Cyclodextrins as enabling excipients in ophthalmic drug delivery

Manisha Prajapati

Thesis for the degree of Philosophiae Doctor

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Ágrip

Sýklódextrín eru hjálparefni sem notuð eru lyfjaform svo sem vatnslausnir (t.d. stungulyf) og föst lyfjaform (t.d. töflur), til að auka leysanleika, stöðugleika og aðgengi lyfjanna. Í þessu verkefni var notkun sýklódextrína sem hjálparefni í augndropum rannsökuð. Rannsakað var hvernig sýklódextrín hafa áhrif á eðlisefnafræðilega eiginleika (þ.e. leysanleika og stöðugleika) lyfja og hvernig þau auka frásog lyfja í gegnum lífrænar himnur. Einnig voru rannsökuð áhrif sýklódextrína á frumur augans, svo sem áhrif þeirra á sjónhimnu augans.

Aðgengi lyfs sem gefið er á yfirborð augans er oftast aðeins 3 til 5%, það er aðeins 3 til 5% af lyfinu frásogast inn í augað. Helstu ástæður lélegs aðgengis augnlyfja frá yfirborði augans er takmarkað frásog þeirra í gegnum lífrænar himnur, svo sem í gegnum hornhimnuna. Flest lyf eru einnig fitusækin og hafa því mörg hver takmarkaðan leysanleika í vatni. Þau leysast illa upp í augndropum, sem oftast eru vatnslausnir lyfja, og tárahimnunni sem hylur yfirborð augans. Önnur stór áskorun er efnafræðilegur óstöðugleiki margra lyfja í vatnslausnum sem takmarkar geymsluþol augndropa. Hægt að nota sýklódextrín til að auga bæði stöðugleika og leysni lyfja. Í vatnslausnum mynda sýklódextrín fléttur (þ.e. komplexa) með fitusæknum lyfjum. Fitusæknar sameindir, eða fitusæknir hlutar sameindanna, setjast í op í miðju sýklódextrínsameindanna sem þá verja lyfin fyrir utanaðkomandi áhrifum í lausninni. Sýklódextrín geta einnig aukið aðgengi lyfjanna að lífrænum himnum og þar með aðgengi þeirra inn í augað. Að lokum hafa nýlegar rannsóknir sýnt að sýklódextrín geta myndað fléttur með ýmsum lífrænum efnasamböndum eins og kólesteróli. Því er ekki ósennilegt að hægt verði að nota sýklódextrín við meðhöndlun á sjónhimnusjúkdómum, eins og Stargardt-sjúkdómi og slagæðastíflu í sjónhimnu. Rannsóknir á eiturverkunum sýklódextrína á sjónhimnu eru þó takmarkaðar.

Áhrif ýmissa sýklódextrína á leysanleika og stöðugleika takrólímus og díflúprednats í vatni voru rannsökuð. Áhrifum sýrustigs (pH), sýklódextrína og fjölliða á niðurbrotsferla og niðurbrotshraða lyfjanna voru rannsökuð með vökvagreini (HPLC) og massagreini (MS). Myndun flétta lyfja og sýklódextrín var könnuð með ýmsum aðferðum svo sem NMR, DSC og FTIR. Áhrif sýklódextrína á flæði lyfja í gegnum himnur voru rannsökuð. Að lokum voru áhrif 2-hýdroxýprópýl-β-sýklódextríns (HPβCD) og metýl-β-sýklódextríns (RMβCD) á frumur sjónhimna úr músum rannsökuð.

Rannsóknir á niðurbrotshraða sýndu að takrólímus hefur hámarks stöðugleika í vatnslausn við pH á milli 4 og 6, á meðan díflúprednat er stöðugast við pH um 5. Niðurbrotið beggja lyfjanna eykst verulega ef pH-ið fer yfir 6. MS rannsóknir sýndu að vatnsrof var aðalorsök niðurbrots takrólímus í HPβCD vatnslausnum. Leysanleikarannsóknir sýndu að β-sýklódextrín (βCD) og afleiður þess mynda vatnsleysanlegar fléttur með takrólímusi og eru bestu sýklódextrínin til að auka leysanleika lyfsins í vatni, en γ-sýklódextrín (γCD) og afleiður þess voru bestar fyrir díflúprednat. Ennfremur sýndu stöðugleikarannsóknir með sýklódextrínum og mismunandi fjölliðum að stöðugleiki takrólímus í vatni jókst með blöndu HPβCD og tyloxapóli. Rannsóknir á díflúprednati gáfu svipaðar niðurstöður þar sem blanda af 2-hýdroxýprópýl-γ-sýklódextríni (HPγCD) og póloxamer 407 reyndist best. Þá voru einkenni flétta díflúprednats og HPγCD rannsökuð á bæði föstu formi og í lausn, það er díflúprednat/HPγCD fléttu og díflúprednat/HPγCD/póloxamer fléttu. NanoSight var notað til að mæla myndun díflúprednat/HPγCD nanóagna og díflúprednat/HPγCD/póloxamer nanóagna, stærð þeirra og stærðardreifingu sem hlutfall af styrk póloxamers. Niðurstöðurnar voru síðan staðfestar með rafeindasmásjárrannsóknum (TEM). Eiginleikar fléttanna á föstu formi voru rannsakaðar með DSC og FTIR. Rannsóknir á flæði hýdrókortisóns í gegnum himnur sýndu að styrkur mismunandi leysanleika aukandi efna (þ.e. HPβCD, etanóls og natríumlárýlsúlfats) hafði markverð áhrif á flæði hýdrókortisóns í gegnum himnur. Áhrifin voru tengd varmafræðilegu ástandi lyfsins í vatnslausninni. Rannsóknir á eituráhrifum sýndu að RMβCD hafði meiri eituráhrif en HPβCD, sem sjónhimnan þoldi í allt að 10 mM HPβCD styrk. Auk þess sýndu rannsóknir sem gerðar voru á flúrljómandi afleiðum sömu sýklódextrína að bæði RMβCD og HPβCD geta farið djúpt inn í sjónhimnuna.

Þótt að HPβCD hafi reynst besta sýklódextrínið fyrir takrólímus og aukið bæði leysanleika þess og stöðugleika, var aukningin ekki nægjanleg til að hægt væri að mynda takrólímus augndropa. Leysaleiki díflúprednats í vatnslausnum sem innihéldu HPγCD nægði til að mynda augndropa sem innihalda 0,1% díflúprednat, sem er tvöfalt meira magn en í þeim díflúprednat augndropum sem nú eru á markaði. Frekari rannsóknir þarf þó að gera til að fullhanna augndropana.

Abstract

Cyclodextrins (CDs) have been widely used as pharmaceutical excipients for formulation purposes for different drug delivery systems. Recent developments have explored their use in ophthalmic drug delivery to overcome the challenges faced due to the anatomical and physiological barriers of the eye. Other big challenges in the ocular delivery are the poor aqueous solubility and chemical instability of many drugs in aqueous solutions and the short retention time. As we know, CDs can be used to improve the stability and solubility of hydrophobic drugs in aqueous CD solutions through the formation of drug/CD complexes. The lipophilic molecules of parts of larger drugs can enter the CD cavity and hence can be protected from external factors in the solution. Not only this but the use of CDs as solubilisers has helped in the permeation properties of different hydrophobic drugs. In addition to this, recent studies have shown that CDs are able to form complexes with a variety of biomolecules, such as cholesterol. This has subsequently paved the way for the possibility of using CDs in retinal diseases, such as Stargardt disease and retinal artery occlusion, where CDs could absorb cholesterol pumps, but the studies on the retinal toxicity of CDs are limited.

This study aimed to explore the applicability of CDs in ophthalmic delivery as solubilisers/stabilisers for various hydrophobic drugs (tacrolimus and difluprednate) to develop a vehicle for aqueous eye drops, their concentration effect on important parameters for formulation like permeation, and to examine the retinal toxicity of different CDs and their localisation within retinal tissues.

Phase-solubility and kinetics studies were performed in the presence of different CDs at different pH values for tacrolimus and difluprednate. A mass spectrometric (MS) study was also done to elucidate the degradation mechanism of the tacrolimus in an aqueous CD solution. Furthermore, stabilisation studies were done with CD and different polymers to further improve the stability of these drugs. After this, the characterisations of drug/CD complexes were done using techniques like NMR, DSC, and FTIR. Permeation studies were done with hydrocortisone to study the effect of different solubiliser concentrations on the permeation. Finally, cytotoxicity studies with 2-hydroxypropyl-βCD (HPβCD) and randomly methylated β-cyclodextrin (RMβCD) were done using wild-type mouse retinal explants, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, and fluorescence microscopy.

Kinetics studies showed that tacrolimus has a maximum stability between pH 4 and 6 while difluprednate has one at around pH 5. The degradation is

vigorous in basic conditions after pH 6 for both drugs. The MS studies showed that hydrolysis was the main cause of tacrolimus degradation in aqueous HPβCD solutions. Phase-solubility studies revealed that βCD and its derivatives were good solubilisers for tacrolimus, while γCD and its derivatives for difluprednate. Furthermore, stability studies with CD and different polymers showed that the stability of tacrolimus was improved with the combination of HPβCD and tyloxapol. But still, tacrolimus degradation was around more than 30% with 5% HPβCD and 2% tyloxapol. A similar trend was observed in the case of difluprednate where the drug degradation was less than 1% in the combination system with 15% HPγCD and 4% poloxamer 407. Studies with tacrolimus were not continued due to the unsatisfactory stability results. After this, the characterisations of drug/CD complex in solid- and solution-states were done in binary (difluprednate/HPγCD) and ternary (difluprednate/HPγCD/poloxamer) systems. NanoSight was used to measure the drug/CD and drug/CD/polymer aggregates with observed increasing micelles aggregate size with an increasing poloxamer concentration. This was confirmed by transmission electron microscopy (TEM) studies. The solid-state characterisation of these binary and ternary complexes done by using DSC and FTIR showed the disappearance of the characteristic peaks confirming the presence of an inclusion complex in our system.

Permeation studies showed that the concentration of different solubilisers (HPβCD, ethanol, and sodium lauryl sulfate; SLS) had a pronounced effect on the permeation of hydrocortisone across different membrane barriers. This was shown by the flux profiles where we observed decreasing flux values when the concentration of HPβCD and ethanol was increased/decreased from the concentration when hydrocortisone was in a saturated solution. The permeation of hydrocortisone in SLS system revealed a different profile with decreasing flux over increasing SLS concentration.

Cytotoxicity studies showed that RMβCD was more toxic to retinal explants when compared to HPβCD, which the retina can safely tolerate at levels as high as 10 mM. Additionally, studies conducted with fluorescent forms of the same CDs showed that both CDs can penetrate deep into the inner nuclear layer of the retina, with some uptake by Müller cells.

To conclude, even though HPβCD was a better solubiliser/stabiliser for tacrolimus among the CDs tested, tacrolimus was not adequately chemically stable to be formulated as aqueous eye drops. In the case of difluprednate, it was possible to solubilise 0.1% difluprednate in an aqueous HPγCD solution, which is twice as much as the commercially available eye drops, and stabilise

in an aqueous solution using a combination of CD and polymer. However, in order to formulate into eye drops, further studies with different excipients and different parameters are needed. In addition to this, the solubiliser concentration does play a critical role in the permeation capability of the drug molecule across the barrier membrane which is, in turn, related to the thermodynamic activity of the drug molecule. Lastly, HPβCD was found to be a safer option than RMβCD for retinal drug delivery and may advance the use of CDs in the development of drugs in this field. Hopefully, these obtained results could be beneficial in further exploration of CDs in various fields of ophthalmic drug delivery either as drug carriers or the drug itself.

Keywords:

Cyclodextrins

Stability

Solubility

Permeation

Cytotoxicity

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Contents

List of abbreviations.

αCD	α-cyclodextrin
βCD	β-cyclodextrin
γCD	γ-cyclodextrin
CD	cyclodextrin
CE	Complexation efficiency
D_2O	Deuterium oxide
DSC	Differential Scanning Calorimetry
EDTA	Ethylenediaminetetraacetic acid disodium salt
FITC-HPβCD	6-deoxy-6-[(5/6)-fluoresceinylthioureido]-2-hydroxypropyl-β-cyclodextrin
FTIR	Fourier Transform Infrared Spectroscopy
GCL	Ganglion cell layer
HC	Hydrocortisone
HPLC	Reverse-phase high performance liquid chromatography
HPαCD	2-hydroxypropyl-α-cyclodextrin
HPβCD	2-hydroxypropyl-β-cyclodextrin
HPγCD	2-hydroxypropyl-γ-cyclodextrin
INL	Inner nuclear layer
J_{ss}	Steady-state flux
$K_{1:1}$	Stability constant of the complex
K_c	Equilibrium constant
k_c	Rate constant for the drug degradation within the complex
K_d	Dissociation constant
k_f	Rate constant for the degradation of free drug

k_{obs}	Observed rate constant
MS	Mass Spectroscopic
MWCO	Molecular weight cut-off
NMR	Nuclear magnetic resonance
ONL	Outer nuclear layer
Poloxamer 407	poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol)
RBITC-HPβCD	6-deoxy-6-[(5/6)-rhodaminylthioureido]-2-hydroxypropyl-β-cyclodextrin
RBITC-RMβCD	6-deoxy-6-[(5/6)-rhodaminylthioureido]-randomly methylated β-cyclodextrin
RMβCD	Randomly methylated β-cyclodextrin
S_0	Intrinsic solubility
SBEβCD	Sulfobutyl ether β-cyclodextrin
SBEγCD	Sulfobutyl ether γ-cyclodextrin
SLS	Sodium lauryl sulfate
TEM	Transmission Electron Microscope
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UHPLC	Ultra-high performance liquid chromatography

List of figures

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List of original papers

This thesis is based on the following original publications:

I. Stability characterization, kinetics and mechanism of tacrolimus degradation in cyclodextrin solutions. Manisha Prajapati, Finnur Freyr Eriksson, Thorsteinn Loftsson. *International Journal of Pharmaceutics*, 2020. 586: p119579.

II. Cytotoxicity of β-cyclodextrins in retinal explants for intravitreal drug formulations. Manisha Prajapati, Gustav Christensen, François Paquet-Durand, Thorsteinn Loftsson. *Molecules* 2021, 26(5), 1492.

III. Stabilization and solubilization of difluprednate in aqueous cyclodextrin solution and its characterization for ophthalmic delivery. Manisha Prajapati, Thorsteinn Loftsson. *Journal of Drug Delivery Science and Technology*, 2022. 69, 103106.

Some unpublished data are presented.

In addition, other scientific publications as a co-author:

IV. Antifungal activity of econazole nitrate/cyclodextrin complex: Effect of pH and formation of complex aggregates. Phatsawee Jansook, Manisha Prajapati, Patamoporn Prusakorn, Thorsteinn Loftsson. *International Journal of Pharmaceutics*, 2020, 574: p118896

V. Drug Stability: the basics. Manisha Prajapati, Thorsteinn Loftsson in (A. Porter, Ed.) *Drug stability: what do we need to know?* Hospital Pharmacy Europe. Handbook, pages 4-7

Declaration of contribution

The doctoral student, Manisha Prajapati planned the research work for these studies, applied for appropriate ethical and research approvals, drafted the manuscripts and wrote this thesis with the guidance of her supervisor, the doctoral committee and in close co-operation with the co-authors of each study.

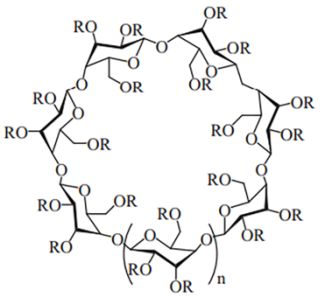
1 Introduction

1.1 Cyclodextrins: Structure and Physiochemical properties

Cyclodextrins (CDs) are enzymatic degradation products of starch which were first discovered and described in the 19th century by Antoine Villers; he described them as “cellulosines”. However, it was not until the early 20th century when Franz Schardinger did most of the work on further characterisation, methods of preparation and purification of CDs [1-3]. He was also the first one to state them as cyclic polysaccharides. Friedrich Cramer later in the 1940s worked more on purification and separation of natural CDs and was responsible for them being finally called cyclodextrins [2].

Cyclodextrins are cyclic oligosaccharides consisting of α-D-glucopyranose with a hydrophobic central cavity and a hydrophilic outer surface. The glucopyranose units are in the chair conformation hence the cyclodextrins are shaped like a truncated cone rather than perfect cylinders. The hydroxyl functions are oriented to the cone exterior while the central cavity is lined by the skeletal carbons and ethereal oxygen of the glucose residues giving it a lipophilic character [4]. They are less susceptible to enzymatic degradation and are better solubilising and complexing agents even though they have identical physicochemical and biological features as analogous linear dextrins because of their cyclic configuration [1, 5].

The natural α, β, and γ-cyclodextrins consist of six, seven, and eight glucopyranose units, respectively [4]. CD molecules are relatively large, with several hydrogen donors and acceptors and do not permeate lipophilic membranes [6]. The natural CDs, particularly βCD, have limited aqueous solubility due to relatively strong intermolecular hydrogen bonding in the crystal state. These confine the usage of natural CDs in some applications [4, 7, 8].



αCD, n=0, R =H

βCD, n=1, R =H

γCD, n=2, R =H

Figure 1. Schematic representation of αCD, βCD, and γCD. Based on [9].

Several hydrophilic CD derivatives have been synthesised through the random substitution of CD´s hydroxyl groups so as to overcome the limitations of the physiochemical properties of these CDs, such as aqueous solubility, stability, and complex formation abilities. The amorphous mixture of CD isomeric derivatives was formed when substituted by hydrophilic or even hydrophobic groups. This resulted in marked water solubility enhancements of these CDs and their complexes. The CD derivatives such as methylated, hydroxypropylated, and sulfobutyl ether are hydrophilic and form highly water-soluble complexes with lipophilic drugs [1, 4, 10-12].

Some important physicochemical characteristics of natural CDs and their derivatives are outlined in Table 1.

Table 1. Physicochemical properties of native CDs and their hydroxypropyl derivatives [4, 9, 13-16].

Properties	**αCD**	**βCD**	**γCD**
No. of glucose units	6	7	8
Molecular weight in grams per mol	972.8	1135	1297
Water solubility at approx. 25°C	145	18.5	232
Log $P_{oct/water}$	-13	-14	-17
No. of water molecules in the cavity	6	11	17
Diameter of cavity (nm)	0.5-0.6	0.6-0.8	0.8-1.0
Hydroxypropylated derivatives			
Properties	**HPαCD**	**HPβCD**	**HPγCD**
Degree of substitution	3.6	4.2	4.2
Molecular weight (g/mol)	1180	1380	1540
Water solubility at 25°C	>600	>600	>600
Log $P_{oct/water}$	-11	-11	-13

1.2 CD complexation

The central cavity of the cyclodextrin molecule is lined with skeletal carbons and ethereal oxygen of the glucose residues, which makes it lipophilic. The polarity of the cavity has been estimated to be similar to that of an aqueous ethanolic solution. It provides a lipophilic micro-environment into which appropriately sized drug molecules may enter [11]. CDs are able to form

inclusion complexes with several drugs provided that their structure (or part of it) fits in the CD cavity. No covalent bonds are formed or broken during drug/CD complex formation in the aqueous solution and the complexes are readily dissociated [5, 17].

Free drug molecules are in equilibrium with the molecules bound within the CD cavity. In all of the complexation process associated with CDs, the measurement and knowledge of the stability of equilibrium constant (K_c) or its inverse, and the dissociation constant(K_d) are crucial since these values provide an index of change of physicochemical properties that result upon host-guest binding [11, 17]. It has been thought that the main driving force for drug/cyclodextrin complex formation is the release of enthalpy-rich water molecules from the cyclodextrin cavity [18], whereas other important forces involved are van der Waals interactions, hydrogen bonding, hydrophobic interactions, the release of ring strain in the cyclodextrin molecule, and changes in solvent-surface tension [11].

In aqueous solutions, CDs self-assemble to form nano-sized aggregates. The fraction of CD molecules forming aggregates is insignificant but the aggregation increases rapidly with increasing CD concentration. Also, the formation of CD complexes can increase the tendency of CDs to form aggregates and can lead to the formation of micellar-type CD aggregates, capable of solubilising poorly soluble compounds that do not readily form inclusion complexes [2, 19].

Drug/CD complexes in the solution can be prepared by an adding excess amount of the drug to an aqueous CD solution, the suspension agitated for up to 1 week at the desired temperature to which the suspension is filtered/centrifuged to form a clear drug/CD complex solution. While the solid drug/CD complexes are prepared by removing the water from the aqueous drug/CD solution by evaporation or sublimation [20].

The CD complexation can affect many physiochemical properties of drugs such as their chemical stability and aqueous solubility [3, 21, 22]. CD based inclusion and non-inclusion complexes are widely used to improve oral bioavailability and enable parenteral dosage form configuration for molecules with less optimum physicochemical properties. They have very low toxic potential, are not orally bioavailable making them true oral carriers, and are affordable [7]. They are also widely used in the food and cosmetic industries to protect labile molecules against light and oxidation, stabilise emulsions, mask unpleasant odors, protect flavors and remove cholesterol from eggs and dairy products [3, 21, 22].

1.3 Cyclodextrins as drug solubilisers and stabilisers

1.3.1 Drug solubilisation

CDs interact with poorly water-soluble drugs to increase their apparent solubility. One of the most prominent mechanisms by which solubilisation occurs is inclusion complex formation. The guest and host molecules are in dynamic equilibrium with the complex [17].

CDs are usually preferred to organic solvents because of their favorable toxicological profile [17]. CDs often solubilise compounds as a linear function of their concentration based on their inclusion complex formation. This means that both the drug and CD concentrations are reduced in a linear manner, suggesting that the precipitation after either oral or i.v. dosing is not likely, at least theoretically. Meanwhile, this is highly likely in organic solvents where a solubilising solute is a log function of their concentration (Hildebrand equation). As the solvent comes across the aqueous environment, the solubilising power of the formulation is lost and precipitation can occur [23-25]. The drug/CD complexes are continuously being formed and broken apart, and the rates of formation and dissociation of drug/CD complexes are very close to the diffusion-controlled limits [25].

1.3.1.1 Phase-solubility analysis

Phase-solubility analysis of the effect of the complexing agents on the compound being solubilised has been widely used to determine the stability constant and to better understand the stoichiometry of the equilibrium as well. In practice, it is performed by adding an excess of a poorly water-soluble drug to several vials with a constant volume of an aqueous vehicle containing a successively larger concentration of the CD. The purpose of using an excess drug is to maintain as high a thermodynamic activity of the drug as possible. The vials are shaken at the desired temperature until equilibrium is established. The suspensions are filtered and the total drug concentration of the drug is quantified using necessary analytical techniques. The phase-solubility profile is then made by evaluating the effect of the CD on the apparent solubility of the drug [17].

Higuchi and Connons (1965) have described the different phase-solubility profiles: A-type phase-solubility profiles can be related to the water-soluble CD derivatives and the B-types to the less soluble natural CDs [26].

The drug solubility increases with increasing CD concentration in the A-type profiles due to the formation of water-soluble drug/CD complexes. There are

further three subtypes of A-type: A_L profiles indicate a linear increase in solubility as a function of solubiliser concentration, A_P systems indicate an isotherm wherein the curve deviates in a positive direction from linearity (i.e. the solubiliser is proportionally more effective at higher concentrations), and A_N relationships indicate a negative deviation from linearity (i.e. the CD is proportionally less effective at higher concentrations) [17].

B-type profiles are related to the formation of complexes that have limited solubility in water. For B-type profiles, an initial increase in drug solubility is observed with increasing CD concentration, then a plateau is formed, where the dissolved drug concentration is at its maximum, followed by a decrease in the total concentration of dissolved drugs [27]. Two subtypes have been described. The B_s type denotes some limited soluble complexes, and the B_I type is indicative of insoluble complexes where the initial ascending component of the isotherm has not risen [17].

The different types of phase-solubility diagrams are shown in Figure 2.

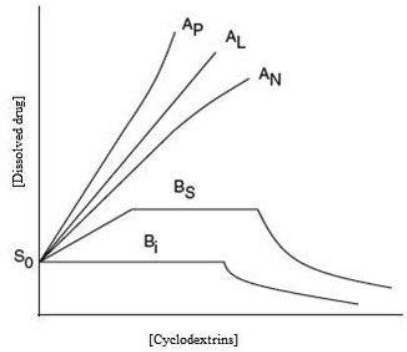


Figure 2. Different types of phase-solubility diagrams and classification of guest/CD complexes according to Higuchi and Connors [26].

The most common complex observed is the 1:1 drug/CD complex where one drug molecule (D) forms a complex with one CD molecule:

$$D + CD \overset{K_{1:1}}{\longleftrightarrow} D/CD \quad (1)$$

Such 1:1 complex display A_L-type phase-solubility profiles and the stability constant of the complex ($K_{1:1}$) can be calculated from Equation (2) where S_0 is the apparent intrinsic solubility of the drug in the complexation media when no CD is present. The value of $K_{1:1}$ is frequently between 50 and 2000 M^{-1} with a reported mean value of 490 M^{-1} for βCD [1, 28].

$$K_{1:1} = \frac{Slope}{S_0\,(1 - Slope)} \quad (2)$$

When a drug molecule forms a complex with more than one CD molecule, a consecutive complexation is assumed, thus stability constants of higher-order complexes ($K_{1:n}$) should be calculated using a different model [17]. The most common stoichiometry is the formation of 1:2 D/CD complexes, where one drug molecule forms a complex with two CD molecules:

$$\mathrm{D/CD} + \mathrm{CD} \overset{K_{1:2}}{\longleftrightarrow} \mathrm{D/(CD)_2} \quad (3)$$

A_P phase-solubility types are usually observed under such conditions. Equation 4, which is a quadratic model, allows the estimation of both stability constants ($K_{1:1}$ and $K_{1:2}$). The value of $K_{1:2}$ is often in the range of 10 to 500 M^{-1} or significantly lower than that of $K_{1:1}$ [1, 17].

$$[S_t] = [S_0] + K_{1:1}\,[S_0]\,[\mathrm{CD}] + K_{1:1}\,K_{1:2}\,[S_0]\,[\mathrm{CD}]^2 \quad (4)$$

The determination of the complexation efficiency (CE) can be a better alternative to $K_{1:1}$ for comparing the solubilising effect of CDs [4]. The CE determination (Equation 5) has less variation because it can be calculated from only the slope of the linear phase-solubility diagram [17, 28, 29].

$$CE = \frac{[D/CD]}{[CD]} = \frac{Slope}{1-Slope} \quad (5)$$

1.3.2 CDs as drug stabilisers

Drug complexation can be considered as molecular encapsulation where the drug molecule is at least partly shielded from various reactive molecules by the CDs, i.e. CDs can insulate a labile compound from a potentially corrosive environment. Hence, CD complexation can affect drug stability by influencing processes like hydrolysis, oxidation, isomerisation, photodegradation, racemisation, and even enzyme-catalysed degradation [11].

Chemical stability can be enhanced when the reactive groups are accommodated in the cyclodextrin cavity, thereby preventing inter- or intra-molecular reactions [30]. Inclusion complex generally leads to drug protection and Table 2 below shows some of the examples of the effect of CD complexation on drug decomposition caused by different degradation pathways in aqueous solution.

Table 2. The effect of complexation on the stability of drugs in aqueous solution.

Drug	CD(s)	Observations	Ref.
Hydrolysis			
Novocaine	αCD, βCD	The presence of both studied CDs retarded the hydrolysis of the drug in alkaline solution.	[31]
Tauromustine	HPαCD, HPβCD, maltosylβCD	All studied CDs stabilised the drug in an acidic environment (pH 1.98 and 4.06) with the highest effect for HPαCD, however in neutral conditions (pH 6.38), no stabilisation was observed.	[32]
Drug oxidation			
Sulfamethoxazole	HPβCD	The stability of the drug within the complex under oxidation stress at 50°C was improved 11-fold when compared with the drug subjected to the same conditions.	[33]
Trimethoprim	HPβCD	Complexation reduced the degradation of the drug under oxidation stress at 50°C 4.2-fold.	[33]
Photostability			
Bufexamac	SBEβCD	Complexation improved the photostabilty of bufexamac while CD presence increased the drug degradation constant by 30%.	[34]
Doxycycline	βCD	Complexation increased the photostabilty of doxycycline with exposure to light ten-fold.	[35]
Epimerisation and racemisation			
S-(-)-scopolamine	αCD, βCD, RMβCD, HEβCD, HPβCD, γCD	αCD enhanced the racemisation of scopolamine while other studied CDs stabilised the drug with the following order: γCD < βCD < HeβCD < HPβCD < RMβCD.	[36, 37]

Even though CD complexation generally increases the stability in solution, it might have an opposite effect in some cases. E.g., Complexation of vitamin K with βCD accelerates decomposition in a solution where labile groups might reside in a different part of the CD cavity and are not protected by encapsulation even though the hydrophobic part accommodates in the CD cavity [38].

The inclusion phenomenon affects not only the drug in aqueous media but also in the solid-state where the drug/CD complexes occur in the amorphous state. However, the drug degradation kinetics in the solid-state progress slowly and are more complicated compared to in aqueous solutions. Solid drug/CD complexes are more water-soluble than pure hydrophobic drugs. So, the solid-state composition caused by the moisture is in turn accelerated by the formation of water-soluble drug/CD complexes. This was observed in carmofur, an anticancer drug when complexed with βCD. Meanwhile, CD complexation increases both their chemical and physical stability under dry conditions [11].

Yet, there are some studies on the solid-state where CDs display the stabilising effect of the complexed drug e.g., limaprost/CD (binary and ternary complexes) where the limited water mobility results in increased stability of limaprost in the solid-state [39].

1.3.2.1 Calculation of the drug degradation constants in CD solutions

The complex formation process by the inclusion of the guest within the CD cavity is a reversible process and the free drug (D) is in dynamic equilibrium with the drug/cyclodextrin complex (D/CD).

In diluted media, one guest molecule forms a complex with one host (CD) molecule, which means 1:1 complexes are formed and $K_{1:1}$ is the stability constant for the complex.

$$\begin{array}{ccc} D + CD \rightleftharpoons D/CD & & \\ \downarrow k_f & \downarrow k_c & \\ \text{Degradation products} & & \end{array} \qquad (6)$$

where k_c is the observed first-order rate constant for the drug degradation within the complex (D/CD) and k_f represents the observed first-order rate constant for the degradation of the free drug (D). Here D represents the drug.

The observed first-order rate constant (k_{obs}) for the drug degradation is the weighted average of k_f and k_c:

$$k_{obs} = k_f \cdot f_f + k_c \cdot f_c \quad (7)$$

where f_f is the fraction of drug in solution that is unbound (i.e. free) and f_c is the fraction of drug in solution that is bound in a CD complex. Further manipulation of the mathematical equations gives:

$$k_{obs} = \frac{k_f + k_c K_{1:1}\,[CD])}{(1 + K_{1:1}\,[CD])} \quad (8)$$

where [CD] is the concentration of the free (i.e. unbound) CD in the aqueous medium. If the total CD concentration (i.e. $[CD]_T$ = [CD] + [D/CD]) is much greater than the total drug concentration (i.e. $[D]_T$ = [D] + [D/CD]) then [CD] ≈ $[CD]_T$:

$$k_{obs} = \frac{k_f + k_c K_{1:1}\,[CD]_T)}{(\,1 + K_{1:1}\,[CD]_T)} \quad (9)$$

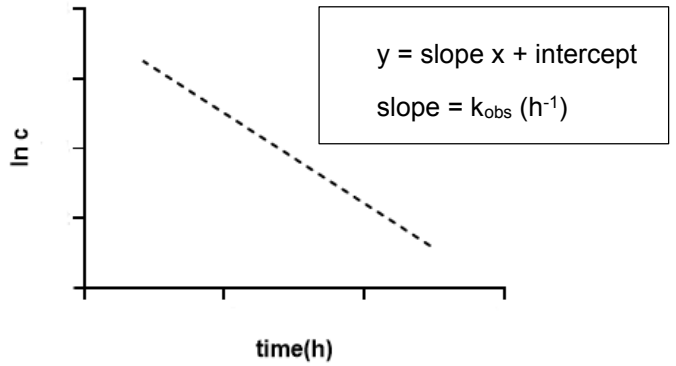


Figure 3. Experimental determination of observed rate constant (k_{obs}).

The k_{obs} is determined experimentally at each concentration of CD. The plot of the natural logarithm of the remaining drug concentration against time gives a straight line where the slope gives k_{obs}.

The k_f is also determined when no CD is present in the aqueous medium.

Rearrangement of Equation (9) gives Equation (10) [40]:

$$\frac{1}{k_f - k_{obs}} = \frac{1}{K_{1:1}(k_f - k_c)} \cdot \frac{1}{[CD]_T)} + \frac{1}{k_f - k_c} \quad (10)$$

Knowing k_f, both k_c and $K_{1:1}$ can be calculated after the construction of the Lineweaver-Burk plot (Figure 4) using Equation (10). The value of k_c is obtained from the ordinate intercept and k_f, and $K_{1:1}$ is obtained by dividing the slope into the ordinate intercept.

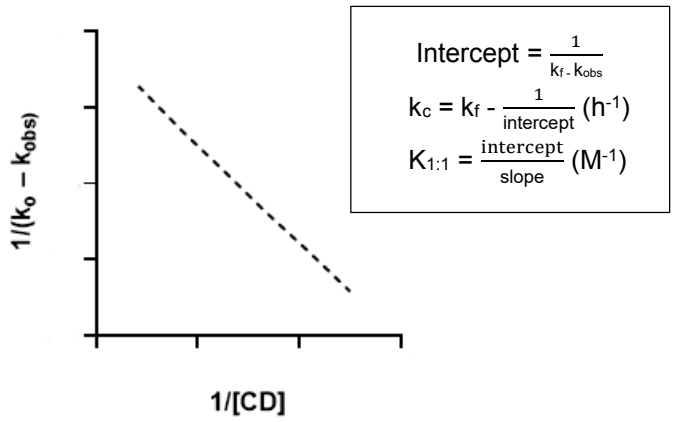


Figure 4. Lineweaver-Burk plot.

1.4 Cyclodextrin and polymer system for drug stabilisation

Even though the exact mechanism of how polymers interact with the CDs is not known, many studies have shown that at low concentrations, polymers increase the complexing abilities of CDs. Not only this, they enhance the bioavailability of drugs in aqueous CD solutions, solubilise CD and its complexes and enhance the formation of complexes between CDs and drugs.

The addition of a small amount of various water-soluble polymers to an aqueous complexation medium, followed by the heating of the medium in an autoclave, significantly increase the apparent stability constant of the drug/CD complex. Such an effect has been obtained through the formation of drug-hydroxy-acid ternary complexes of salts with basic drugs [11, 41].

Similarly, the encapsulation properties of CDs can be improved by forming ternary complexes. The ternary complexes are supramolecular systems composed of three distinct molecular entities and polymers are the most widely used ternary components in drug/CD systems. The combined use of polymer and CD was more effective in enhancing the aqueous solubility of the drug compared to the corresponding drug/CD or drug/polymer binary systems and the effect was synergistic compared to additive. Besides these, the combination of CDs and polymer systems not only can enhance the properties of both the CDs or polymers but also modify the physicochemical, chemical,

and bioavailability properties of guest molecules including the stability [42]. It is believed that the addition of a ternary component to the binary complex leads to an improved macromolecular assembly [28, 42-48] and has been used to improve the bioavailability as well [49].

Results have been reported where the combination of HPβCD and PEG-4000 helped in improving the stability of the drug, glimepiride tablets, and showed consistent therapeutic efficacy [50]. Also, nano emulsion emulsified by tween 80 and HPβCD showed good stability [51]. Likewise, the combination of HPβCD and poloxamer 407 was used to prepare a stable thermally sensitive hydrogel for a potentially effective ophthalmic doxycycline delivery system [52]. Poloxamer 407 has been used as a carrier of both poorly water-soluble and amphiphilic drugs, and it spontaneously forms nano-sized micellar aggregates with a hydrophobic core and a hydrophilic shell. The drug/γCD/HPγCD complex was stabilised by poloxamer 407 through the formation of multi-component aggregates [53]

In this project, we also try to use the combination of CD and different polymers to better stabilise the chemically unstable drug in an aqueous solution.

1.5 Guest/Cyclodextrin complex formation and its characterisation

We have previously described that the peculiar shape of the CDs provides a hydrophilic exterior and a lipophilic cavity, which helps in creating a hydrophobic microenvironment in an aqueous solution. Due to this property, CDs can entrap completely or a part of poorly water-soluble drugs as represented in Figure 5 [3, 5]. The main driving forces involved in this phenomenon are weak interactions such as the hydrophobic effect, van der Walls and London dispersion forces, hydrogen bond interactions, and the release of enthalpy-rich water molecules from the cavity [4, 54-57].

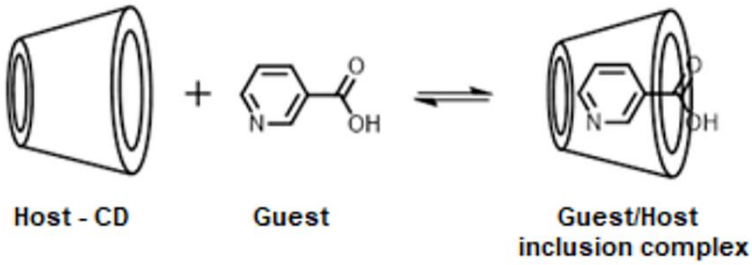


Figure 5. Schematic drawing of the formation of 1:1 guest/host CD complex.

In an aqueous solution, the inclusion complexes are constantly in fast dynamic equilibrium [9, 15, 25] and these interactions are fragile and can fall apart easily like under physiological conditions [15]. The inclusion formation is dependent upon several factors like the type of CD, its cavity size, method of preparation, temperature, etc. [46]. Generally, CDs form inclusion complexes following a simple 1:1 stoichiometry (i.e. one guest molecule is included by one CD molecule) [28], but the formation of higher-order inclusion complexes [58] and non-inclusion complexes should not be excluded [59-62].

Phase-solubility study is the most common method to study the complex formation and determine their stoichiometry in the solution. However, there are other different analytical methods which have been widely used to confirm their formation, both in solution- and solid-states [1, 28, 63, 64] and they are described in Table 3.

Table 3. Examples of CD complexes confirmed by various analytical methods.

Analytical method		Guest/CD system
Phase-solubility studies		Cyclosporine A/native CDs, HPαCD, RMβCD, and HPγCD [65] Hydrocortisone/native CDs, HPβCD, SBEβCD, and HPγCD [66]
Ultraviolet/Visible (UV)		Naproxen/native CDs, HPβCD, MβCD [64], Itraconazole/HPβCD [67]
Circular dichroism spectroscopy		Biphenylacetic acid/βCD, HPβCD, MβCD [68]
Fluorescence spectroscopy		Benzocaine/βCD [69], Warfarin/βCD [70]
NMR	^{1}H-NMR ^{3}C-NMR 2D ROESY	Gemfibrozil/γCD, HPγCD [71], Danazol/βCD [72] Naproxen/native CDs, HPβCD, MβCD [73], Ascorbic acid/ HPβCD [74] Benzopenicillin/βCD, RMβCD [75] Lidocaine/HPβCD [76]
Potentiometry		Arsthinol/HPβCD, RMβCD [77]
HPLC		Betamethasone-related steroids/βCD, γCD [78]
CE		Nicardipine/native CDs, HPαCD, HPβCD, HPγCD [79]
DSC		Salbutamol/ native CDs, MβCD [80], Meloxicam/native CDs [81]
TGA		Taxifolin/βCD, γCD, HPβCD [82], Furosemide/βCD [83]
HSM		Ibuproxam/native CDs [84]
X-Ray Diffraction	SCXRD PXRD	S-ibuprofen/βCD [85], Bornerol enantiomers/αCD, βCD [86] Naproxen/RMβCD, DIMEβCD [87], Quercetin/βCD, HPβCD [88]
FTIR		Dioclein/βCD [89], ibuproxam/βCD [90]
Raman spectroscopy		Bexaldehyde, vanillin, cinnamaldehyde/βCD [91], Diclofenac sodium/βCD [92]
SEM		Meloxicam/native CDs [81], Sulfamethazine/βCD, MβCD [93]
TEM		Itraconazole/HPβCD [94], Hydrocortisone/HPγCD [66]

1.6 CD applications

The CD complexation process (inclusion or non-inclusion) changes various physicochemical properties of the guest molecules which are different from the properties of the free guest molecules. CDs have been widely used to increase the guest molecule´s solubility, stability, dissolution rate, to improve their permeability, protect flavors, protect labile compounds from degradation, modify the chemical reactivity of guest molecule, increase bioavailability and/or biological activity of the guest, catalyse reactions and separate isomers/enantiomers, and for the production of different drug delivery systems [2, 3]. Because of this versatility of the complexation process, researchers have broadly used CDs in different fields like the agrochemical industry, the food and beverage industry [15, 22], environmental protection, the chemical industry [54, 95], and the cosmetic and pharmaceutical industry [2, 21].

There are many marketed pharmaceutical formulations containing cyclodextrin which are summarised in Table 4.

Table 4. Examples of some marketed pharmaceutical formulations containing cyclodextrin. Based on[4, 5, 7].

Formulation	Drug/CD	Trade name and Company
Oral delivery	Limaprost/αCD	Opalmon, Ono
	Omeprazole/βCD	Omebeta, Betafarm
Sublingual delivery	PGE2/βCD	Prostarmon E, Ono
	Nitroglycerin/βCD	Nitropen, Nihon Kayaku (Japan)
Ocular delivery	Diclofenac sodium/HPγCD	Voltaren Ophtha (Novartis)
	Chloramphenicol/RMβCD	Clorocil, Oftalder
Nasal delivery	17β-Estradiol/RMβCD	Aerodiol, Servier
Intravenous	Voriconazole/SBEβCD	Vfend, Pfizer
	Mitomycin/HPβCD	MitoExtra, Novartis
Intramuscular	Aripiprazole/SBEβCD	Abilify, Bristol-Myers Squibb
	Ziprasiodne mesylate/SBEβCD	Geodon, Pfizer
Rectal	Cisapride/HPβCD	Propulsid, Janssen
Dermal	Dexamethasone/βCD	Glymesason, Fujinaga

1.6.1 Topical delivery to the eyes

1.6.1.1 The eye anatomy and physiology

The eye consists of an anterior segment that comprises the cornea, aqueous humor, iris, and lens, and a posterior segment that includes the vitreous humor, retina, sclera, and optic nerve. There are two main routes of drug penetration into the eye; the corneal route (cornea → aqueous humor → intraocular tissue) and the scleral route (conjunctiva → sclera → choroid/retinal pigment epithelium) [96-99].

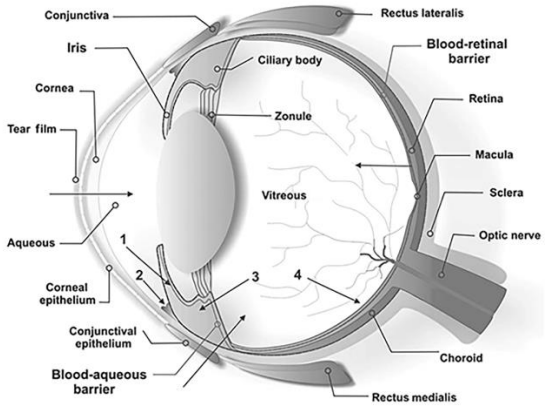


Figure 6. Anatomy of the human eye [100].

The main barrier layer towards the drug penetration through the cornea is the lipophilic epithelium which contributes about 90% of the barrier towards hydrophilic drugs and about 10% of the barrier towards lipophilic drugs. The epithelium consists of three to six layers of tightly adherent epithelial cells. The epithelial surface is covered with microvilli. Drugs penetrate the epithelium either transcellularly (i.e. through the cells) or paracellularly (i.e. through pores between the cells); the dominant route for lipophilic drug molecules [101].

The sclera is composed of mainly collagen fibers in the mucopolysachharides matrix. The primary route for drug permeation through the sclera is passive diffusion through an aqueous pathway. The permeability of the sclera does not display any apparent dependence on the drug lipophilicity but displays a strong dependence on the molecular weight of the drug, i.e. the hydrodynamic radius of the permeating drug molecule, with the permeability coefficient decreasing with increasing molecular weight [102].

The surface of the eye is covered with mucus, a gel-like fluid containing mostly water and mucins, which form hydrogen bonds with the surrounding water molecules increasing the thickness and viscosity of the tear fluid, referred to at unstirred water layer [103].

1.6.1.2 Obstacles to topical delivery

Passive drug diffusion into the eye is affected by three major obstacles [103, 104]. The first is aqueous drug solubility. Only dissolved drugs can permeate into the eye and increasing the solubility of poorly water-soluble drugs will increase their concentration gradient over the unstirred water layer and consequent passive diffusion into the eye. The second obstacle is the drug´s short contact time with the eye surface. When an eye drop is applied to the eye, the drop is mixed with the tear fluid and dispersed over the eye surface, the majority of which is drained from the surface. The drug concentration decreases due to dilution by tear turnover and corneal and non-corneal absorption. The third major obstacle is the lipid membrane barrier, the cornea, and conjunctiva/sclera. The permeation rate through the lipophilic membrane is affected by the drug´s ability to partition from the aqueous exterior into the lipophilic membrane. In addition to that, the permeation rate is also affected by whether or not the drug is charged [105].

Various formulation approaches have been employed in order to improve the bioavailability of ophthalmic drugs and cyclodextrins have been widely used as solubilising systems to enhance the drug solubility in the aqueous tear fluid [98, 106, 107]. However, for effective delivery to the eye, all these obstacles should be addressed.

1.6.1.3 Toxicological considerations

Safety and toxicity are the main concerns with using CDs as pharmaceutical excipients. The route of CD administration can influence the degree of these parameters. Natural CDs are not absorbed through the gastrointestinal tract; thus their oral administration is practically non-toxic [108]. Parenterally administered CDs are rapidly eliminated from the body without undergoing metabolism [16]. Natural CDs are considered to be non-corrosive excipients in topical formulations, however they are more toxic than their derivatives. (αCD > SBEβCD = HPβCD > γCD > HPγCD) [109].

1.6.1.4 CDs as drug carriers in eye drops

Among various applications of CDs, they have been used to form aqueous eye drop solutions with many lipophilic drugs. This has been possible not only

because CDs increase the water solubility of the drug but also enhance drug absorption into the eye and reduce local irritation [110]. Their use as useful excipients in the formulation of eye drops covers various drugs including steroids, carbonic anhydrase inhibitors, pilocarpine, and cyclosporins [65, 110-112]. These selections will likely expand owing to their extensive use in ophthalmology.

Drugs in aqueous eye drops have very low bioavailability and have to be administered frequently. This makes them difficult to administer, especially for older patients, and tend to deliver much more of the drugs to the general blood circulation than to the eye. Despite all these drawbacks, patients prefer low-viscosity aqueous eye drops over other forms of ophthalmic drug delivery system [105].

The drug molecules in the CD cavity may dissociate from the CD molecules through complex dilution in the aqueous tear fluid. Not only this, but the included drug can be replaced by some other suitable molecule (e.g., lipids), or when the complex is located in close approximation to a lipophilic biological membrane (such as the eye cornea), the drug may be transferred to the matrix for which it has the highest affinity [110].

The different studies on the effects of cyclodextrin on permeability and delivery through biological membranes showed that hydrophilic CDs act as true carriers by keeping the lipophilic drug molecules in the solution and delivering them to the membrane surface. There, the drug molecules partition from the CD cavity into the lipophilic membrane. The lipophilic membrane has a low affinity for the large hydrophilic CD molecules and the drug/CD complexes and hence they will remain in the aqueous exterior e.g., tear fluid [25, 55, 113-116]. CDs also do not contribute to disrupting the ophthalmic barrier like the conventional penetration enhancers as CDs enhance drug penetration into the eye by carrying the lipophilic drug molecules through the mucin layer and increasing the availability at the lipophilic eye surface [113]. E.g., the formation of CD complexes with corticosteroids, chloramphenicol, diclofenac, cyclosporine, and sulfonamide carbonic anhydrase inhibitors demonstrate significant corneal penetration [105, 115]. Not only this, but cyclodextrin-based dexamethasone eye drops are well tolerated in the eye and seem to provide a higher degree of bioavailability and clinical efficiency than the steroid eye drop formulations presently available [110]. Another advantage of using CDs would be, it is possible to increase the drug concentration and bioavailability and formulate with more effective and less frequent dosing.

They have been shown to reduce irritation after topical administration to the eye and also enhance the chemical stability of the drugs in aqueous solutions. For optimum bioavailability, it is important to add excess CD to the aqueous vehicles more than needed, to dissolve the drug dose in the vehicle [105].

1.6.1.5 CD and drug permeation through biological membranes

Conventional penetration enhancers, such as fatty acids, surfactants, and quaternary ammonium compounds, enhance drug permeation into and through bio-membranes, such as the cornea, by permeating into the bio-membrane where they temporary decrease its barrier properties, while the permeation enhancing behavior of CDs is different. They are only able to enhance the permeation of relatively lipophilic drug molecules that have limited solubility in water and only from aqueous media [117-119]. Many studies show that depending on the vehicle composition and the drug/CD molar ratio CDs can both enhance and hamper drug permeation through bio-membranes [119, 120].

Topical drug delivery to the eye via eye drops is affected by drug availability at the eye surface, that is in the tear fluid and the epithelia (i.e. cornea and conjunctiva). The consequent increase in drug permeation through inner tissues is a direct result of enhanced drug availability in the cornea and conjunctiva epithelium [121].

Thus, the permeation from the surface of the eye can be described as a permeation through a simple bilayer mathematically. Here, the train drainage and first-pass effect of the conjunctiva and choroid are omitted. The mucus layer is the first barrier that forms an aqueous diffusion barrier and the drug flux through the mucus follows Fick´s first law. The drug partition from the aqueous exterior into the lipophilic membrane is the second barrier and the partition coefficient represents the drug distribution between the aqueous exterior to the membrane and the external layer of the epithelium.

The epithelium is the third barrier that forms a lipophilic membrane and the drug flux through it also follows Fick´s first law. Fick's law states that passive drug diffusion through the two barriers is driven by the concentration gradient of the dissolved drug within the mucus layer and the epithelium. Likewise, the drug partition coefficient between the epithelium and the mucus layer is the drug concentration ratio between those two layers [9, 122-124].

Along with these, the thermodynamic activity of the drug should also be considered [125, 126]. The thermodynamic activity is equal to one in a saturated solution and under such conditions, the drug has the highest

potential for leaving the aqueous exterior and entering the cornea. The activity is greater than one in supersaturated solutions but these are physically metastable states [127].

The thermodynamic activity increases with the increasing drug concentration, approaching unity in a drug saturated medium. The addition of solubilisers, such as CDs and surfactants, to an aqueous drug solution containing a fixed amount of dissolved drug will lower the drug activity. Thus, under normal conditions, CDs will lower the potential of the drug exiting the tear fluid and entering the cornea or conjunctiva/sclera, and hence the ability to partition into the epithelium is decreased [128].

However, adding a solubiliser to an aqueous drug suspension in such a way that, while the concentration of the dissolved drug is increased and the tear fluid kept saturated with the drug, the drug activity will not lower. The thermodynamic activity of the drug is kept at its maximum under such conditions and hence the dissolved drug molecules are at their highest exiting potential and the drug flux through the bilayer membrane barrier will increase. The high thermodynamic activity of the dissolved drug molecules in the external mucus layer will enhance their ability to partition into the epithelium and create a steep concentration gradient within the epithelium [105, 121].

Such effect was observed in the study of hydrocortisone permeation from the HPβCD solution, where the drug concentration was kept constant and the CD concentration was increased and decreased from the concentration, where the activity of the drug was maximum as it was in a saturated condition [121]. Likewise, Flynn *et al.* used a water-soluble co-solvent instead of a solubiliser and obtained similar results [129].

Here, we have tried to study more in detail the thermodynamic activity of the permeating drug molecules using different solubilisers, co-solvents, and surfactants in different membranes.

1.6.2 CDs in various retinal diseases and the related toxicities

Drugs for the treatment of retinal diseases are most often delivered via intravitreal injections or implants where the drug is administered directly into the vitreous humor, the hydrogel-type fluid that occupies the space between the lens and the retina. The vitreous consists mainly of (99%) water in a network of collagen and hyaluronic acid. In humans, the volume of the vitreous humor is about 4 mL. Drug molecules must be dissolved in the aqueous vitreous to permeate into the retinal tissue. After intravitreal injection, hydrophilic and high molecular weight drugs (e.g., proteins and peptides) are

known to be excreted via an anterior route to the aqueous humor while small lipophilic drugs easily pass the retina and are removed via the posterior choroidal flow [130]. The half-life of a dissolved drug in the vitreous humor is typically less than 10 to 24 hours, where small molecules have a shorter half-life than biomolecules like proteins [131, 132]. It is expected that the hydrophilic CD molecules (molecular weight between about 1000 and 2000 Da) are readily removed from the vitreous humor after intravitreal injection. CDs might be able to enhance retinal delivery of poorly soluble lipophilic drugs after intravitreal administration.

In ophthalmic drug delivery, CDs have mainly been applied topically to the eye with little or no reports on their intravitreal administration [133-135]. Hydrophilic CDs, such as HPβCD and SBEβCD, have been shown to be well tolerated when applied topically to the eye with no detectable side effects [108].

Moreover, CDs tend to form complexes with biomolecules as well. αCD and βCD are better suited to solubilising phospholipids and cholesterol respectively while γCD is less lipid-selective. The studies done on the erythrocyte membranes showed that the affinity of CDs to solubilise follows the order γ-CD << β-CD < α-CD for phospholipids and α-CD < γ-CD << β-CD for cholesterol [136]. And, the hemolytic activity was reduced in the presence of hydrophilic substituents (e.g., hydroxypropyl, sulfobutyl ether, etc.) compared to the parent CDs, while the lipophilic ones (e.g., methylated CDs) demonstrated the strongest hemolytic activities. The hemolysis is related to the removal of erythrocyte membrane components, especially phospholipids and cholesterol hence the CDs complexation with lipids can be very relevant [137]. Similar reports were found with βCD derivatives where the cytotoxic effect, hemolytic activity, and the cholesterol complexation of the CDs are correlated [138, 139]. Therefore, most biological effects of CDs are based on their interaction with membranes rich in lipids and their ability to extract lipids (e.g., phospholipids and cholesterol) from the plasma membrane. This particular behaviour has been employed in many applications like cholesterol manipulation, the control of viral and bacterial infections, the treatment of Alzheimer´s diseases, etc. [140].

Furthermore, Nociari *et al*. observed that βCD extracted lipofuscin bisretinoids from the retinal pigment epithelium (RPE) [141]. Based on all these observations, it was proposed that CDs can be used to develop therapeutic candidates for many retinal degenerative diseases like Stargardt disease that is an inherited form of macular degeneration causing central vision loss and sometimes referred to as juvenile macular degeneration. The

cause of Stargardt disease is characterised by an abnormal accumulation of lipofuscin in the retina [142]. Similarly, topical administration of HPβCD as eye drops over 3 months has shown significant efficacy in reducing amyloid-beta and inflammation in aged mouse retina and consequently improving retinal function by elevating retinal pigment epithelium-specific protein 65 (RPE 65), a key molecule in the visual cycle [143]. Even oral HPβCD treatment in mice exhibited a reduction in the retinal cholesterol content and changes in the retinal sterol, gene, and protein levels [144].

1.7 Model drugs

Tacrolimus (FK506) is a 23-membered macrolide lactone produced by the bacterium *Streptomyces tsukubaensis.* It is a potent immunosuppressant used to prevent graft rejection after organ transplants [145]. Recent studies have found that immunomodulators like tacrolimus are especially effective for the treatment of anterior inflammatory ocular disorders and can replace corticosteroids that frequently cause cataracts and induce glaucoma [146]. Similarly, in diseases like atopic dermatitis and dry eyes, topical tacrolimus formulations have been noted to have significant therapeutic efficacy [147]. However, tacrolimus is a highly lipophilic compound (LogP 4.8) and has a water solubility of only about 1 µg/mL. In addition to this, the drug is susceptible to hydrolysis resulting in very low stability in aqueous solutions [146].

Difluprednate is a synthetic glucocorticoid that is rapidly hydrolysed to 6α,9-difluoro-11β,17,21-trihydroxypregna-1,4-diene-3,20-dione 17-butyrate, a deacetylated metabolite of difluprednate, in the aqueous humor after penetration into the eye [148, 149]. It is used for the treatment of postoperative inflammation and inflammation and pain associated with endogenous uveitis [150]. It is lipophilic (LogP 2.9) and has a very limited water solubility (less than 1 µg/mL) [149, 151] and stability and the only commercially available formulation of difluprednate (Durezol®, Alcon, the United States of America) is an oil-in-water ophthalmic emulsion with 0.05% difluprednate [152].

Hydrocortisone is a widely used corticosteroid to treat various inflammation disorders of the eye. It has a water solubility of around 0.4 mg/mL and a log P value of 1.61 [153].

Tacrolimus and difluprednate were used for the studies presented in this thesis in order to stabilise these drugs into aqueous eye drop solutions using different CDs and polymers and provide a basis for an optimum formulation for ophthalmic delivery. Hydrocortisone was chosen as a model drug for the permeation studies to study the effect of the thermodynamic activity of the

permeating drug molecule primarily because of its high solubility in different solubilisers, co-solvents, and surfactants.

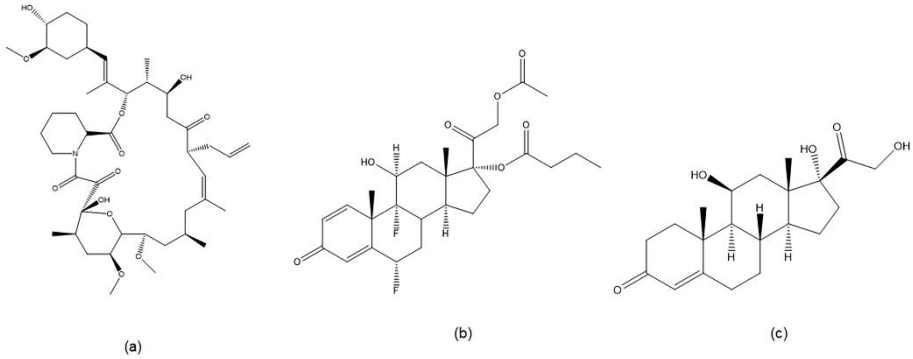


Figure 7. Chemical structure of (a) tacrolimus, (b) difluprednate, and (c) hydrocortisone

2 Aims

The major aims of this project were: the investigation of CDs and CD-polymer system for the stabilisation of poorly water-soluble and chemically unstable drugs in aqueous solutions in order to provide a solid foundation for stable, safe, and effective formulations for opthalmic delivery; study the thermodynamic considerations of the permeation drug molecule when permeating through different membrane barriers; and the toxicity of different CDs on retinal explants to explore their applicability for future ophthalmic formulations.

The research was divided into the following tasks:

1. Stability and kinetics studies of drug (tacrolimus and difluprednate) degradation in CD solutions. The purpose was to investigate the chemical stability and kinetics of tacrolimus and difluprednate in various CD solutions, elucidate the degradation mechanism, and provide a mode of stabilisation in aqueous solutions.
2. Solubilisation in various CDs and stabilisation in a CD-polymer system to further improve the solubiilty and stability of tacrolimus and difluprednate.
3. Characterisation of selected drug/CD systems by complementary methods (NMR, FTIR, DSC) in order to confirm complexation and evaluate the complexation pattern.
4. Studying the permeation of a guest molecule (hydrocortisone) in different systems (drug/CD, drug/co-solvent, drug/surfactants) across different barrier membranes to provide more insight into the thermodynamic effect of the permeating molecule.
5. Cytotoxicity studies of different CDs on retinal explants to further explore the applicability in various retinal diseases.

3 Materials and methods

3.1 Materials

Model drugs, (tacrolimus and difluprednate) were purchased from Shanghai Huirui Chemical Technology Co., Ltd., China while hydrocortisone from Fagron Chemicals, Netherlands.

α-Cyclodextrin (αCD), β-cyclodextrin (βCD), γ-cyclodextrin (γCD) and 2-hydroxypropyl-β-cyclodextrin (HPβCD) with degree of substitution(DS) 4.2(MW 1380) were kindly provided by Janssen Pharmaceutica, Belgium, 2-hydroxypropyl-γ-cyclodextrin (HPγCD) with DS 4.0-5.6 (MW 1540) by Chemical Marketing Concepts Europe, the Netherlands, and sulfobutyl ether β-cyclodextrin (SBEβCD) (sodium salt) with DS 4.8 (MW 2163) by CyDex Pharmaceuticals, Lenexa. 2-hydroxypropyl-α-cyclodextrin (HPαCD) with DS 0.6(MW 1180) and randomly methylated β-cyclodextrin (RMβCD) with DS 12.6(MW 1312) were purchased from Wacker Chemie, Munich, Germany, and sulfobutyl ether γ-cyclodextrin (SBEγCD) with DS 4.2(MW 1961) from CYDEX Pharmaceuticals, the United States of America. 6-deoxy-6-[(5/6)-fluoresceinylthioureido]-RMβCD, 6-deoxy-6-[(5/6)-fluoresceinylthioureido]-HPβCD and 6-deoxy-6-[(5/6)-rhodaminylthioureido]-HPβCD were kindly provided by Cyclolab, Hungary. Similarly, we purchased tyloxapol reagent grade and poloxamer 407 from Sigma-Aldrich, the United States of America, and tween 80 from Tokyo Chemical Industry Co., Ltd., Japan.

A Spectra/Por semi-permeable membrane with molecular cut-offs (MWCOs) of 6-8 KDa was purchased from Spectrum Europe, the Netherlands. A permeapad barrier membrane was purchased from innoME GmbH, Germany.

Milli-Q water (Millipore, the United States of America) was used to prepare both the CD solutions and mobile phase for UHPLC measurements. Reagents for quantitative analysis by ultra-high performance liquid chromatography (UHPLC) were commercial products of analytical grade. Acetonitrile (ACN), methanol (MeOH), ethanol, dimethyl sulfoxide (DMSO), trifluoroacetic acid (TFA), ammonium bicarbonate, and ammonium hydroxide were purchased from Sigma-Aldrich, St. Louis Missouri, the United States of America. Formic acid, acetic acid, and phosphoric acid were purchased from GmbH chemicals

GmbH, Germany. All other chemicals were commercially available products of special reagent grade.

3.2 Quantitative analysis

3.2.1 Quantitative analysis of model drugs (tacrolimus, difluprednate, and hydrocortisone)

Quantitative determination of tacrolimus was performed on a reversed-phase ultrahigh-performance liquid chromatographic (UHPLC) component system from Thermo Fisher Scientific Vanquish HPLC system consisting of a VF-P10-A pump, a VF-A10-A autosampler, a VH-C10-A column compartment, a VWD-3100 UV-Vis detector operated at 205 nm, and a Phenomenex Kinetex C18 1.7µm 100 x 2.1 mm with a security guard ULTRA HOLDER. The column temperature was 50°C and the mobile phase consisted of acetonitrile (ACN) and Milli Q water containing 0.1% (v/v) trifluoroacetic acid (60:40). The flow rate was 0.4 mL/min, the sample injection volume was 10 µL, and the retention time (R_t) was 3 min.

Quantitative determination of difluprednate was performed on a reversed-phase UHPLC component system from Thermo Fisher Scientific Vanquish HPLC system consisting of a VF-P10-A pump, a VF-A10-A autosampler, a VH-C10-A column compartment, a VWD-3100 UV-Vis detector operated at 240 nm, and a Phenomenex Kinetex C18 1.7µm 100 x 2.1 mm column with a security guard ULTRA HOLDER. The column temperature was 40°C. The mobile phase consisted of ACN and Milli Q water containing 0.1% (v/v) ortho-phosphoric acid (50:50). The flow rate was 0.3 mL/min, the sample injection volume was 5 µL, and the retention time (R_t) was 3 min.

Quantitative determination of hydrocortisone was performed on a reversed-phase UHPLC component system from Thermo Fisher Scientific Vanquish HPLC system consisting of a VF-P10-A pump, a VF-A10-A autosampler, a VH-C10-A column compartment, a VWD-3100 UV-Vis detector operated at 240 nm, and a Symmetry C18 3.5 µm 100 x 2.1 mm column. The column temperature was 25°C. The mobile phase consisted of ACN and Milli Q water containing 0.1% (v/v) acetic acid (50:50). The flow rate was 0.25 mL/min, the sample injection volume was 5 µL, and the retention time (R_t) was 1.2 min.

Quantitative determination of cholesterol was performed on a reversed-phase HPLC system from Merck-Hitachi, Germany consisting of an L 4250 UV-Vis detector operated at 203 nm, an L 6200 A Intelligent pump, an AS-2000A Autosampler, a D-2500 Cromato-Integrator, and a Phenomenex Luna 5µm

C18 reversed-phase column (150 x 4.6 mm). The mobile phase consisted of methanol, acetonitrile, isopropyl alcohol, and tetrahydrofuran (50:25:25:0.1).

3.3 Solution-state experiments

3.3.1 Kinetics studies

3.3.1.1 Buffers

Hydrochloric acid-potassium chloride buffer (pH 1-2), citrate buffer (pH 3-6), phosphate buffer (7-8), and carbonate-bicarbonate buffer (pH 9) were prepared by mixing aqueous solutions of the acid with the aqueous solutions of the corresponding salt. The concentration of the buffer salts was 0.1 M. The ionic strength of the media was not adjusted. Also, volatile buffers like 20 mM ammonium bicarbonate, ammonium hydroxide, and formic acid were used in the mass spectroscopic (MS) studies. Various amounts (expressed as % w/v) of different CDs were added to the buffer solutions when the effects of CDs were investigated.

3.3.1.2 Degradation kinetics in drug/CD system

The drug degradation (tacrolimus and difluprednate) was investigated by adding stock solution (100 µL) of the drug in methanol to aqueous buffer solution (5 mL), previously equilibrated at 40°C in a heating block, and mixed thoroughly. The initial concentration was 2.48 mM for tacrolimus and 3.93 mM for difluprednate. The pH of the final reaction mixture was determined at the end of each experiment with a pH meter standardised at 40°C. All reactions were run under pseudo-first-order conditions. Aliquots (5 µL) were injected into the column at various time intervals, and the first-order rate constant (k_{obs}) was determined by linear regression of the natural logarithm of the remaining drug concentration vs time plots.

3.3.2 Identification of degradation products using MS Quad/LC-MS

The degradation products of tacrolimus in aqueous CD solution were identified using LC-Ms. All samples for mass spectrometer (MS) studies were prepared as described above in the kinetics studies (*3.3.1*) using MS-compatible buffers. The samples were diluted with the mobile phase before analysing by Waters ACUITY UPLCTM (Waters Corporation, the United States of America) coupled to Waters QT_oF SYNAPT G1 mass spectrometer (Waters MS Technologies, the United Kingdom). The UPLC system was equipped with a binary solvent delivery system and autosampler. A chromatographic analysis of tacrolimus

degradation products was conducted on an ACQUITY UPLC BEH C18 column (2.1 mm x 100 mm, 1.7 µm; Waters corp., the United States of America). The mobile phase consisted of solvent A: 10 mM ammonium acetate in water pH 5.5, and solvent B: 10 mM ammonium acetate in ACN pH 5.5. A gradient elution was used at a flow rate of 0.50 mL/min as follows: initial 40%B 0-0.1 min, linear gradient from 0.1-5 min from 40%B to 100%B, holding at 100%B 5-5.5 min, linear gradient from 100%B to 40%B 5.5-5.6 min and holding at 40%B 5.6-7 min.

The injection volume was 4 µL. The Synapt G1 QT_oF-MS mass spectrometer was operated in positive electrospray ionisation mode (capillary voltage 3.2 kV, source temperature 120°C, desolvation temperature 400 °C, cone gas flow 50 L/h, desolvation nitrogen gas flow 800 L/h). Ions with mass range 50–1000 m/z (mass-to-charge ratio) were scanned. All samples were analysed in triplicates. The UPLC-QT_oF-MS system and data acquisition were controlled by the MassLynx v4.1 software (Waters Corp., the United States of America).

3.3.3 Phase-solubility studies

Solubility studies were determined by adding an excess amount of drug to aqueous solutions containing various concentrations of CD at a pH of maximum stability (around pH 5 for both tacrolimus and difluprednate). The suspensions formed were sonicated in an ultrasonic bath (Edmund Buhler GmbH) for 90 minutes for tacrolimus and 60 minutes for difluprednate. The vials containing these suspensions were then shaken at room temperature. After equilibrium (24 hours for tacrolimus and 7 days for difluprednate), the aliquots were filtered through a 0.45 µm membrane filter unit (Phenomenex, the United Kingdom), diluted with 50% aqueous acetonitrile solution (whenever necessary), and analysed by UHPLC.

Phase-solubility studies were performed according to the method of Higuchi and Connors [26]. The stability constant ($K_{1:1}$) and the complexation efficiency (CE) were calculated from the slope of the linear part of the phase-solubility profiles previously described in the introduction chapter (*section 1.3.1.1*).

$$K_{1:1} = \frac{Slope}{S_0\,(1 - Slope)} \quad (11)$$

$$CE = S_0 . K_{1:1} = \frac{[D/CD]}{[CD]} = \frac{Slope}{1 - Slope} \quad (12)$$

3.3.4 Stabilisation with cyclodextrin and polymers

Poloxamer 407, tween 80, and tyloxapol polymers were used in these studies. Aqueous solutions containing 5% (w/v) HPβCD and polymers (from 0 to 5%

w/v) were prepared to which 100 µL of tacrolimus stock solution (2.48 mM) was added. While, aqueous solutions containing 15% (w/v) HPγCD and polymers (from 0 to 4% w/v) were prepared with difluprednate concentration of 0.1% (w/v). These solutions were subjected to one cycle of autoclaving and the remaining drug concentration was measured by using the UHPLC method.

3.3.5 NanoSight Wave for particle size measurement

The laser-based light scattering analysis of CD particles was performed with NanoSight NS300 (Malvern), fitted with an O-ring top plate. Nanoparticle tracking analysis (NTA) software was used to capture images and process data, representing the concentration, size distribution, and intensity of particles in the sample. A sample measurement was done in static mode using a capture time of 60 seconds and 5 repeats. The camera level was adjusted to 11 so that all particles were visible. The same camera level was used for all of the samples. A suitable detection level was selected for data analysis to limit the detection of non-particles and was between levels 4-12. The result for each sample was based on the average of five measurements obtained from the NTA and represented by average particle concentration, average particle size (i.e. mean size), and mode size (i.e. the size that displays the highest peak).

3.3.6 ^{1}H-NMR (nuclear magnetic resonance) spectroscopy

Solutions of the pure compounds (i.e. difluprednate and HPγCD), difluprednate/HPγCD, and the difluprednate/HPγCD/poloxamer complexes were prepared by dissolving the freeze-dried solid complex in D_2O. Their spectrum and chemical shift values were recorded by using a 400 MHz ^{1}H-NMR spectrometer (BRUKER™ model AVANCE III HD, Brucker Biospin GmbH, Germany). The resonance at 4.8000 ppm, due to residual solvent (D_2O), was used as an internal reference. The ^{1}H-NMR chemical shift change ($\Delta\delta^{*}$) was calculated as

$$\Delta\delta^{*} = \delta_{complex} - \delta_{free} \quad (13)$$

3.3.7 Morphological analysis by Transmission Electron Microscope (TEM)

The morphology of HPβCD aggregates, RMβCD aggregates, difluprednate/HPγCD, and difluprednate/HPγCD/poloxamer nanoaggregates was studied visually by TEM. Samples were prepared using 4% of uranyl acetate as a negative staining agent. Firstly, 3 µL of each sample were loaded into a coated grid on Parafilm® located inside a petri dish, and left to dry for 30 min at 37-40°C. After centrifugation of uranyl acetate at 10,000 rpm for 5 min,

a drop of 26 µL of the dye was transferred to another petri dish containing a Parafilm® flip-loaded grid onto uranyl acetate and left for 5 min. The excess dye was removed and the grid was dried with filter paper and left at room temperature for 12 hours. Finally, the samples were analysed using a Model JEM 1400 TEM (JEOL, Japan).

3.3.8 In vitro permeation studies

Permeability studies were used to study the permeation of hydrocortisone (HC) solubilised in different systems like HPβCD, ethanol, and sodium lauryl sulfate (SLS) and the effect of these solubilisers´ concentration on permeation.

These studies were carried out in unjacketed Franz diffusion cells with a diffusion area of 1.77 cm2 (SES GmBH – Analyse system, Germany). The donor phase (DP) consisted of 2 mL of different samples (different HC/CD complexes from phases solubility experiments, HC/ethanol, and HC/SLS) while the receptor phase consisted of 2% (w/v) HPβCD solution (degassed previously to remove the dissolved air). Two mL of donor phase were added to the donor compartment, while the receptor compartment was filled with 12 mL of the degassed HPβCD solution. Different types of membranes (cellulose and permeapad membranes) were used to physically separate the donor and receptor compartments. These experiments were operated at room temperature, with continuous magnetic stirring of the receptor phase at 300 rpm.

200 µL aliquot (receptor phase) were collected at different time intervals and immediately replaced with the same volume of the fresh receptor phase. The samples were collected over a period of 4 hours and were then quantified using the UHPLC validated methods (*section 3.2.1*). The calculation of the steady state flux (J_{ss}) of the drug was obtained from the slope (d_q/d_t) applying the linear regression relationship between time and the amount of the drug in the receptor chamber and is given by Equation (14).

$$J_{ss} = \frac{d_q}{A \cdot d_t} = P_{app} \cdot C_d \qquad (14)$$

Where A is the diffusion area (1.77 mc2), P_{app} is the apparent permeability constant and C_d is the total drug concentration in the donor phase.

In order to assure sink conditions were maintained, common guidelines were followed and the volume change and the final concentration of the components in the donor phase were assessed.

3.4 Solid-state experiments

3.4.1 Preparation of solid inclusion complex

Samples were prepared using a freeze-drying method [3, 154]. Clear supernatant solutions from phase solubility studies of difluprednate in HPγCD solutions, that had displayed A_L-type profiles and solutions containing difluprednate, HPγCD, and poloxamer 407, were used to confirm the presence of difluprednate/CD complexes. Ten mL of the samples were placed in the vials and freeze-dried at −55 °C for 3 days in a Snijdersscientific 2040 Freeze dryer (Snijders Labs, the Netherlands).

3.4.2 Differential Scanning Calorimetry (DSC)

The DSC thermograms of pure difluprednate, pure CD, and their freeze-dried complexes were recorded on a Netzsch DSC 214 polyma (Netzsch GmbH, Germany). Samples were weighed in an aluminium closed pierced crucible and an identical empty was one used as reference. Using a constantly purged nitrogen atmosphere, samples were heated up at a rate of 10 °C/min over 25-200 °C temperature range.

3.4.3 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of pure difluprednate, pure CD, and their freeze-dried complexes were measured with an FTIR spectrometer (Thermo Fisher Scientific model Nicolet iS10, Waltham, the United States of America) using an Attenuated Total Reflectance (ATR) technique. Data were obtained in the range of 500-4000 cm^{-1}. Analyses were performed at room temperature.

3.5 Assessment on the retinal cytotoxicity of β-cyclodextrin derivatives

3.5.1 Animals

The C3H wild-type mouse line was used in all studies [155]. Animals were used irrespective of gender and were housed with free access to food and water under standard white light with 12 hour light/dark cycles. They were sacrificed at postnatal day (P) 13 by CO_2 asphyxiation followed by cervical dislocation. All procedures were performed in accordance with the German law on animal protection (*Tierschutzgesetz*) and approved by the institutional animal welfare office of the University of Tübingen.

3.5.2 Culturing and treatment of organotypic retinal explant cultures

To study the cytotoxicity of CDs on the retinal tissue, retinas from mice were isolated for culturing for an extended time period. The detailed protocol is described elsewhere [156] but will be summarised here. Immediately after animal sacrifice, the eyes were enucleated and incubated for 5 min at RT in R16 serum-free culture medium (Gibco, the United States of America). To promote the removal of the sclera and choroid, the eyes were transferred to a preheated (37°C) solution of 0.12% proteinase K (MP Biomedicals, France) and incubated for 15 min. Afterwards, the eyes were soaked in 1:4 mixtures of 10% FBS/medium to stop the protease reaction. The eyes were dissected under sterile conditions. The retina with the retinal pigment epithelium (RPE) attached was isolated and cultured on a transwell membrane (polycarbonate, 0.4 µm pore size, COSTAR, the United States of America) with the RPE side facing down in a 6-well plate. One mL of complete medium (CM, R16 medium with supplements; detailed under [156] was added to each well. The explants were allowed to recover from the explantation procedure in a sterile incubator (37°C, 5% CO_2) for 48 hours before the treatment began. The CM was exchanged every second day by removing 0.7 mL of the CM in the plate and adding 0.9 mL fresh CM to account for evaporation and conserve neuroprotective agents produced by the retinal cultures. The cultures were treated at P15 and incubated for 48 hours. Treatment was done by placing 20 µL of an isotonic CD solution (adjusted by NaCl) carefully on the top of the retina to cover the whole tissue. Either 10 mM or 100 mM CD were used. For rhodamine- or fluorescein-labelled CD, 5 mM were applied. Alternatively, a 0.9% NaCl solution was added as a control. All treatment solutions were passed through sterile filters (PES, 0.22 µm, Merck Millipore, Ireland) before being introduced to the culture.

3.5.3 Preparation of retinal tissue sections

After treatment of organotypic retinal explant cultures with CD or saline, the cultures were fixed in a 4% paraformaldehyde/PBS solution for at least 45 min. The explants were cryoprotected by introducing an incremental amount of sucrose to the well plate, i.e. 10% sucrose, 20% sucrose, and 30% sucrose for 10 min, 20 min, and 30 min (at RT), respectively. Afterwards, the area of the retina culture attached to the transwell membrane was cut out with fine scissors, and the membrane piece was submerged in an embedding medium (Tissue-Tek O.C.T. Compound, Sakura Finetek Europe, the Netherlands), followed by snap freezing in liquid nitrogen. The frozen specimens were

sectioned on an NX50 cryostat (ThermoFisher, the United States of America) to produce 14 µm thick sections on Superfrost Plus object slides (R. Langenbrinck, Germany) used for direct imaging or further staining.

3.5.4 Assessing cell death in retinal sections using the TUNEL assay

A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to stain the nuclei with damaged (nick-end) DNA [157]. This was done to quantify the number of dying cells in retinal cultures after treatment with CD or saline to assess the cytotoxicity of the CDs. Firstly, microscopy slides with retinal sections were rehydrated with PBS. A proteinase K solution preheated to 37°C (0.21 µg/mL, TBS) was added and incubated for 5 min at 37°C. After washing with TBS, a solution of ethanol/acetic acid was added and incubated for 5 min before washing. A blocking solution consisting of 1% bovine serum albumin, 10% normal goat serum, 3% PBST, and 2.5% fish gelatin in PBS was incubated on the sections (1 hour, RT). A TUNEL reaction solution consisting of the enzyme solution and labelling solution from the *In Situ* Cell Death Detection Kit (TMR red, Product No. 12156792910, Sigma Aldrich) was prepared in a 1:9 ratio, diluted in blocking solution (1:1), and incubated with the slides at 37°C for 1 hour. The slides were washed with PBS, and a mounting medium with DAPI (Vectashield, Vector Laboratories, the United States of America) was added. Samples were kept at 2-8°C for at least 30 min before imaging with fluorescent microscopy (Axio Imager Z2 ApoTome, Carl Zeiss Microscopy GmbH, Germany), using a CCD camera with a 20X objective. Ex./Em. of 548/561 nm was used to detect TUNEL labelling at random locations on the section. Image acquisition was done by recording z-stacks, each with 10 images 1 µm apart. Values like exposure time for the red (TUNEL) channel, binning, and brightness/contrast of each image were kept consistent. To quantify the number of dying cells, in either the inner or outer nuclear layer in the tissue section, the following equation was used:

$$\text{TUNEL positive cells (\%)} = \frac{\text{\#TUNEL positive nuclei}}{\text{Area of layer / Average area of nuclei}} \text{ X } 100\% \qquad (15)$$

To analyse statistical significance within the dataset, one-way ANOVA with Tukey's multiple comparisons test ($\alpha = 0.05$) was performed using GraphPad Prism 8.

3.5.5 Determining retinal uptake of fluorescently labelled β-cyclodextrin derivatives

Fluorescently labelled CD (fluorescein-HPβCD, rhodamine-HPβCD, and

fluorescein-RMβCD) was added to organotypic retinal explant cultures as described previously. Slides with retinal tissue sections were rehydrated in PBS for 10 min before mounting medium with DAPI (Vectashield, Vector Laboratories, the United States of America) was added. Images were recorded with fluorescent microscopy as described above. Green (Ex./Em. 493/513) and red (Ex./Em. 558/575) channels were used to detect fluorescein and rhodamine, respectively. Image acquisition was done by recording z-stacks, each with 14 images 1 µm apart. Values like exposure time for the green and red channels, binning, and brightness/contrast of each image were kept consistent. Sections from the saline-treated retinas were imaged using the same parameters to determine the level of auto-fluorescence from the cultures. Alternatively, tile pictures showing the entire retinal section were recorded by stitching together adjacent projected z-stacks in the image acquisition software (ZEN 2.6, Carl Zeiss Microscopy GmbH, Germany).

4 Results and Discussion

4.1 Stability, kinetics, and mechanism of drug degradation in CD solutions

4.1.1 Effect on CD concentration and pH on drug degradation in the CD solution

The degradation of the drugs (tacrolimus and difluprednate) has been shown to follow pseudo-first-order kinetics in aqueous buffer CD solutions. This kinetic behavior was not affected even by the introduction of up to 7.5% (w/v) CD to the reaction medium as a linear relationship was obtained in all cases between the logarithms of the percent of the remaining drug concentration and time. The representative first-order plots are given in Figure 4.

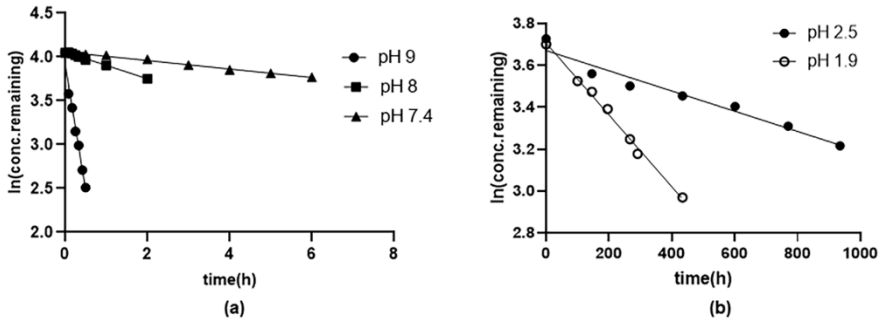


Figure 8. Representative first-order plots (ln (drug concentration remaining) against time) for the degradation of (a) tacrolimus in 2.5 % (w/v) HPβCD solution pH 7.4, 8 and 9; (b) difluprednate in 5% (w/v) HPβCD pH 1.9 and 2.5

These results are consistent with a kinetic system where a drug degrades at a higher rate outside of the CD inclusion complex than within the complex.

$$\begin{array}{ccc} D + CD \rightleftharpoons D/CD & & \\ \downarrow k_f \qquad\qquad \downarrow k_c & & \\ \text{Degradation products} & & \end{array} \tag{16}$$

Where $K_{1:1}$ = complex stability constant

k_f = observed rate constant of the free drug degradation

k_c = observed rate constant for the drug degradation within the complex

$K_{1:1}$, k_c, and k_f were calculated as described previously (*section 1.3.2.1)*

The influence of pH on the degradation of tacrolimus and difluprednate in aqueous HPβCD solution was investigated over the pH range of 1-9. The ionic strength of the buffer was not controlled. The pH-rate profiles for the observed first-order degradation of tacrolimus in aqueous HPβCD solutions at 40°C are shown in Figure 9.

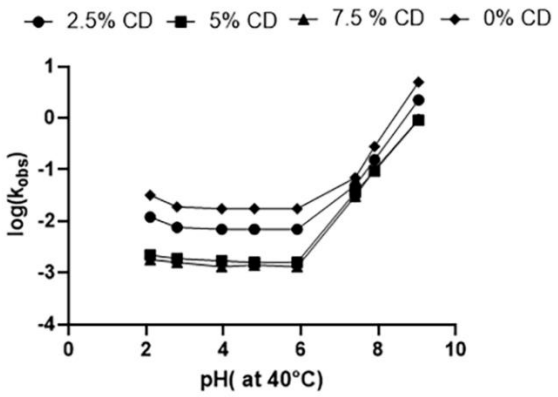


Figure 9. pH rate profile for tacrolimus in HPβCD solution at 40°C

The pH rate profile of tacrolimus in CD solution showed a plateau region in the pH range of 4-6, a small increase in degradation rate between pH 2 and 3, and a sharp increase at pH 6. The degradation pattern was similar for all the CD concentrations tested where we observe a decrease in the observed rate constant for higher CD concentrations. Degradation was massive when there was no CD present. This clearly shows that the tacrolimus degradation decreases with increasing CD concentration. It is most stable at pH between 4 and 6, both in CD and CD-free media.

Tacrolimus is in the unionised form at all pH values tested and all of the k_c and k_f values for tacrolimus in HPβCD solution are presented in Table 5.

Table 5. Values of k_f, k_c, and $K_{1:1}$ for tacrolimus in aqueous HPβCD solutions in the pH range of 2-9.

pH	2	3	4	5	6	7.4	8	9
k_f (h^{-1})	0.032	0.019	0.0175	0.0175	0.0175	0.07	0.28	5
k_c (h^{-1})	0.013	0.0071	0.0070	0.0069	0.0071	0.0072	0.037	1.18

k_c/k_f	0.40	0.37	0.40	0.40	0.40	0.10	0.13	0.24
$K_{1:1}(M^{-1})$	47.47	46.57	44.91	45.29	44.49	21.53	66.35	47.35

$k_f > k_c$ at all of the pH values tested showed that the drug degraded at a higher rate outside the CD complex than within the complex at all pH tested. Also, the both k_c and k_f curves follow similar pathways shown by Figure 10.

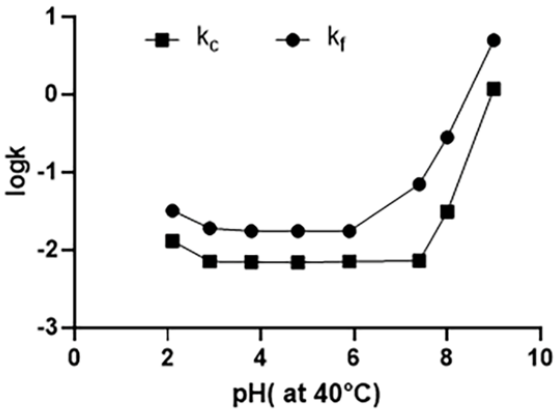


Figure 10. Log k_f (●) and log k_c (○) of tacrolimus in HPβCD solution at 40˚C. The rate constants (i.e. k_f and k_c) are first-order and have the unit h^{-1}.

The shapes of both curves (i.e. for k_f and k_c) show that the hydrolysis reaction of tacrolimus in aqueous CD solutions and CD free media consists of three regions, that is the specific acid-catalysed (i.e. H_3O^+ catalysed) region at pH below about 3, an uncatalysed region or plateau between pH 3 and 7, and a specific base-catalysed (i.e. OH^- catalysed) region at pH above about 7. Hence, the rate of hydrolysis is dependent upon the pH of the medium and both k_c and k_f are composed of three terms as shown by Equations (17) and (18).

$$k_f = k_H[H^+] + k_o + k_{OH}[OH^-] \quad (17)$$

$$k_c = k'_H[H^+] + k'_o + k'_{OH}[OH^-] \quad (18)$$

where k_H and k'_H are acid-catalysed, k_o and k'_o uncatalysed and k_{OH} and k'_{OH} being basic-catalysed rate constants.

The curves have a negative slope below pH values 3 and the k_c and k_f in Equations (17) and (18) are dominated by k_H and k'_H, respectively. This hydrolysis reaction is catalyzed by H_3O^+ ions. For pH values between 4 and 6,

the curves have zero slopes and k_o and k'_o were dominating. Lastly, from pH 7 onwards, we observe a positive slope where k_{OH} and k'_{OH} were dominating as the hydrolysis was catalysed by HO^- ions.

The values of k_H, k'_H, k_o, k'_o, k_{OH} and k'_{OH} for different reaction pathways that constitute the whole hydrolysis process were determined (Table 6).

Table 6. Values of k_H, k'_H, k_o, k'_o, k_{OH}, and k'_{OH} for tacrolimus in aqueous HPβCD solution.

k_H	k_{OH}	k_o
3.2 $M^{-1}h^{-1}$	1.73 x 10^5 $M^{-1}h^{-1}$	0.016 h^{-1}
k'_H	k'_{OH}	k'_o
1.3 $M^{-1}h^{-1}$	4.09 x 10^4 $M^{-1}h^{-1}$	6.76 x 10^{-3} h^{-1}

The definitive expression of the k_f and k_c at 40°C is now given by Equations (19) and (20).

$$k_f = 3.2\,[H^+] + 0.016 + 1.73 \times 105\,[OH^-] \quad (19)$$

$$k_c = 1.3\,[H^+] + 6.76 \times 10\text{-}3 + 4.09 \times 104\,[OH^-] \quad (20)$$

All of these results demonstrated that the hydrolysis reaction of tacrolimus followed an acid-base catalysis mechanism in CD and CD free media. The degradation was the fastest in basic medium as shown by the dominant reaction pathway catalysed by HO- ions.

However, difluprednate shows a V-shaped profile in the pH range of 1-9 (Figure 11). The maximum stability was obtained at pH 5 with a sharp inflection below or above pH 5. It has a pka value of 13.55 and is in unionised form at all pH values tested.

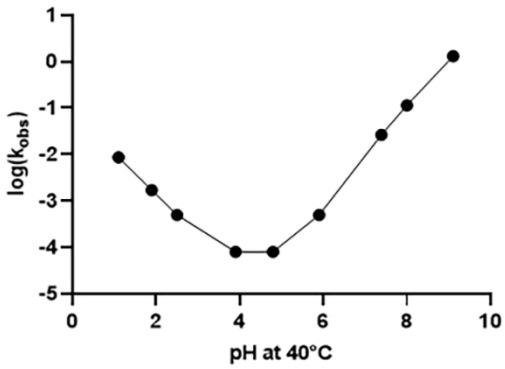


Figure 11. pH rate profile for difluprednate in aqueous HPβCD solutions at 40°C.

The slope of the curve increases sharply at lower and higher pH values suggesting efficient catalysing by hydronium and hydroxide ions at acidic and basic pH respectively as observed in the case of tacrolimus as well. So, the experimental results could be fitted to the following equation:

$$k_{obs} = k_H[H^+] + k_o + k_{OH}[OH^-] \quad (21)$$

k_H is dominant below pH 4 where the slope of the curve is negative and k_{OH} is dominant for pH values greater than 6 where the slope of the curve is positive, and k_o is dominating at a pH between 4 and 5. These three rate constants were determined to be $k_H = 0.109\ M^{-1}\ h^{-1}$, $k_{OH} = 2.6 \times 10^4\ M^{-1}\ h^{-1}$, and $k_o = 2.94 \times 10^{-5}\ h^{-1}$, and inserting these values into Equation (21) gives:

$$k_{obs} = 0.109\ [H^+] + 2.6 \times 10^{-5} + 2.6 \times 10^4\ [OH^-] \quad (22)$$

4.1.2 Effect of different cyclodextrins on drug stability

Tacrolimus is most stable at a pH between 4-6 while the degradation rate is accelerated under basic conditions (pH above 7). The effect of different natural CDs such as αCD and βCD on the tacrolimus degradation was tested (Table 7). Tacrolimus did not readily form a complex with γCD so this CD was not regarded in this study. k_f and k_c values were calculated to see the stabilisation provided by different CDs and are given in Table 7.

Table 7. Values of k_c, k_f and $K_{1:1}$ for tacrolimus at 40˚C and pH 5 or 9 in various aqueous CD solutions.

pH	**5**			**9**		
CD	αCD	βCD	HPβCD	αCD	βCD	HPβCD
k_f (h^{-1})	0.0175	0.0175	0.0175	5	5	5
k_c (h^{-1})	0.0035	0.003	0.00716	1.02	0.97	1.18
k_c/k_f	0.20	0.17	0.41	0.20	0.19	0.23
$K_{1:1}$ (M^{-1})	65.63	1170	44.9	419	2515	47.35

k_c values for αCD and βCD were comparatively lower than that of HPβCD at both pH values tested. Tacrolimus was stabilised by the CD complexation under all conditions given by the k_c/k_f ratio. βCD provided better stabilization in both conditions among the CDs tested as shown by lower k_c values and the highest $K_{1:1}$ values.

Similarly, difluprednate was most stable at a pH of about 5 with a higher degradation rate both below and above pH 5. In order to compare the stabilisation provided by different CDs, the observed rate constants (k_{obs}), the acid-catalysed rate constant (k_H), and the basic-catalysed rate constant (k_{OH}) were calculated at 30 mM concentration except for βCD (maximum solubility used, equivalent to 13.2 mM) under acidic and basic conditions and are listed in Table 8.

Table 8. Observed rate constant (k_{obs}), acid-catalysed rate constant (k_H), and basic-catalysed rate constants (k_{OH}) of difluprednate at 40°C in different CDs at 30mM concentration, except βCD at 13.2mM.

	Acidic conditions			**Basic conditions**		
Samples	pH	k_{obs} (h^{-1})	k_H (M^{-1} h^{-1})	pH	k_{obs} (h^{-1})	k_{OH} (M^{-1} h^{-1})
No CD	1	0.028	0.28	9	2.70	91,000
HPβCD	1.06	0.022	0.25	9.07	1.24	35,200
HPγCD	1.04	0.017	0.18	9.13	2.49	62,400
αCD	1.01	0.026	0.26	9.09	1.85	51,000
βCD	1.00	0.025	0.26	9.11	1.67	43,800
γCD	1.01	0.021	0.21	9.09	7.25	200,000

All of the CDs were able to stabilise difluprednate in aqueous solution except γCD in basic media where the rate constant was almost 3 times more than the observed rate constant when no CD is present. It seems like γCD was catalysing difluprednate degradation in basic conditions which were supported by a very high k_{OH} value.

Under basic conditions, HPβCD gave the lowest k_{OH} values among all the CDs tested displaying that difluprednate was better stabilized by HPβCD under such conditions. However, HPγCD gave the lowest k_H value followed by the natural γCD and HPβCD gave the lowest k_{OH} value meaning that that the HPγCD was able to better stabilise the drug under acidic conditions. After this, we calculated the value of k_c (degradation constant from within the CD complex) of these two CDs (HPβCD and HPγCD) under acidic and basic conditions using the Lineweaver plot (*section 1.3.2.1*) to compare the stability provided by these CDs in specifics.

Table 9. Values of k_c, k_f, and $K_{1:1}$ for difluprednate at 40˚C at pH 1 and 9 in aqueous HPβCD and HPγCD solutions.

pH	**1**		**9**	
CD	HPβCD	HPγCD	HPβCD	HPγCD
k_f (h^{-1})	0.028	0.028	2.70	2.70
k_c (h^{-1})	0.019	0.009	2.40	0.85
k_c/k_f	0.71	0.32	0.88	0.31
$K_{1:1}$ (M^{-1})	18.16	52.73	193.17	13.88

k_c is lower than k_f under all conditions for both the CDs, suggesting that the degradation from within the complex was slower in all cases. Difluprednate degraded more than 3- and 1.2-folds slower within HPβCD and HPγCD complex respectively in basic media (pH 9). In acidic media (pH 1), difluprednate degraded more than 1.3-folds slower within HPβCD complex and more than 3-folds slower within HPγCD complex shown by the lower k_c/k_f ratio and stability constant values for HPγCD.

In brief, βCD provided better stabilisation for tacrolimus in both acidic and basic conditions. Difluprednate was better stabilised by HPγCD under acidic conditions. Since difluprednate was most stable at pH 5 (i.e. acidic conditions), HPγCD seemed like a better host for difluprednate in terms of stability.

4.1.3 Drug degradation mechanism in cyclodextrin solution

Preliminary studies with tacrolimus showed that oxidation might not be a major degradation pathway in an aqueous CD solution. This was concluded because no significant difference in rate constant (Table 10) was observed when the degradation studies were done with and without purging the reaction media with nitrogen and also with and without 0.5% EDTA. EDTA forms complexes with metal ions that can catalyse oxidation degradation of tacrolimus.

Table 10. Values of observed rate constants (k_{obs}) of tacrolimus in HPβCD solution at pH 5 and 9 with and without 0.1% EDTA.

k_{obs} (h^{-1})	**pH 5**	**pH 9**
With 0.1% EDTA	0.0014	0.96

Without EDTA	0.0016	0.91

UHPLC-MS studies were then used to profile and identify the degradation products of tacrolimus in CD solution. Parameters like mass/charge (m/z) values, fragmentation pathway, and chromatographic properties were employed to identify the degradation products.

Under acidic conditions, tacrolimus (R_t 4.04 min) degradation was relatively slow. Tacrolimus degradation in HPβCD buffer solution at pH 2.5 yielded a mixture of two compounds that were more polar than tacrolimus with retention times (R_t) of 2.88 and 2.90. Both had identical masses 844 [M $^{+}$Na] $^{+}$. These two compounds could be isomers as the mass spectra and fragmentation data were quite similar and practically indistinguishable from each other. The MS data of these compounds coincided with the hydrolysed form of tacrolimus at its lactone group when analysed by MassLynx software. Il'ichev *et al*. mentioned similar results where the lactone group is hydrolysed during the degradation of tacrolimus-related compounds like everolimus and sirolimus [158]. Another compound was obtained at 4.38 R_t with m/z 808, which was less polar than tacrolimus. The MS data indicated that dehydration might have occurred with a loss of water molecules resulting in the formation of a less polar compound.

Under basic conditions (pH 10), tacrolimus degraded completely within 1 hour. Here, it also generated two compounds, with the retention time of 2.90 and 2.99 with identical masses 844 [M $^{+}$Na] $^{+}$. These compounds presented the same elemental composition and similar fragmentation data as given by the hydrolysed form of tacrolimus under acidic conditions which suggest that all these compounds could be isomers. Another major product slightly more polar than tacrolimus was observed with a retention time (R_t 3.38) and 826 [M $_{+}$Na] $^{+}$. This was identified as the open-chain form of a compound formed by the dehydration of the tacrolimus molecule by the elemental composition from the MS data. Skytte *et al*. observed the formation of the same compound when they treated tacrolimus with 1,5-diazabicyclo [4.3.0] nonene (DBN) in dichloromethane (basic conditions) [159]. It seems like tacrolimus has undergone hydrolysis at the lactone group and a dehydration reaction to from a double bond based on the structure of this compound. The proposed degradation mechanism of tacrolimus in HPβCD solution can be represented in Figure 12.

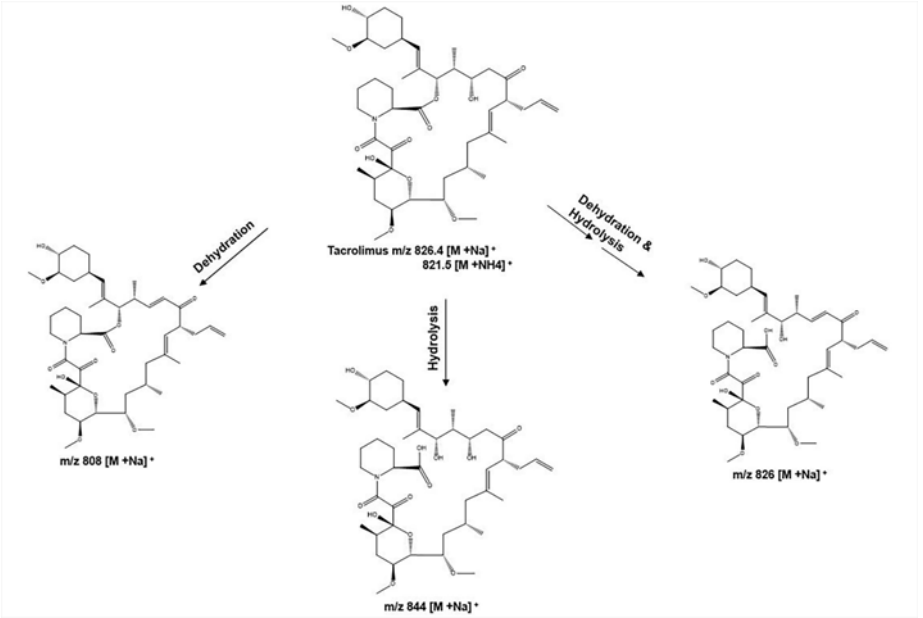


Figure 12. Proposed tacrolimus degradation pathways in aqueous HPβCD solutions.

4.1.4 Phase-solubility studies

Different CDs were used to determine the solubility of tacrolimus and difluprednate in aqueous solutions by the phase-solubility method of Higuchi and Connors [26]. The interaction of different CDs with tacrolimus and difluprednate usually led to an increase in their apparent aqueous solubility due to the formation of drug/CD complexes. Phase solubility plots were constructed by plotting the CD concentration versus the concentration of the drug in the solution. The slopes were calculated using different portions of the graph depending upon the phase solubility type (A-types: all CD concentration ranges; B-types: just the initial linear portion). These slopes and the determined intrinsic solubility (S_0) were used to calculate other parameters like the stability constant ($K_{1:1}$), the complexation efficiency (CE), and the molar ratio (MR).

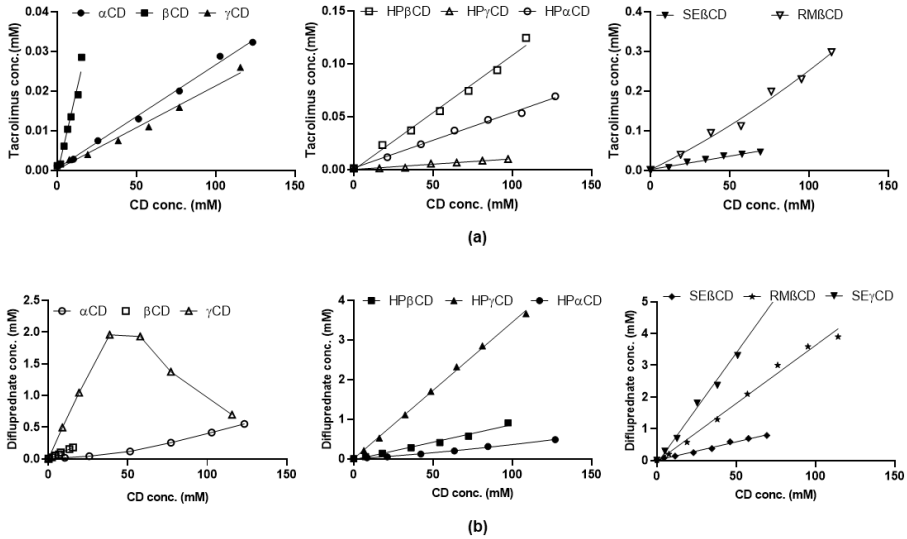


Figure 13. Phase-solubility diagrams of (a) tacrolimus and (b) difluprednate in different CDs in pure water at room temperature. Each point represents the mean of triplicate experiments.

Natural CDs have lower water solubility compared to their hydroxypropylated derivatives and tend to form aggregates. This aggregate formation can contribute to the definition of phase solubility profiles for many drugs [160, 161].

We observe three different types of phase solubility diagrams, that is A_L-type diagram where a strict linear relationship is observed, the A_P-type diagram with positive deviation from the linearity, and the B_s-type diagram where the solubility of the drug/CD complex is limited in the aqueous media. Distinguishing between the two A-types was done by comparing the correlation coefficient values (r^2) where the solubility curves with r^2 values greater than 0.99 are regarded as A_L type and less than 0.99 are regarded as A_P [28].

αCD, HPαCD, and SEBβCD displayed A_L phase-solubility type while the rest of the CDs displayed A_P phase-solubility type for tacrolimus. Contrasting results were observed for diflupredate where αCD and HPαCD showed A_P type and the rest of the CDs showed A_L type except for γCD. γCD with diflupredate exhibited B_S type where the solubility decreased gradually after increasing γCD concentration from 5% (w/v) onwards. This is common behavior for native CD, especially γCD because of their relatively limited water solubility and also the aggregation properties where the CD molecules and drug/γCD complexes self-assemble to form aggregates that precipitate from the solution [162].

Table 11. Stability Constants ($K_{1:1}$ and $K_{1:2}$) and complexation efficiency (CE) of tacrolimus/CD and difluprednate/CD complexes in pure water at room temperature (pH about 5).

	Tacrolimus					Difluprednate				
	Type	Slope	$K_{1:1}$	$K_{1:2}$	CE	Type	Slope	$K_{1:1}$	$K_{1:2}$	CE
αCD	A_L	0.0003	500	***	0.0003	A_P	0.016	172	30	0.016
βCD	A_P	0.0017	571	66	0.0017	A_L	0.011	2006	***	0.011
γCD	A_P	0.0002	50	9	0.0002	B_s	0.0498	9036	***	0.052
HPαCD	A_L	0.0005	278	***	0.0005	A_P	0.0056	464	19	0.002
HPβCD	A_P	0.001	174	4	0.001	A_L	0.0082	1425	***	0.008
HPγCD	A_P	0.00009	55	7	0.00009	A_L	0.0369	6605	***	0.038
RMβCD	A_P	0.0025	500	3	0.0025	A_L	0.0366	6550	***	0.038
SEBβCD	A_L	0.0007	269	***	0.0007	A_L	0.0116	2024	***	0.011
SBEγCD	NA	NA	NA	NA	NA	A_L	0.0696	13358	***	0.075

Among the natural CDs, βCD had the highest stability constant for tacrolimus and γCD for difluprednate. suggesting that these CDs´ cavities were of appropriate size for the respective drugs. The hydroxypropyl derivatives of the natural αCD and βCD had inferior stability constants compared to natural CDs. Similar results were obtained in the case of difluprednate where the hydroxypropyl derivatives of βCD and γCD have inferior stability constants. The reduced ability of these hydroxypropyl derivatives might be due to the steric hindrance of the substituent groups at the CD cavity [163].

RMβCD showed a comparatively high stability constant for both tacrolimus and difluprednate explained by the increased hydrophobic cavity of the CD upon methylation of the OH-groups.

The use of complexation efficiency (CE) is a better approach for comparing the solubilising potential of different CDs as the determination of the stability constant values is strongly affected by the accuracy of the intercept (S_{int}) and intrinsic solubility (S_o) obtained from the phase-solubility plots (theoretically S_{int} should be equal to S_o) [27-29]. In our study, the CE values are higher for γCD and its derivatives, especially for SBEγCD for difluprednate and RMβCD for tacrolimus, which are reflected in the stability constant values of these CDs as well.

From both the stability and solubility studies with different CDs, βCD provided better stabilisation for tacrolimus in both acidic and basic conditions. However, the limited solubilisation of tacrolimus by βCD was problematic while HPβCD provided better solubilisation. Therefore, further studies of tacrolimus were conducted with HPβCD. Moreover, HPγCD seemed to be a better host for difluprednate in terms of both solubilisation and stabilisation. Even though these CDs were able to stabilise the drugs in solution, the stability obtained was not sufficient, as required for the commercially available dosage forms. So, different surfactants/polymers were used in combination with these CDs to further improve the chemical stability of tacrolimus and difluprednate in aqueous media.

4.1.5 Solubilisation and stabilisation of drugs in CD-polymer system

An aqueous solution containing CD and polymers like poloxamer 407, tyloxapol, and tween 80 were used (from 0 to 5% w/v) for these studies. The media contained 5% (w/v) HPβCD for the tacrolimus study to which 100 µL of tacrolimus stock solution (2.48 mM) were added. Commercially available difluprednate emulsion has 0.05% (w/v) drug while it was possible to solubilise

0.1% with 15% (w/v) HPγCD. Hence, on the contrary, the combination of polymers and 15% (w/v) HPγCD were prepared to solubilise 0.1% difluprednate. These solutions were subjected to one cycle of autoclaving and the remaining drug concentration was measured by using the UHPLC methods explained before. Drug degradation was observed in both the combination of CD and polymer and only CD and polymer, respectively, to observe the stabilising effect of both the components alone and their combination.

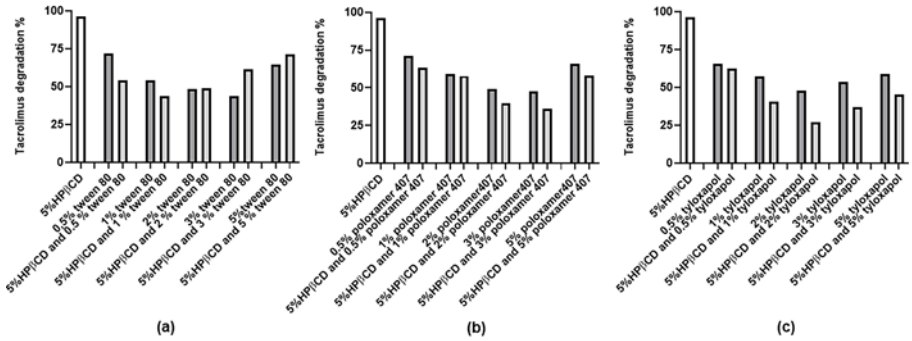


Figure 14. Tacrolimus degradation % after one cycle of autoclaving with 5% (w/v) HPβCD and various % (w/v) of polymers (a) tween 80, (b) poloxamer 407, and (c) tyloxapol.

Tacrolimus degradation in aqueous HPβCD solution showed more than 90% drug degradation within one autoclave cycle which is massive. The drug stabilised by polymers individually and by the HPβCD-polymer system was far better compared to only HPβCD. The combination system provided better stability in all cases compared to individual components except in the case of tween 80-HPβCD system from 2% tween 80 onwards, as shown in Figure 14.

The drug degradation decreased with increasing poloxamer concentration up to 3% where the degradation again increased from 5% which applied to when only poloxamer or the combination of poloxamer-HPβCD were used. A similar trend was observed in the case of tyloxapol where tacrolimus degradation was at a minimum at 2% tyloxapol and then increased upon increasing tyloxapol concentration (for both only tyloxapol and tyloxapol-HPβCD combination). The best result in our studies was with the combination of HPβCD and 2% tyloxapol where about 30% of tacrolimus was degraded which was still high for drug degradation.

However, promising results were observed in difluprednate stabilisation with HPγCD and these polymers. The drug degradation % in the case of difluprednate with these polymers is given in Figure 15.

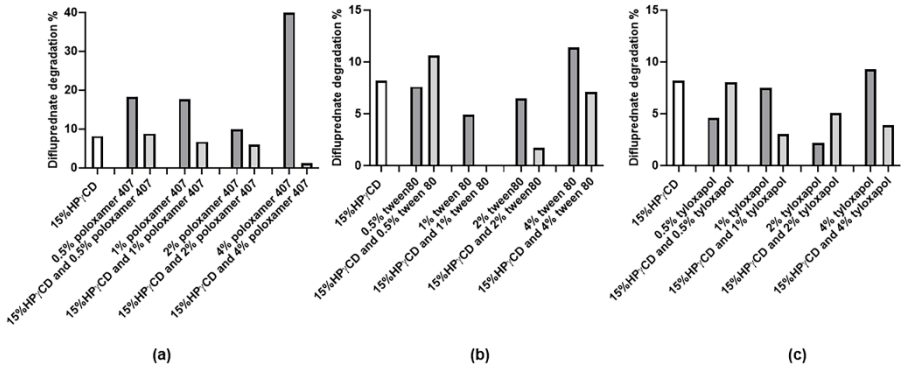


Figure 15. Difluprednate degradation % after one cycle of autoclaving with 15%(w/v) HPγCD and various % (w/v) of polymers: (a) poloxamer 407, (b) tween 80, and (c) tyloxapol.

The difluprednate degradation in the poloxamer-HPγCD system decreased with increasing poloxamer concentration where the degradation was the least at 4% combination (less than 1% drug degradation). Difluprednate degradation was massive when only poloxamer was present in the solution, especially at 4%. Difluprednate was better stabilized in the combination system compared to only HPγCD or poloxamer, and also HPγCD was a better stabiliser than poloxamer when compared individually.

The tween 80-HPγCD combination showed that the degradation decreased when the concentration of tween 80 was increased from 0.5 to 1% but again increased from 2% onwards. The combination did not provide better stabilisation compared to the individual components in all cases. One percent tween 80 in 15% HPγCD solution exhibited no drug degradation but the solubility of the drug was greatly reduced in the presence of tween 80. The tyloxapol-HPγCD combinations followed the same trend as with tween 80, where difluprednate degradation decreased up to 1% tyloxapol and then increased again from 2%.

Overall, the minimum degradation was observed with the combination of 4% poloxamer and HPγCD as well as 1% tween and HPγCD. The tween 80-HPγCD combination was not viewed as beneficial as solubility was heavily reduced (only 0.37 mg/mL, which was less solubilisation than by pure HPγCD). The reduction in the solubility might be due to the competitive displacement of the drug molecules from the CD cavity by the polymers contributing to this solubility problem [164].

Furthermore, tyloxapol and poloxamer 407 were used in an attempt to increase the solubility of the tacrolimus in combination with 5% HPβCD. Figure 16 shows the solubility of tacrolimus in 5% HPβCD along with various % of poloxamer and tyloxapol.

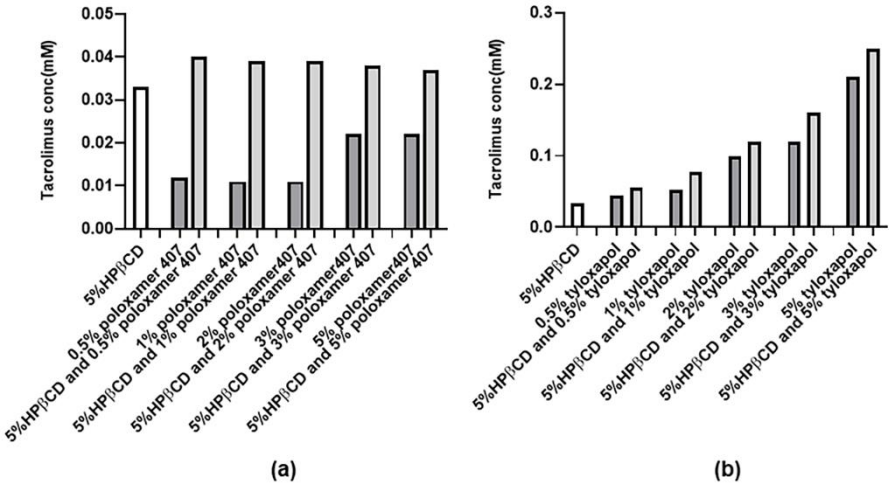


Figure 16. Solubility studies with 5% (w/v) HPβCD and various % (w/v) of (a) poloxamer407 and (b) tyloxapol

The solubility of tacrolimus was increased by about 1.2-folds by poloxamer-HPβCD combination but did not show an increasing trend with increasing poloxamer concentrations. The addition of poloxamer to the complexation media resulted in competition with drug molecules for the CD cavity and a consequent displacement of the drug molecules from the CD cavity [164]. The competitive displacement was probably compensated by the solubilising effect of polymer micellization, hence we observed a slight increase in solubility.

In the case of tyloxapol-HPβCD, tacrolimus solubility increased with increasing tyloxapol concentration in the ternary system. The solubilisation by the combination system was more than the individual component in all cases giving the maximum solubility of 0.3 mM at 5% tyloxapol-HPβCD. There might have been no or little influence of drug-polymer competition for the CD cavity in this case and has been explained previously [165].

In summary, the stability of tacrolimus and difluprednate could be further improved with the drug/CD/polymer ternary system. The CD-poloxamer system was found to be effective for difluprednate while CD-tyloxapol for tacrolimus. The use of the CD-polymer system for drug stabilisation has been studied before and thus our attempts to increase the stability of these drugs.

CD stabilises the drug by the complexation process while polymers like poloxamer and tyloxapol by micelle formation and protection of the hydrophobic drug within the micelles [164, 166, 167]. Generally, the combination of CD complexation and micellar solubilisation can lead to a synergistic effect resulting in enhanced drug stability [17, 53, 168]. The formation of micellar-type CD aggregates enabled the solubilization of very poorly water-soluble drugs [19, 53, 169]. Tyloxapol, a non-ionic surfactant oligomer, has been known to improve the solubility and stability of drugs by either improving drug wettability or micellar incorporation of drug and drug/CD complexes in the case of HPβCD [170]. Similarly, poloxamer 407 formed nano-sized micellar aggregates with a hydrophobic core and a hydrophilic shell [53] and acted as a stabiliser in dispersed systems [171].

In the case of difluprednate, increased stability with increased % of poloxamer could indicate the formation of micellar CD aggregates with an increase in % of polymers. A similar result was obtained with better stability when the combination of CD and poloxamer was used [50, 169]. It has been shown that the addition of a ternary component (polymers in our case) to the binary complex (drug/CD) leads to improved macromolecular assembly [28, 42-48] and has been used to improve stability.

Even though slight improvements in terms of stability and solubility were observed with tacrolimus using a CD-polymer system, tacrolimus was not adequately chemically stable and hence no further studies were conducted.

In contrast, the stability of difluprednate in an aqueous solution could be improved without compromising the target solubility with HPγCD and poloxamer 407. Four percent (w/v) poloxamer and 15% (w/v) HPγCD was found to be the best combination in terms of stability without compromising the drug solubility intended, hence, further characterisation studies were done with this combination.

4.2 Characterisation of drug/CD complex

4.2.1 Particle size measurement and morphological analysis (NanoSight and TEM)

The poloxamer-HPγCD combination provided better difluprednate stabilisation in an aqueous solution. To better understand the poloxamer-HPγCD system and the drug/CD complexes, we tried to determine the particle size of the poloxamer-HPγCD aggregates containing difluprednate by using Nanosight Wave. The aggregate size of 15% HPγCD and its combination with different % of poloxamer containing difluprednate are given in Table 12.

Table 12. Aggregate size of difluprednate/HPγCD/poloxamer aggregates in solutions containing different concentrations (% w/v) of poloxamer 407.

Poloxamer 407 concentration (%w/v)	Aggregate size (nm)
0.0%	125.9
0.5 %	160.7
1.0 %	194.1
2.0 %	217.1
4.0 %	234.1

The aggregate size of aqueous 15% HPγCD solution containing 0.1% (w/v) difluprednate is approximately 161 nm. The aggregate size of the samples increased when the combination of poloxamer 407 and HPγCD was used and increased with an increasing percentage of poloxamer 407.

Studies have shown that CDs interact with the poloxamer unimer, preferably by the inclusion of the hydrophobic propylene oxide segment into the CD cavity [172]. The ethylene oxide unit of poloxamer has relatively high hydrophilicity and hence has less tendency to penetrate into the cavities [173, 174]. When poloxamer is introduced to the CD media, reorganisation of the whole system will occur [175-177]. Similar changes were observed when poloxamer was added to an aqueous medium containing budesonide and HPβCD [165].

The morphology and size of these samples were further confirmed by TEM as shown in Figure 17. Aggregates with larger sizes (from 100 nm) do not have a spherical shape like the ones below 100 nm. Instead, they look like clusters of smaller spherically shaped aggregates [178]. The size of aggregates observed from the TEM is somewhat smaller than the size of the aggregates determined by NanoSight, as expected. This can be explained from the TEM sample preparation, which can change the aggregate size or structure due to the drying process involved.

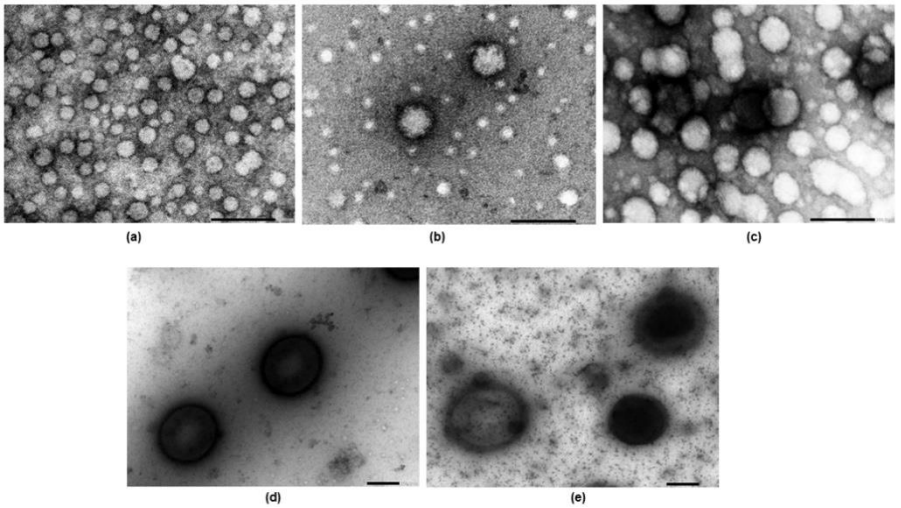


Figure 17. Transmission electron microscopic images of (a) difluprednate/HPγCD and difluprednate/HPγCD/poloxamer complexes with different %(w/v) of poloxamer 407 (b) 0.5% poloxamer 407, (c) 1% poloxamer 407, (d) 2% poloxamer 407, and (e) 4% poloxamer 407, at magnitude of 60 K.

4.2.2 ^{1}H-NMR (nuclear magnetic resonance) spectroscopy

^{1}H-NMR spectroscopy has become one of the most important methods for structural elucidation of organic compounds in the solution state [179]. These studies do not just provide the information on the characteristics of guest/CD inclusion complexes but also the orientation of the guest molecule inside the hydrophobic CD cavity [180, 181].

When an inclusion complex is formed, chemical shifts ($\Delta\delta^{*}$) in the ^{1}H-NMR spectra of the guest and the CD molecule are observed. The observed chemical shifts of a specific host or guest can provide evidence for the formation of inclusion complexes in solution, as major changes in the microenvironment are known to occur between the free and bound states [182]. In our study, the difference in the chemical shifts of the difluprednate/HPγCD and difluprednate/HPγCD/poloxamer was noted and $\Delta\delta^{*}$ calculated using the following equation:

$$\Delta\delta^{*} = \delta_{complex} - \delta_{free} \quad (23)$$

where $\delta_{complex}$ and δ_{free} are chemical shifts between free and bound CD molecules, respectively.

HPγCD has 6 protons located at different positions: on the interior of the cavity (i.e. H3 and H5) and on the exterior part (H1, H2, H4, and H6) based on

the molecular shape of CDs. Depending on the protons where the shifts are more produced, different types of complexes can be formed: inclusion or non-inclusion. More pronounced shifts at H3 and/or H5 proton mean that the drug forms inclusion complexes with CD (incorporation inside the CD cavity), while the H-protons on the outer CD surface will mean that the non-inclusion complexes are formed [183-185]. Similarly, the shifts of H5 represent a 'deep' inclusion complex since its position in the CD cavity is deeper than H3 while the shifts of H3 indicate a 'shallow' complex or partial inclusion [117, 186].

All these protons displayed significant resonance alternation in the presence of guest molecules (difluprednate) and the ^{1}H-NMR chemical shifts corresponding to HPγCD in free-state and complexes are shown in ppm and listed in Table 13.

Table 13. The ^{1}H-chemical shifts of HPγCD alone and in the presence of difluprednate and poloxamer 407.

Protons	**HPγCD**	**difluprednate/HPγCD ($\Delta\ \delta^{*}$)**	**difluprednate/HPγCD/ poloxamer 407($\Delta\ \delta^{*}$)**
H1	5.151	-0.0025	-0.0014
H2	3.6571	0.0005	0.0014
H3	4.0548	-0.0043	-0.0043
H4	3.5032	0.0010	0.0034
H5	3.8895	-0.0028	-0.0021

The changes in ^{1}H-chemical shifts (Δδ*) of the H1, H3, and H5 protons were -0.0025, -0. 0043 and -0.0028, respectively, displaying upfield shifts. This can be explained by the fact that water was replaced by the hydrophobic aromatic ring(s) of the difluprednate molecule inside the cavity as these effects are an indication of reduced hydration due to steric hindrance or hydrogen bonding [187]. The upfield shift of the H3 was observed with higher changes than the H5, where H5 was situated at the inner surface of the cavity of the primary hydroxyl group side and H3 was located on the inner surface at the secondary hydroxyl group side. So, the higher shielding effect on H3 with respect to H3 suggests that the difluprednate formed a shallow complex with HPγCD [117, 186]. Likewise, it has been explained that the upfield effects experienced by the host molecules were most probably due to ring-current and magnetic anisotropy effects created by the aromatic drug [188].

H2 and H4 showed downfield shifts ($\Delta > 0$) when difluprednate formed a binary complex with HPγCD. This can be probably due to the de-shielding effects of the van der Waals interaction between HPγCD and difluprednate molecules or due to variation of local polarity upon complex formation [182, 186, 189, 190].

In the case of HPγCD in difluprednate/HPγCD/poloxamer ternary complex, resonance protons in both internal and external cavities underwent upfield and downfield shifts similar to the binary complex of HPγCD with difluprednate. The addition of the polymer increased the shielding effect of the characteristic protons in the outer cavity shown by greater $\Delta\delta^*$ values of H2 and H4. In conclusion, we observe the shielding effect on both the internal and external cavities of HPγCD in binary and ternary complexes while the presence of polymer enhanced the interaction of difluprednate with the external part of the HPγCD molecule.

4.2.3 Fourier transform infrared spectroscopy (FTIR)

A complex formation between drug and a CD usually leads to a shifting/disappearing of the individual components characteristic bands or a decrease in their intensity [191]. So, FTIR was used to elucidate the interaction between difluprednate and HPγCD to observe the shifts of the vibrational wavelengths of the components due to the presence of inclusion complexes [191].

HPγCD was characterised by bands at 2930 cm^{-1} due to the C-H stretching vibrations, bands at 3370 cm^{-1} related to the symmetric and antisymmetric O-H stretching mode, and other bands at lower frequencies. Concerning difluprednate, it had characteristic bands at 1660.44 cm^{-1}, which are representative of the unsaturated ketone or secondary carbonyl, and other bands from 1720.41 cm^{-1} to 1750.53 cm^{-1} related to the acyclic ketone and esters.

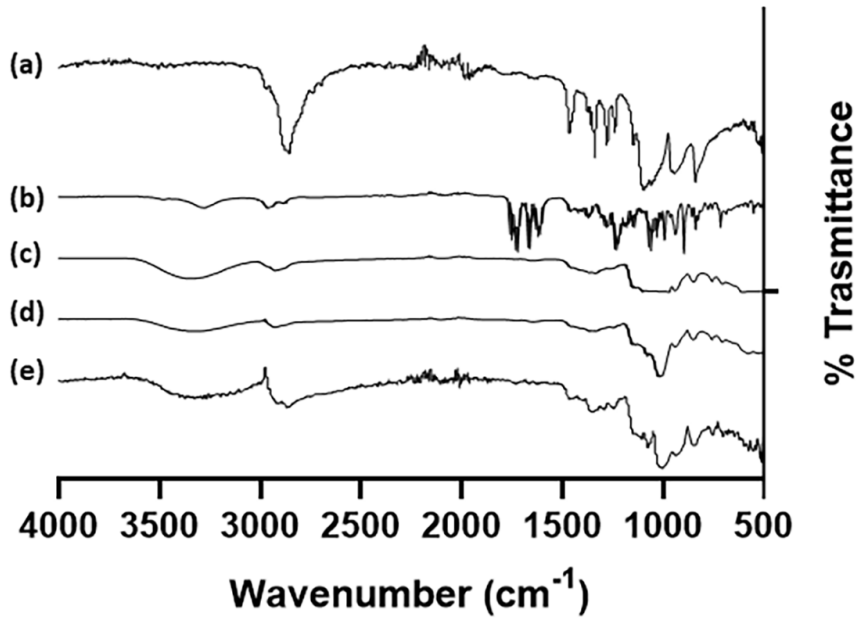


Figure 18. FTIR spectra of (a) poloxamer 407, (b) difluprednate, (c) HPγCD, (d) difluprednate/HPγCD complex, and (e) difluprednate/HPγCD/poloxamer complex

From the FTIR results of the complex state, all of the characteristic peaks of difluprednate at 1660 and 1720 cm^{-1} are not observed. This may suggest that all of these carbonyls and acylic ketone/ester sections are complexed with HPγCD. All of these disappearances of the absorption frequencies indicate that HPγCD formed a complex with the drug, difluprednate. Similarly, the characteristic peaks of difluprednate disappeared in the complex in the presence of poloxamer. These changes indicated that HPγCD formed a complex with the drug that included the polymer.

4.2.4 Differential Scanning Calorimetry (DSC)

Generally, the interaction between CD and drug can be revealed either by the shifting or the disappearing of the individual components´ melting point. Here, DSC was applied to evaluate the solid-state characterisation of HPγCD, pure difluprednate, and their complexes. The DCS thermograms of pure difluprednate, HPγCD, and the complex of difluprednate and HPγCD are shown in Figure 19.

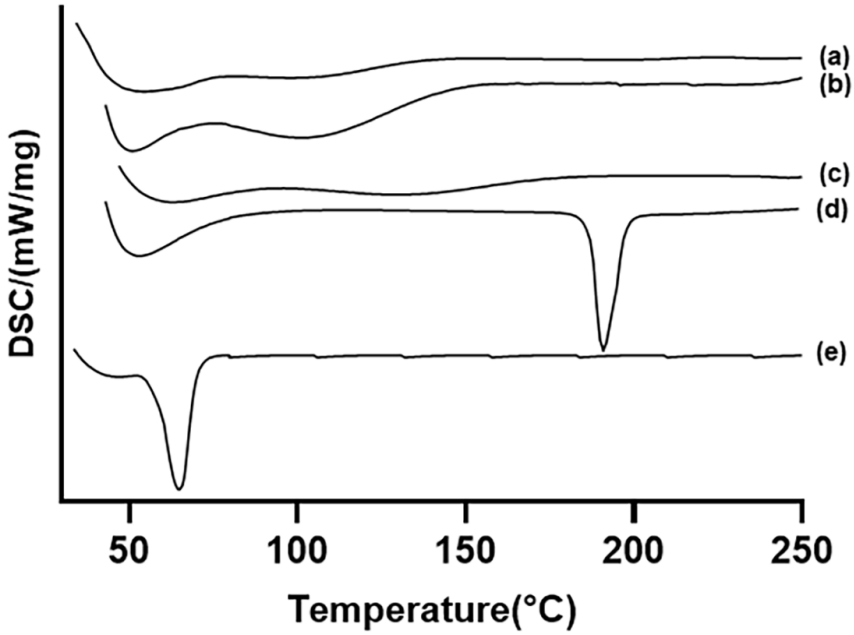


Figure 19. DSC of (a) difluprednate/HPγCD/poloxamer complex, (b) difluprednate/HPγCD complex, (c) HPγCD, (d) difluprednate, and (e) poloxamer 407.

When a complex is formed between drug and CD molecules, the disappearance or shifting of the individual endothermic peak to other temperatures observed [192] indicates a change in the crystal lattice, melting, boiling, or sublimation points [193]. The DSC results showed the typical thermal curves from crystalline difluprednate with a well-defined sharp endothermic peak at 191°C corresponding to the difluprednate melting point with the decomposition of the drug. The CDs themselves do not display any melting peak but decompose at a temperature above 300°C [194-196]. The temperature range in this study was 0 to 250°C. However, HPγCD displayed broad endothermic peaks between 60-130°C, which is indicative of dehydration or the loss of water molecules from the CD cavity, upon heating. The difluprednate peak has completely disappeared in the complex solid-state with HPγCD and the one that has HPγCD and poloxamer 407 suggesting the inclusion-complex formation and the existence of a new solid phase.

4.3 Effect of thermodynamic activity of the permeating drug molecule: different solubiliser and different barrier systems

Different factors affect the permeation of drug molecules through the biological

barrier including aqueous drug solubility, a drug´s short residence time, the lipophilic membrane barrier, and lastly the thermodynamic activity of the dissolved drug molecule. Sripetch *et al*. have already described the theory behind the critical role of the thermodynamic activity of the permeating molecule and its effect [121]. Here, we have tried to explain that further in detail.

Hydrocortisone (HC) was the model drug used for this purpose. The permeation of hydrocortisone in three different solubiliser systems such as cyclodextrin, ethanol, and SLS was studied in different barrier systems like cellulose membrane and permeapad membrane. A permeapad membrane is a biomimetic artificial barrier with cellulose hydrate foil supports coated with a mixture of phospholipids and additives [197]. It proved to maintain high integrity over time and in different pH environments [198].

4.3.1 Solubility profiles of hydrocortisone in different solubiliser systems

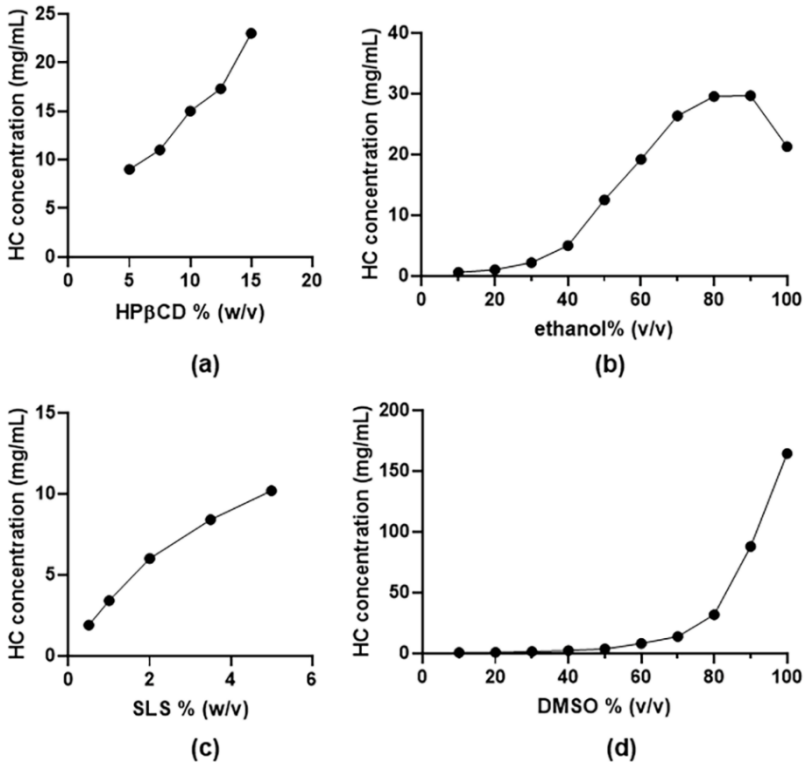


Figure 20. Solubility profiles of hydrocortisone in different solubiliser systems (a) HPβCD, (b) ethanol, (c) SLS, and (d) DMSO.

All the solubility studies were carried out at room temperature and it was observed that different solubilisers have various solubilising capacity with respect to hydrocortisone. HPβCD could solubilise hydrocortisone greatly leading to a concentration of about 25 mg/mL in 15% (w/v) HPβCD.

In the case of ethanol, solubility increased with the concentration of ethanol reaching the maximum at 80%, and then it showed a declining trend. It was concurrent with the results obtained from Ali. *et al.* where they reported the maximum solubility of hydrocortisone at value fractions of 80% of the ethanol [199]. Sodium lauryl sulfate was also found to be a good solubiliser but only a low concentration of up to 5% was used with respect to their toxicity in high concentration and it has limited their high concentration use in various formulation purposes [200]. However, DMSO was found to be a poor solubiliser of hydrocortisone. That made it difficult to see the permeation effects on the Franz cells and thus the studies were not regarded.

For our studies, the drug concentration was kept saturated at 10% (w/v) HPβCD, 30% (v/v) ethanol, and 2% (w/v) SLS. The concentrations of the solubiliser were increased and decreased further, keeping the drug concentration the same. Solutions were obtained at higher concentrations while suspension was obtained at the lower concentration. The flux was calculated through the slope applying the linear relationship between the time and the amount of the drug in the receptor chamber. Finally, the flux obtained was plotted against the different concentrations of the solubiliser used.

4.3.2 Hydrocortisone in HPβCD system

Figure 21 shows the effect of HPβCD concentration on the flux of hydrocortisone from an aqueous donor vehicle through different membrane barriers.

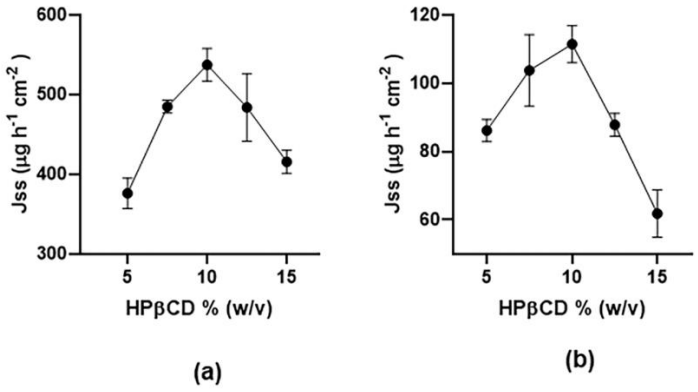


Figure 21. Effect of HPβCD concentration on the flux of hydrocortisone through (a) cellulose membrane (MWCO 6000-8000) and (b) permeapad barrier.

All of the flux profiles show similar trends across all of the artificial membranes with maximum flux at 10% HPβCD and a decreasing flux trend when the HPβCD concentration is either decreased or increased. The total hydrocortisone concentration was kept at 15 mg/mL in the donor compartment but the concentration was increased from 5 to 15%. The concentration of dissolved hydrocortisone increased with increasing HPβCD concentration until all of the drugs were in solution and then remained constant at 15 mg/mL.

At 10% HPβCD, hydrocortisone was in the saturated condition in solution with the thermodynamic activity at its maximum, so it was able to permeate through the membrane at its highest potential giving the maximum flux. When the HPβCD concentration was increased, it will lower the drug activity resulting in a decreased permeability coefficient. So, CDs will decrease the potential of the drug to enter the membrane when HPβCD was decreased even though the concentration of dissolved hydrocortisone remained the same.

Cyclodextrin and cyclodextrin complexes are known to form aggregates or micellar-like structures [54, 201]. Aggregates consisting of more than 2 to 8 hydrocortisone/HPβCD complexes will be unable to permeate membranes depending upon the MWCO of the membrane [202]. So, in the cellulose membrane, the decrease in flux at higher concentrations might be argued with the formation of bigger cyclodextrin aggregates at higher concentrations and their ability of not able to permeate through the membrane.

However, permeapad has a lipid layer sandwiched between the cellulose membrane so in theory, no cyclodextrin molecules should enter the membrane as cyclodextrin are very hydrophilic molecules that are unable to permeate the

lipophilic membrane. The flux is solely from the permeation of drug molecules itself.

Even though similar profiles were observed in all the barrier systems studied, the flux was variable from one to another, and was especially high for the cellulose membrane and low for the permeapad membrane. As explained before, the comparatively high flux for the cellulose membrane might be due to the flux from both the permeation of drug/cyclodextrin complexes and the drug molecule itself. Consequently, relatively low flux in the permeapad was observed as expected, as only the lipophilic drug molecule entered through the membrane barrier.

4.3.3 Hydrocortisone in ethanol

The study was continued with hydrocortisone in the ethanol system and Figure 22 shows the effect of ethanol concentration on the flux of hydrocortisone from an aqueous donor vehicle through different permeapad membrane barriers.

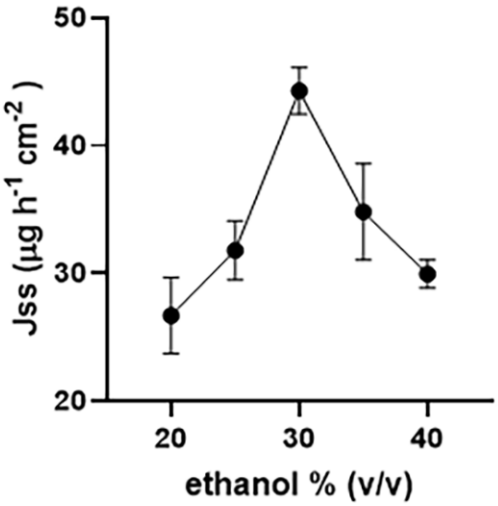


Figure 22. Effect of ethanol concentration on the flux of hydrocortisone through the permeapad barrier.

As expected, a similar profile was also observed in ethanol to that of HPβCD. Here, the total hydrocortisone concentration was kept constant at 2 mg/mL in the donor compartment and the concentration of ethanol was increased from 20% to 40%. The thermodynamic activity of the drug was at its maximum at 30% when it was in saturated solution and hence the highest flux.

Increasing or decreasing the ethanol concentration led to a decrease in drug activity and consequently lower flux as observed.

Ethanol is generally used as a penetration enhancer and the enhancing effect was found to be concentration-dependent especially for skin permeation [203, 204]. That means that a higher flux will be observed with a higher ethanol concentration, and that was the case found to be in the cellulose membrane and hence the study was not regarded.

However, in the case of the permeapad, studies have shown that the membrane better tolerated ethanol up to 40%, maintaining the integrity over a period of 5 hours [205]. Hence, it can be expected that the flux profile we have observed was also due to the thermodynamic activity of the drug molecule.

4.3.4 Hydrocortisone in sodium lauryl sulfate

The flux profile in SLS was different to what we have observed in the case of HPβCD and ethanol. Here, the total hydrocortisone was kept constant at 6 mg/mL and the concentration of the SLS was increased from 0.5 % to 5% SLS. The highest flux was observed in the low concentration of SLS and the flux continued to decrease with increasing concentration of SLS.

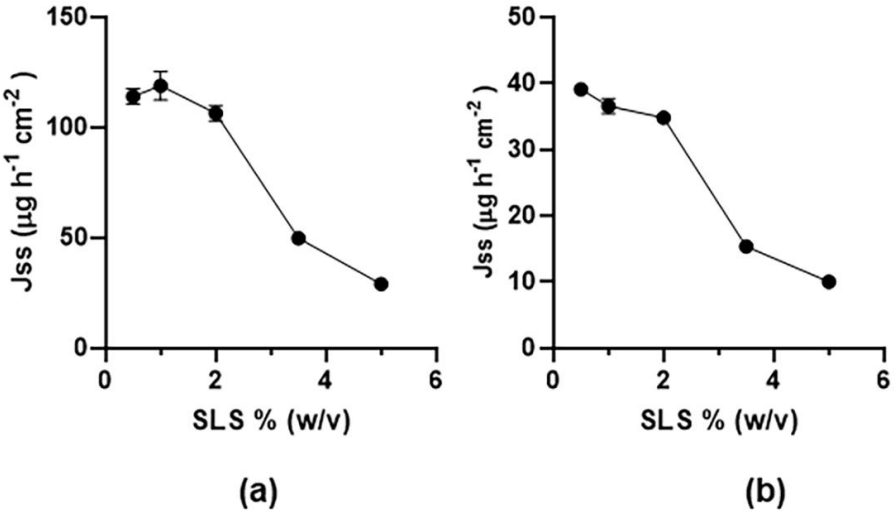


Figure 23. Effect of SLS concentration on the flux of hydrocortisone through (a) cellulose membrane (MCO 6000-8000) and (b) permeapad barrier

But here, we have to consider that the critical micelle concentration of SLS is 0.2% [206] and the formation of micelles might have effects on the drug permeation as well. There could be an interaction between SLS micelles (with

or without hydrocortisone) and the surface of the barrier depositing the micelles and increasing the resistance of the diffusion layer that hydrocortisone has to pass. This was explained by Bibi *et al*. in the permeapad barrier system in Polysorbate 80 system [205].

Therefore, we could explain the role of the thermodynamic activity on the permeation of hydrocortisone from solubiliser systems like HPβCD and ethanol across different artificial membrane barriers supporting our hypothesis.

4.4 Cytotoxicity of β-cyclodextrin derivatives in retinal explant cultures

4.4.1 Particle size and TEM data analysis

CDs self-assemble to form nano-sized aggregates and the formation of the aggregates is concentration-dependent. The aggregate formation increases with increasing CD concentration. Natural CDs like α, β, and γCD form aggregates in low concentrations while hydrophilic CDs like hydroxypropylated derivatives have less tendency to form aggregates at low concentrations [19]. Although the aggregation in pure aqueous CD solutions is generally low, the aggregation is frequently enhanced by the formation of drug/CD complexes. Furthermore, the aggregation and the size of the aggregates increase with an increasing CD concentration [207].

In order to observe aggregation, we have measured the particle size of the highest concentration of CD used, i.e. 100 mM using NanoSight, and confirmed by TEM.

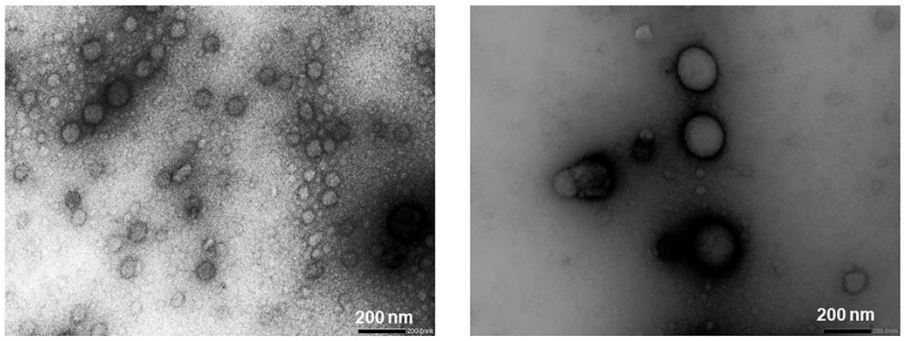


Figure 24. Transmission electron microscopy images of cyclodextrin (CD) aggregates at a magnitude of 30 K. (a) 100 mM HPβCD and (b) 100 mM RMβCD. The average diameter of RMβCD aggregates appeared to be around two times larger than that of HPβCD.

CDs are enabling pharmaceutical excipients widely used in pharmaceutical formulations and the concentration of CDs used will depend upon the type of CDs. However, based on their favorable toxicological and pharmacological profiles, they can be used in relatively high concentrations [208]. Some limitations to this can be natural CDs, especially βCD where they have limited solubility compared to hydroxypropyl or sulfobutylether derivatives [4].

Both CDs used in our study (HPβCD and RMβCD) have high water solubility hence high concentrations of these CD derivatives could be used in order to observe their potential toxicity on retinal explants. Aggregates were observed in aqueous HPβCD and RMβCD solutions. The average diameter of the HPβCD aggregate, as determined by NanoSight, was 148 nm and that of RMβCD was 305 nm. Larger aggregates (diameter >100 nm) do not have a spherical shape like smaller ones (<100 nm). Instead, they look like clusters of smaller, spherically-shaped aggregates [178]. The aggregate diameters observed with TEM are smaller than those determined by NanoSight. This can be explained by the TEM sample preparation, where the aggregate size or structure can change during sample preparation.

Generally, it is thought that only the free drug molecules, which have dissociated from the CD complex, are able to permeate cell membranes [4]. However, recent findings have revealed that CD molecules can enter the cells by endocytosis [209] and this may also be true for drug/CD complexes [210]. However, it should be noted that it is highly unlikely that the large and hydrophilic CD aggregates will enter cells.

4.4.2 Cytotoxicity of β-Cyclodextrin derivatives in retinal explant cultures

The TUNEL assay was employed to quantify the number of dying cells in histological sections from retinal explant cultures incubated with CDs (Figure 25).

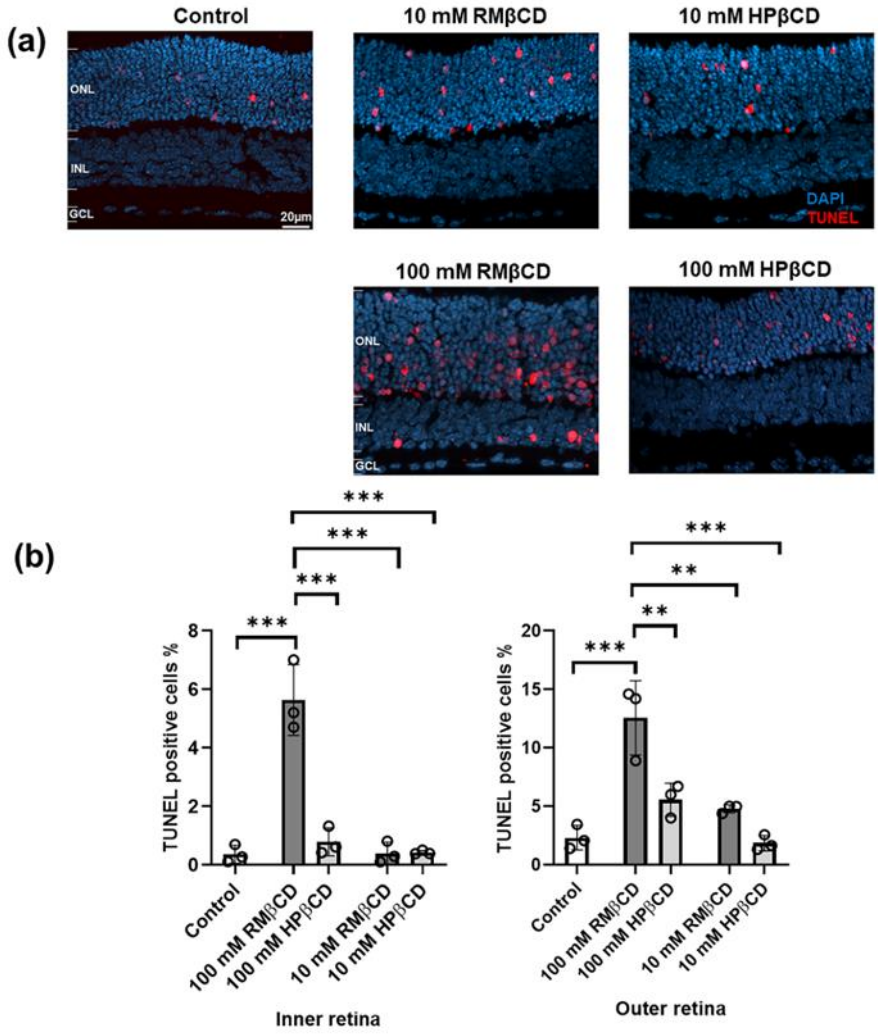


Figure 25. Cytotoxicity of β-derivatives of cyclodextrin in retina explant cultures. (a) Sections of retinal cultures to which different CD solutions were applied; the saline solution was used as the control. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (red) was used to detect dying cells. DAPI (blue) was used as a nuclear counterstain. Cultures were derived from wild-type mice at postnatal day (P) 13. CDs were added at P15 and incubated with the cultures for a duration of 48 hours. ONL = outer nuclear layer, INL = inner nuclear layer, and GCL = ganglion cell layer. (b) Analysis of average TUNEL positive cells (%) in both INL and ONL from cultures with different CD solutions and the control. Results represent the mean ± SD for $n = 3$ explant cultures per group. A statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons tests ($\alpha = 0.05$) and asterisks represent the significant difference (** = $p \leq 0.01$ and *** = $p \leq 0.001$).

The relative cytotoxicity of the CDs was expressed as the percentage of TUNEL positive cells in the respective part of the retinal tissue section. They were counted in both the outer retina (outer nuclear layer, ONL) and inner retina (inner nuclear layer, INL) (Figure 25a). Most of the cells typically degenerate quickly after explant tissue preparation in the ganglion cell layer (GCL) due to the cutting of the optic nerve, hence, TUNEL positive cells were not counted there.

The top of the retinal cultures (side closest to the GCL) was applied with 10 and 100 mM CD solutions and this is the route the CDs would naturally follow after intravitreal injection.

For 100 mM, the TUNEL positive cell values in the INL were about 0.5%, 5%, and 0.6% for the control, RMβCD, and HPβCD, respectively, while for the ONL, they were about 3%, 12%, and 6% for the control, RMβCD, and HPβCD, respectively (Figure 25b). This showed that both CDs were toxic compared to the control when used in 100 mM concentrations. RMβCD was significantly more toxic compared to HPβCD, both in INL and ONL. HPβCD was predominantly toxic to ONL cells, i.e. where the cell bodies of photoreceptors are located.

For 10 mM, the TUNEL positive cell values in the INL were about 0.4% and 0.4% for HPβCD and RMβCD, respectively and for the ONL, they were about 1.8% and 4.8% for HPβCD and RMβCD, respectively. Here, RMβCD still exhibited significant toxicity and killed cells, especially in ONL, while the number of TUNEL positive cells for HPβCD was similar to the control. This exhibited that the retina could safely tolerate levels as high as 10 mM HPβCD.

RMβCD displayed higher toxicity at both concentrations studied compared to HPβCD. RMβCD is a modified βCD where about two-thirds of the hydroxy groups have been replaced by methoxy groups, while in the case of HPβCD, only a few of the CD-hydroxyl groups have been substituted by 2-hydroxypropyl groups. This contributes to high lipophilicity in RMβCD, with a logP value of -6 [1, 211, 212] while the logP value of HPβCD is about −11. Different properties of CDs like the solubilising capacity, the tissue irritating effect, the hemolytic activity, and the surface activity are vastly affected by their lipophilicity. The more lipophilic the compound, the easier it penetrates the cell layer. However, because of the size and hydrophilicity of the CD molecule, it has been explained that the permeability of CDs through biological membranes is negligible [1].

It has been shown recently that CD was able to penetrate every cutaneous layer of the human skin [213]. In addition, relatively high amounts of HPβCD and dimethyl-β-cyclodextrin were absorbed via the rectum of rats and excreted into the urine, suggesting CD complexes may be absorbable through the rectal mucosa [214]. However, the latest findings regarding the endocytosis of CDs gave a whole new perspective of CDs being able to enter cells [209].

CD molecules can easily form complexes with natural hydrophobic molecules including the cellular components based on the host-guest interaction. Phospholipids are the preferred cellular target for αCD and cholesterol for βCD [215]. Because of this property, they have the capacity to remove membrane components such as cholesterol, phospholipids, and proteins directly affecting cells and biological barriers. Both α and β CDs can cause dose-dependent hemolysis and cytotoxicity and the substituents on the CD ring greatly impact these effects [108, 136, 216].

Cholesterol is one of the major components of the cell membrane constituting 30% of total lipids and plays an important structural role in membrane stability [217]. Since βCD has an affinity for cholesterol, this CD can induce the release of cellular cholesterol inducing lysis of the cell membrane [216]. Consequently, the cholesterol content of the membranes can decrease thus affecting the function of the cell membrane and disrupting the barrier function of the cell layers [215]. Additionally, it was found that cholesterol extraction caused the destabilisation of tight-junction protein complexes, which are localised in lipid rafts [215].

Methylated β-cyclodextrins tend to interact strongly with lipids [218, 219], and there is a correlation between the cytotoxic effect and the cholesterol complexation properties of βCD derivatives [138]. The higher complexation with cholesterol will impose more toxicity on the cells.

Cholesterol represents >98% of the total sterols in the retina [220]. The stronger interaction of CD with the lipids/cholesterol in retinal cells can aid in more vigorous cell membrane destabilisation and more cell death. Likewise, there are similar reports on Müller glial cells where the cholesterol status plays an important role. Low cholesterol is harmful to the retinal cells; hence, more cholesterol extraction by RMβCD might have caused more toxicity [220]. This impression was further supported by our phase-solubility studies of cholesterol with different β-cyclodextrin derivates (Figure 26).

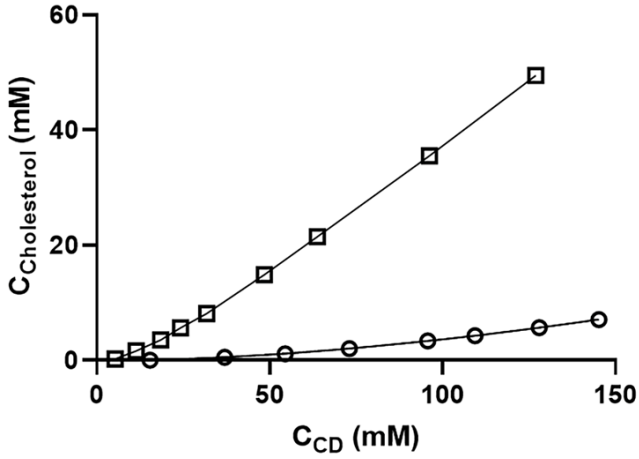


Figure 26. Phase-solubility diagrams of cholesterol in various aqueous β-cyclodextrin derivatives at room temperature. The diagram shows the CD concentration plotted against the cholesterol concentration. Overall, cholesterol was solubilised about five-fold more by RMβCD than by HPβCD. Each point represents the mean of triplicate experiments. Key: o = HPβCD and □ = RMβCD.

Cholesterol was solubilised five-folds more effectively by RMβCD than HPβCD (Figure 26). Due to the higher lipophilicity of RMβCD as explained before, cholesterol had a higher affinity for them compared to hydrophilic cyclodextrin like HPβCD. The cholesterol solubilisation was also affected by the structure of the CD derivative, like the number and position of the methyl groups and the presence of ionic groups [138]. Similar results have been obtained when the toxicity of these CDs was tested on different cell lines. The cytotoxicity of methylated βCDs was found to be very high in pulmonary Calu-3 cells while hydroxypropylated βCD was proven to be safe for pulmonary drug delivery [221].

In another study, a high concentration of HPβCD up to 200 mM presented no cytotoxicity on Caco-2 cell lines, and the cholesterol extraction capacity was considered a predictive factor for βCD derivatives cell toxicity [138].

Furthermore, RMβCD possesses surface-active properties [222, 223] and it even shows a detergent-like mechanism of lipid solubilisation when interacting with lipid vesicles. Here, RMβCD was first adsorbed onto the vesicle surface, which was followed by RMβCD partitioning from the aqueous medium into the phospholipid bilayers forming lipid-RMβCD mixed assemblies and finally the lipid solubilisation into micelle like aggregates [224]. The cells in the ONL are photoreceptor cells, and cholesterol is an important component of

photoreceptor membranes, relevant for the cells´ function [225, 226]. Hence, photoreceptors might suffer more from the cholesterol extraction capacity of the CDs, something that might be particularly relevant for the higher toxicity observed with RMβCD. Additionally, the cell death in the ONL might be exacerbated by an overall higher sensitivity of photoreceptors, when compared to INL cells.

4.4.3 Fluorescent microscopy of fluorescently-labelled cyclodextrin derivates to study cellular uptake in retinal cultures

The overall distribution of fluorescently-labelled CDs was investigated after the cytotoxicity studies on explant cultures. For this purpose, RBITC-HPβCD (rhodamine-labelled HPβCD), FITC-HPβCD (fluorescein-labelled HPβCD), and FITC-RMβCD (fluorescein-labelled RMβCD) were used and the fluorescent intensity in the inner and outer retina was quantified (Figure 27) and compared to the control specimen to account for auto-fluorescence coming from the tissue itself.

No difference in fluorescent intensity between FITC-labelled CDs was observed for the inner and outer retina, indicating that the HPβCD and RMβCD distribute similarly within the retinal tissue. Therefore, the difference in cytotoxicity between the two compounds was likely due to their respective effects on the cells, as discussed above, and not because of differences in the overall tissue distribution.

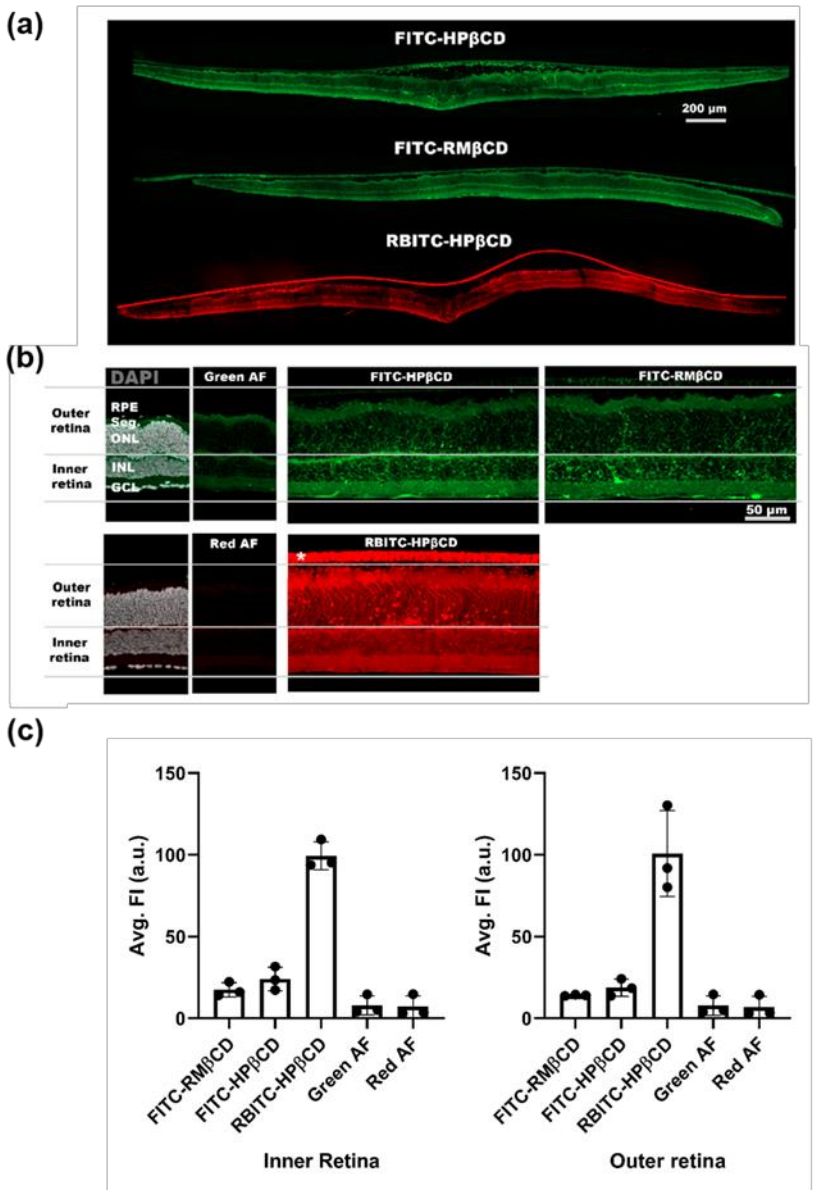


Figure 27. Uptake of fluorescently-labelled CDs in the retina explant culture. (a) Overview of tissue sections from wild-type(WT) mice explant cultures to which three different fluorescently-labelled CDs were added to the side facing down in the image. (b) Close-up images of the sections were taken for an analysis of the fluorescent signal. Images from control sections (without added CDs) were used to measure the intensity of the red and green autofluorescence (AF) coming from the tissue itself. For this analysis, the outer retina is defined as the area from the outer nuclear layer (ONL) to the retinal pigment epithelium (RPE), while the inner retina encompasses the area from the ganglion cell layer (GCL) to the inner nuclear layer (INL). Seg. = segments of the photoreceptors (inner and outer segment) membrane on which the retinal explant was

cultured. (c) Analysis of the average fluorescent intensity coming from either the inner or outer retina. For both inner and outer retina, a high signal was detected from RBITC-labelled CDs, while the signal was lower and similar for FITC-labelled CDs. Results represent the mean ± SD for $n = 3$, where n is the number of animals.

Some subtle differences between FITC-labelled CDs were observed. For FITC-HPβCD, a string-like structure was seen, spanning across the inner and outer retina. This was not as prominent in cultures where FITC-RMβCD was applied (Figure 27a). This type of staining suggests that the CDs had partly been taken up by the Müller glial cells.

When using a rhodamine-labelled HPβCD, we observed an elevated signal in the retina. The signal-to-noise ratio was higher for RBITC-HPβCD than for FITC-HPβCD (Figure 27, c). This allowed us to detect the specific uptake in photoreceptors, which supported the result from the cytotoxicity analysis, where HPβCD mainly killed cells in the outer retina.

However, it should be noted that the dye labelling could also have affected cell uptake. First of all, we did not observe the same Müller cell uptake in retinas with RBITC-HPβCD, compared with retinas with FITC-HPβCD. Since the rhodamine molecule is positively charged, the rhodamine-conjugated CDs might bind to negatively charged cell membranes and extracellular matrix elements, possibly facilitating cellular uptake. FITC and RBITC CD derivatives behave differently and are internalised by different processes. The labelling increases the molecular weight and may alter the properties of the parental CDs, while these still retain high water solubility and cannot cross the cell membrane by passive diffusion. However, there are reports suggesting that endocytosis was observed in fluorescent CDs as well [210]. Müller cells have the capacity to assemble and secrete lipoproteins which can be utilised by photoreceptors or inner retinal neurons, serving as an intraretinal source of cholesterol [227]. This could explain the Müller cell uptake of HPβCD. While the fluorescent CDs do not behave exactly as their non-fluorescent form, these studies enhance the understanding of the behavior of labelled CD derivatives at the tissue level [228].

5 Summary and conclusions

This dissertation focused on different topics on the applicability of cyclodextrins in ophthalmic delivery whether as stabiliser/solubiliser for hydrophobic drugs or as the drug entity itself and the toxicity related with it. Also, it covered a small part on the effect of the thermodynamic activity of the permeating drug molecule across different barrier systems in different solubilisers including CDs. Firstly, tacrolimus and difluprednate were selected as model drugs. The study aimed at investigating the chemical stability and kinetics of tacrolimus and difluprednate in various CD solutions to provide a mode of stabilisation. Different kinetics studies and phase solubility studies were performed in aqueous solutions containing different CDs at different pH values. Mass spectrometry studies were also performed to elucidate the degradation mechanism of tacrolimus in CD solution. After all these, the CD which was found to be the best solubiliser and stabiliser was selected to study with different polymers like poloxamer 407, tween 80, and tyloxapol to further improve the stability for both tacrolimus and difluprednate. Then, characterisation of the drug/CD complexes was done using techniques like NMR, DSC, and FTIR. Secondly, the toxicity of different βCD derivatives was studied on retinal explants to explore their use in various retinal diseases. Finally, investigations on the effect of solubiliser concentrations on the permeation of the drug molecule was performed.

The following observations were made in this dissertation:

- The stability of tacrolimus and difluprednate was determined as the function of the medium acidity. CDs tend to stabilise both drugs under pH conditions tested (1-9) as observed by comparing the rate constants of the free and unbound drug (i.e. within the complex). The stabilisation was more enhanced under acidic conditions. Both drugs were found to be stable at around pH 5 and the degradation was vigorous in basic conditions.
- Tacrolimus degradation in CD solutions was mainly due to hydrolysis of the lactone linkage both under acidic and basic conditions, dehydration or simultaneous hydrolysis, and dehydration to yield the final product. This was confirmed by mass spectroscopic studies using the MassLynx software.
- Phase-solubility studies were done with various natural CDs and their derivatives. It showed that βCD and its derivatives increased tacrolimus solubility much more than the other CDs tested while γCD

and its derivatives could solubilise difluprednate more compared to other CDs.

- Based on the described solubility and stability studies, HPβCD and HPγCD were found to be the best CDs in terms of stability and solubility for tacrolimus and difluprednate, respectively.
- Further stabilisation of tacrolimus was done with HPβCD and different polymers. The stability and solubility were improved when the combination of CD and polymers were used, particularly with poloxamer 407 and tyloxapol. However, tacrolimus was not adequately chemically stable to be formulated as aqueous eye drops.
- Promising results were obtained with HPγCD-polymers systems in the case of difluprednate. The stability was greatly improved with the combination of HPγCD and poloxamer 407 system especially with 15% HPγCD and 4% poloxamer 407. This system could solubilise 0.1% difluprednate and the drug degradation was less than 1%.
- Difluprednate/HPγCD and difluprednate/HPγCD/poloxamer system in solution were characterised by measuring the micelle aggregate size using NanoSight and confirmed by TEM. Likewise, ^{1}H-NMR confirmed the presence of drug/CD complexes in both binary (difluprednate/HPγCD) and ternary (difluprednate/HPγCD/poloxamer) systems confirmed by the observed shifts in proton peaks. Solid-state characterisation studies using different methods like DSC and FTIR further confirmed the presence of drug/CD complexes in both binary and ternary systems. Even though difluprednate could be stabilised in an aqueous solution consisting of CD and polymers, various other parameters like viscosity, tonicity, etc., need to be considered and, further studies with different excipients should be done to formulate this drug into aqueous eye drops.
- Permeation studies of hydrocortisone in different solubiliser systems across different artificial membrane barriers showed that the concentration of these solubilisers do affect the thermodynamic activity of hydrocortisone in the solution and consequently, the permeation. This was shown by the flux profile against solubiliser concentration with the decrease in flux when the solubiliser concentration is increased/decreased from the saturated conditions.
- Cytotoxicity studies of HPβCD and RMβCD on retinal explants showed that both CDs were toxic to the retina when the concentration was as high as 100 mM. RMβCD was significantly toxic even at a low concentration while the retina could safely tolerate as high as 10 mM HPβCD.
- From the cytotoxicity studies done by using TUNEL assay, it can be concluded that the higher toxicity of RMβCD might be due to its high lipophilicity, more vigorous cholesterol extraction capacity, and surface-active properties. Fluorescent microscopy of fluorescently-

labelled cyclodextrin derivatives accounted that the difference in cytotoxicity was likely due to their respective effects on the cells rather than their distribution.

To summarise, our results suggested that CD can be used as a good solubiliser and stabiliser for poorly water-soluble and chemically unstable drugs in an aqueous solution. This can be further improved by using the combination of drug and polymers systems. However, detailed studies should be done to better understand the drug/CD/polymer system and the interactions within them. Permeation through the biological membrane is one of the important factors that need to be considered while developing any kind of formulation. We could provide more insight on the effect of the thermodynamic activity of the drug molecule when permeating across different barriers. This could help in choosing different solubilisers and their concentration for the optimum formulation. Lastly, the use of CDs for various retinal diseases has been explored in the last decades and our toxicity analysis together with fluorescent studies could be employed in further developing CDs as drugs or drug carriers for the treatment of retinal diseases.

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