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Stability characterization, kinetics and mechanism of tacrolimus degradation in cyclodextrin solutions

Manisha Prajapati [a], Finnur Freyr Eiriksson [b], Thorsteinn Loftsson [a],[*]

[a] *Faculty of Pharmaceutical Sciences, University of Iceland, Hofsvallagata 53, 107 Reykjavik, Iceland*
[b] *ArticMass, Sturlugata 8, 101 Reykjavik, Iceland*

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ABSTRACT

Tacrolimus is a macrolide lactone and potent immunosuppressant. It is highly lipophilic and has very limited aqueous solubility. Tacrolimus is highly susceptible to hydrolysis which results in very limited stability in aqueous solutions. Besides this, tacrolimus also undergoes dehydration and epimerization. Cyclodextrin (CD) complexation can increase the solubility and stability of hydrophobic drugs in aqueous solutions through the formation of drug/CD complexes. The aim of this study was to investigate degradation kinetics, mechanism and stability of tacrolimus in aqueous CD solutions, with the ultimate goal of developing an aqueous vehicle for ophthalmic delivery. For this, phase-solubility and kinetic studies in aqueous solutions containing different CDs at different pH values were performed. Mass spectrometry studies were also performed to elucidate the degradation mechanism of the drug in aqueous CD solution. The study showed that the drug has maximum stability between pH 4 and 6 and hydrolysis was the main cause of tacrolimus degradation in aqueous 2-hydroxypropyl-βCD (HPβCD) solutions. βCD and its derivatives were the better CD solubilizers for tacrolimus. The solubility and stability studies were further conducted with CD and surfactants, which is tyloxapol, tween 80 and poloxamer 407, where the combination provided better results compared to individual components.

1. Introduction

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 (Akashi, 1996). 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 cataract and induce glaucoma (Siegl, 2019). Similarly, in diseases like atopic dermatitis and dry eyes, topical tacrolimus formulations have been noted to have significant therapeutic efficacy (Arima, 2001). However, tacrolimus is a highly lipophilic compound and has 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 (Siegl, 2019).

Cyclodextrins (CDs) are cyclic oligosaccharides of α-D-glucopyranose with hydrophobic central cavity and a hydrophilic outer surface. They are able to form inclusion complexes with several drugs provided that their structure (or part of it) fits in the CD cavity (Loftsson, 1989). No covalent bonds are formed or being broken during the complexation and drug molecules in the complex are in rapid equilibria with free molecules in the complexation media (Loftsson, 2005). The complexation affects many physicochemical properties of drugs such

as their chemical stability and aqueous solubility (Loftsson, 1989). The usage of natural CDs as drug carriers is restricted by their limited aqueous solubility but several hydrophilic CD derivatives have been synthesized such as methylated, hydroxypropylated and sulfobutyl ether CD derivatives (Arima, 2001). These hydrophilic CD derivatives can form highly water-soluble complexes with lipophilic drugs.

No ophthalmic dosage formulation is commercially available for tacrolimus. Though, many researchers have recently studied the efficacy of topical tacrolimus for various allergic ocular diseases. Vichayon et al. stated marked clinical responses with 0.1% tacrolimus ointment. Hideshi et al. reported that 0.1% tacrolimus ophthalmic suspension was viable to treatment of severe allergic conjunctivitis (Zhai, 2011; Shoughy, 2017; Ohashi, 2010). These are the few of the reported dosage for tacrolimus for ophthalmic use which showed efficacy. For commercial eye drops, shelf-life of at least 3 years is desired (Baranowski, 2014). However, there is a lack of proper and extensive stability data on the available studies. Surprisingly, it was mentioned that 0.1% tacrolimus ophthalmic solution was stable only for 20 days when stored at 25 °C and for at 85 days or more when stored at 2–8 °C (Ezquer-Garin et al., 2017). Besides solubility and stability, various factors affect the physicochemical properties of eye drops like pH, drug concentration, osmolality and viscosity (Sharma, 2016). There are few reports on the use of CDs to improve the pharmaceutical characteris-

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tics of tacrolimus, especially its solubility (Benelli, 1996; Mills, 1995). Mills et al. (Mills, 1995) assessed the efficacy of topical CD-encapsulated tacrolimus to prevent experimental corneal allograft rejection (Ezquer-Garin et al., 2017). Arima et al. (2000) have reported improvement of tacrolimus solubility through complexation with various βCD derivatives ultimately leading to improved oral bioavailability supported by faster dissolution rate of tacrolimus (Arima, 2001). Nonetheless, the development of aqueous eye drop formulation containing tacrolimus is still a challenge, particularly due to its low chemical stability and solubility.

Evaluation of drug degradation is important during the development of pharmaceutical formulations to determine chemical degradation pathways and products as well as to estimate the product shelf-life. Knowing the degradation pathways can facilitate stabilization of the drug as degradation products can cause toxic side effects and other unwanted effects. Knowledge of drug stability and its degradation products are essential during development of any pharmaceutical formulation (Campos, 2017). Taking this into consideration, the objective of this study was to investigate the chemical stability and kinetics of tacrolimus in various CD solutions, elucidate the degradation mechanism and provide mode of stabilization. The ultimate goal was designing and developing a tacrolimus ophthalmic formulation containing CDs.

1.1. Theory

Tacrolimus is a complex 23-membered macrolide lactone with L-pipecolic acid moiety adjacent to a masked tricarbonyl functionality. It has 14 stereocenters, 3 double-bonds and a number of free hydroxyl groups and other functionalities (Skytte, 2013). There exists a solvent-dependent equilibrium between cis and trans rotamers in solution due to restricted rotation of the amide bond in the pipecolic acid moiety. Besides these, a different kind of equilibrium exists in polar solvents with respect to cyclic ketal moiety. This equilibrium is explained by tautomerism of tacrolimus where tacrolimus epimerizes to an intermediate tautomer I(ring-opened tacrolimus) which is then converted to tautomer II to reach an equilibrium containing the three forms (Skytte, 2013; Namiki, 1993; Peterka, 2019).

Tacrolimus can undergo several degradation and transformation pathways such as dehydration, epimerization, rearrangement and isomerization of double bonds due to its structural characteristics (Myers, 2016). It is also highly susceptible to lactone hydrolysis under acidic and basic conditions leading to formation of several products (Higuchi, 1965).

2. Materials and methods

2.1. Materials

Tacrolimus was purchased from Shanghai Huirui Chemical Technology Co., Ltd. (China) and tacrolimus monohydrate (European Pharmacopoeia (EP) Reference Standard) from Sigma-Aldrich. α-Cyclodextrin (αCD), β-cyclodextrin (βCD), γ-cyclodextrin (γCD) and 2-hydroxy-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, Netherland 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). Similarly, we purchased Ethylenediaminetetraacetic acid (EDTA), tyloxapol reagent grade and poloxamer 407 from Sigma-Aldrich, USA and tween 80 from Tokyo Chemical Industry Co., Ltd. Japan. Milli-Q water was used for the preparation of all solutions and the mobile phase for UHPLC measurements. All other chemicals were commercially available products of special reagent grade.

2.2. Methods

2.2.1. Chromatographic conditions

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 VF-P10-A pump, a VF-A10-A autosampler, VH-C10-A column compartment, VWD-3100 UV–Vis detector operated at 205 nm and a Phenomenex Kinetex C18 1.7 μm 100 × 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, sample injection volume was 10 μl and the retention time(RT) was 3 min.

2.2.2. Buffers

Hydrochloric acid–potassium chloride buffer (pH 2), citrate buffer (pH 3–6), phosphate buffer (7–8) and carbonate-bicarbonate buffer (pH 9) was 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 studies.

Various amounts (expressed as % w/v) of different CDs were added to the buffer solutions when the effects of CDs were investigated.

2.2.3. Kinetic studies

The tacrolimus degradation 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 tacrolimus concentration was 2.48 mM. The pH of the final reaction mixture was determined at the end of each experiment with a pH meter standardized at 40 °C. All reactions were run under pseudo-first-order conditions. Aliquots (10 μl) were injected into the column at various time intervals, and the pseudo-first-order rate constant (k_{obs}) determined by linear regression of natural logarithm of the remaining drug concentration vs time plots.

2.2.4. MS quad/LC-MS studies for degradation products

All samples for mass spectrometer (MS) studies were prepared as described above in the kinetics studies (Section 2.2.3) except for the buffers where only MS-compatible buffers were used. The samples were diluted with the mobile phase before analyzing by Waters ACUITY UPLCTM (Waters Corporation, Milford, MA, USA) coupled to Waters QT_oF SYNAPT G1 mass spectrometer (Waters MS Technologies, Manchester, UK). The UPLC system was equipped with a binary solvent delivery system and autosampler. Chromatographic analysis of tacrolimus degradation products was conducted on an ACQUITY UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm; Waters corp., Milford, MA, USA). 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. Gradient elution was used at a flow rate of 0.50 ml/min as follows: initial 40%B 0–0.1, linear gradient from 0.1 to 5 from 40%B to 100%B, holding at 100%B 5–5.5, linear gradient from 100%B to 40%B 5.5–5.6 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 ionization 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 analyzed in triplicates. The UPLC-QT_oF-

MS system and data acquisition were controlled by the MassLynx v4.1 software (Waters Corp., Milford. USA).

2.2.5. Solubility studies

Solubility studies were determined by adding an excess amount of tacrolimus to aqueous solutions containing various concentrations of CD at around pH 5. The suspensions formed were sonicated in an ultrasonic bath (Edmund Buhler GmbH) for 90 min. The vials containing these suspensions were then shaken at room temperature. After equilibrium for 24 hrs, and aliquots were filtered through a 0.45 μm membrane filter unit (Phenomenex, UK), diluted with 50% aqueous acetonitrile solution (whenever necessary) and analyzed by UHPLC.

Higuchi and Connors (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 (Saokham, 2018). The drug solubility increases with increasing CD concentration in the A-type profiles due to formation of water-soluble drug/CD complexes. While B-type profiles are related to 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 (Jansook et al., 2018). The most common complex observed is the 1:1 drug/CD complex where one drug molecule (D) forms a complex with one CD molecule:

$$D + CD \overset{K_{1:1}}{\leftrightarrow} D/CD \tag{1}$$

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 the 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 (Loftsson et al., 2005; Brewster and Loftsson, 2007).Fig. 1

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

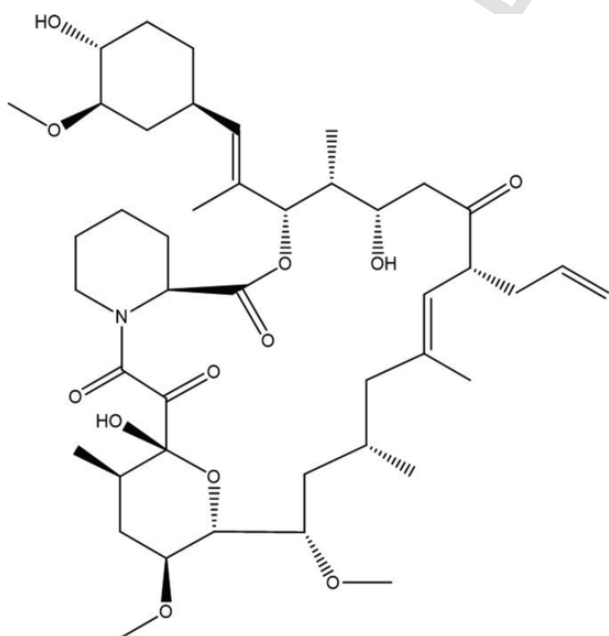When a drug molecule forms a complex with more than one CD molecule, a consecutive complexation is assumed, thus stability constants of higher-order complexes ($K_{1:n}$) should be calculated using a different model (Loftsson et al., 2005). The most common stoichiometry is the formation of 1:2 D/CD complexes, where one drug molecule forms a complex with two CD molecules:

$$D/CD + CD \overset{K_{1:2}}{\leftrightarrow} D/(CD)_2 \tag{3}$$

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 10 to 500 M^{-1} or significantly lower than that of $K_{1:1}$ (Loftsson et al., 2005; Loftsson et al., 2005)

$$[S_t] = [S_0] + K_{1:1}[S_0][CD] + K_{1:1}K_{1:2}[S_0][CD]^2 \tag{4}$$

Determination of the complexation efficiency (CE) can be a better alternative to $K_{1:1}$ to compare the solubilizing effect of CDs (Loftsson et al., 2007). The CE determination (Equation (5)) has less variation because it can be calculated from only the slope of the linear phase-solubility diagram (Loftsson et al., 2005; Loftsson, 2014; UEKAMA and HIRAYAMA, 1987).

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

3. Results and discussions

3.1. Influence of cyclodextrin concentration

The degradation of tacrolimus has been shown to follow pseudo-first-order kinetics in aqueous buffer CD solutions at constant pH and temperature. This kinetic behavior was not affected even by introduction of up to 7.5% (w/v) HPβCD 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 (Fig. 2).

Increasing the HPβCD concentration in the reaction medium decreases the rate of degradation of tacrolimus and a non-linear relationship is obtained between the pseudo-first-order rate constants calculated from the slopes is plotted against the HPβCD concentration (Fig. 3). The rate decreases fast when the HPβCD concentration is increased from 2.5% to 5% but then levels off at 7.5%. These results are consistent with a kinetic system where a drug degrades at a higher rate outside the CD inclusion complex than within the complex (Lineweaver and Burk, 1934; Il'ichev et al., 2007):

$$D + CD \rightleftharpoons D/CD \tag{6}$$

Fig. 1. Chemical Structure of Tacrolimus.
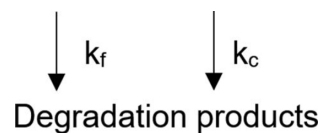
where $K_{1:1}$ is the complex stability constant, k_c is the observed first-order rate constant for the drug degradation within the complex (D/CD) and k_f represents the observed first-order rate constant for the degradation of the free drug (D). Here D represents the drug tacrolimus. The observed first-order rate constant (k_{obs}) for the drug degradation is the weighted average of k_f and k_c:

$$k_{obs} = k_f \cdot f_f + k_c \cdot f_c \tag{7}$$

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:

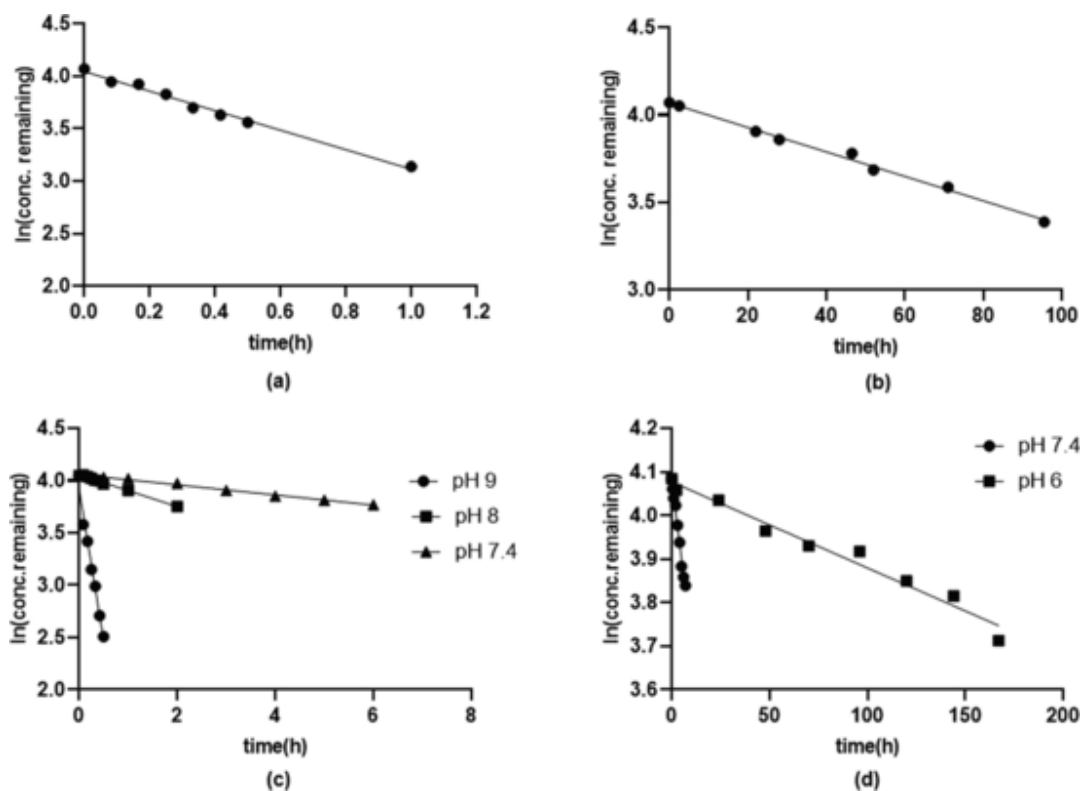$$k_{obs} = \frac{k_f + k_c K_{1:1}[CD]}{(1 + K_{1:1}[CD])} \tag{8}$$

Fig. 2. Representative first-order plots (ln (drug concentration remaining) against time) for the degradation of tacrolimus in aqueous a) 5%HPβCD at pH 9, (b) 2.5%HPβCD at pH 5, (c) 2.5%HPβCD pH at 7.4,8 and 9 and (d) 5%HPβCD at pH 6 and 7.4 at 40 ˚C.
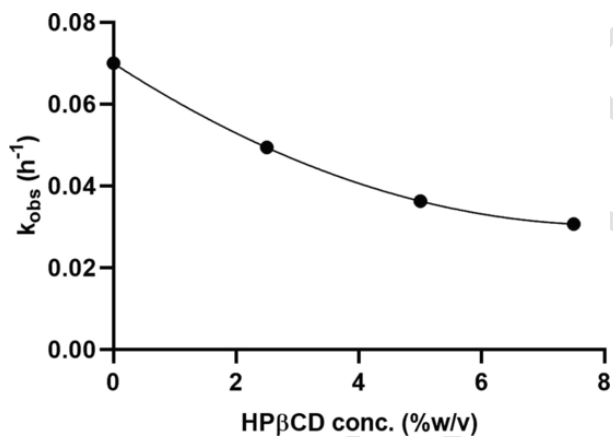

Fig. 3. The effect of HPβCD concentration on the observed rate constant for tacrolimus degradation in aqueous buffer solution at pH 7.4 at 40 ˚C. The initial tacrolimus concentration $[D]_T$ was kept constant at 2.48 mM but the HPβCD concentration $[CD]_T$ ranged from 0 to 7.5% (w/v).

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 $[CD] \approx [CD]_T$:

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

Rearrangement of Equation (9) gives Equation (10) (Ruth and Chika, 2018):

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

Knowing k_f, both k_c and $K_{1:1}$ can be calculated after construction of Lineweaver-Burk plot (Fig. 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. The values of k_c were smaller but were affected by the media pH like those of k_f. The $K_{1:1}$ was less affected by pH, being almost identical at all pH values tested. Tacrolimus does not contain ionizable moiety and is, thus, unionized at all pH values tested. However, the buffer salts and ionic strength can affect the value of $K_{1:1}$.

Fig. 4
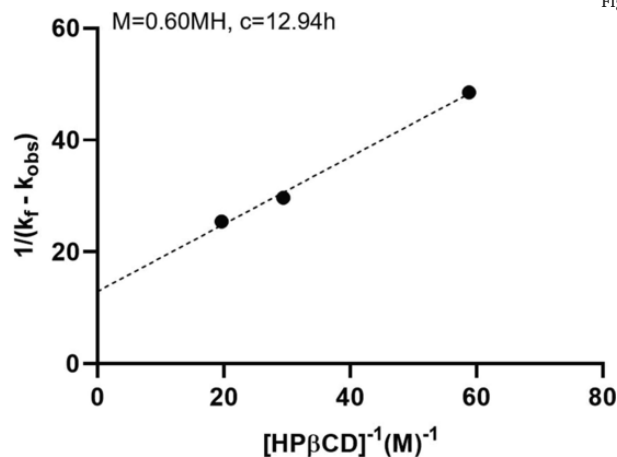
Lineweaver-Burk plot for tacrolimus degradation in aqueous buffer solution at pH 7.4 at 40 ˚C.

3.2. Degradation profile of tacrolimus in aqueous cyclodextrin solution and proposed degradation mechanism

First, the degradation rate of tacrolimus was calculated in aqueous 5% HPβCD solutions with and without 0.1% EDTA. EDTA forms complexes with metal ions that can catalyze oxidative degradation of tacrolimus. No significant difference in rate constant was observed as shown in Table 1. Similar results were observed when the degradation studies were done with and without purging the reaction media with nitrogen. This showed that oxidation is probably not a major degradation pathway in aqueous CD solutions.

Profiling and identification of degradation products was carried out using UHPLC-MS. Degradation products were identified in our study by determining the mass/charge (m/z) values, fragmentation pathway and chromatographic properties. Under acidic conditions, tacrolimus (RT 4.04 min) degradation was relatively slow. Tacrolimus degradation in CD buffer solution at pH 2.5 yielded a mixture of two compounds that were more polar than tacrolimus with retention times (RT) of 2.88 and 2.90. Both had identical masses 844 [M + Na] $^+$. Mass spectra and fragmentation data of the two compounds were similar, practically indistinguishable from each other and thus the two compounds could be isomers. The MS data of these compounds when analyzed by Mass Lynx software coincided with the hydrolyzed form of tacrolimus at its lactone group. Similar results have been observed during degradation

Table 1
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

of tacrolimus related compounds, like everolimus and sirolimus, where the lactone group is hydrolyzed as explained by Il'ichev et al. (Il'ichev et al., 2007) (Nogueiras-Nieto, 2012). Another less polar compound was obtained at 4.38 RT with m/z 808. The MS data indicated that dehydration might have occurred with a loss of water molecule resulting in formation of a less polar compound.

Tacrolimus degradation appeared to be completed within 1 h at pH 10. The basic condition also yielded a mixture of two compounds that are more polar than tacrolimus with retention time of 2.90 and 2.99. Both had identical masses 844 [M + Na] $^+$. The compounds were similar to the one obtained under acidic conditions. This suggests that the hydrolyzed form of tacrolimus obtained at acidic and basic conditions could all be isomers since all gave the same elemental composition and similar fragmentation data. Another major degradation product formed under basic condition had a longer retention time (RT 3.38) than the other degradation compounds but was slightly more polar than tacrolimus (RT 4.04) with m/z 826 [M + Na] $^+$. It was identified as the open-chain form of compound formed by dehydration of tacrolimus molecule under acidic conditions by the elemental composition from the MS data. Skytte et al (2012) observed the formation of same compound when they treated tacrolimus with 1,5-diazabicyclo [4.3.0] nonene (DBN) in dichloromethane (basic conditions) (Skytte, 2013). Based on the structure of this compound, it looks like tacrolimus has undergone hydrolysis at lactone group and a dehydration reaction to form a double bond. The proposed degradation mechanism of tacrolimus in HPβCD solution can be represented in Fig. 5.

3.3. Effect of pH (pH rate profile)

The influence of pH on the degradation of tacrolimus in aqueous HPβCD buffer solutions was investigated over the pH range of 2–9. The ionic strength of the buffer was not controlled. The pH-rate profiles for the observed first-order degradation of tacrolimus in aqueous solu-
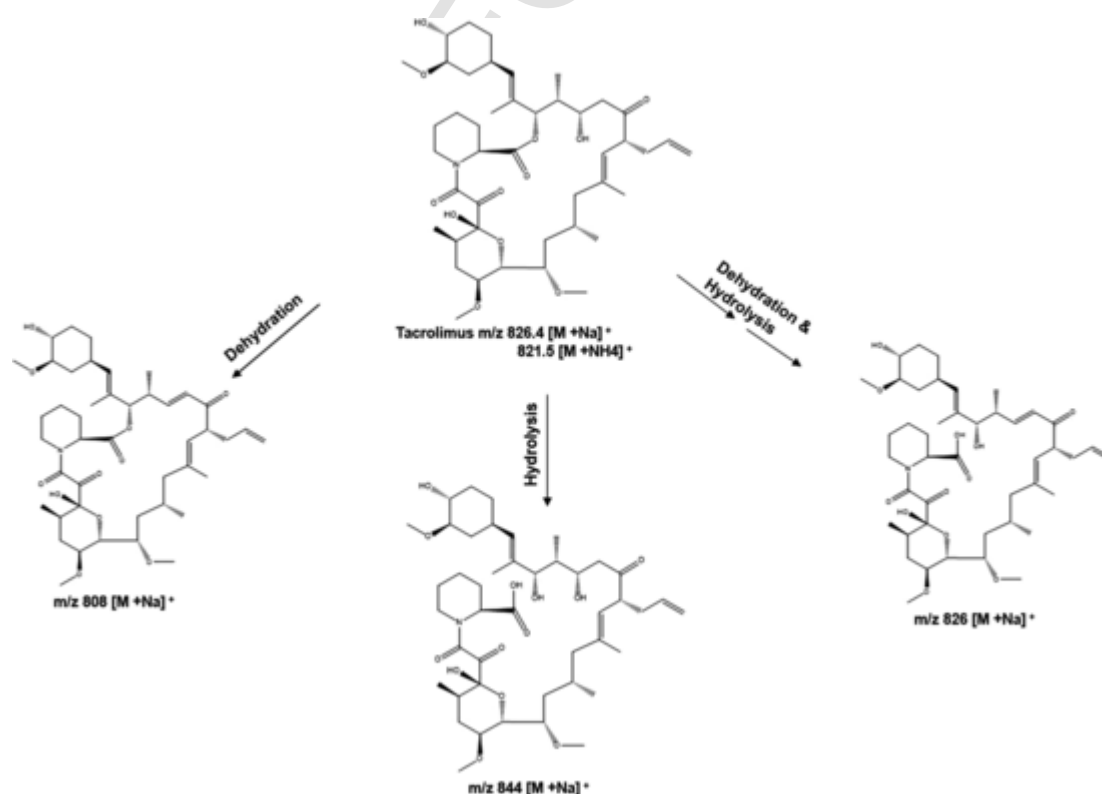
Fig. 5

Proposed tacrolimus degradation pathways in aqueous HPβCD solution.

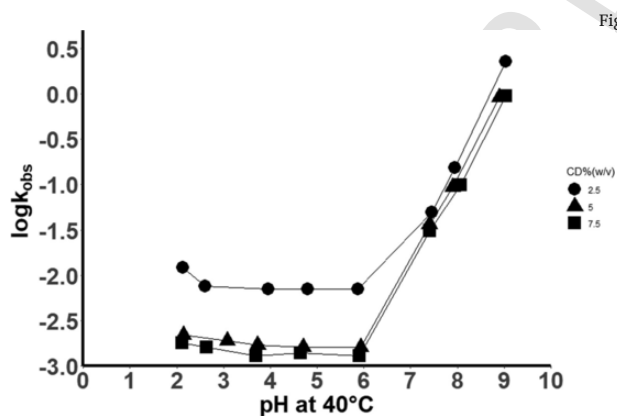tions containing 2.5, 5.0 and 7.5% (w/v) of HPβCD at 40 °C are shown in Fig. 6.

The kinetics of the drug degradation in CD solution is sensitive to the medium acidity as shown by the pH-rate profile in Fig. 6. The pH-rate profile consisted of plateau region in the pH range of 4–6, small increase in degradation rate between pH 2 and 3, and a sharp increase at pH 6. The drug (pka 10) is in its unionized form at all pH values tested. Table 2 shows the k_c and k_f for tacrolimus in HPβCD solutions at different pH values and 40 °C.

This clearly shows that the tacrolimus degradation decreases with increasing CD concentration, with degradation being relatively fast when there is no CD present. The drug is most stable at pH between 4 and 6, both in aqueous CD solutions and in CD free medium. We can also see that $k_f > k_c$ at all pH values showing that drug degrades at a higher rate outside the CD complex than within the complex at all pH tested. k_c and k_c follow similar profile as shown in Fig. 7. Consequently, the drug degradation within the CD complex and outside the complex follow similar reaction pathways.

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 consist of three regions, that is the specific acid-catalyzed (i.e. H_3O^+ catalyzed) region at pH below about 3, an uncatalyzed region or plateau between pH 3 and 7, and a specific base-catalyzed (i.e. OH^- catalyzed) region at pH above about 7.4. For pH values below 3, both the curve has negative slope and the k_c and k_f in Equations (11) and (12) are dominated by k_H and $\acute{k}_H$, respectively, and this hydrolysis reaction proceeds according to the reaction pathway catalyzed by H_3O^+ ions. The zero slope of the curves, presented for pH values 4–6 indicates that in this pH range, the k_o and $\acute{k}_o$ are dominating, and from pH 7.4 onwards, k_{OH} and $\acute{k}_{OH}$ are dominating since the hydrolysis is catalyzed by HO^- ions. 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 (11) ad 12.

$$k_f = k_H [H^+] + k_o + k_{OH}[OH^-] \tag{11}$$

$$k_c = k'_H [H^+] + k'_o + k'_{OH}[OH^-] \tag{12}$$

where k_H and $\acute{k}_H$ are acid-catalyzed, k_o and $\acute{k}_o$ uncatalyzed and k_{OH} and $\acute{k}_{OH}$ being basic-catalyzed rate constants.

The values of k_H, $\acute{k}_H$, k_o, $\acute{k}_o$, k_{OH} and $\acute{k}_{OH}$ for different reaction pathways that constitute the whole hydrolysis process were determined. Table 3 shows the values for these constants and the definitive expression of the k_f and k_c at 40 °C is given by Equations (13) and (14).

$$k_f = 3.2 [H^+] + 0.016 + 1.73 \times 10^5 [OH^-] \tag{13}$$

$$k_c = 1.3 [H^+] + 6.76 \times 10^{-3} + 4.09 \times 10^4 [OH^-] \tag{14}$$

These results show that the hydrolysis reactions of tacrolimus (both in CD and CD free solutions) follow acid-base catalysis mechanism where the reaction pathway catalyzed HO^- ions is dominant and the degradation of tacrolimus is fastest in basic medium.

3.4. Influence of different Cyclodextrins

The stability studies above in aqueous HPβCD media show that the drug is most stable at pH about 5 but the degradation rate is accelerated under basic conditions or at pH above 7.4. The effect of αCD and βCD on the tacrolimus degradation was also tested (Table 4). However, tacrolimus does not readily form a complex with γCD and, thus, this CD was omitted from this part of the study.

αCD and βCD give lower k_c values than HPβCD at both pH values tested. The k_f /k_c ratios show that under all conditions tacrolimus is stabilized by the CD complexation. βCD results in the lowest k_c values and the highest $K_{1:1}$ values in comparison to αCD and HPβCD and, thus, is the best stabilizer of the three CDs tested.

3.5. Phase-solubility studies

Different CDs were used to determine the solubility of tacrolimus in aqueous solutions by the phase-solubility method of Higuchi and Connors (1965) (Saokham, 2018). A preliminary study indicated that tacrolimus degraded during autoclaving and was not chemically stable in aqueous solution during a 7-day equilibration at room temperature. Thus, the solubility studies were carried out by sonicating the aqueous CD media containing excess of tacrolimus for 90 min and equilibration in a rotary shaker at room temperature for 24 hrs. Fig. 8 shows the phase-solubility diagrams of tacrolimus in various CD solutions in pure (i.e. unbuffered) water at around pH 5 at room temperature.

The solubility of tacrolimus in pure water is extremely low (1.58 μM in water at 25 °C) (Arima, 2001). Two different types of phase-solubility diagrams were observed, A_L-type where strictly linear relationship is observed and A_P where positive deviation from linearity is observed. A_L and A_P were distinguished by comparing the correlation coefficient squared values (r^2). The solubility curves with r^2 values greater than 0.99 were regarded as A_L-type and those displaying r^2 values of less than 0.99 were regarded at A_P-type (Loftsson, 2014)[26].

αCD, HPαCD and SBEβCD gave r^2 values of greater or equal to 0.99, regarded as A_L-type and the stability constants ($K_{1:1}$) calculated using Equation (2). Whereas, all the other CDs gave r^2 values less than 0.99, the phase-solubility diagrams considered to be of A_P-type and the stability constants, $K_{1:1}$ and $K_{1:2}$, calculated using Equation (2) and (4), respectively.
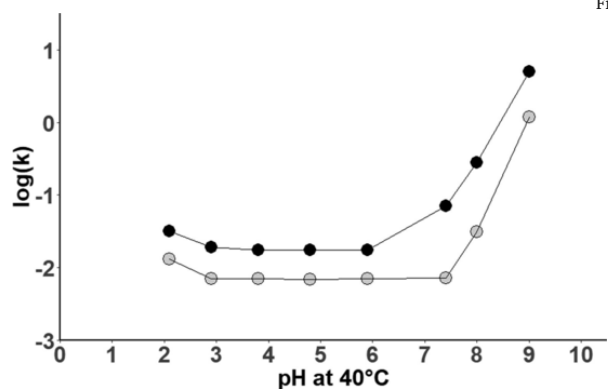
Fig. 6

pH rate (k_{obs} h^{-1}) profile for tacrolimus in HPβCD solution at 40°C.

Table 2
Values of k_f, k_c and $K_{1:1}$ for tacrolimus in 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_f/k_c	2.5	2.7	2.5	2.5	2.5	9.7	7.6	4.2
$K_{1:1}$ (M^{-1})	47.47	46.57	44.91	45.29	44.49	21.53	66.35	47.35

Fig. 7

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

Table 3
Values of k_H, $\acute{k}_H$, k_o, $\acute{k}_o$, k_{OH} and $\acute{k}_{OH}$ in aqueous HPβCD solution.

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

Table 4
Values of k_c, k_f and $K_{1:1}$ of tacrolimus at 40℃ and pH 5 or 9 in 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_f/k_c	5	5.8	2.5	4.9	5.1	4.2
$K_{1:1}$ (M^{-1})	65.63	1170	44.9	419	2515	47.35

The analysis of the 1:3 and 1:4 (guest: host) inclusion models gave negative values for the stability constants suggesting that tacrolimus predominantly forms 1:1 and 1:2 complexes with these CDs under mentioned conditions. The stability constant for all the CDs tested are listed in Table 5. It should be noted that the values of the stability constants given in Table 5 are obtained from phase-solubility profiles at room temperature where aqueous CD solutions are saturated with the drug while the values in Table 4 are obtained at 40 °C in dilute solutions. In general, the values of the stability constants decrease with increasing temperature.

Among the natural CDs, βCD had the highest stability constant suggesting that the βCD cavity was of appropriate size. The hydroxypropyl derivatives of the natural αCD and βCD had inferior stability constant compared to natural CDs. While of the CD derivates RMβCD had

the highest stability constant ($K_{1:1}$). This high value for the RMβCD complex was consistent with other results of other drugs and is due to the increase in hydrophobic space of the βCD cavity upon methylation of the OH-groups. The $K_{1:1}$ value for RMβCD was marked higher than the $K_{1:2}$ value, showing that the 1:1 complex to be favored at the RMβCD concentrations tested.

Interestingly, SBEβCD gives A$_L$-phase-solubility diagram while HPβCD gives an A$_P$ diagram. This may be because of the negative charges on the SBEβCD molecule which might reduce the possibility of formation of tacrolimus/SBEβCD 1:2 complexes due to charge repulsion. The reduced ability of HPβCD and HPαCD to form complex with tacrolimus compared to the natural CDs might be due to steric hindrance of the substituent groups at the CD cavity.

3.6. Effect of different surfactants on tacrolimus stability and solubility in HPβCD solution

Even though the CDs were able to stabilize tacrolimus in solution, the stability obtained was not sufficient to move the drug toward the formulation step. So, different surfactants were also tested in combination with the CDs to improve further the chemical stability of tacrolimus in aqueous media. Poloxamer 407, tyloxapol and tween 80 were used for this purpose. Aqueous solutions containing 5% (w/v) HPβCD and surfactant (from 0 to 5% w/v) were prepared to which 100 μl of tacrolimus stock solution (2.48 mM) was added. These solutions were subjected to one cycle of autoclaving and the remaining drug concentration was measured by using the UHPLC method (Fig. 9).

Fig. 9b shows that drug degradation decreases with increasing poloxamer concentration up to 3% where the degradation again increases from 5%. This was observed either when only poloxamer or combination of poloxamer and CD were used. Similar trend is observed in Fig. 9c showing the drug degradation in aqueous CD solutions containing tyloxapol. In case of tyloxapol, drug degradation is minimum at 2% and then increases upon increasing tyloxapol concentration (for both, the combination of HPβCD and tyloxapol and only tyloxapol). Likewise, maximum tacrolimus stability was observed in 5% HPβCD solution containing 1% tween 80(Fig. 9a). Pure aqueous solutions containing more than 2% tween 80 individually provided more stability than when combined with HPβCD while the combination of poloxamer 407 and tyloxapol with HPβCD provided more stability in all cases than the pure polymers and CD. More than 90% of drug is degraded upon heating in an autoclave (121 °C for 20 min) when only 5%HPβCD is present in the degradation media but the tacrolimus degradation dropped to about 30% and 40% when 2% tyloxapol and 3% poloxamer were present in the HPβCD media, respectively. The stabilizing effect of the surfactants might be due to micelle formation and protection of tacrolimus within the micelles (Santos Akkari, 2016).

Tyloxapol and poloxamer 407 were then used to evaluate the solubility of the drug along with 5% HPβCD as the combination of HPβCD with them increased the stability of the drug. Fig. 10 shows the solu-
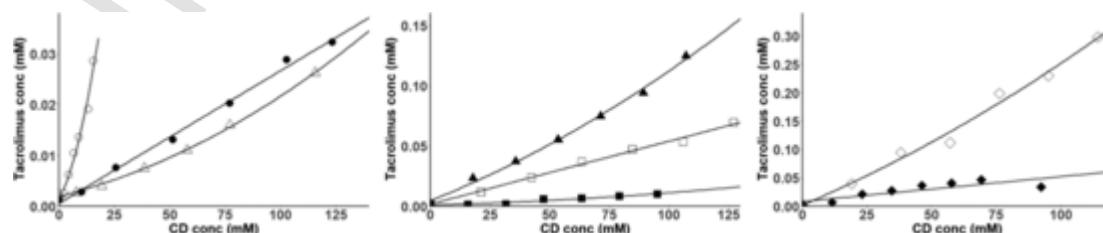
Fig. 8

Phase-solubility diagrams of tacrolimus in CD in pure water at room temperature. Each point represents the mean of triplicate experiments. Key: (●) αCD; (○) βCD; (△) γCD; (▲) HPβCD; (□) HPαCD; (■) HPγCD; (◇) RMβCD and (♦) SBEβCD.

Table 5
Stability Constants (K$_{1:1}$ and K$_{1:2}$) [a] of tacrolimus/CD complexes in pure water at room temperature.

System	K$_{1:1}$ (M^{-1})	K$_{1:2}$ (M^{-1})
αCD	500	***
βCD	571	66
ϒCD	50	9
HPαCD	278	***
HPβCD	174	4
HPϒCD	55	7
RMβCD	500	3
SBEβCD	269	***

[a] K$_{1:1}$: Stability constant of 1:1 complex, K$_{1:2}$: Stability constant of 1:2 complex

bility of tacrolimus in 5% HPβCD along with various % of poloxamer and tyloxapol.

We showed that poloxamer was able to slightly increase (about 1.2 folds) the solubility of tacrolimus. The solubility did not increase with increasing concentrations of poloxamer

(Fig. 10a). Addition of poloxamer to the complexation media results in competition with drug molecules for the CD cavity and consequent displacement of the drug molecules from the CD cavity (Muankaew, 2014). The slight increase in solubility may indicate that a decrease in solubility due to the competitive displacement is proba-

bly compensated by the solubilizing effect of polymer micellization (Fig. 10a). On the contrary, Akkari et al.(2015) observed that the increase in aqueous solubility of hydrophobic drugs, in the presence of polymer and CD (compared to isolated systems), suggesting no or little influence of drug-polymer competition for the HPβCD cavity [34]. Those observations go in the line with our results where tacrolimus solubility in aqueous 5% HPβCD solution is improved by the addition of tyloxapol (Fig. 10b). The tacrolimus solubility increased with increasing tyloxapol concentration. The combination of tyloxapol and HPβCD solubilize more than the individual component giving the maximum solubility of 0.3 mM at 5% tyloxapol. Tyloxapol, a non-ionic surfactant oligomer, might improve the solubility of drug by either improving drug wettability or micellar incorporation of drug and drug/CD complexes in case of HPβCD [35].

4. Conclusions

The stability of tacrolimus in CD solution was determined as a function of the medium acidity and tacrolimus shown to be more stable at acidic pH than at basic pH. Moreover, CD has a stabilizing effect at all pH values tested as observed by comparing the rate constants of the free and bound drug (i.e. within the complex). Tacrolimus degradation in CD solutions is mainly due to hydrolysis of the lactone linkage (occurred under both acidic and basic conditions), dehydration, or simultaneous hydrolysis and dehydration to yield the final product as confirmed by the MS studies. βCD and its derivates increased
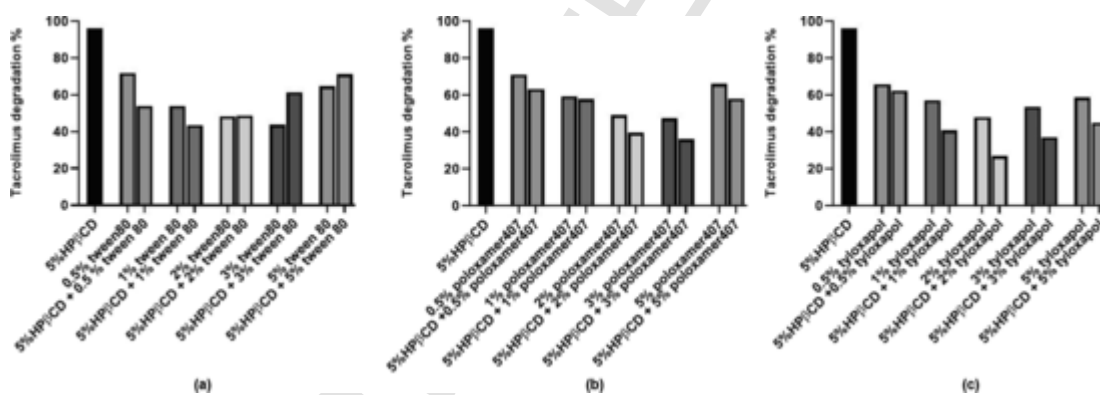
Fig. 9

Drug degradation % after one cycle of autoclaving with 5%(w/v) HPβCD and various % (w/v) of surfactants (a) with tween 80, (b) with poloxamer 407 and (c) with tyloxapol.
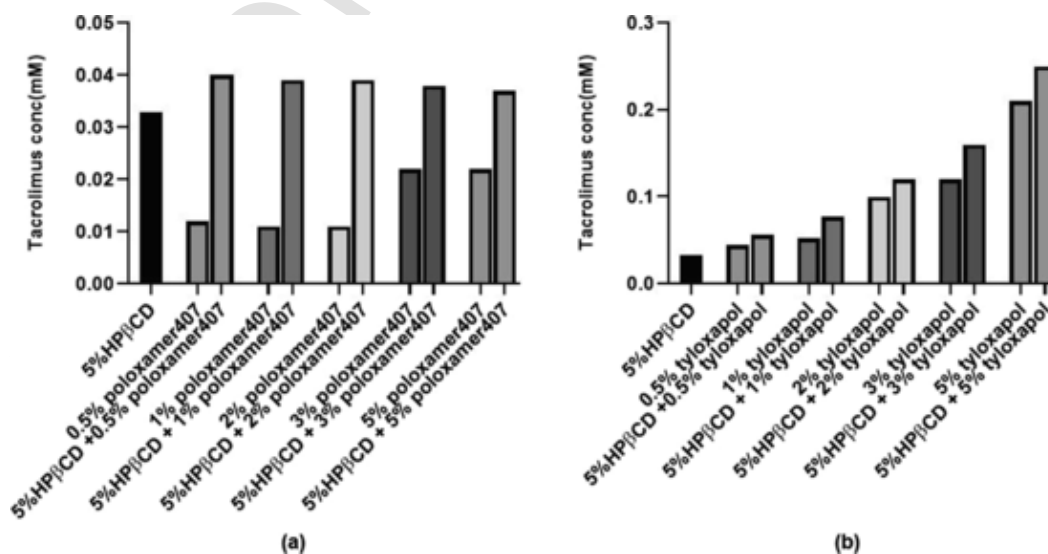
Fig. 10

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

tacrolimus solubility much more than the other CDs tested. The stability and solubility were improved when combination of CD and surfactants was used, particularly with HPβCD and poloxamer 407 or tyloxapol. However, tacrolimus was not adequately chemically stable to be formulated as aqueous eye drops.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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