 nm) and reported no significant particle size increase in EL14 NP when increasing the drug payload from 10 to 30%, although the entrapment efficiency (EE) tended to decrease. Conversely, PLGA NP exhibited an increased particle size and increased EE. CsA release *in vitro* was found to be over 90% for both PLGA and EL14 NP, albeit much slower for PLGA NP. *In vivo* pharmacokinetic studies in rats showed increased C_{max}, faster T_{max} and enhanced tissue levels with EL14 NP compared to PLGA NP, and higher bioavailability for both nanoparticles compared to Neoral®. Despite the promising results obtained for EL14, the increased C_{max} and T_{max} compromises the safety of the formulation and, in concrete, might promote the CsA associated nephrotoxicity. Consequently, the authors carried out further studies evaluating the associated nephrotoxicity of CsA-loaded PLGA NP [74]. This study concluded that PLGA NP could reach Neoral® C_{max} while decreasing CsA associated nephrotoxicity. The absence of toxicity when administering CsA within PLGA NP was confirmed by Venkatpurwar *et al*. [75] after a long-term dosing of CsA NP (daily for 28 days). Interestingly, no differences in terms of toxicity, or bioavailability, were observed between PLGA NP or MP, despite the significantly different particle size. These results are in agreement with Andrysek [32], who reported no particle size-CsA bioavailability correlation. More recently, PLGA MS have been applied orally for the treatment of inflammatory bowel disease (IBD) [78]. CsA PLGA MS thoroughly ameliorated IBD in a DSS-induced murine model while decreasing the total dosage of CsA and, thus the elevation of serum levels of CsA. This is very important again for safe CsA treatment, since it reduces CsA-associated nephrotoxicity. These data support previous reports on PLGA's safety while demonstrating an efficient *in vivo* effect.

Table 2. Polymer-based formulations encapsulating CsA *via* different routes of administration

Route of Administration	Type of carrier	Ligand grafting /coating	Composition	Outcomes
Oral	NP	-	CsA EL14 (50 mg) 0.25% (w/v) DMAB 2.5 mL ethyl acetate: DCM (1:4)	• Higher C_{max}, faster T_{max} and enhanced tissue CsA levels compared to PLGA NP *in vivo* in rats • Bioavailability similar to Neoral® [73]
		-	CsA (5, 10 or 15 mg) PLGA (50 mg) 2.5 mL ethyl acetate 0.25% (w/v) DMAB	• Bioequivalent C_{max} compared to Neoral® in rats • Significant lower nephrotoxicity [74]
		-	CsA (75 mg) PLGA (500 mg) 25 mL ethyl acetate 1% (w/v) PVA	• Increased serum drug concentrations despite particle size, exhibiting no toxicity [75]
		-	CsA (2 mg/mL) GCPQ (15 mg/mL)	• Significantly increased CsA absorption compared to free drug in suspension (~5-fold) or Neoral® (~2-fold) in rats [76]
	pH-sensitive NP	-	CsA Eudragit® S100 Sylysia 350 (1/5/5 wt/wt/wt %)	• Delayed CsA absorption with T_{max} varying from 3.7 to 9 h and significantly lower C_{max} compared to Neoral® in rats [8]
	Cubic NP	-	CsA GMO (500 mg) Poloxamer 407 (40-100 mg)	• Higher C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ (178%) compared to Neoral® *in vivo* in Beagle dogs • Facilitated absorption over increased release [77]
	Microspheres	-	CsA (10 mg) PLGA (200 mg) 1 mL methylene chloride 1 wt% PVA	• Inhibited expression of IL-1β, IL-6 and TNF-α *in vitro* in LPS-activated macrophages • Significant colitis amelioration compared to untreated group in a DSS-induced murine colitis model [78]
	Micelles	-	Soluplus®/CsA (ratios ½-1/30)	• Significantly increased oral bioavailability compared to Neoral® (~1.35-fold) in rats [79]
Intravenous	NP	mPEG	CsA Soybean lecithin mPEG-chitosan Poloxamer	• Elimination half-life of the NP was 21-fold longer compared to a CsA solution and the AUC ~26-fold larger in rabbits • Improved stabilizing properties due to PEG moieties [7]
		mPEG	CsA-PLA (10 mg) PLA-mPEG (10 mg)	• Targeted immunosuppression to the lymph nodes in mice after intravenous administration of CsA NP-loaded DCs [80]
		LTP	CsA (5 mg) PLGA (50 mg) 2% PVA PEG LTP	• Selective accumulation in the liver • Absence of toxicity compared to free CsA treatment • Decreased immunosuppressive effect compared to free CsA • Inhibited HCV replication in a HCV mouse model [81]
	Micelles	-	CsA (9 mg) PEO-*b*-PCL (30 mg)	• Equivalent immunosuppressant effect in mice compared to Sandimmune® [5]
Ocular	NP	Carbopol®	CsA (10 mg) 1% and 0.36% (w/v) PVA PLGA (50 mg) or Eudragit® RL (50 mg) or PLGA+Eudragit® RL (75:25, 50:50, 25:75% (w/w)) 0.05% (w/v) Carbopol®	• Significant CsA concentration with PLGA+Eudragit® RL (25:75) in rabbit tears compared to Restasis® (AUC_{0-24} 972.59 *vs* 514.24 ng h/g, respectively; C_{max} 366.30 *vs* 299.02 ng/g, respectively) [82]
		-	CsA (10 or 20% of the polymer) Chitosan (2 g) 2% (v/v) acetic acid solution 96% ethanolic solution	• CsA estimation in both the aqueous and the vitreous humor 72 h after topical administration in sheep [83]
	Micelles	-	CsA (26.4 mg) mPEG-hexPLA (120 mg)	• Significant lower edema and increased transparency in a rat cornea transplanted model compared to the untreated group • Higher CsA cornea levels compared to the systemic treatment [84]

Siew *et al*. [76] have formulated CsA within NP made of a chitosan amphiphile: GCPQ. *In vivo*, these NP increased CsA C_{max} almost 5-fold (380%) when compared to free drug in suspension and 2-fold (80%) when compared to Neoral® in rats. They attributed the increased CsA absorption to (i) the enhanced drug dissolution from the NP, (ii) the bioadhesive properties of the NP and (iii) the enhanced transcellular transport, based on an *in vitro* mechanistic study carried out in Caco-2 cells.

Cubic NP, made of GMO and poloxamer 407, encapsulating CsA have been also evaluated toward increased CsA bioavailability [77]. The relative $AUC_{0-\infty}$ of CsA in dogs compared to Neoral® was found to be 178%. The authors correlated the enhanced CsA bioavailability with facilitated absorption rather than improved drug release.

Yu *et al*. [79] evaluated supersaturated micelles made of Soluplus®, a graft amphiphilic polymer. Following an *in vivo* pharmacokinetic study in rats, the authors reported an increased AUC_{0-24}, T_{max} and C_{max} with a relative bioavailability of 134%, compared to Neoral®. However, this was only achieved with one of four supersaturation degrees, in concrete, with 3.53 (drug/ Soluplus® ratio, 1/7), and increasing supersaturation degrees led to decreased oral absorption ($p<0.01$). These data illustrate a dissolution and solubility-limited oral absorption of CsA.

pH-sensitive NP have been described as promising for oral peptide/protein delivery. Different examples, including CsA loading pH-sensitive NP, are included in the review written by Wang *et al*. [85]. Dai *et al*. [8] developed pH-sensitive NP made of medical-grade nanoporous silica (Sylysia 350) and Eudragit®. CsA-loaded NP exhibited a delayed T_{max} and a C_{max} significantly lower than Neoral® after orally administered to rats. However, it was worth noting that the CsA blood concentrations detected within the Neoral®-treated group were found to be beyond the CsA concentrations that have been reported to lead to severe nephrotoxicity [86] and thus, pH-sensitive NP might also represent an alternative toward innocuous CsA oral formulations.

All in all, polymeric-based drug delivery systems encapsulating CsA for oral delivery present increased bioavailability compared to marketed CsA formulations. However, the high accumulation of CsA in different organs and the rapid absorption might compromise the safety of these formulations. The associated CsA nephrotoxicity has only been evaluated in few cases, and mainly for PLGA-based NP. Exhaustive long-term dose-toxicity studies should be carried out prior to the translation of these formulations to clinical practice. Nevertheless, the increased CsA absorption within these formulations is undeniable.

4.2.2. Intravenous route

Probably the most widely exploited strategy to achieve prolonged circulation time of the formulations in the bloodstream is PEGylation, as PEG chains are known to provide ''stealth'' properties [87]. As an example, by PEGylating NP surface, the NP half-life is prolonged and it is known to decrease their recognition by the reticulo-endothelial system (RES). This approach has also been exploited to deliver CsA following the intravenous route. This is the case of the study reported by Zhang *et al*. [7]. These authors grafted mPEG to chitosan and then prepared mPEG-chitosan nanoparticles encapsulating CsA and lecithin in their inner core. After being intravenously administered, the PEG-modified chitosan NP exhibited an elimination half-time 21-fold longer than CsA in solution and an AUC ~26-fold higher. Additionally, the authors reported that PEG chains (i) provided the NP with stabilizing properties, (ii) hindered the interaction with plasma proteins, (iii) reduced the number of NP taken up by the RES, (iv) prolonged the retention time of the NP and (v) improved the bioavailability of the NP. Jyothi *et al*. [81] conjugated a liver-targeting peptide (LTP) to PEGylated CsA-encapsulated PLGA NP. The authors used these NP to treat hepatitis C virus (HCV) in a HCV murine model, thus using CsA as antiviral agent. The HCV-NP treated group showed a sustained anti-HCV effect after a short-term treatment (21 days) while minimizing the liver and kidney toxicity compared to free CsA treatment. These are promising data as the applicability of CsA as antiviral agent is hampered by its related nephrotoxicity and hepatotoxicity that have limited its use in clinical practice.

An innovative and smart alternative for targeted immunosuppression was described by Azzi *et al*. [80]. In order to exploit the ability of CsA in suppressing T-cell mediated-responses, the authors aimed at targeting PLGA containing CsA-PLA to the lymph nodes, which represent the primary site where naive T cells meet antigen presenting cells inducing them to become alloreactive. Following the presumption that dendritic cells (DCs) would phagocyte the NP and then migrate into the lymph nodes, the researchers coupled dendritic cells (DCs) with CsA-NP. The conjugated NP technique on CsA would protect DCs from cell death. The authors successfully demonstrated CsA-NP internalization by DCs *in vitro*, exhibiting no apoptosis, in contrast with free CsA-treated DCs. After the injection of coupled DCs into mice footpads, the authors showed efficient trafficking of DC to the lymph nodes. Interestingly, compared to uncoupled DCs, CsA-NP-treated DCs efficiently suppressed the proliferation and activation of CD8 T cells in the lymph nodes.

Hamdy *et al*. [5] developed PEO-*b*-PCL micelles encapsulating CsA and evaluated their

immunosuppressive effect *in vitro* and *in vivo*. *In vitro*, the inhibitory effect of CsA on the allostimulatory ability of DCs was assessed. However, the effect was comparable to that observed for Sandimmune®. The same effects, and the results, were further confirmed *in vivo*. Nevertheless, CsA-micelles represent an alternative for the delivery of CsA with the advantages of prolonged drug release and reduced risk of nephrotoxicity.

These results call for further studies focused on targeted immunosuppression using CsA as immunomodulator.

4.2.3. Ocular route

In the case of ocular formulations, a major goal is to maintain the therapeutic effects for an adequate period of time as the liquid forms can be easily removed from the eye. The rapid renewal rate of the lachrymal fluid (1–3 μl/min) and the blinking reflex, restrict the residence time of drugs in the precorneal space (<1 min) and, as a consequence, the ocular bioavailability of the instilled drugs (<5%) [88]. In order to increase CsA residence time, Aksungur *et al*. [82] prepared PLGA NP and PLGA-Eudragit®RL blended NP. Eudragit®RL was added within the formulation as this polymer provides positive charges, which could interact with the mucins present in the mucus layer, thus increasing NP residence time at the surface of the eye. In addition, the researchers also coated PLGA NP with Carbopol® in order to increase also the adhesiveness of the NP. Different ratios of PLGA:Eudragit® were evaluated. When these NP were tested *in vivo* in rabbits, the drug concentration in the rabbits´ tear film was higher at all time intervals with a PLGA: Eudragit® NP (25:75) followed by Restasis®. Regarding the kinetic parameters calculated to determine CsA elimination from the precorneal area, the $AUC_{0\text{-}24}$ was again significantly higher for PLGA: Eudragit® NP (25:75). Interestingly, this parameter was highly dependent on the nature of the polymers used. The authors attributed the efficacy of the nanocarrier to an increased interaction with the eye surface, rendering an enhanced formulation-mucosa contact and prolonged residence time and thus, increased CsA concentration in the tear film. Following the aforesaid hypothesis, Başaran *et al*. [83] prepared positively charged chitosan NP using the spray-drying method. CsA was detected in both the vitreous and the aqueous humor even 72 h after topical administration in sheep.

CsA is routinely used in clinical practice to prevent the cornea rejection after a cornea transplantation using a systemic treatment. Di Tommaso *et al*. [84] evaluated a micelle-based formulation encapsulating CsA in a rat model for the prevention of cornea graft rejection after a keratoplasty procedure. Following a 14-day topical CsA treatment, three parameters

were evaluated: (i) cornea transparency, (ii) edema and (iii) neovascularization. Compared to the untreated group, the micelle-treated group presented significant higher cornea transparency and lower edema 7 and 13 days post-surgery. This effect was comparable to that observed for the systemic treatment, without CsA systemic-related side effects.

4.3. Other types of carriers

There are quite a number of examples in the literature describing drug delivery systems other than nanocarriers for CsA delivery, mainly *via* the ocular route. Wu *et al*. [89] described a CsA thermosensitive *in situ* forming gel. The gel consisted of hyaluronic acid and a temperature-sensitive polymer (PNIPAAm). Compared to commercial eye drops, the gel exhibited no irritation after it was topically administered to rabbits. Moreover, the conjunctival concentrations of CsA after 24 h of topical administration were significantly higher than those of castor oil solution and commercial eye drops. However, the concentrations were found to be below 10 ng/mL, thus indicating a limited absorption, which could avoid the related systemic side effects.

Eperon *et al*. [90] prepared CsA and triamcinolone-loaded discs and loaded them into an intraocular lens, aiming at inhibiting uveitis after cataract surgery. This drug delivery system exhibited reduced ocular inflammation after more than 3 months post-implantation.

Gupta *et al*. [91] developed a novel punctual plug consisting of a hydroxyl ethyl methacrylate core loaded with microparticles and surrounded by a silicone shell. These plugs were able to deliver CsA for 3 months at zero-order at a 3 μg/day rate.

Rodriguez-Aller *et al*. [92] evaluated concentrated eye drops containing a CsA prodrug, soluble in water. The prodrug solutions were tested *in vivo* at increasing CsA concentrations (0.05-2% w/v CsA). Each prodrug formulation was compared to conventionally used CsA eye drops at an equivalent concentration. The *in vivo* results showed that the prodrug formulation led to higher corneal and conjunctival levels than the CsA formulations.

5. Conclusions and future perspectives

Developing novel drug delivery systems for CsA administration remains a challenge. The balance between efficacy and safety in CsA therapy has not been resolved yet and therefore,

the costly and unpleasant monitoring for patients is still required. The scientific community has made an enormous effort to improve the available CsA formulations. The major concerns still remain its variable pharmacokinetics and the excipients used in the formulation of this drug. It is obvious that there is increasing interest in this immunosuppressant for use in daily clinical practice: researchers are looking for an ideal vehicle able to give the maximum CsA efficacy after local or systemic delivery while avoiding as far as possible its related side effects.

The literature is rich on publications concerning formulations which successfully encapsulated CsA *via* different routes of administration, using different types of drug delivery systems and for treating different diseases. The examples included in this manuscript specially highlight nano- and microcarrier-based drug delivery systems as promising alternative formulations to those currently being marketed. The examples of CsA-containing formulations herein described broaden the applicability of CsA. An example is the success of CsA as an antiviral agent in treating hepatitis C.

Although examples of CsA drug delivery systems *via* percutaneous and pulmonary route have not been extensively described in recent studies, the skin and the lungs represent promising routes of administration for CsA local therapy, as has been reported [93-96].

Neoral®, NeuroSTAT® and CiCloMulsion® contain CsA in solution as a lipid emulsion (lipid formulations). Interestingly, more efforts have been made within the last five years in order to foster the advancement in polymeric rather than lipid formulations. The number of formulations containing Cremophor® EL has been dramatically decreased and has been replaced mainly by polymers that have exhibited no related side effects *in vivo*.

One of the major hurdles in CsA delivery is its innate toxicity that induces, among other effects, nephrotoxicity and liver toxicity. This has represented a major concern and is currently a matter for investigation. Fortunately, most of the examples described in this manuscript have overcome these limitations, enhancing CsA's safety profile. More importantly, the toxicity evaluation of CsA-containing formulations has become a must and is present in almost all the studies herein described.

The advances in defeating CsA formulation barriers have led to several clinical trials [36,97,98]. Baiza-Durán *et al*. [97] substituted the ocular CsA oily emulsion with a micellated aqueous solution, benzalkonium chloride free. Shi *et al*. [98] implanted polylactide-*co*-glycolide-*co*-caprolactone (PGLC) CsA drug delivery systems in the anterior chamber of the

eye for suppressing the occurrence of rejection after high-risk keratoplasty. Ehinger *et al*. [36] assessed bioequivalence after the intravenous administration of CicloMulsion®, a Cremophor® EL-free emulsion, exhibiting reduced side effects.

Almost all the drug delivery systems included within this manuscript at least provide comparable *in vivo* CsA concentrations with regard to Neoral®, if not increasing CsA concentrations, while improving its toxicological profile. There are some examples of clinical trials including newly developed CsA formulations and in view of the data herein included, one might hypothesize that the number of clinical trials including new delivery systems will be increased in the near future.

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HYPOTHESIS and OBJECTIVES

HYPOTHESIS and OBJECTIVES

Lipid nanoparticles have been proposed during recent years as a promising strategy for the oral delivery of poorly water soluble drugs, such as cyclosporine A.

The lipophilic nature and the small particle size of these nanosystems improve the solubility of lipophilic substances in the gastrointestinal tract and enhance their permeability through the intestinal epithelium. In addition, lipid nanoparticles enable a more consistent and reproducible bioavailability of the drug by promoting drug transport to the bloodstream by intestinal lymphatic systems, thus avoiding the P-glycoprotein efflux and the presystemic metabolism.

Along with this, the use of biocompatible materials for lipid nanoparticle preparation prevents any possible adverse effects related to the excipients used in the formulation. In addition, the feasibility of using these nanoparticles to obtain solid powders would prolong the long term storage stability of the final products.

In this context, the use of lipid nanoparticles to incorporate cyclosporine A would provide a suitable oral delivery system, stable at storage conditions, which enhances the pharmacokinetic characteristics of the drug as well as minimizing the toxicity associated with the active agent and also the excipients contained in the commercial formulations.

Therefore, the main objective of this thesis was to design and develop lipid nanoparticles for oral administration of cyclosporine A capable of providing a safe and stable formulation with an efficient pharmacokinetic profile as an alternative to the formulations currently on the market.

In order to achieve this aim, the following partial objectives were addressed:

1. To develop and characterize cyclosporine A lipid nanoparticles appropriate for oral delivery and to study the physicochemical storage stability of the new delivery systems.

2. To explore the performance of cyclosporine A incorporated into the lipid nanoparticles developed when delivered orally, using Caco-2 cell model to assess the *in vitro* permeability across the simulated intestinal barrier, and Balb/c mice to evaluate the bioavailability and biodistribution profile.

3. To evaluate the pharmacodynamic effect of the cyclosporine A lipid nanoparticles in a murine model using peripheral blood lymphocytes as biological marker, and to investigate the safety profile of the novel formulations.

4. To assess the efficacy of the cyclosporine A delivery systems in a murine dextran sulfate-induced colitis model.

CHAPTER 1

LIPID NANOPARTICLES FOR CYCLOSPORINE A ADMINISTRATION: DEVELOPMENT, CHARACTERIZATION AND *IN VITRO* EVALUATION OF THEIR IMMUNOSUPPRESSION ACTIVITY

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LIPID NANOPARTICLES FOR CYCLOSPORINE A ADMINISTRATION: DEVELOPMENT, CHARACTERIZATION AND *IN VITRO* EVALUATION OF THEIR IMMUNOSUPPRESSION ACTIVITY

Melissa Guada[1,2], Victor Sebastián[3,4], Silvia Irusta[3,4], Esperanza Feijoó[1], María del Carmen Dios-Viéitez[1], María José Blanco-Prieto[1,2*]

[1] *Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Navarra, Pamplona, Spain.*

[2] *Instituto de Investigación Sanitaria de Navarra, IdiSNA, Irunlarrea 3, 31008, Pamplona, Spain.*

[3] *Chemical & Environmental Engineering Department & Nanoscience Institute of Aragon, University of Zaragoza, Zaragoza, Spain.*

[4] *Networking Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, Madrid, Spain.*

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***Corresponding author:**

María José Blanco-Prieto, PhD

Department of Pharmacy and Pharmaceutical Technology

School of Pharmacy, University of Navarra, C/Irunlarrea 1, E-31008 Pamplona, Spain

Office phone: + 34 948 425 600 ext. 6519

Fax: + 34 948 425 649

e-mail: mjblanco@unav.es

ABSTRACT

Cyclosporine A (CsA) is an immunosuppressant commonly used in transplants for prevention of organ rejection as well as in the treatment of several autoimmune disorders. Although commercial formulations are available, they have some stability, bioavailability and toxicity related problems. Some of these issues are associated with the drug or excipients and others with the dosage forms. With the aim of overcoming these drawbacks, lipid nanoparticles (LN) have been proposed as an alternative, since excipients are biocompatible and also large amount of surfactants and organic solvent can be avoided. CsA was successfully incorporated into LN using the method of hot homogenization followed by ultrasonication. Three different formulations were optimized for CsA oral administration, using different surfactants: Tween® 80, phosphatidylcholine, taurocholate and Pluronic® F127 (either alone or mixtures). Freshly prepared Precirol nanoparticles showed mean sizes with a narrow size distribution ranging from 121 to 202 nm, and after freeze-drying were between 163 and 270 nm, depending on the stabilizer used. Surface charge was negative in all LN developed. High CsA entrapment efficiency of around 100% was achieved. Transmission electron microscopy (TEM) was used to study the morphology of the optimized LN. Also, the crystallinity of the nanoparticles was studied by X-ray powder diffraction (XRD) and differential scanning calorimetry (DSC). The presence of the drug in LN surfaces was confirmed by X-ray photoelectron spectroscopy (XPS). The CsA LN developed preserved their physicochemical properties for three months when stored at 4ºC. Moreover, when the stabilizer system was composed of two surfactants, the LN formulations were also stable at room temperature. Finally, the new CsA formulations showed *in vitro* dose-dependent immunosuppressive effects caused by the inhibition of IL-2 levels secreted from stimulated jurkat cells. The findings obtained in this paper suggest that new lipid nanosystems are a good alternative to produce physicochemically stable CsA formulations for oral administration.

Keywords: cyclosporine A, lipid nanoparticles, oral administration, stability, immunosuppressive activity, jurkat cells

1. INTRODUCTION

Cyclosporine A (CsA) is a well-known immunosuppressive agent widely used in the prevention of allograft organ rejection and several autoimmune disorders, such as psoriasis, rheumatoid arthritis, dry eye and ulcerative colitis. CsA was an important discovery in the immunotherapy field since it was the first immunosuppressant with selective action on lymphocyte inhibition avoiding myelotoxicity. The molecule was isolated from the fungal extract of *Tolypocladium inflatum.* [1] CsA is a neutral cyclic peptide consisting of 11 amino acid residues. As a result of this peculiar structure and its high molecular weight (1203 Da), CsA presents poor biopharmaceutical properties, including hydrophobicity, and low permeability through biological barriers (ie gastrointestinal tract, skin and cornea). These characteristics mean that CsA is classified as Class IV according to the Biopharmaceutics Classification System. [2] Nonetheless, this molecule has also been classified as Class II into the same system. [3]

CsA was initially marketed as a conventional oil based form for oral administration (Sandimmune®; oral solution or soft gelatin capsules). This formulation presented some inconveniences associated with low and unpredictable drug bioavailability, leading to an erratic relationship between oral dose and total exposure. Subsequently, with the aim of achieving a more consistent pharmacokinetic profile, a reformulated product consisting of a microemulsion was developed (Sandimmune Neoral®; oral solution or soft gelatin capsules). This product has made it possible to enhance oral absorption and reduce variability compared to the first mentioned formulation. [4] However, Sandimmune Neoral ® is not capable of sustaining constant levels of the drug in blood within the narrow therapeutic window, and therefore CsA monitoring is still required. [5] In addition, there are some other safety issues that remain unsolved: the dose-dependent nephrotoxicity attributed to the pronounced initial peak blood drug concentration, gastrointestinal disorders caused by Cremophor RH40, and the ethanol content, which is contraindicated in a certain patient population. Along with these, pharmaceutical issues associated with the microemulsion dosage forms have also been raised. High concentrations of emulsifying agents and organic solvents lead to incompatibility with the shells of soft gelatin capsules as well as precipitation of components when stored at certain temperatures. [6,7]

During the last decade, lipid nanoparticles (LN), which consist of a solid lipid matrix stabilized by surfactants, have gained considerable interest as suitable oral delivery systems for drugs that exhibit poor and variable gastrointestinal absorption, not only because of their

adequate *in vivo* performance but also as a result of their versatility in manufacturing processes. Their numerous advantages combine those presented by oil-based formulations and polymeric colloidal carriers. Within the benefits offered by LN we may mention the physiological and biocompatible excipients in their composition, low surfactant quantities required for their stabilization, avoidance of organic solvents, enhancement of physicochemical stability by lyophilization or spray drying, scale-up feasibility and relatively low cost production. In addition, LN enable us to enhance drug absorption, protect the drug from possible biological fluid degradation, and allow controlled drug release and drug targeting. [8,9] Considering the aforementioned attributes, LN seem an attractive alternative to design a suitable CsA oral delivery system.

Therefore, the main purpose of this study was to develop and characterize safe and stable LN for CsA oral administration. The influence of different surfactants in the properties of the nanosystems and the physicochemical stability of the new CsA formulations developed were also investigated. The biological activity of the optimized lipid nanosystems was studied *in vitro* by measuring the inhibition of interleukin-2 (IL-2) production of jurkat cells after treatment with the nanoparticles and concanavalin A (Con A) stimulation.

2. MATERIAL AND METHODS

2.1 Reagents

CsA and Tween® 80 (Tw) were provided by Roig Farma S.A. (Barcelona, Spain). Precirol® ATO 5 was a gift from Gattefossé (Lyon, France). L-α-phosphatidylcholine from egg yolk (Lec), taurocholic acid sodium salt hydrate (TC), Pluronic® F127 (PL), D-(+)-trehalose dihydrate, formic acid 98% for mass spectroscopy, chloroform (HPLC grade), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Con A were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol (HPLC gradient grade) was supplied by Merck (Barcelona, Spain). Ammonium acetate (HPLC grade) was purchased from Scharlau (Sentmenat, Spain). Roswell Park Memorial Institute 1640 cell culture media, heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin antibiotics were obtained from Gibco® by Life Technologies, (Barcelona, Spain). All other chemicals and solvents were analytical grade.

2.2 Development and optimization of lipid nanoparticles

2.2.1 Lipid nanoparticles preparation

LN were prepared by the hot homogenization followed by ultrasonication method. Firstly, 200 mg of lipid (Precirol® ATO 5) and different amounts of drug (CsA) were melted at 70ºC (slightly above the lipid melting point). Then, 10 mL of an aqueous solution containing 2% (w/v) of surfactant/co-surfactant preheated at the same temperature were added to the lipid phase and immediately homogenized by ultrasonication with a Microson™ ultrasonic cell disruptor (NY, USA) for 4 min at 10-12 Watts. The emulsion formed was cooled in an ice bath to obtain a nanoparticle suspension by lipid solidification. Then, the excess of surfactant aqueous solution and free drug were removed by diafiltration using Amicon® Ultra-15 10,000 MWCO filters at 4500 x *g* for 30 min and washed twice with distilled water. Finally, LN suspension was kept at -80ºC and lyophilized to concentrate the LN and obtain a nanoparticulate powder. Trehalose was used as cryoprotective agent. Blank LN were prepared following the same procedure as described above without the drug incorporation step.

2.2.2 Effect of surfactant/co-surfactant on particle properties

Different types of surfactants (Tw, Lec, TC, PL) were used to prepare the LN to assess their influence on the mean particle diameter, size distribution and drug entrapment efficiency. Different surfactant combinations were also investigated to optimize the formulation quality. For this study, the amount of drug was maintained constant at 2.5% (w/w) of the lipid content.

2.2.3 Effect of initial drug loading on particle properties

The effect of the amount of drug incorporated in the selected formulation was evaluated at different concentrations: 2.5, 3.75, 5.0, 6.25, 7.5 and 10% (w/w) of the lipid content. All other components were kept at the same concentration. Each formulation was prepared in duplicate. Particle size, size distribution and entrapment efficiency were systematically analyzed.

2.3 Characterization of cyclosporine A lipid nanoparticles

2.3.1 Particle size, polydispersity index and zeta potential

The mean particle diameter and polydispersity index (PDI) of the formulations developed were measured at 25°C by dynamic light scattering (DLS; Zetasizer Nano, Malvern Instruments, UK) at an angle of 173°. LN suspensions were diluted with ultrapure water until an appropriate particle concentration was achieved. Each sample was measured in triplicate. Values were expressed as a mean ± standard deviation.

The surface charge of the nanoparticles was investigated by zeta potential measurements using laser Doppler velocimetry (Zetasizer Nano, Malvern Instruments, UK) at 25°C. Analysis was carried out in triplicate and each measurement was an average over at least 12 runs.

2.3.2 Drug Entrapment Efficiency

Entrapment efficiency (EE) was determined by quantifying the amount of CsA incorporated in the lyophilized LN using an ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) method previously validated. [10] Briefly, 500 µL of chloroform were added to 5 mg of LN and vortexed for 30 s, then 1.5 mL of methanol were added to the mixture and vortexed for 1 min. After centrifuging at 15,000 x *g* for 10 min, the supernatant was diluted with methanol (1:10) and a 2 µL aliquot was injected into the UHPLC system for drug analysis.

2.3.3 Morphological characterization

The morphological examination of LN formulations was performed by Transmission electron microscopy (TEM). TEM images were taken on a FEI Tecnai T20 microscope at INA-LMA (Zaragoza, Spain). To prepare the LN samples for TEM observation, the LN suspension was first dispersed for 30 s in an ultrasonic bath. A drop of this suspension was applied to a copper grid (200 mesh) coated with carbon film. Then, samples were air-dried for 30 min at room temperature (RT) after removing the excessive sample with filter paper. The microscope was operated at 80 kV to preserve the LN morphology and diminish radiation damage.

2.3.4 Crystallinity studies

X-ray powder diffraction analysis (XRD) was performed to study the crystalline properties of blank and CsA LN. Lyophilized LN formulations were analyzed using RIKAGU D/Max-2500

(Japan). The XRD analysis range was scanned at 2.5–50º over 2θ with a step angle of 0.03º and a count time of 1 s at a constant temperature of 25ºC, tesp = 0.03º, with 40 kV voltage and current intensity level of 80 mA.

Pure lipids and pure CsA were studied and those XRD spectra were used as references in evaluating the LN formulations. Bragg spacing was determined by the Bragg equation which relates the wavelength of the X-ray beam to both the angle of incidence and the interatomic distance.

2.3.5 Thermal analysis

Temperature-dependent structure and crystallinity changes in the lipids were analyzed using Differential scanning calorimetry (DSC). DSC was performed using accurately weighed samples of bulk lipids and drug loaded and unloaded LN. These accurately weighed samples were sealed in aluminum pans (50 µL) and heating curves were recorded with a scan rate of 10ºC/min in the 25 – 300ºC temperature range using Differential Scanning Calorimeter (DSC 822, Mettler Toledo, Japan).

2.3.6 Surface elemental analysis

The surface composition of the LN as well as the individual components was analyzed by X-ray photoelectron spectroscopy (XPS). The analysis was performed with an Axis Ultra DLD (Kratos Tech.). The spectra were excited by the monochromatized AlKα source (1486.6 eV) run at 15 kV and 10 mA. For the individual peak regions, pass energy of 20 eV was used. Peaks were analyzed with the CasaXPS software, using a weighted sum of Lorentzian and Gaussian components curves after background subtraction. The binding energies were referenced to the internal C 1s (285.1 eV) standard.

2.4 Physicochemical stability studies of cyclosporine A lipid nanoparticles

After lyophilization, approximately 100 mg of each formulation were stored in closed glass vials at three different conditions: 4±2ºC (refrigeration), 25±2ºC (RT) and 40±2ºC (accelerated conditions). The physical stability of the nanosystems was evaluated by periodically measuring the mean particle diameter, size distribution and zeta potential over a period of 3 months. Just after lyophilization and every 30 days 10 mg of the dried powder were resuspended in 1 mL of distilled water and sonicated for 10-15 s. Then, samples were analyzed in triplicate as described above. The data are expressed as mean values ± standard

deviation. Chemical stability of the formulations was studied by quantifying CsA in the dried powder over the same period of time using the UHPLC–MS/MS. [10]

2.5 *In vitro* biological activity of cyclosporine A lipid nanoparticles

2.5.1 Cell culture

Jurkat cells were obtained from American Type Culture Collection (ATCC) cultured in suspension at a concentration between $1x10^5$ and $1x10^6$ viable cells/mL in RPMI 1640 cell culture medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Cells were subcultured every 3-4 days depending on cell density to an initial concentration of $2x10^5$ viable cells/mL.

2.5.2 Cell viability study

The cytotoxicity of blank and CsA LN was determined on jurkat cells by a colorimetric method using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay). For the experiment, 100 µL of cells were seeded in a 96-well plate at a density of $4x10^5$ cells/well in fresh culture media and were incubated with the nanosystems at increasing drug concentrations up to 1.5 µg/mL in a humidified 5% CO_2 atmosphere at 37°C. After 20 hours, 20 µL of MTT solution at 5 mg/mL in complete cellular media were added to the wells and incubated for 4 h in the same conditions. After centrifuging at 200 x *g* for 10 min, supernatant was carefully removed, blue formazan crystals were dissolved with DMSO and the absorbance was measured at 540 nm with a microplate reader (Labsystems iEMS Reader MF, Finland). Culture medium was used as negative control (100% cell viability) and a 10% DMSO solution as a positive control (0% cell viability).

2.5.3 Inhibition of interleukin-2 (IL-2) production by concanavalin A stimulated jurkat cells

The biological effect of CsA incorporated in LN was assessed on human T lymphocyte cell line (jurkat cells) and Sandimmune Neoral® was used as reference. For this study, $4x10^5$ cells/well were seeded in a 96-well plate and cells were treated with CsA loaded LN (equivalent to 10 and 25 ng/mL of drug) and unloaded LN (equivalent to the highest concentration). Then, Con A solution was added to the wells at a final concentration of 20 µg/mL and the plate was incubated in a humidified 5% CO_2 atmosphere at 37°C. Con A-stimulated and non-stimulated cells without treatments were used as positive and negative

controls, respectively. After 24 h of incubation, microplate was centrifuged at 200 x *g* for 10 min and the supernatants were collected and stored at -20ºC until analysis. Human IL-2 levels were measured by ELISA (BD OptEIA™, BD Biosciences Pharmingen, CA, USA) following the manufacturer instructions. Absorbance measurements were carried out at 450 nm on a microplate reader (PowerWave XS, Biotek, USA).

2.6 Statistical analysis

Mann-Whitney U test was performed for statistical comparison between different groups considering statistically significant differences when $p < 0.05$. Data analysis was conducted using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

3. RESULTS AND DISCUSSION

3.1 Lipid nanoparticles preparation and characterization

Over the years it has been a challenge to incorporate CsA in a suitable oral drug delivery system. Researchers have spent major efforts on developing alternatives, such as self-nano-emulsifying drug delivery systems, [6] lipid based nanoparticles, [11,12] polymeric based nanoparticles, [13] micelles, [14,15] liposomes, [16,17] pH sensitive nanoparticles, [7] etc. These novel design CsA carriers offer several advantages compared to the formulations commercialized previously. These include enhancement of drug bioavailability, avoidance of the blood peak concentration, lower risk of nephrotoxicity and controlled release of the drug, among others.

The main objective of this study was to design CsA lipid based nanoparticles for oral administration as an alternative to the currently marketed Sandimmune Neoral® in order to overcome some concerns about stability, safety and pharmacokinetic behavior associated with the drug, excipients or dosage forms.

CsA was successfully incorporated into LN using Precirol® ATO 5 as the lipid matrix. Precirol is a Generally Recognized as Safe (GRAS) fatty ester with long acid chain length (palmitic acid) composed of a mixture of mono-, di- and triglycerides. When this type of lipid is used to prepare LN, it has the ability to form less perfect crystals with many imperfections on the matrix and therefore offers more space for drug accommodation. [18] The hot homogenization followed by ultrasonication method was selected for nanoparticle preparation since it is an

organic solvent free melting process, easy to scale up, in which no complex equipment is needed, and which avoids the need for high concentrations of surfactants and co-surfactants. As previously mentioned, preventing the use of organic solvent and large amounts of surfactants in the final product is directly related to the safety of the dosage form, which is one of the main drawbacks of the marketed formulation.

To obtain suitable LN, the influence of different variables such as type of surfactant and their combination, and also the amount of CsA incorporated into the system, were investigated in terms of particle size, size distribution and entrapment efficiency. In this work, Tween® 80 (Tw) and Pluronic® F127 (PL) were chosen as nonionic surfactants, phosphatidylcholine (Lec) as amphoteric surfactant and taurocholate (TC) as anionic surfactant, all of them usually used as stabilizing agents in manufacturing LN.

In this study, two types of stabilizer systems were investigated. One set of formulations was prepared with a single surfactant (Tw, TC, Lec) and in the other set mixtures of Lec, TC and/or PL were employed to optimize LN characteristics.

Table 1 summarizes some of the physicochemical properties of the formulations developed including mean particle diameter, size distribution and drug entrapment efficiency.

Table 1. Effect of surfactant/co-surfactant on cyclosporine A lipid nanoparticles characteristics loaded with 2.5% (w/w) of drug according to the lipid content

Formulation	Surfactant	Co-surfactant	Ratio	Size (nm)	PDI	EE (%)
LN 1	Tw	-	-	120.87±8.24	0.163±0.012	96.16±2.51
LN 2	TC	-	-	537.70±14.86	0.194±0.014	93.91±1.63
LN 3	Lec	-	-	Gelation		
LN 4	Lec	TC	3:1	201.27±6.96	0.207±0.014	98.60±6.93
LN 5	PL	TC	3:1	89.51±1.39	0.158±0.004	66.14±6.56
LN 6	PL	TC	1:1	114.68±2.02	0.173±0.014	99.16±4.31
LN 7	PL	Lec	3:1	78.52±0.12	0.151±0.002	45.47±8.13
LN 8	PL	Lec	1:1	129.00±1.99	0.232±0.006	46.83±6.56

Regarding LN 1, Tw was capable of stabilizing the lipid system producing particles around 121 nm and a monodisperse size distribution (PDI 0.163) along with high CsA entrapment efficiency. These results are in good agreement with those obtained by Estella-Hermoso de Mendoza *et al,* [19] who developed good quality Precirol LN for oral administration containing

edelfosine and Tween® 80 as surfactant. Given its optimal characteristics, LN 1 was selected for further analysis, hereafter referred to as LN Tw-CsA.

TC also led to a submicron particle diameter (538 nm) with a narrow size distribution and good incorporation of the drug, as can be seen in the case of LN 2 (Table 1); however, particle size was above the limit advisable for oral administration [20] and thus this formulation needed to be optimized.

Where Lec was used as a surfactant (LN 3), the formulation became a gel at the cooling step of the manufacturing process. This gelation has been attributed to the limited mobility of phospholipid molecules that leads to incomplete coverage of the particle interface. [21] In order to overcome this limitation, the addition of co-surfactants with high mobility (eg bile salts) has been proposed to retard or prevent gel formation during nanoparticle preparation when using phospholipids such as Lec as stabilizing agents. [22] Besides, the combination of surfactants could boost the effect of lowering the surface tension of the emulsion leading to a reduction in particle size and also may enhance long-term stability of lipid nanosystems.

It has been described that CsA is very well solubilized by mixtures of lecithin and bile salts and that this solubility is enhanced at a higher lecithin concentrations due to hydrophobic interaction with phospholipid molecules. [14]

In this regard, LN 4 was prepared with a blend of Lec and TC at proportion 3:1. This surfactant combination led to stable nanoparticle dispersions with high drug loading capacity, and a particle size and polydispersity index suitable for oral administration (Table 1). Therefore, this formulation was used for further studies (henceforth referred to as LN Lec:TC-CsA).

In addition, mixtures of PL:Lec and PL:TC at different ratios were also evaluated (Table 1). In both cases a high influence of PL on the mean particle diameter of the lipid nanosystems was observed (LN 5 – LN 8). Higher concentrations of PL led to a decrease in particle size. It appears that this nonionic surfactant is potent for lowering surface tension. Although particle size and size distribution were optimal in all the formulations containing PL, drug entrapment efficiencies were low when PL:Lec was employed as emulsifier. It is possible that the presence of PL in the aqueous phase improves CsA solubility in this phase and thus decreases its incorporation in the lipid matrix. Indeed, this fact has been previously reported for encapsulation of lipophilic drugs in solid lipid nanoparticles (SLN). [23] Formulations consisting of PL:TC showed higher CsA entrapment than the ones containing PL:Lec, CsA

incorporation being higher when increasing TC concentration. This phenomenon could be due to the ability of bile salts to disturb the hydrophobic chains of the lipid phase, thus improving the solubility of lipophilic drugs in the system. [16] Accordingly, LN 6 showed optimal characteristics for this study and was selected for further analysis. This formulation is hereafter referred to as LN PL:TC-CsA.

The influence of the initial amount of drug used to prepare the nanosystems was investigated in terms of particle size, polydipersity index and drug entrapment efficiency. For this study LN Lec:TC was selected since Lec:TC are known to solubilize CsA to a greater extent. Size ranges between 195.55±5.16 nm and 208.55±3.18 nm with PDI below 0.219±0.011 were obtained for all the studied drug concentrations. Results showed that increasing concentration of CsA from 2.5% to 10% (w/w) added to the lipid matrix did not notably change the mean particle diameter and size distribution. However, a slight reduction in drug entrapment efficiency was observed from 98.60±6.93% to 71.30±5.76% as the CsA concentration was increased from 2.5% to 10% (w/w), respectively. The appearance of macroscopic agglomerates in the highest concentration tested was also observed, possibly due to the limited ability of the system to incorporate the drug. This phenomenon has already been reported. [12] Reduction of spaces in the matrix owing to rearrangements of the lipid causes drug expulsion and thus agglomerate formation.

Another important characteristic of LN is the particle surface charge, which is measured by zeta potential. This parameter is essential to predict the dispersion stability. In general, dispersions with high absolute zeta potential values are considered stable systems due to the electric repulsion forces generated among charged particles preventing aggregation. Nonetheless, it has been reported that in the case of nonionic surfactants, the situation is more complex and stability is reached by steric repulsion. [18] In this study, zeta potential values of the optimized LN, measured before lyophilization process, were -27.8±1.5 mV (LN Lec:TC-CsA), -20.6±2.5 mV (LN PL:TC-CsA) and -14.6±1.9 mV (LN Tw-CsA). Variation in zeta potential values among the developed LN may be explained by structural changes in the surface caused by differences in the emulsifier utilized to produce them. In all cases particles were negatively charged due to the fatty acid in the lipid matrix, [24] those containing ionic surfactants (Lec and TC) being more negative.

Blank LN were prepared and characterized to compare the physical properties of the nanosystems (CsA loaded and unloaded LN) and to evaluate possible changes in particle size and zeta potential value caused by the drug incorporation. As can be observed in Table 2,

unloaded formulations showed similar characteristics to those obtained by loaded LN regarding particle size, PDI and surface charge. To that effect, it appears that CsA, as a lipophilic and neutral molecule, was completely solubilized in the lipid phase without producing any change in these particle properties.

3.2 Morphological characterization of the lipid nanoparticles

TEM characterization was performed in order to explore the particle morphology and size distribution. The TEM images (Figure 1) revealed that the optimized LN are dispersed as individual particles with a well-defined spherical shape.

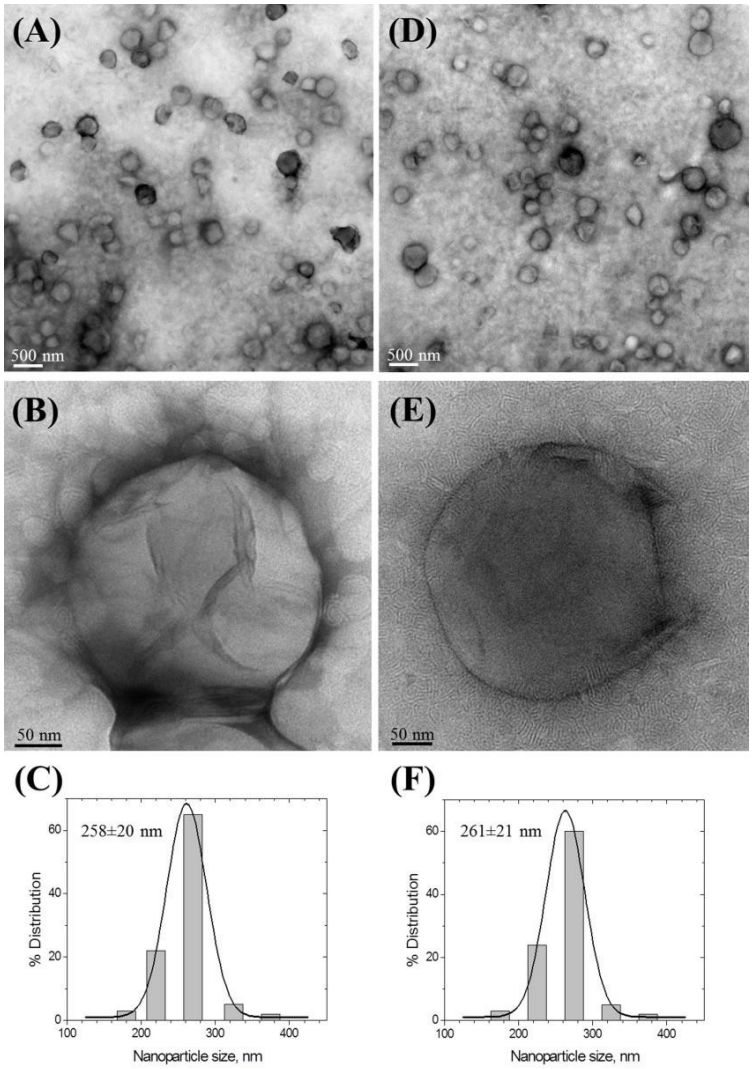

Figure 1. Transmission electron microscopy micrographs and particle size distribution of LN Lec:TC formulations. Blank **(A)**, **(B)**, **(C)** and Cyclosporine A lipid nanoparticles **(D)**, **(E)**, **(F)**.

Figures 1A and 1B depict LN Lec:TC-Blank, whereas Figures 1D and 1E show LN Lec:TC loaded with CsA. We can infer from TEM micrographs that the morphology is not altered by the presence of the drug. Particle size distribution histograms estimated from TEM images clearly evidence that the particle size distribution of both formulations is governed by a Gaussian distribution (Figures 1C and 1F). The sizes obtained ranged from 258±20 nm and 261±21 nm in LN Lec:TC unloaded and loaded with CsA, respectively. Consequently, TEM analysis indicates that the presence of CsA has no effect either on the morphology or the size of nanoparticles. This statement is consistent with the results obtained from the particle size measurement by DLS (Table 2).

Table 2. Physical characteristics of the optimized cyclosporine A loaded and unloaded lipid nanoparticles

Formulation	Size (nm)	PDI	Zeta potential (mV)
LN Lec:TC-CsA	201.27±6.96	0.207±0.014	-27.8±1.5
LN Lec:TC-Blank	202.43±8.65	0.210±0.012	-30.0±1.4
LN PL:TC-CsA	114.68±2.02	0.173±0.014	-20.6±2.5
LN PL:TC-Blank	111.11±2.20	0.165±0.007	-22.7±2.7
LN Tw-CsA	120.87±8.24	0.163±0.012	-14.6±1.9
LN Tw-Blank	117.96±6.39	0.164±0.022	-16.2±2.9

3.3 Crystallinity and thermal analysis of the lipid nanoparticles

XRD analysis enables us to identify the crystalline or amorphous state of LN, as well as revealing the spacings in the solid lipid lattice. [18] This is particularly important since lipids are very often polymorphic substances. XRD patterns of pure lipid and CsA were used as references for the evaluation of LN spectra (Figure 2A). Reference spectra indicated that Precirol and CsA existed in a crystalline state before being processed to give rise to the LN Lec-TC formulation. LN prepared with and without CsA and further lyophilized exhibited the same peaks with the starting lipid material, despite the hot homogenization conditions. On the other hand, the intensity peaks that belong to CsA could not be identified in the diffractograms of CsA loaded LN, suggesting the presence of an amorphous CsA payload. The Bragg-spacing values calculated for the reflections show the presence of two types of spacings: long spacings (depending on the fatty acid chain length and the angle tilt) and short spacings (non-dependent on fatty acid chain length). Short spacings correspond to reflections at high angles, originating from the packing of lipids, and are related with the presence of polymorphs. The most stable form, the β polymorph, has a triclinic subcell with a characteristic spacing at 4.6 Å. The β´ form has an orthorhombic subcell structure with

characteristic spacings at 3.8 Å and 4.2 Å. Finally, the α polymorph has a hexagonal subcell with a characteristic spacing at 4.15 Å. [25] Figure 2A shows the existence of 4.61, 4.22 and 3.86 Bragg-spacings, which implies that both the bulk lipid and the nanoscale counterpart are a mixture of β and β´ polymorphs. The polymorphs differ in stability, melting point, density and melting enthalpy. The β polymorph is the most stable and has the highest melting point and melting enthalpy.

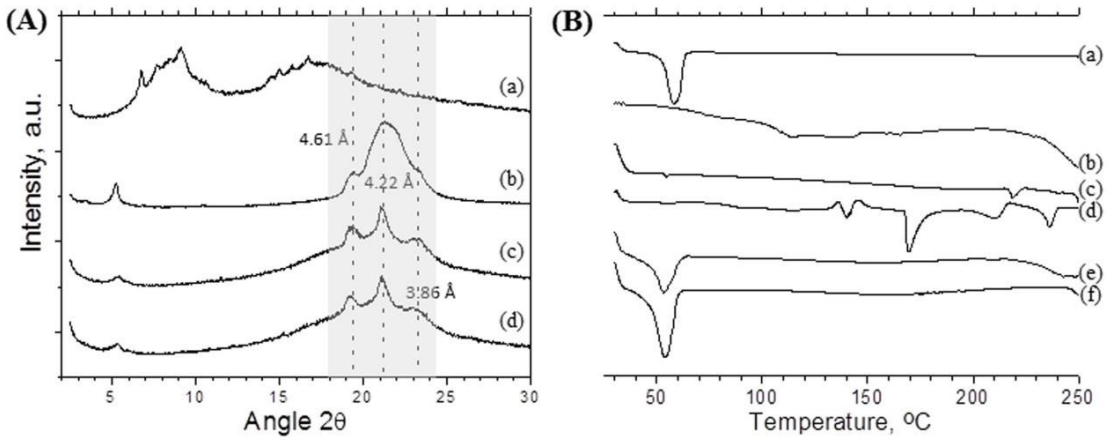

Figure 2. (A) X-ray difractograms of: (a) Cyclosporine A, (b) Precirol, (c) LN Lec:TC-Blank, (d) LN Lec:TC-CsA. **(B)** Differential scanning calorimetry thermograms of: (a) Precirol, (b) Cyclosporine A, (c) Phosphatidylcholine, (d) Taurocholate, (e) LN Lec:TC-Blank, (f) LN Lec:TC-CsA. Grey shadow corresponds to Bragg-spacing.

XDR results can be corroborated by DSC analysis. The DSC thermogram of pure Precirol exhibited a melting endothermic peak at 58ºC, while pure CsA, Lec and TC peaked at 140ºC, 225ºC and 140ºC, respectively (Figure 2B). The observed melting peak of LN, with and without CsA payload, was found to be 53ºC. This depression cannot be attributed to any polymorph transition, since XRD did not reveal any Bragg-spacing modification. On the other hand, it is stated that the presence of surfactants in the melted lipid phase during the production process could distort crystals resulting in a lower melting energy. [26] This fact can also be explained by the Kelvin effect, where a reduced particle size and increased surface area led to a decrease in the melting enthalpy compared to the bulk lipid. [26] The CsA melting endothermic peak of loaded LN disappeared, indicating the existence of amorphous CsA or molecularly dispersed within the Precirol matrix, confirming the results obtained by XRD.

3.4 *Surface analysis of the lipid nanoparticles*

The presence of CsA in the LN surfaces was studied by XPS. Surfaces of loaded and unloaded nanoparticles as well as some of the components were characterized. Beside carbon (C) and oxygen (O), phosphorus (P) was detected in formulations containing Lec. Nitrogen (N) is a component of CsA, Lec and TC and was detected in samples containing them, except for the unloaded LN PL:TC-Blank, probably because N concentration was below the detection limit of the technique. The absences of sulfur (S) signals also present in the TC would support the low concentration of TC on this sample surface. The N/C atomic ratio obtained from C 1s and N 1s peaks is shown in Table 3.

Table 3. X-ray photoelectron spectroscopy surface characterization of cyclosporine A loaded and unloaded lipid nanoparticles: N/C atomic ratio obtained from N 1s and C 1s levels and O 1s peak components

Formulation	N/C Atomic ratio	O 1s Binding energy (eV) (Atomic %)		
		PO_4^-	O-C/O-H	O=C
LN Lec:TC-Blank	0.006	531.0 (6)	532.7 (53)	533.7 (41)
LN Lec:TC-CsA	0.010	531.1 (8)	532.5 (60)	533.8 (32)
LN PL:TC-Blank	0.000	-	532.4 (48)	533.8 (52)
LN PL:TC-CsA	0.004	-	532.4 (71)	533.7 (29)
LN Tw-Blank	0.000	-	532.5 (47)	533.6 (53)
LN Tw-CsA	0.002	-	532.5 (61)	533.8 (39)

For samples loaded with CsA there is an important increase in N content compared to the unloaded ones due to the presence of the drug. The most important increase in N signal was observed in the LN Lec:TC-CsA (Table 3). For this sample two different N 1s signals were identified, with binding energies of 402.8 and 400.0 eV (Figure 3A).

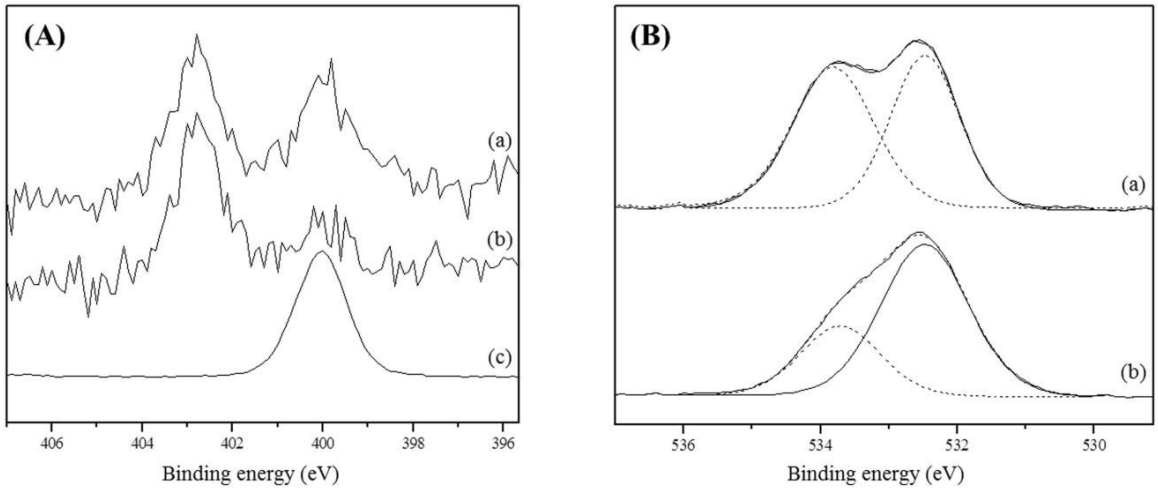

Figure 3. (A) N 1s core level spectra of LN Lec:TC formulations: (a) Cyclosporine A lipid nanoparticles, (b) Blank lipid nanoparticles, (c) Cyclosporine A. **(B)** O 1s core level spectra of LN PL:TC formulations: (a) Blank and (b) Cyclosporine A lipid nanoparticles.

The peak at higher binding energy would be associated with quaternary ammonium cations, [27,28] while the peak at lower binding energy could be assigned to hydrogen-bonded amines. [29] The intensity ratio between the low- and the high-binding-energy peaks increases from 0.38 in the unloaded LN to 0.95 for the loaded LN, due to the presence of CsA.

Another interesting feature is the change in the O 1s peak for loaded samples (Table 3). The O 1s peak was decomposed into two peaks for samples containing PL:TC (Figure 3B) and Tw. The component at low binding energy, 532.4-532.7 eV, is attributed to O single bonded to C in C-O-H and/or in C-O-C groups while the peak at 533.6-533.8 eV would be related to O in carboxyl function. [30] The third peak that appears in Lec:TC formulations would be related to the presence of PO_3^{2-} groups present in the Lec. [31] The atomic concentration decrease of O in carboxyl functions (peaks at 533.6-533.8 eV) in samples containing the drug compared to the unloaded ones suggests that the CsA would be interacting with these functional groups of the lipid.

3.5 Physicochemical stability studies of cyclosporine A lipid nanoparticles

The physicochemical stability of the optimized CsA LN was studied after the lyophilization of the formulations in order to preserve their characteristics for an extended period of time. Lyophilization prolongs the physicochemical stability of lipid nanosystems by transforming the liquid nanodispersion into a dry product. Besides, a solid form allows the incorporation of

the LN into capsules, tablets or pellets bearing a feasible dosage form for oral administration. [8] For the lyophilization, cryoprotectant was added to the nanodispersions to reduce the LN aggregation and to obtain better particle redispersions after the freeze-drying process. Trehalose was used as the cryoprotective agent since it has been reported as being most effective in preventing particle growth in SLN. [20,32]

First, the effect of the freeze drying process on the resuspension properties of the nanoparticles was studied (Figure 4).

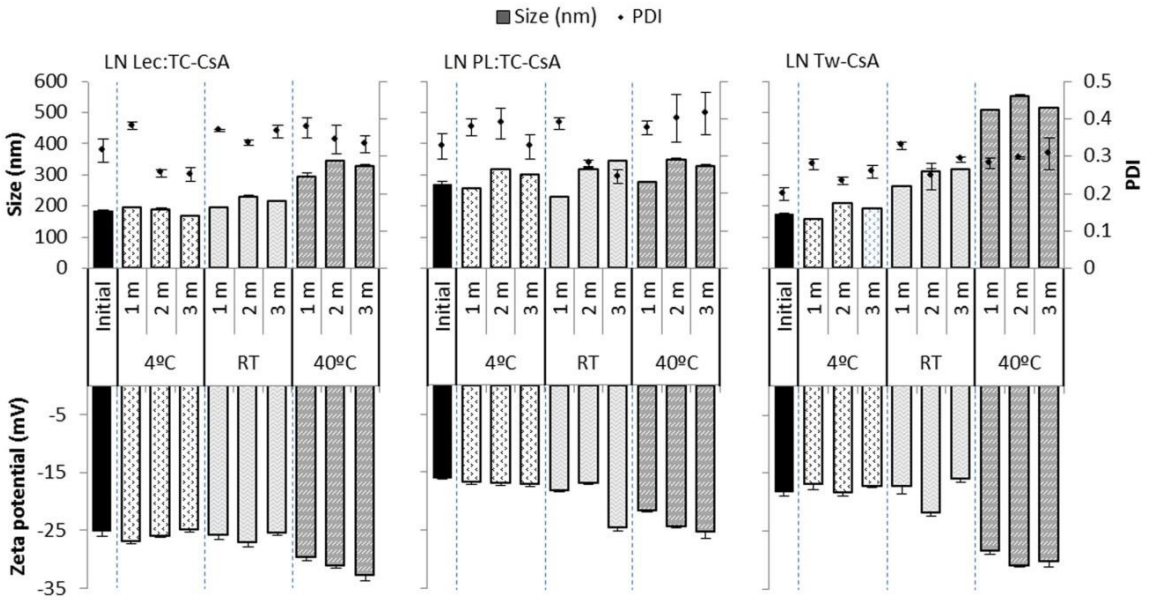

Figure 4. Particle size (top bars), polydispersity index (rhombus symbols) and zeta potential (bottom bars) characterization of the lyophilized cyclosporine A lipid nanoparticles measured at different time points during 3 months of stability evaluation. Results are represented by mean value ± standard deviation (n=3).

With regard to LN Lec:TC-CsA, particle characteristics remained practically unchanged after lyophilization. On the other hand, LN PL:TC-CsA and LN Tw-CsA redispersed in ultra-pure water showed a 2.35 and 1.43 fold increase in particle size, respectively. This particle growth has also been observed by other authors in SLN production. [32,33] This observed variation in particle size was attributed to the different stabilizing ability of the surfactants employed in each case. In some cases, it is possible that the freeze-drying process causes a modification in the surfactant layer properties by increasing the particle concentration after water removal, leading therefore to agglomeration. [8] Despite this size increase, lyophilized LN diameter was appropriate for oral delivery. Particle size distribution was also considered acceptable in the three developed LN with PDI values around 0.3. In addition, slight changes in the measured

zeta potential of the lyophilized formulations compared to those freshly prepared were observed. The presence of trehalose solubilized in the dispersion medium (ultra-pure water) may produce modification of its conductivity characteristics. [34]

Once the redispersion properties of the LN were evaluated, the storage stability of the three developed nanosystems (LN Lec:TC-CsA, LN PL:TC-CsA and LN Tw-CsA) was studied at various conditions (4ºC, RT and 40ºC) over a period of 3 months in terms of physical and chemical properties. The formulations presented a fine, loose powder appearance in the different storage conditions, except in the case of LN Lec:TC-CsA, that after one month at 40ºC started to lose this characteristic. This event can be promoted by larger amounts of lipid in its composition (Precirol and Lec) that are likely to melt when exposed to high temperatures.

Figure 4 summarizes the physical characteristics (size, PDI and zeta potential values) of the lyophilized LN after their storage at different temperature conditions and resuspension in distilled water. As can be seen in the figure, in the case of LN Lec:TC-CsA particle size was practically unaltered at 4ºC and RT at the end of the 3 months. However, samples at 40ºC showed a marked size increase after the first month of storage (up to 1.8 fold). With respect to LN PL:TC-CsA, a negligible progressive increase in particle size over the time (below 1.3 fold) was observed, which was slightly higher at RT and 40ºC. Nonetheless, these particle size changes seem to be less influenced by temperature. In contrast, a different particle growth behavior was observed with LN Tw-CsA. In this case, particle size increase was obvious from the first month at both RT and 40ºC (up to 1.8 and 3.2 fold, respectively), although at 4ºC storage no evident change was observed in particle size after three months. The particle size enlargement may be attributed to damage to the stabilizer layer causing incomplete coverage of the particle surface leading to aggregates in the system. In fact, the presence of few agglomerates in the lipid nanosystems developed could be confirmed by the size distribution with PDI mean values ranging from 0.2 to 0.4.

So far, physical stability observations sustain the hypothesis that a mixture of surfactants has a synergistic effect in extending the long-term stability of LN. These results also suggest the good performance of Lec:TC and PL:TC in stabilizing CsA lipid nanosystems, probably by the formation of a stable layer on the particle surface with an excellent repulsion effect.

Moreover, in general terms, the zeta potential of all nanosystems remained unaffected for 3 months when stored at 4ºC and RT. However, a slight increment of the absolute zeta potential value was observed in samples kept at 40ºC (Figure 4). This increment may be explained by a possible degradation of the lipid which occurs when the product is stored under stress

conditions. This storage may cause rupture of the ester bonds, resulting in negative charge of the free fatty acid in the system along with lipid rearrangement which probably modify the surface charge of the particles, resulting in a more negative zeta potential.

Finally, in order to assess the chemical stability of the CsA LN developed, the drug content of the formulations was quantified. During the period of the study under different conditions the three formulations conserved the amount of entrapped CsA above 92% when compared to the initial drug content (data not shown), except LN Tw-CsA kept at 40ºC, which showed reduction of drug content up to 20% after the second month. This destabilization of the system can be explained by rearrangement of the lipid crystal lattice caused by interaction with the emulsifier, leading to drug expulsion. [8]

Summing up, the optimized LN presented good physical and chemical stability and it can be stated that the best storage condition to preserve the physicochemical properties of the developed CsA lipid nanosystems was under refrigeration at 4±2ºC. However the nanosystems could be stable at RT for a certain period of time.

3.6 *In vitro* biological activity of cyclosporine A lipid nanoparticles

The immunosuppressive activity of CsA is attributed to selective T-lymphocyte inhibition. The drug, which belongs to the calcineurin inhibitor group, forms a complex on the surface of lymphocytes with the cytosolic protein cyclophilin A impeding the T cell activation, and consequently blocking the expression of IL-2. [2,4] The biological activity of the lipid nanosystems developed was evaluated by measuring the IL-2 production of jurkat cells, a cell line derived from human T cell leukemia, after their stimulation with the T cell activator Con A.

In order to ensure that the inhibition of IL-2 production was due to the effect of the drug rather than to the toxicity of the treatments, it was necessary to determine the influence of the formulations on cell viability. The MTT assay revealed negligible cytotoxicity in the range of concentrations studied (data not shown) since after 24 h of incubation, cells exposed to the different treatments showed viabilities of over 90% compared to the negative control.

The ability of the CsA loaded LN to inhibit cytokine production was studied at concentrations equivalent to 10 and 25 ng/mL CsA. These concentrations were chosen based on previous work. [35] As shown in Figure 5, IL-2 secretion was significantly suppressed by CsA LN in a

dose-dependent manner compared to the positive control. The same effect was observed with Sandimmune Neoral®.

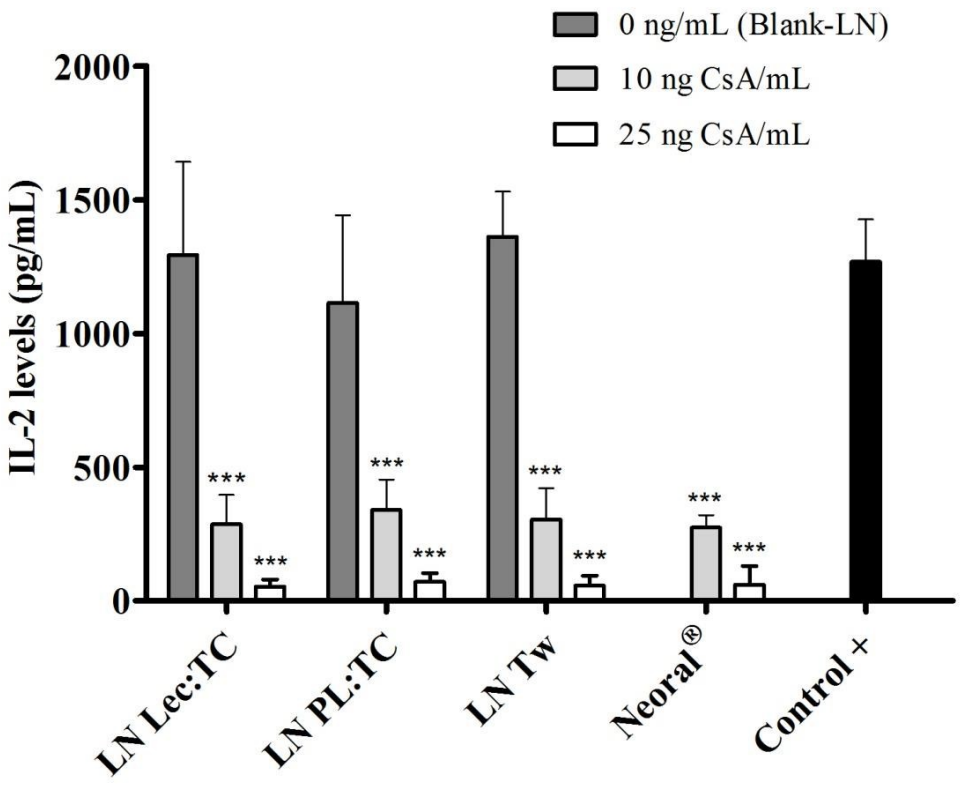

Figure 5. Inhibitory effect of the cyclosporine A loaded and unloaded lipid nanoparticles on IL-2 secretion from jurkat cells stimulated with 20 µg/mL concanavalin A. esults are represented by mean value ± standard deviation (n=3). Statistical differences are represented by *** = $p < 0.001$ compared to positive control.

Indeed, no significant differences were obtained when comparing the CsA LN formulations with the reference formulation, indicating that our CsA nanosystems might be as effective as the marketed formulation. Similar inhibitory activity was observed among the different CsA loaded nanosystems, ruling out possible influence of the surfactants on the biological activity of the formulations. Moreover, since blank LN did not exhibit any significant difference in IL-2 levels compared to the stimulated control, the immunosuppressive effect of the formulations could be attributed to the incorporated CsA alone. These results are in accordance with those obtained for CsA polymeric nanoparticles using similar *in vitro* models [35,36] and confirm that the immunosuppressive effect of the drug was conserved after its production process.

4. CONCLUSION

Two crucial approaches were obtained with this study. First, the CsA formulations were prepared with low surfactant concentration and avoiding organic solvents, so they are likely to have low toxicity compared to commercial formulations. And second, the CsA delivery systems were dried to obtain a powder formulation which could be easily incorporated in a conventional dosage form and also enhance the long-term stability of the final product. Interestingly, the developed formulations showed immunosuppressive effects in a stimulated human T lymphocyte cell line. *In vivo* studies are in progress in order to investigate the pharmacokinetic behavior of CsA incorporated into the lipid nanosystems developed.

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Disclosure

The author reports no conflicts of interest in this work.

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CHAPTER 2

LIPID NANOPARTICLES ENHANCE THE ABSORPTION OF CYCLOSPORINE A THROUGH THE GASTROINTESTINAL BARRIER: *IN VITRO* AND *IN VIVO* STUDIES

CHAPTER 2

Guada, M., Lana, H., Dios-Viéitez, M. D. C., Blanco-Prieto, M. J., & Gil, A. G. (2016). Cyclosporine A lipid nanoparticles for oral administration: Pharmacodynamics and safety evaluation. European Journal of Pharmaceutics and Biopharmaceutics, 101, 112–118. https://doi.org/10.1016/j.ejpb.2016.01.011

CHAPTER 3

CYCLOSPORINE A LIPID NANOPARTICLES FOR ORAL ADMINISTRATION: PHARMACODYNAMICS AND SAFETY EVALUATION

CHAPTER 3

Guada, M., Beloqui, A., Alhouayek, M., Muccioli, G. G., Dios-Viéitez, M. D. C., Préat, V., & Blanco-Prieto, M. J. (2016). Cyclosporine A-loaded lipid nanoparticles in inflammatory bowel disease. International Journal Of Pharmaceutics, 503(1-2), 196–198. https://doi.org/10.1016/j.ijpharm.2016.03.012

CHAPTER 4

CYCLOSPORINE A-LOADED LIPID NANOPARTICLES IN INFLAMMATORY BOWEL DISEASE TREATMENT

CHAPTER 4

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GENERAL DISCUSSION

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Cyclosporine A (CsA) is a well-established drug indicated to prevent the rejection of organ transplants. In addition, the use of CsA for other indications such as rare diseases related to the immune system is becoming wide spread. Evidence of the clinical interest of CsA is revealed by the numerous published data including clinical trials and case reports with promising outcomes for various pathologies such as pyoderma gangrenosum [1], aplastic anemia [2], severe refractory immune thrombocytopenia [3], idiopathic pulmonary fibrosis [4], etc. Preclinical studies and clinical trials are ongoing aimed to prove its cardio- and neuroprotective effect and thus, widening the use of CsA for mitochondrial dysfunctions, including stroke [5] and traumatic brain injury [6], which currently lack treatment protocols. Although CsA provides evident efficacy benefits, the balance between efficacy and safety remains an important problem that cannot be ignored, as a consequence of its narrow therapeutic index and the side effects associated with the drug, and also with the excipients used in the commercial formulations.

CsA is a challenging drug to formulate as an optimal dosage form due to its poor pharmaceutical properties. Moreover, its low permeability through biological barriers and its variable bioavailability lead to an unpredictable relationship between the administered dose and the total exposure. Consequently, low CsA blood levels might results in a lack of therapeutic effect whereas high levels can induce toxicity.

So far, the most popular formulation available on the market for CsA oral administration is Sandimmune Neoral® (hereafter Neoral®), which consists of a preconcetrate microemulsion supplied as oral solution and soft gelatin capsules. Its composition is mainly based on corn oil as lipid matrix, Cremophor RH 40 as surfactant and ethanol as co-solvent. This product improves drug absorption and reduces variability compared to the first marketed formulation (Sandimmune®). However, there is no evidence that Neoral® either reduces risk of toxicity nor achieves sustained drug blood levels within the therapeutic range, and therefore CsA monitoring is still mandatory [7]. Additionally, undesirable excipients in high concentrations, such as Cremophor RH 40 and ethanol, may induce gastrointestinal irritation and, more importantly, alcohol is contraindicated in certain patient population, which restricts its use. Other inconveniences associated with the dosage forms mentioned are related to their poor stability under certain storage conditions: the high content of

surfactants and co-solvent in the composition may lead to compatibility problems with the shell of the soft gelatin capsules as well as to the precipitation of the components [8,9]. In view of this situation, the need to develop new biocompatible CsA formulations with reduced adverse effects is clear.

In this context, the present study focuses on designing new alternative delivery systems to the existing CsA dosage forms, intended for oral administration, as this is the most convenient route for patient compliance, and with enhanced pharmacokinetic behavior, with the ultimate objective of achieving the maximum benefit in its different indications along with an improvement in its safety profile.

The development of novel drug delivery systems for known drugs is gaining considerable interest, given the great advantages this offers over the introduction of new chemical entities. On the one hand, drug delivery technology might overcome limitations associated with old drugs, improving their safety and efficacy, while on the other it reduces the time and cost involved in developing new molecules as well as the risks associated with obtaining approval for the active agent [10].

Nanotechnology is one of the most attractive approaches used in drug delivery technology. The success of this technology in the pharmaceutical world is mainly based on the reduction of the system to the nanometric size, which helps to increase the dissolution rate and permeability of compounds and thus enhances their biological performance [11]. Within this field, lipid based nanosystems, especially lipid nanoparticles (LN), have been widely explored for the oral delivery of poorly soluble compounds thanks to their numerous benefits [12]. These nanoparticulate systems consist of a solid lipid matrix, made of biodegradable and biocompatible materials, which is stabilized by surfactants and presents a high physicochemical stability. Low concentrations of surfactants are needed to stabilize the lipid matrix and the use of organic solvents can be avoided since the lipophilic drugs are successfully dissolved in this matrix. These aspects contribute to diminish the risk of toxicity related to the excipients used in the formulation. In view of the ease of manufacture and stability of the product, the lipid matrix solid at room temperature is expected to overcome the agglomeration and drug leakage limitations encountered in oil-based systems. Similarly, lyophilization of LN is possible in order to increase long-term stability. Scale-up feasibility and relatively low cost production are also advantages of this type of lipid nanosystem [13,14]. Regarding biopharmaceutical features, LN enhance the bioavailability of drugs that exhibit erratic absorption by means of different mechanisms that include: inhibition of P-

glycoprotein-mediated drug efflux, avoidance of the hepatic first pass metabolism by promotion of lymphatic transport, ability to solubilize the drug into the system and higher permeability across the gastrointestinal barrier through adhesiveness to the intestinal epithelium. In addition, LN offer drug protection from possible biological fluids degradation, as well as allowing controlled drug release and drug targeting [12,15].

Owing to the previously mentioned attributes, LN were selected to develop novel CsA formulations for oral administration as an alternative to the currently marketed Neoral® with the aim of overcoming limitations related to instability, toxicity and pharmacokinetic behavior associated with drug characteristics, excipients or dosage forms.

Chapter 1 has focused on the development and characterization of three CsA LN formulations, the evaluation of their storage stability as well as the assessment of their *in vitro* biological activity.

As a first step and in order to ensure the accuracy and precision in the measurement of the drug concentration throughout the research, the development and validation of an optimal analytical method for CsA quantification in various types of samples was required (***Annex***). The method developed was based on ultra-high performance liquid chromatography combined with tandem mass spectrometry and allowed the quantification of drug concentrations contained in lipid matrix, cell culture medium and various biological matrices.

LN were prepared using the hot homogenization method followed by ultrasonication, since that is an easy, time saving process, and no complex equipment is required for particle production. More importantly, this technique does not require neither high quantities of surfactant and co-surfactant for particle stabilization or organic solvent, which are critical points to consider in order to minimize the excipient-related toxicity. In addition, Precirol® ATO 5, a Generally Recognized as Safe (GRAS) product, was used as solid lipid matrix, and the stabilizing agents selected for the different formulations were Tween® 80 (Tw), Pluronic® F127 (PL), phosphatidylcholine (Lec) and taurocholate (TC), all of which are biocompatible ingredients commonly used in manufacturing LN.

The selection of the surfactant plays an important role in the particle size and also in the long term stability of the system. Therefore, the optimization process of the final CsA LN formulations was mainly focused on the type of surfactants used and their combination at different ratios (***Chapter 1***, Table 1). The best LN were selected based on mean particle diameter, size distribution and drug entrapment efficiency, the optimal physicochemical

characteristics being found with 2% aqueous solution of Tw (LN Tw), mixture of Lec:TC at ratio 3:1 (LN Lec:TC) and mixture of PL:TC at ratio 1:1 (LN PL:TC). Since the nanoparticles were designed for oral administration, sizes below 300 nm were preferred to facilitate intestinal transport [15]. Table 1 and 2 in ***Chapter 1*** summarize in detail the physicochemical properties of the LN developed. Freshly prepared Precirol nanoparticles showed a monodisperse size distribution (polydispersity index < 0.3) with mean diameters ranging from 121±8 to 202±7 nm, depending on the stabilizer used. Surface charge was negative in all LN developed, attributed to the fatty acid content in the lipid matrix [16], which was slightly more negative in those containing ionic surfactants (Lec and TC). High CsA entrapment efficiency values close to 100%, were achieved, indicating the high solubility of the drug in the system.

The nanosystems were characterized as individually dispersed particles with a well-defined spherical shape (***Chapter 1***, Figure 1), in which CsA occurs in its amorphous state or molecularly dispersed in the Precirol matrix. This situation might benefit drug solubility enhancement and promote its absorption across the intestinal epithelium. Also, CsA was found in the surface of the particles, which could influence the drug release after LN administration.

In order to prolong the physicochemical stability of lipid nanosystems, lyophilization was used to transform the liquid nanodispersions into dry products. Obtaining the solid form of LN facilitates their incorporation into conventional oral dosage forms, such as capsules, tablets or pellets, and also enhances the long term stability of the final product [13].

After the lyophilization process, an enlargement of particle size for LN PL:TC and LN Tw was observed (2.35 and 1.43 fold, respectively), whereas mean diameter for LN Lec:TC was unaltered. The size increase can be attributed to the ability of the surfactants to stabilize the system. Despite this particle increase, the diameters of lyophilized LN remained between 163±11 and 270±9 nm which is appropriate for oral administration.

Once the optimized LN had been well-characterized and the resuspension properties had been studied, the physicochemical stability was evaluated under various storage conditions (4ºC, room temperature and 40ºC) over a period of 3 months. In general terms, the CsA LN developed preserved their physicochemical properties for the whole period under study when stored at 4ºC. Moreover, when the stabilizer system was composed of two surfactants (LN Lec:TC and LN PL:TC), the LN formulations were also stable at room temperature for a certain period of time (***Chapter 1***, Figure 4). This observation supports the hypothesis that

the mixture of surfactants has a synergistic effect in extending the long-term stability of LN [13]. These results also indicate the good stabilizing capacity of Lec:TC and PL:TC in CsA lipid nanosystems, associated with the formation of a stable surfactant layer on the particle surface with an excellent repulsion effect.

The next step was the evaluation of the *in vitro* biological activity of the LN formulations in order to confirm that the drug efficacy was maintained after the production process. The immunosuppressive activity of CsA is attributed to selective T-lymphocyte inhibition. CsA, which belongs to the calcineurin inhibitor group, forms a complex on the surface of lymphocytes with the cytosolic protein cyclophilin A, impeding the T cell activation, and consequently blocking the expression of IL-2 [17]. Therefore, the production of IL-2 by jurkat cells, a cell line derived from human T cell leukemia, was evaluated as a measurement of CsA activity. Interestingly, the new CsA formulations showed *in vitro* dose-dependent immunosuppressive effects caused by the inhibition of IL-2 levels secreted from stimulated cells (***Chapter 1***, Figure 5). This biological activity was totally attributed to the drug, regardless of the vehicle used, which remained active after the manufacturing process. Since the same effect was observed with Neoral®, our CsA LN developed formulations may be as effective as the commercial formulation.

Considering that the developed LN were designed for CsA oral delivery, ***Chapter 2*** was aimed to assess the *in vitro* and *in vivo* behavior of the new formulations after their exposure to gastrointestinal conditions.

The Caco-2 cell monolayers were used to evaluate the *in vitro* permeability of CsA incorporated into the LN. Since Caco-2 cells are able to differentiate to enterocyte-like cells and mimic the intestinal barrier, this cell line has been widely used to predict the *in vivo* absorption of novel drug delivery systems and also to study their transport mechanism. However, in this study we found a lack of *in vitro/in vivo* correlation: Precirol nanoparticles containing Lec:TC turned out to be the best vehicle for promoting CsA absorption across the simulated intestinal epithelium (***Chapter 2***, Figure 2), whereas nanoparticles containing Tw showed the best performance *in vivo* (***Chapter 2***, Table 3). This finding can be explained by certain limitations of the classic Caco-2 intestinal model [18]. With this model, there is a gap in simulating the complexity of the gastrointestinal environment. In this regard, the impact of pH changes was investigated as one of the biological situations that occur during oral delivery, and the influence over the physical properties of the LN was demonstrated (***Chapter 2***, Table 2). LN containing Lec:TC lose their integrity under gastrointestinal pH, while the

other two nanosystems containing Tw and PL:TC remain practically unchanged when exposed to the same conditions. As a consequence, the drug permeability might vary *in vivo* compared to the *in vitro* results. Moreover, the action of bile salt and digestive enzymes is a crucial factor in the lipid digestion of the nanomatrix and therefore can influence the drug release and at the same time its absorption. Along with this, the underexpression of the uptake transporters in Caco-2 monolayers and the lack of lymphatic system as one of the suggested pathways for LN absorption might be another critical factor responsible for the discrepancies found between the *in vitro* and *in vivo* results. Nonetheless, the *in vitro* model gave us an insight into the transport mechanism involved in the absorption of the lipid systems across the intestinal barrier. As observed in Figure 1 (***Chapter 2***), trans-epithelial electrical resistance (TEER) values and confocal microscopy images indicated that the integrity of the Caco-2 cell monolayers was preserved after the treatment with CsA in its different formulations, without opening the intercellular tight junctions. Hence, the cellular uptake mechanisms involved in the transport of the tested formulations were other than the paracellular route. In fact, the transcellular transport has been broadly proposed as the pathway through which LN move efficiently from one side of the cell to the other. This is based on the partitioning of lipid bilayers of the membrane via vesicular carriers due to the lipophilic nature of the nanosystems [19].

On the other hand, as far as the *in vivo* evaluation is concerned, the studies performed in Balb/c mice following the administration of a single oral dose of the novel CsA formulations, showed relevant results that should be highlighted. The blood levels curve of CsA after the administration of lyophilized and freshly prepared LN was investigated with the aim to demonstrate that the above-mentioned particle enlargement occurring after the freeze-drying process does not compromise drug bioavailability (***Chapter 2***, Figure 3, Table 3). LN formulations showed a C_{max} in an earlier absorption stage comparable to Neoral®, being higher in the case of LN containing Tw. The fast CsA peak levels in whole blood have been attributed to the rapid digestion of the LN by the pancreatic lipases due to the lipid nature of the systems [20]. Besides, the presence of drug at the surface of the particle previously mentioned contributes to an early release of CsA. These features are favorable for an optimal CsA delivery system since they contribute to shorten the time to reach the steady-state concentration and translate into a prompt pharmacological response. Interestingly, LN stabilized with Tw showed an improved bioavailability when compared to Neoral®. This absorption enhancement was associated with the protective effect of the surfactant against the fast degradation of the lipid system by the digestive enzymes, due to the stearic hindrance provided by Tw [21]. Additionally, this formulation proved to be the most stable vehicle in

gastric and intestinal pH over time. In this regard, the lipid matrix stabilized with Tw probably prevents the release of the drug and avoids its precipitation in the intestinal lumen in a greater extent. Therefore, the drug incorporated into the intact nanoparticles goes into the bloodstream, reaching higher levels of CsA in whole blood compared to the other lipid nanosystems. Although PL is also known to be slowly degraded by the pancreatic lipases, it was used in combination with TC, which similarly to Lec, is rapidly digested in the gastrointestinal environment [21], probably causing a partially degradation of the lipid matrix before its absorption through the gut wall. Consequently, a similar performance for CsA bioavailability was observed for LN containing PL:TC and Lec:TC compared to the liquid lipid system (Neoral®).

Similar tissue distribution profiles of CsA were found at 24 h post dosing among the tested formulations. High accumulation was observed in fatty organs like liver and kidney, these organs being responsible for the metabolism and elimination of CsA. Likewise, significant levels of CsA were obtained in spleen which may be advantageous for improving CsA therapeutic efficacy, since the spleen is one of the major organs involved in the immune system.

Once the blood levels curve and biodistribution profile of CsA incorporated into the different nanosystems had been evaluated, the next step was to inquire about their pharmacodynamics and safety profile in mice after multiple administrations (***Chapter 3***). For this purpose, two LN formulations were selected, the one containing Lec:TC, considering the physiological nature of its surfactants, and the one containing Tw, given its better CsA bioavailability.

Given the ability of CsA to block the activation of lymphocytes entailing the inhibition of cytokine production (mainly IL-2) essential for proliferation, differentiation and maturation of T-helper cells [22], the pharmacological effect of the novel drug delivery systems was assessed using the lymphocyte count in peripheral whole blood as an indicator of immunosuppression. Moreover, CsA blood levels were monitored to study the relationship between drug concentration and effect. The results obtained demonstrated a superior capacity of the CsA LN formulation to produce a pharmacodynamic response that was not observed with Neoral® over the studied period (***Chapter 3***, Figure 3). Interestingly, LN containing Tw achieved an immediate effect that was sustained for up to 10 days, which was associated with the higher drug levels found in blood in earlier stage (***Chapter 3***, Figure 4). However, in the case of LN containing Lec:TC, no correlation was observed between CsA blood levels and pharmacodynamic response. This result suggests that the absorption

mechanism of the LN through the mesenteric lymphatic pathway [23] and further accumulation in organs related to the immune system as well as the affinity of negative surface particles for T-cells [24] might be also implicated in the enhanced biological activity. Novel formulations provided a more sustained CsA blood levels over time compared to Neoral® as observed on day 5 and 10 after administration (***Chapter 3***, Figure 4). These results suggest that LN provide a more predictable pharmacokinetic behavior, and therefore facilitate dose adjustments to reduce the risk of toxicity. This finding is crucial in particular when CsA is administered in transplantation, where subtherapeutic exposures can lead to risk of organ rejection and overexposure can result in acute or chronic toxicity.

Moreover, toxicity issues of the LN were addressed. As is widely known, in CsA therapy the most serious side effect is nephrotoxicity [25]. *In vivo* toxicity studies showed kidney alterations after 15-day treatment with 15 mg/kg of CsA, the highest dose used for humans, administrated orally in its different formulations. Renal damage caused by Neoral® and LN containing Lec:TC was characterized by a severe tubulonephrosis along with abnormalities in the convoluted tubules and glomerular hypercellularity (***Chapter 3***, Figure 5). In contrast, LN containing Tw resulted in less evident renal alterations, which indicates a certain protective effect of this vehicle against CsA-associated nephrotoxicity. This result might be explained by the drug exposed to the kidney. As previously mentioned, LN stabilized with Lec:TC are more susceptible to pH changes and these surfactants are digested faster by lipase/co-lipase system, so the majority of the drug is released and reaches the renal system in its free form, as does the liquid dosage form (Neoral®). Conversely, the resistant characteristics of LN containing Tw against the gastrointestinal environment translate into a slower release of the drug. This phenomenon could minimize the formation of metabolites which can be associated with renal injury [26], and thus diminishes CsA-related nephrotoxicity.

Finally, ***Chapter 4*** was focused on the assessment of the *in vivo* therapeutic efficacy of the novel CsA LN formulations as an important aspect to consider in the development of drug delivery systems. For this purpose, the dextran sodium sulfate (DSS)-induced colitis mouse model was selected since this immunosuppressant has been used in clinical practice as rescue therapy in severe steroid-refractory ulcerative colitis. Unfortunately, in this study, none of the tested formulations containing CsA induced an effect on decreasing the colon inflammation in terms of the parameters investigated, including myeloperoxidase expression (MPO), tumor necrosis factor (TNF)-α expression, or histological scoring at the CsA dose administered in the acute stage of the disease (***Chapter 4***, Figure 1). Our result disagreed with those observed by Fukata *et al*. [27] who reported an effect of their CsA polymeric

microspheres orally given to mice in the reduction of the colonic inflammation using the same animal model and the same CsA dose. This favorable observation has been explained by the increased retention of the microspheres in the damaged tissue and the decreased in drug permeability trough the intestinal barrier that unlike to our CsA formulations, these nanoparticles showed high drug permeability and good oral bioavailability. However, further studies are needed in order to optimize the experimental design and corroborate the efficacy of these formulations in the chronic phase of the disease.

In conclusion, CsA LN formulations appropriate for oral delivery were successfully developed and characterized. The use of biocompatible components for their preparation, the low quantities of surfactants and the avoidance of organic solvents in their composition contributed to overcome the problems inherent in the excipients contained in formulation that is currently being marketed. In addition, the new nanosytems are dried powders that could be easily incorporated in a conventional dosage form and also enhance the long-term stability of the final product. Moreover, the enhanced CsA bioavailability and the nephroprotective effect observed with LN containing Tw as well as the advantages provided by both LN formulations (LN Lec:TC and LN Tw) regarding the early pharmacological activity and the more predictable pharmacokinetics facilitate dose adjustment, and therefore minimize the risk of side effects. In this context, the novel CsA delivery systems improve the balance between effectiveness and toxicity, which represents one of the major concerns for ensuring patient safety and compliance in clinical practice. Furthermore, the novel CsA delivery systems developed in this study could be exploited for other routes of administration considering the clinical applications of CsA, in view of the versatility that LN offer when it comes to improving permeability through the various physiological barriers; this is the case, for example, with percutaneous delivery of CsA for the treatment of psoriasis or atopic dermatitis.

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CONCLUSIONS

CONCLUSIONES

1. The UHPLC-MS/MS method developed in this work for the quantification of cyclosporine A in methanol and in various biological matrices provided adequate accuracy and precision. This method was successfully applied to quantify the drug in whole blood and in various tissues after administration to mice, and also to perform quality control of lipid nanosystems.

2. Cyclosporine A was successfully incorporated into lipid nanoparticles using the method of hot homogenization followed by ultrasonication. Three nanosystems based on biocompatible materials using different stabilizing systems were developed and optimized for oral delivery. These formulations are likely to diminish the risk of excipient-related side effects compared to commercial formulations.

3. The lipid nanoparticles formulated in this study were stable for three months at 4°C. Moreover, the formulations were also stable at room temperature when the stabilizer system was composed of two surfactants. The production process provided a dried powder which can be easily incorporated into conventional oral dosage forms without compromising the immunosuppressive activity of the cyclosporine A, as suggested by the results found *in vitro.*

4. Among the three types of lipid nanoparticles developed, the nanoparticles containing phosphatidylcholine/taurocholate showed the best *in vitro* performance of cyclosporine A permeability through the Caco-2 cell monolayer compared to the marketed formulation. However, this result did not correlate with the permeability measured *in vivo.*

5. Cyclosporine A formulated in lipid nanoparticles containing Tween® 80 showed higher bioavailability than the commercial product Sandimmune Neoral®. Nevertheless, the bioavailability of the drug formulated in nanoparticles containing either Pluronic® F127/taurocholate or phosphatidylcholine/taurocholate was similar to that in the commercial formulation.

6. The distribution of cyclosporine A in mice was found to be similar for all tested formulations 24 h after a single dose administration. Given its lipophilic nature, cyclosporine A was widely distributed, accumulating mainly in liver, kidney and spleen. The accumulation in spleen may be favorable to enhance drug effectiveness.

7. The pharmacodynamic studies showed that the nanoparticles containing either Tween® 80 or phosphatidylcholine/taurocholate exhibited a superior biological response compared to Sandimmune Neoral®. Among the lipid nanosystems, the formulation containing Tween® 80 had an immediate effect, associated with higher drug levels found in blood at an earlier stage. On the other hand, these novel formulations provided a more sustained drug levels over time, which facilitates dose adjustments, thus reducing the risk of toxicity.

8. Compared to Sandimmune Neoral® or particles containing phosphatidylcholine/taurocholate, lipid nanoparticles containing Tween® 80 produced less cyclosporine A-related nephrotoxicity despite their enhanced bioavailability. This formulation therefore offers a promising alternative to the currently marketed formulations for oral administration of this immunosuppressive agent.

9. The efficacy studies of cyclosporine A lipid nanoparticles performed in the dextran sodium sulfate-induced colitis model were not conclusive. Therefore, the optimization of the experimental design or the use of an alternative animal model might be necessary to evaluate the therapeutic effectiveness of the developed nanoformulations.

1. El método de UHPLC-MS/MS desarrollado en este trabajo para la cuantificación de ciclosporina A en metanol y en diversas matrices biológicas presentó exactitud y precisión suficientes. Este método resultó adecuado para cuantificar el fármaco en sangre total y en distintos tejidos tras su administración a ratones, así como para realizar el control de calidad de los nanosistemas lipídicos preparados.

2. La ciclosporina A se incorporó con éxito en nanopartículas lipídicas empleando el método de homogeneización en caliente seguido por ultrasonicación. Se desarrollaron y optimizaron tres nanosistemas para la liberación oral del fármaco utilizando materiales biocompatibles y diferentes medios estabilizantes. En comparación con las formulaciones comerciales es probable que con estas nuevas formulaciones se disminuya el riesgo de efectos adversos asociados a los excipientes.

3. Las nanopartículas lipídicas preparadas en este trabajo fueron estables durante tres meses a 4ºC. Además, cuando el medio estabilizador contenía dos agentes tensioactivos también eran estables a temperatura ambiente. Tras el proceso de preparación se obtenía un polvo seco que podría incorporarse fácilmente a formas de dosificación oral convencionales sin comprometer la actividad inmunosupresora de la ciclosporina A, tal como sugieren los resultados obtenidos *in vitro*.

4. De los tres tipos de nanopartículas lipídicas preparados, el que contenía fosfatidilcolina/taurocolato incrementaba la permeabilidad *in vitro* de la ciclosporina A a través de la monocapa de células Caco-2 en comparación con la formulación comercial. Sin embargo, no se encontró correlación entre este resultado y la permeabilidad obtenida en los estudios realizados *in vivo*.

5. La ciclosporina A formulada en las nanopartículas lipídicas que contenían Tween® 80 presentó mayor biodisponibilidad que cuando estaba formulada como Sandimmune Neoral®. Sin embargo, la biodisponibilidad del fármaco formulado en las nanopartículas que contenían Pluronic® F127 y en las que contenían fosfatidilcolina/taurocolato fue similar a la que presentaba en dicha formulación comercial.

6. 24 h después de administrar una única dosis de cada una de las formulaciones ensayadas, la distribución de la ciclosporina A en el organismo fue similar en todos los

casos. Debido a sus características lipófilas se distribuyó ampliamente, acumulándose principalmente, en hígado, riñón y bazo. La acumulación en este último órgano podría favorecer la eficacia del fármaco.

7. Los estudios farmacodinámicos revelaron que la ciclosporina A formulada en las nanopartículas que contenían Tween® 80 y en las que contenían fosfatidilcolina/taurocolato tenía mayor capacidad para producir una respuesta biológica que si estaba formulada como Sandimmune Neoral®. Tras la administración de esos dos nanosistemas se encontró que con el primero se obtenía un efecto inmediato, que parece corresponderse con la existencia de niveles sanguíneos elevados a tiempos iniciales, a diferencia de lo observado con el segundo. Por otro lado, esos nanosistemas proporcionaron niveles sanguíneos de ciclosporina A más sostenidos a lo largo del tiempo, lo que puede facilitar los ajustes de la dosis, reduciendo así el riesgo de toxicidad.

8. Tras la administración de la ciclosporina A formulada en las nanopartículas que contenían Tween® 80 la nefrotoxicidad producida por el fármaco era menor que cuando se administraba formulado como Sandimmune Neoral® o en las nanopartículas que contenían fosfatidilcolina/taurocolato, y esto a pesar de que su biodisponibilidad era mayor cuando se administraba en la primera de dichas formulaciones. Por lo tanto, esta formulación podría ser una posible alternativa a las formulaciones existentes en el mercado para la administración oral de este agente inmunosupresor.

9. Los estudios de eficacia de las nanopartículas lipídicas de ciclosporina A, llevados a cabo en un modelo de colitis inducido por dextran sulfato sódico, no mostraron resultados concluyentes. Con el fin de evaluar la eficacia terapéutica de las nanoformulaciones desarrolladas podría ser necesario optimizar el diseño experimental o usar un modelo animal alternativo.

ANNEX

ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY METHOD FOR CYCLOSPORINE A QUANTIFICATION IN BIOLOGICAL SAMPLES AND LIPID NANOSYSTEMS

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Ultra high performance liquid chromatography–tandem mass spectrometry method for cyclosporine a quantification in biological samples and lipid nanosystems☆

M. Guada, E. Imbuluzqueta, A. Estella-Hermoso de Mendoza, H. Lana, M.C. Dios-Viéitez, M.J. Blanco-Prieto*

Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Navarra, C/Irunlarrea 1, E-31008 Pamplona, Spain

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ABSTRACT

Cyclosporine A (CyA) is an immunosuppressant cyclic undecapeptide used for the prevention of organ transplant rejection and in the treatment of several autoimmune disorders. An ultra high performance liquid chromatography–tandem mass spectrometry method (UHPLC–MS/MS) to quantify CyA in lipid nanosystems and mouse biological matrices (whole blood, kidneys, lungs, spleen, liver, heart, brain, stomach and intestine) was developed and fully validated. Chromatographic separation was performed on an Acquity UPLC® BEH C18 column with a gradient elution consisting of methanol and 2 mM ammonium acetate aqueous solution containing 0.1% formic acid at a flow rate of 0.6 mL/min. Amiodarone was used as internal standard (IS). Retention times of IS and CyA were 0.69 min and 1.09 min, respectively. Mass spectrometer operated in electrospray ionization positive mode (ESI+) and multiple reaction monitoring (MRM) transitions were detected, m/z 1220.69 → 1203.7 for CyA and m/z 646 → 58 for IS. The extraction method from biological samples consisted of a simple protein precipitation with 10% trichloroacetic acid aqueous solution and acetonitrile and 5 μL of supernatant were directly injected into the UHPLC–MS/MS system. Linearity was observed between 0.001 μg/mL–2.5 μg/mL ($r \geq 0.99$) in all matrices. The precision expressed in coefficient of variation (CV) was below 11.44% and accuracy in bias ranged from −12.78% to 7.99% including methanol and biological matrices. Recovery in all cases was above 70.54% and some matrix effect was observed. CyA was found to be stable in post-extraction whole blood and liver homogenate samples exposed for 6 h at room temperature and 72 h at 4 °C. The present method was successfully applied for quality control of lipid nanocarriers as well as *in vivo* studies in BALB/c mice.

1. Introduction

Cyclosporine A (CyA) is a neutral cyclic peptide consisting of 11 aminoacid residues, widely used for the prevention of transplant organ rejection and also for the treatment of autoimmune disorders such as psoriasis, rheumatoid arthritis and nephrotic syndrome. The importance of this drug as immunosuppressant was due to its selective lymphocyte inhibition action. The molecule was first isolated from the fungal extract of *Tolypocladium inflatum* [1]. Its structure and its high molecular weight (1203 Da) confer poor biopharmaceutical properties on this substance such as low water solubility and low permeability through biological barriers (gastrointestinal tract, skin and cornea). It is therefore a challenge to formulate an appropriate delivery system that improves its bioavailability and thus its efficacy. To date, lipid nanosystems (LN) seem to be a promising strategy to overcome the limitations associated with certain drug characteristics including low solubility, poor permeability, instability in the gastrointestinal medium, P-glycoprotein efflux and presystemic drug metabolism [2]. Scientific efforts have therefore been employed to design novel delivery systems based on LN for CyA oral administration leading to better alternatives to those currently available on the market, which will be capable of enhancing its oral bioavailability and thus its efficacy [3–5].

In the field of novel dosage form development, it is important to have a suitable drug quantification method that allows us to evaluate its behavior in animal models and its determination in the new nanosystems as quality control. Several analytical techniques for monitoring CyA levels, including immunoassays and chromatography methods, have been reported in the literature so far [6–11]. Although immunoassays offer rapid analysis and easy handling, there are many concerns about these methods

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* Corresponding author. Tel.: +34 948 425 600x6519; fax: +34 948 425 649.
E-mail address: mjblanco@unav.es (M.J. Blanco-Prieto).

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because of the cross-reactivity of the antibodies used with inactive CyA metabolites that may result in overestimation of the drug values [12–14]. For this reason, chromatography-based methods such as high performance liquid chromatography with ultraviolet detection (HPLC-UV), high performance liquid chromatography coupled to mass spectrometry detection (HPLC–MS) and ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS), are considered the best options for the quantification of CyA in biological matrices [15–17]. HPLC-UV has some limitations related to specificity and sensibility. The lack of chromophores in CyA structure implies the use of short-wavelength light detection (e.g. 205 nm, 210 nm) where many molecular species also absorbs, yielding to interferences with the quantification of the drug studied [18]. Consequently, laborious and time-consuming extraction procedures and chromatographical separations are required to improve the limit of drug quantification. During the past decade mass detection has attracted more interest since quantification is based on the relationship of mass/electric charge of the molecule and thus all compounds with different mass to the target do not interfere in the measurement [16]. This leads to both reduced sample preparation and chromatographical separation efforts in complex matrices. Currently, UHPLC–MS/MS has become an attractive alternative to the quantification of this immunosuppressant because it offers multiple advantages: shorter analysis time, lower solvent consumption, minimal sample pretreatment and also a higher range of measurement and throughput of samples. In addition, this technique is considered the best choice in pharmacokinetic studies due to its sensitivity to detect low concentrations of drug after a unique dose and in a long period of time and its specificity to detect it in complex biological matrices [19]. There are so far no validated UHPLC/MS–MS methods that allow the assessment of CyA tissue distribution in animal models when the drug is administered encapsulated in novel delivery nanosystems. Based on this, the main objective of the present research work was to develop and validate a simple, rapid, sensitive and specific UHPLC/MS–MS method to quantify CyA in lipid matrices and also in biological samples (whole blood, kidneys, lungs, spleen, liver, heart, brain, stomach and intestine) using the same analysis conditions, and to study the pharmacokinetic and biodistribution behavior of the drug *in vivo* as well as the quality control of the new lipid nanocarriers developed.

2. Experimental

2.1. Chemicals and reagents

CyA was provided by Roig Farma S.A. (Barcelona, Spain). Amiodarone hydrochloride (the internal standard, IS) and formic acid 99% were obtained from Sigma–Aldrich (Madrid, Spain). Methanol and acetonitrile (both HPLC grade) were supplied by Merck (Barcelona, Spain). Ammonium acetate (HPLC grade) was purchased from Scharlau (Sentmenat, Spain). Trichloroacetic acid was obtained from Panreac Quimica S.A. (Barcelona, Spain). Sandimmun Neoral® 100 mg/mL oral solution and Sandimmun® 50 mg/mL intravenous solution were provided by Novartis Pharmaceutical (Barcelona, Spain). Polyvinyl alcohol (PVA) (87%–89% hydrolyzed, typical MW 13,000–23,000) was obtained from Sigma–Aldrich (Madrid, Spain). Precirol® ATO 5 was a gift from Gattefossé (Lyon, France). Other reagents employed for analysis were of analytical grade. Type I deionized water (18.2 MΩ resistivity) was obtained using a water purification system (Wasserlab, Pamplona, Spain). Nitrogen gas (ultra-pure, >99%) was produced by a Domnick Hunter LCMS series (Madrid, Spain). Argon gas (ultra-pure, >99.9%) was provided by Praxair (Madrid, Spain).

2.2. Instruments and analysis conditions

The UHPLC system consisted of an Acquity UPLC™ system (Waters Corp., Milford, USA) with thermostatized autosampler and a column compartment. Chromatography was performed on an Acquity UPLC® BEH C18 column (50 mm × 2.1 mm, 1.7 μm; Waters, USA) equipped with an Acquity UPLC® BEH C18 VanGuard™ pre-column cartridge (5 mm × 2.1 mm, 1.7 μm; Waters, USA), using a gradient elution program. The mobile phase system consisted of 2 mM ammonium acetate aqueous solution with 0.1% formic acid (A) and methanol (B). The gradient program was: initial – 0.6 min 80% B; 0.61–2 min 95% B, 2.1–3 min 80% B with a flow rate of 0.6 mL/min. Total run time was 3 min. Column temperature was maintained at 50 °C and the autosampler was thermostatized at 4 °C. The volume injected was 5 μL.

Mass spectrometric detection was achieved on an Acquity™ TQD (Triple Quadrupole Detector) mass spectrometer (Waters Corp., Milford, USA) with an electrospray ionization (ESI) interface. The mass spectrometer was operated in positive mode and set up for multiple reaction monitoring (MRM) to monitor the transition of m/z 1220.69 → 1203.7 for CyA and the transition of m/z 646 → 58 for amiodarone with the dwell time of 0.1 s per transition. To optimize the mass parameters, standard solutions of both the analyte and the IS were infused into the mass spectrometer at a concentration of 10 μg/mL. The following optimized mass parameters were employed: 135 °C source temperature, 420 °C desolvation temperature, 2.5 kV capillary voltage and 30 V cone voltage for both CyA and IS. Nitrogen was used for the desolvation and as cone gas at a flow rate of 600 L/h and 60 L/h, respectively. Argon was used as the collision gas. The optimized collision energy was 20 eV and 50 eV, for CyA and IS, respectively. Under these conditions, CyA and IS were eluted at 1.09 ± 0.02 min and 0.69 ± 0.02 min, respectively. Data acquisition and analysis were performed using the MassLynx™ NT 4.1 software with QuanLynx™ program (Waters Corp., Milford, USA).

2.3. Preparation of standard and quality control (QC) solutions

2.3.1. Stock and working solutions

Stock solutions of CyA and IS were prepared in methanol at 100 μg/mL. Further dilutions in methanol were performed to obtain working solutions of CyA at 0.005, 0.0125, 0.025, 0.5, 2.5, 5 and 12.5 μg/mL. Quality controls were prepared in the same way as calibrators at four different concentrations of 0.005, 0.015, 7.5 and 10 μg/mL (Low Limit of Quantification (LLOQ) and low, medium and high concentration, respectively) from independent stock solutions. The working solution of IS was obtained by diluting the stock solution with methanol until a concentration of 0.25 μg/mL. Lastly, the working solutions and QC were stored at 4 °C and were brought to room temperature before use.

2.3.2. Standard solutions and QC samples for whole blood and tissue samples

Calibration samples were prepared by spiking 20 μL of each CyA and IS working solution to 100 μL of mouse blank whole blood or tissue homogenate, therefore providing drug concentrations of 0.001, 0.0025, 0.005, 0.1, 0.5, 1 and 2.5 μg/mL. QC samples were prepared in the same way as calibration samples at concentrations of 0.001 μg/mL (LLOQ), 0.003 μg/mL (low), 1.5 μg/mL (medium) and 2 μg/mL (high). The solutions obtained (calibrators and QC) were then processed following the extraction procedure described in Section 2.4.

2.3.3. Lipid nanoparticles

Calibration samples were prepared by diluting the working solutions of CyA in methanol in order to obtain drug concentrations of

0.001, 0.0025, 0.005, 0.1, 0.5, 1 and 2.5 μg/mL. Next, an aliquot of 20 μL of IS working solution (0.25 μg/mL) was added. QC samples were prepared in the same way as calibration samples, obtaining concentrations of 0.001 μg/mL (LLOQ), 0.003 μg/mL (low), 1.5 μg/mL (medium) and 2 μg/mL (high). Finally, an aliquot of 5 μL of calibrators and QC samples were injected into the UHPLC system.

2.4. Sample preparation

2.4.1. Whole blood samples

Whole blood samples from BALB/c mice were collected in EDTA-K3 surface-coated tubes, stored at −80 °C until analysis and thawed to room temperature before use. To an aliquot of 100 μL of whole blood was added 20 μL of methanol and 20 μL of IS working solution. After vortex mixing for 30 s, 10 μL of 10% trichloroacetic aqueous solution were added and vortex-mixed for 30 s for protein precipitation. Then, 50 μL of acetonitrile were added to the mixture and vortex-mixed for 1 min. Samples were then centrifuged at 14,500 × *g* for 15 min, and the supernatant was filtered (0.22 μm centrifugal filter, Millipore Corp., Billerica, USA). Last, 5 μL were injected into the UHPLC system.

2.4.2. Tissue samples

Tissue samples were weighed and homogenized in 1 mL of phosphate buffered saline 10 mM (PBS) using a Mini-bead Beater (BioSpect Products, Inc., Bartelsville, USA) and centrifuged at 10,000 × *g* for 10 min. The supernatant was separated, stored frozen at − 80 °C until analysis and thawed to room temperature before use. To 100 μL of tissue homogenate were added 20 μL of methanol and 20 μL of IS working solution and votex-mixed 30 s. Then, 10 μL of 10% trichloroacetic aqueous solution were added to the mixture and vortex-mixed for 30 s. An aliquot of 50 μL of acetonitrile was added to the mixture. After vortex-mixing for 1 min and centrifuging at 14,500 × *g* for 10 min the supernatant was filtered (0.22 μm centrifugal filter, Millipore Corp., Billerica, USA) and 5 μL were injected into the UHPLC system.

2.4.3. Lipid nanoparticles

A 500 μL aliquot of chloroform was added to 5 mg of lyophilized nanoparticles. Then, 1.5 mL of methanol were added to the mixture. After vortex-mixing for 30 s and centrifuging at 21,000 × *g* for 10 min, to an aliquot of 100 μL of the supernatant was added 900 μL of methanol. Finally, 100 μL of this final solution were mixed with 20 μL of IS working solution (0.25 μg/mL) and 80 μL of methanol and 5 μL aliquot were injected into the UHPLC system for analysis.

2.5. Method validation

The analytical method was validated on biological matrices and methanol, including selectivity, linearity, precision and accuracy, recovery, matrix effect and stability according to FDA guidelines [20].

2.5.1. Selectivity

The selectivity of the method was evaluated by analyzing blank (whole blood, tissue homogenate and methanol) and spiked samples at LLOQ level to compare interferences at retention times of CyA and IS. Absence of the peaks at the retention times of the analytes in blank samples must be observed. In any case, the response does not exceed 20% of the LLOQ for CyA and 5% for the IS.

2.5.2. Linearity

The linearity of the present method was evaluated by analyzing calibration samples at concentrations ranging from 0.001 μg/mL to 2.5 μg/mL in duplicate on three different validation days, using the internal standard method. Standard curves were calculated using a linear weighted ($1/x^2$) least squares regression between the peak area ratios of the CyA to the IS and the theoretical CyA concentrations on calibration samples. LLOQ was defined as the lowest concentration of CyA quantified with an acceptable precision and accuracy (less than 20%).

2.5.3. Precision and accuracy

Within-day precision and accuracy were assessed with the analysis of five determinations ($n = 5$) of four QC samples at concentrations of 0.001 μg/mL (LLOQ), 0.003 μg/mL (low), 1.5 μg/mL (medium) and 2 μg/mL (high) on a single run. Between-day precision was also assessed with the analysis of four determinations of the four QC samples in five different validation days. Within- and between- day precisions were evaluated as a function of the coefficient of variation (CV), whereas accuracy was expressed as a function of deviation from theoretical values.

2.5.4. Dilution integrity

A dilution integrity experiment was performed in order to validate the dilution to be carried out on samples with CyA concentrations above the upper limit of quantification. Dilution integrity was evaluated by diluting at 1:15 and 1:3 a stock CyA solution in whole blood or stomach homogenate with blank matrix to give a theoretical concentration of 2 μg/mL. The precision and accuracy for diluted samples were determined by analyzing the samples against calibration curve standards. Dilutional integrity was considered acceptable if precision and accuracy of replicate ($n = 5$) values varied by less than 15%.

2.5.5. Recovery and matrix effect

The extraction recoveries and matrix effect were evaluated on whole blood and tissue samples in three replicate measurements ($n = 3$) at three different concentration levels (0.003 μg/mL, 1.5 μg/mL and 2 μg/mL). Extraction recovery was calculated as the ratio of the mean peak areas from blank samples spiked with CyA before extraction and the mean peak areas of blank-processed samples spiked after extraction. Matrix effect was measured as the ratio of the mean of the peak areas obtained from blank-processed samples spiked with CyA after extraction and the mean of the peak areas obtained from water and extraction solutions spiked with CyA corresponding to the equivalent amount injected on the system.

2.5.6. Stability

The stability study of CyA was performed in whole blood and liver homogenate samples after extraction. First, QC samples were prepared in triplicate at two levels of concentration (0.003 μg/mL and 2 μg/mL). Then, samples were stored under different conditions, at room temperature for 6 h and at 4 °C for 72 h. Finally, samples were analyzed and CyA was considered stable when accuracy biases of the stored QC samples were within ±15% of a freshly prepared standard curve.

2.6. Application of the method

2.6.1. Pharmacokinetic and biodistribution studies

To demonstrate the applicability of this validated UHPLC–MS/MS method, the quantification of CyA in whole blood and tissue samples from BALB/c mice was performed. Mice were divided in two groups ($n = 4$) and treated with two commercial available formulations, Sandimmun® intravenously (i.v.) and Sandimmun Neoral® orally (p.o.). The administration in both cases was in a single dose equivalent to 10 mg of CyA per kg of body weight. Whole blood samples were withdrawn at 0 h (only for i.v. administration), 1, 2, 5, 8, 24 and 48 h post-administration, collected in EDTA-K3 surface-coated tubes and stored frozen (−80 °C) until analysis. Then the animals were

sacrificed and kidneys, lungs, spleen, liver, heart, brain, stomach and intestine were collected, weighed and homogenized as previously described in Section 2.4.2 in order to study CyA tissue distribution.

2.6.2. Determination of CyA encapsulated in lipid nanosystems

The present method was also used to evaluate the encapsulation efficiency and loading capacity of a lipid nanoparticle formulation (CyA-LN). The formulation was prepared by hot-homogenization followed by ultrasonication method previously described with slight modifications [21]. Precirol® ATO 5 was used as lipid and a 2% PVA aqueous solution was used as surfactant. Triplicate samples were processed as described previously in Section 2.4.3 and analyzed for drug quantification.

3. Results and discussion

3.1. Development of the analytical method

The main purpose of developing and optimizing the present UHPLC method was to quantify CyA, either in lipid nanoparticles or biological samples, under the same analysis conditions. Moreover, another intended purpose was to reduce, where possible, the lower limit of quantification for samples collected towards the end of the sampling schedule in pharmacokinetic and biodistribution studies after drug administration.

During the preliminary assays, different columns were tested including Zorbax C8, Kinetex HILIC and Acquity UPLC® BEH C18. Likewise, different mobile phases, organic solvents (methanol and acetonitrile) and aqueous solutions containing ammonium acetate with formic acid or acetic acid at different concentrations and different ratios were also tested. In addition, different flow rates, isocratic and gradient elutions and column temperatures were studied. All these conditions were assayed in order to obtain the best peak resolution and shorter retention time of CyA. The optimal chromatography conditions for CyA and amiodarone elution were achieved in an Acquity UPLC® BEH C18 column using as mobile phase methanol and 2 mM ammonium acetate aqueous solution with 0.1% formic acid, in the gradient profile described in Section 2.2, a flow-rate of 0.6 mL/min and column temperature set at 50 °C. Similar mobile phase compositions have been successfully used previously in the literature [22,23]. High peak resolution and symmetry of CyA and IS were achieved. Although the retention times were 0.69 ± 0.02 min and 1.09 ± 0.02 min for IS and CyA, respectively, it was necessary to prolong the total run analysis for 3 min to stabilize the system pressure. Further, the chromatogram showed a rise in the baseline at about 1 min, as is commonly observed when using gradient elution. In the case of both lipid and biological matrices, no significant interferences were observed in the retention times of the analytes in their respective transitions, m/z 1220.69 → 1203.7 for CyA and m/z 646 → 58 for IS (Fig. 1).

Moreover, since cyclosporine structural analogs are not commercially available, it was necessary to investigate other substances with physicochemical properties similar to CyA. Three different analytes, including rifampicin (MW 823), anidulafungin (MW 1140) and amiodarone (MW 645), were tested as internal standards. Evaluation of the fragmentation was performed by the infusion of each analyte into the mass spectrometer at a concentration of 10 μg/mL. Amiodarone showed the highest mass spectrometric response after the molecule fragmentation in the conditions assayed. Moreover, amiodarone has been used as an internal standard in previous studies where CyA was analyzed using HPLC–MS [24].

3.2. Mass spectrometry

Maximum sensitivity was observed by monitoring the fragmentation of CyA ammonium adducts $[M+NH_4]^+$, which by deamination, at low collision energy (20 eV), forms the protonated molecule $[M+H]^+$ in high abundance. The transition monitored was m/z 1220.69 → 1203.7. Moreover, a higher collision energy for amiodarone was needed (50 eV) in order to obtain the fragmentation of the ammonium adduct $[M+NH_4]^+$, resulting in the abundant fragment ion m/z 58 attributed to two ethyl groups attached to the tertiary nitrogen present in the molecule. In this case the transition monitored was m/z 646 → 58.

3.3. Sample preparation

Pre-treatment of the biological matrices is important since an inadequate procedure can, firstly, shorten the life time of the column when a large amount of samples are analyzed and, secondly, interfere with endogenous compounds present in the sample. Even though liquid–liquid extraction was widely used in the past for CyA extraction [11,24,27–29], the protein precipitation procedure was chosen among the extraction techniques, as it is a fast, simple sample preparation process. The first attempt in this study was to use acetonitrile as a unique precipitation reagent and this resulted in damage to the column. As a result, the novel aspect of the sample preparation technique proposed in this paper is the addition of trichloroacetic acid as a precipitation reagent, which has not been previously published in CyA detection methods. It was observed that an aliquot of 10% trichloroacetic acid aqueous solution improved protein precipitation. This led to a reduction in the organic solvent volumes needed to precipitate proteins reported by other authors (methanol with zinc sulfate or acetonitrile) [7,9,18,25,26], enabling the direct injection of the final supernatant for analysis and consequently improved the lower limit of quantification. The use of trichloroacetic acid as precipitation reagent has not been previously published in CyA detection methods. In this case, CyA extraction method was a simple and fast protein precipitation procedure, avoiding time-consuming and labor-intensive extraction steps in solid phase extraction or liquid–liquid extraction (evaporation and reconstitution) used by other authors [11,24,27–29]. Also, the specificity of the MS/MS detector contributed to simplify sample preparation.

3.4. Method validation

3.4.1. Selectivity

Free-drug whole blood and tissue samples from different sources were tested in order to observe if there were endogenous components that could interfere in the analysis. These chromatograms were compared with chromatograms obtained from blank whole blood and tissue samples spiked with IS and CyA at the concentration of the LLOQ. No significant interferences were observed at the retention times of CyA and IS. Fig. 1 shows chromatograms of blank samples, spiked whole blood and tissue samples with IS and CyA at the LLOQ analyzed by the UHPLC–MS/MS technique.

3.4.2. Linearity

The calibration curves of CyA in methanol and biological matrices were linear in the concentration range of 0.001 μg/mL–2.5 μg/mL with r-values ≥0.99 (n = 3). The mean ± SD of the regression curve equations for all the matrices are shown in Table 1. Calibrator concentration calculated from these regression curve equations expressed in terms of coefficient of variation did not exceed 15% in any case. The lower limit of quantification of the

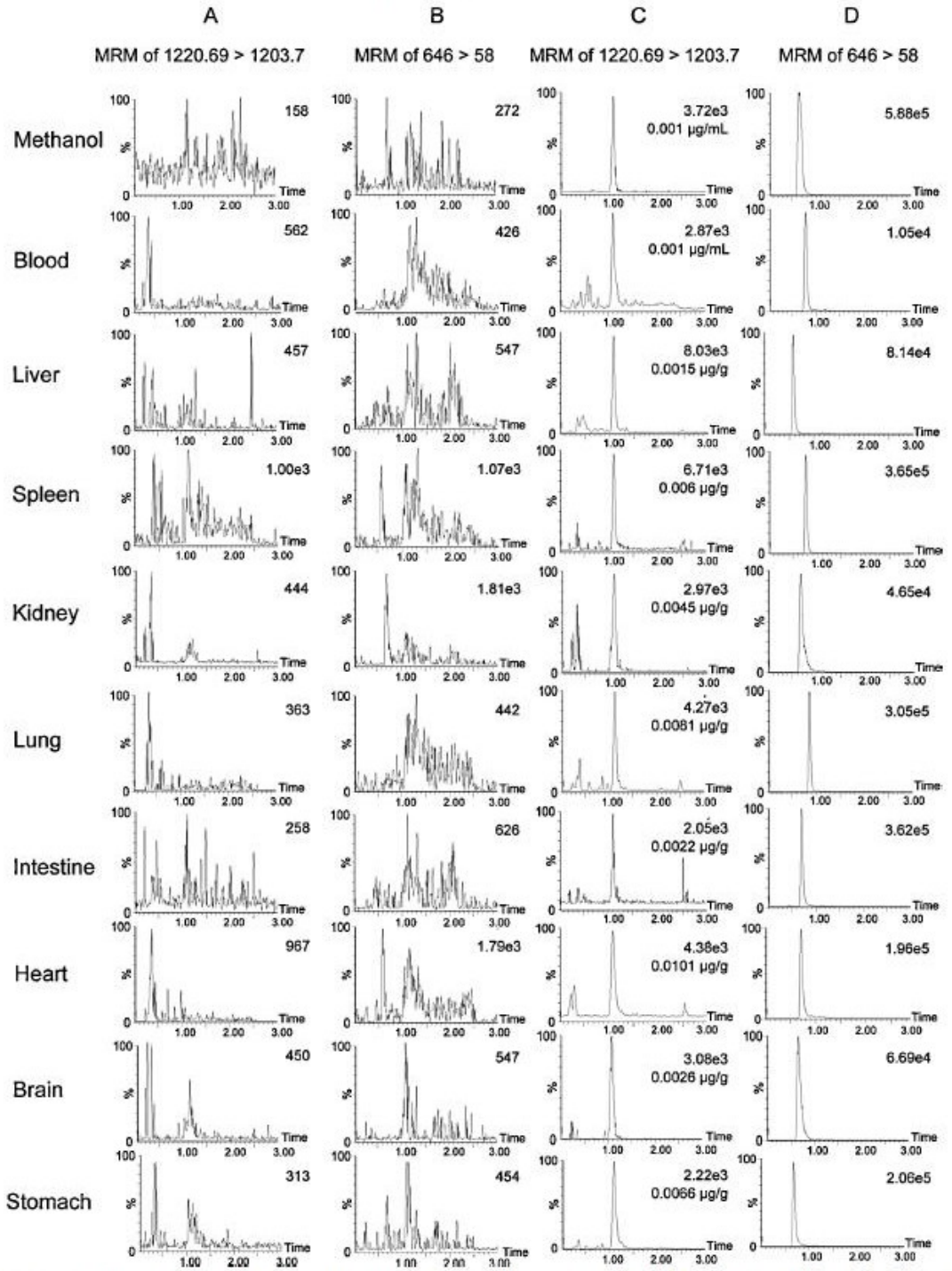

Fig. 1. MRM transition chromatograms of blank samples at (A) *m/z* 1220.69→ 1203.7 and (B) *m/z* 646→ 58; MRM transition chromatograms of blank samples spiked with cyclosporine A at the LLOQ and amiodarone used as internal standard (0.25 µg/mL) at (C) *m/z* 1220.69→ 1203.7 and (D) *m/z* 646→ 58.

Table 1
Standard calibration curves of cyclosporine A calculated by the UHPLC–MS/MS method.

Range	Slope (SD)	Intercept (SD)	r
Methanol 0.001–2.5 (μg/mL)	3.073 (0.127)	0.001 (0.003)	0.994
Blood 0.001–2.5 (μg/mL)	8.149 (0.678)	0.030 (0.028)	0.996
Liver[a] 0.001–2.5 (μg/mL) 0.0015–3.7 (μg/g)	14.097 (1.666)	0.016 (0.006)	0.998
Spleen[a] 0.001–2.5 (μg/mL) 0.0060–15.1 (μg/g)	7.471 (0.314)	0.014 (0.006)	0.998
Kidney[a] 0.001–2.5 (μg/mL) 0.0045–11.2 (μg/g)	14.410 (0.613)	0.012 (0.008)	0.998
Lung[a] 0.001–2.5 (μg/mL) 0.0081–20.3 (μg/g)	8.005 (0.835)	0.009 (0.003)	0.996
Intestine[a] 0.001–2.5 (μg/mL) 0.0022–5.5 (μg/g)	7.836 (1.117)	0.008 (0.005)	0.998
Heart[a] 0.001–2.5 (μg/mL) 0.0101–25.2 (μg/g)	6.450 (1.000)	0.011 (0.004)	0.994
Brain[a] 0.001–2.5 (μg/mL) 0.0026–6.5 (μg/g)	10.720 (1.487)	0.004 (0.004)	0.998
Stomach[a] 0.001–2.5 (μg/mL) 0.0066–16.4 (μg/g)	7.212 (1.536)	0.002 (0.001)	0.998

[a] Concentrations of tissues are expressed as μg/mL tissue homogenate. The equivalence in μg/g tissue is given in the second line.

drug in all samples was 0.001 μg/mL, which is sensitive enough to allow the quantification of CyA in complete pharmacokinetic and biodistribution studies in mice or even in other species. The present method covers a larger range of concentrations compared to those recently published and also decreases the LLOQ [11,22,23,30].

3.4.3. Precision and accuracy

Between- and within- day precision and accuracy values obtained from the four QC samples tested in methanol and biological matrices are summarized in Table 2. The precision varied from 1.44% to 11.44%, while the accuracy varied from −12.78% to 7.99% in all cases. This was within the FDA acceptance criteria (<15%).

3.4.4. Dilution integrity

Dilution integrity was evaluated at two dilution factors, 1:3 for stomach samples and 1:15 for whole blood samples, at five determinations for each factor. The precision for dilution integrity 1:15 and 1:3 were 7.26% and 6.14%, respectively, while the accuracy results were 10.35% and 9.64%, respectively, which are within the acceptance limit of 15%.

3.4.5. Recovery and matrix effect

In general, high extraction recoveries of CyA were obtained from the biological samples studied. This was above 98.72% from whole blood samples, values comparable to those reported by other groups [22,31]. Recoveries from homogenate tissue samples were about 85.12 ± 7.71% (Table 3). Moreover, some matrix effect with variability among the same tissue was observed. Percentages up to 64.61% in whole blood and up to 25.60% in tissue homogenates were obtained, meaning an ion suppression effect (Table 3). This matrix effect could be explained by the presence of undetectable co-eluting compounds with the analytes in the post-extraction sample and, in the case of tissues, also the PBS used to homogenize the samples. Bogusz et al. found a signal suppression caused primarily by zinc sulfate used in deproteinizing solution [26]. However, in the present method CyA diluted in extraction solution was evaluated and showed higher peak intensity than that obtained from blank-processed samples spiked with the drug. Therefore, it was noted that reagents used to precipitate proteins were not responsible for the matrix effect. On the other hand, when a small portion of solvent (1:1) is used in protein precipitation methods endogenous compounds, including fatty acids, triglycerides, nucleotides and salts will be present in the post-treated sample and could interfere in the ionization of the analyte leading to ion suppression [32]. Furthermore, since CyA is mainly bound to erythrocytes,

Table 2
Accuracy, precision and between- and within- day measured concentrations for analysis of cyclosporine A quality controls by the UHPLC–MS/MS method.

Matrix	Conc. (μg/mL)	Measured concentration (μg/mL, mean ± S.D.)				Accuracy (bias %)	Precision (CV)	
		Between-day (5 batches, n = 21)		Within-day (1 batch, n = 5)			Between-day (5 batches, n = 21)	Within-day (1 batch, n = 5)
		Mean	SD	Mean	SD			
Methanol	0.001	0.00100	0.00005	0.00097	0.00006	−2.65	5.50	5.77
	0.003	0.00306	0.00020	0.00295	0.00015	−1.80	6.39	5.18
	1.5	1.46568	0.11242	1.35848	0.05636	−9.43	7.67	4.15
	2	1.91466	0.15061	1.75294	0.03146	−12.35	7.87	1.79
Blood	0.001	0.00099	0.00008	0.00100	0.00008	0.50	8.03	8.02
	0.003	0.00280	0.00018	0.00270	0.00013	−10.01	6.33	4.92
	1.5	1.42530	0.11066	1.40398	0.11017	−6.40	7.76	7.85
	2	1.92910	0.15725	1.98038	0.20177	−0.98	8.15	10.19
Liver[a]	0.001	0.00097	0.00006	0.00099	0.00006	−0.60	6.23	6.39
	0.003	0.00290	0.00019	0.00302	0.00020	0.80	6.47	6.65
	1.5	1.43778	0.06443	1.41420	0.05821	−5.72	4.48	4.12
	2	1.91775	0.10919	1.90058	0.07760	−4.97	5.69	4.08
Spleen[a]	0.001	0.00099	0.00006	0.00102	0.00005	1.56	5.89	4.58
	0.003	0.00305	0.00019	0.00315	0.00013	4.93	6.24	4.09
	1.5	1.54094	0.06850	1.55464	0.07691	3.64	4.45	4.95
	2	2.05017	0.10382	2.09398	0.05937	4.70	5.06	2.84
Kidney[a]	0.001	0.00099	0.00006	0.00098	0.00007	−1.58	5.81	6.77
	0.003	0.00298	0.00019	0.00289	0.00018	−3.64	6.28	6.29
	1.5	1.48875	0.10638	1.40118	0.08605	−6.58	7.15	6.14
	2	1.96596	0.13026	1.83016	0.03118	−8.50	6.63	1.70

Table 2 (*Continued*)

Matrix	Conc. (μg/mL)	Measured concentration (μg/mL, mean ± S.D.)				Accuracy (bias %)	Precision (CV)	
		Between-day (5 batches, n = 21)		Within-day (1 batch, n = 5)			Between-day (5 batches, n = 21)	Within-day (1 batch, n = 5)
		Mean	SD	Mean	SD			
Lung[a]	0.001	0.00104	0.00008	0.00108	0.00006	7.99	7.63	5.17
	0.003	0.00279	0.00016	0.00266	0.00004	−11.32	5.84	1.44
	1.5	1.45115	0.14113	1.37534	0.08889	−8.31	9.73	6.46
	2	1.89224	0.21649	1.74432	0.06739	−12.78	11.44	3.86
Intestine[a]	0.001	0.00099	0.00006	0.00101	0.00004	0.87	5.88	3.69
	0.003	0.00300	0.00018	0.00318	0.00005	5.99	6.13	1.49
	1.5	1.51636	0.08029	1.45500	0.10010	−3.00	5.29	6.88
	2	1.96950	0.11312	1.97050	0.10770	−1.48	5.74	5.47
Heart[a]	0.001	0.00101	0.00006	0.00102	0.00007	1.54	5.55	6.43
	0.003	0.00296	0.00017	0.00299	0.00023	−0.47	5.60	7.85
	1.5	1.53956	0.08761	1.44026	0.10444	−3.98	5.69	7.25
	2	2.06784	0.13069	2.10030	0.15485	5.02	6.32	7.37
Brain[a]	0.001	0.00100	0.00006	0.00097	0.00004	−2.52	5.53	4.18
	0.003	0.00291	0.00013	0.00297	0.00013	−1.13	4.62	4.33
	1.5	1.45232	0.08648	1.48638	0.13211	−0.91	5.95	8.89
	2	1.99141	0.11251	2.11942	0.06915	5.97	5.65	3.26
Stomach[a]	0.001	0.00101	0.00006	0.00101	0.00007	1.03	6.17	6.50
	0.003	0.00308	0.00017	0.00313	0.00016	4.25	5.41	4.97
	1.5	1.48640	0.08477	1.48016	0.05099	−1.32	5.70	3.45
	2	1.98460	0.14326	1.96186	0.10753	−1.91	7.22	5.48

[a] Concentrations of tissues expressed as μg/mL tissue homogenate.

whole blood is the matrix of choice for drug determination [33], so that more interferences are present in the post-treated sample. As was expected, variability in matrix effect occurred because the validation was performed in biological matrices obtained from different subjects, and therefore it was impossible to generate samples containing the same endogenous compounds in the matrices after the CyA extraction [34]. Despite these factors, it was proved that the precision, accuracy, selectivity, sensitivity and even reproducibility of this developed method were not compromised by the matrix effect.

3.4.6. Stability

Post-extraction stability of CyA in whole blood and liver homogenate samples was studied in a variety of situations, including being on bench top for 6 h and in the autosampler (4 °C) for 72 h. The drug was unaltered in extraction supernatant at room temperature for 6 h and at 4 °C for 72 h. All the QC samples both in whole blood and liver homogenate samples yielded accuracy biases within −12.5% and 8.7% of those of a freshly prepared standard curve (Table 4). Previous studies confirmed CyA stability in methanol for 6 months at 4 °C, in blood during three freeze/thaw cycles, up to 24 h at room temperature and in a freezer at or below −15 °C [7].

3.5. Application of the method

As we have already mentioned, there are as yet no validated UHPLC/MS–MS methods that allow the assessment of CyA tissue distribution in animal models. Therefore, this is the first time a method has been described that is sensitive enough to determine not only the biodistribution, but also the whole blood CyA concentrations needed to calculate the pharmacokinetic behavior of the drug.

3.5.1. Pharmacokinetic and biodistribution studies

The present method was successfully applied to quantify CyA in whole blood and tissue samples from BALB/c mice. Figs. 2 and 3 depict the concentration *vs* time profile of CyA in mouse whole blood after a single 10 mg/kg drug administration intravenously and orally, respectively. As shown in Fig. 2, a typical i.v. drug profile was observed; maximum concentrations were achieved almost instantaneously and a steep decline in the curve was observed in the first two hours. Concentrations subsequently declined over time due to drug elimination. The mean Cmax was 21.24 μg/mL and

Table 3
Matrix effect and extraction recovery of cyclosporine A (CyA) in whole blood and tissues.

Matrix	Conc. (μg/mL)	Matrix effect (%)	Extraction recovery of CyA (%)
Blood	0.003	64.61	98.72
	1.5	58.62	98.86
	2	58.27	99.28
Liver	0.003	8.43	89.78
	1.5	7.25	86.08
	2	11.94	92.23
Spleen	0.003	21.80	99.90
	1.5	25.60	89.22
	2	19.04	70.54
Kidney	0.003	0.02	73.30
	1.5	9.67	83.14
	2	4.47	82.64
Lung	0.003	17.97	82.96
	1.5	22.71	87.73
	2	23.78	88.66
Intestine	0.003	15.44	89.48
	1.5	14.67	96.75
	2	14.00	99.99
Heart	0.003	18.56	87.32
	1.5	21.18	83.84
	2	20.95	83.20
Brain	0.003	17.15	77.19
	1.5	16.06	76.48
	2	12.10	73.71
Stomach	0.003	5.47	84.43
	1.5	11.66	82.35
	2	4.60	81.96

Table 4
Post-extraction stability of cyclosporine A in whole blood and liver under different conditions.

	QC2 (0.003 μg/mL)			QC4 (2 μg/mL)		
	Measured concentration (μg/mL) (mean ± S.D.)	CV	Accuracy deviation (%)	Measured concentration[a] (μg/mL) (mean ± S.D.)	CV	Accuracy deviation (%)
Stability in extracted blood sample						
Autosampler stability (72 h)	0.0026 ± 0.0001	2.28	−12.23	1.9003 ± 0.0924	4.86	−5.0
Short-term stability (6 h at room temperature)	0.0033 ± 0.0001	3.16	10.36	2.1735 ± 0.1144	5.27	8.7
Stability in extracted liver sample						
Autosampler Stability (72 h)	0.0030 ± 0.0002	5.68	−0.60	1.7484 ± 0.0493	2.82	−12.5
Short-term stability (6 h at room temperature)	0.0026 ± 0.0002	6.94	−13.13	1.9743 ± 0.0313	1.58	−1.3

[a] Concentrations of liver expressed as μg/mL tissue homogenate.

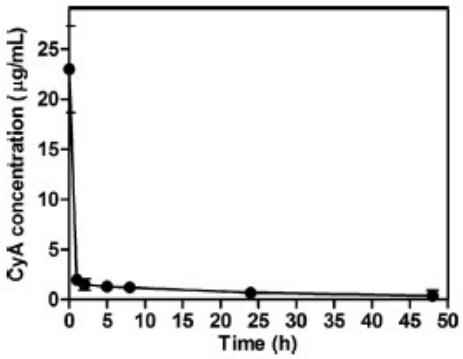

Fig. 2. Whole blood concentration–time profile of cyclosporine A (CyA) over 48 h after a single intravenous administration (10 mg/kg) of Sandimmun® to BALB/c mice (error bars represent SD, $n = 4$).

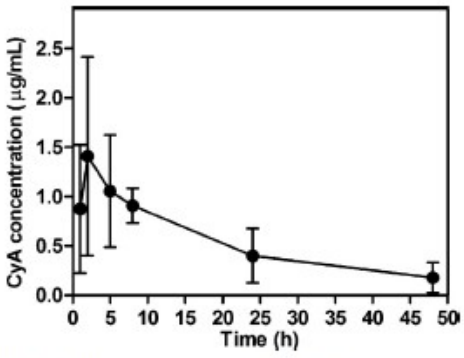

Fig. 3. Whole blood concentration–time profile of cyclosporine A (CyA) over 48 h after a single oral administration (10 mg/kg) of Sandimmun Neoral® to BALB/c mice (error bars represent SD, $n = 4$).

the mean Cmin was 0.39 μg/mL during the 48 h-experiment. Fig. 3 shows a typical concentration-time course when a drug is orally administered. In this case, the time to maximum concentration was influenced by the absorption and distribution rates. The mean Cmax 1.21 μg/mL was observed approximately 2 h post-administration and the mean Cmin was 0.15 μg/mL over the 48 h-experiment. This confirmed the applicability of the UHPLC method for the pharmacokinetic analysis of CyA after its oral administration. Fig. 4 summarizes the CyA distribution to different mouse tissues after intravenous and oral treatment with 10 mg/kg 48 h post-administration. In all cases drug tissue levels were measurable by this method. Mean values ranged from 0.028 μg/g in brain (p.o.) to 3.315 μg/g in stomach (p.o.). CyA concentrations were higher in all organs after intravenous administration compared with oral, except in the stomach.

3.5.2. Determination of CyA encapsulated in lipid nanosystems

The CyA quantification method developed in this work was also employed to determine the drug encapsulated in Precirol® ATO 5 nanoparticles (CyA-LN), thus becoming the first validated UHPLC method applied to the quality control of the preparation of lipid based drug delivery systems. After the analysis, the drug loading of the lipid nanosystem resulted in 11.94 ± 0.35 μg of CyA per mg of formulation with an encapsulation efficiency of 79.72 ± 2.35%.

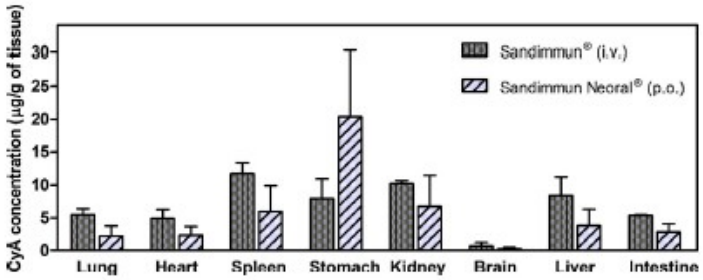

Fig. 4. Tissue distribution of cyclosporine A (CyA) at 48 h after a single intravenous (i.v.) and oral (p.o.) administration (10 mg/kg) of Sandimmun® and Sandimmun Neoral®, respectively, to BALB/c mice (error bars represent SD, $n = 4$) measured by the UHPLC–MS/MS method.

4. Conclusion

An UHPLC–MS/MS method to quantify CyA was developed and validated. The method proved to be simple, rapid, reproducible, sensitive, selective, accurate and precise in methanol and biological matrices (whole blood, kidneys, lungs, spleen, liver, heart, brain, stomach and intestine) from BALB/c mice. The biological sample pre-treatment was found to be easy and reproducible. The method developed has demonstrated its suitability for pharmacokinetic and biodistribution studies and quality control of lipid nanosystems. This bioanalytical method could also be applied in other animal species or even in human matrices.

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