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Review Article

Stability Tests and Analytical Methods of Tacrolimus: A Review

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Abstract

Tacrolimus is an immunosuppressive drug widely used in organ or tissue transplantation. Furthermore, its anti-inflammatory effects are employed in treatments for psoriasis, uveitis, and vernal keratoconjunctivitis. According to the structural characteristics of tacrolimus, different transformations can be occurred on the drug and produces degradation products. Thus, stability and stress tests have a key role in the quality of tacrolimus with a narrow therapeutic index. Up to now, different studies have been developed for the study of tacrolimus stability under different conditions, and the degradation products were detected by different analytical instruments. Therefore, in the current study, available studies about tacrolimus degradation were collected and categorized into five main parts including acidic hydrolysis, alkaline hydrolysis, thermal, oxidative, and photolytic for better survey. The known degradation products with their chemical structures were also discussed in this study. Moreover, the analytical methods that are applied for drug characterization during stability tests were explained.

Introduction

Tacrolimus or FK506 is a potent immunosuppressant used in the prevention of transplant rejection and treatment of atopic eczema and ophthalmic inflammatory diseases.[1-4] It is originally produced by *Streptomyces tsukubensis*.[5] Tacrolimus exerts its action by binding to a protein named FK506 binding protein (FKBP) and inhibits the phosphatase function of calcineurin.[6,7] Calcineurin dephosphorylates the nuclear factor of activated T cells (NF-AT) which is a transcription factor and promotes the production of IL2 and other inflammatory cytokines.[8] Tacrolimus avoids the transcription of IL2 and the other cytokines of T lymphocytes by inhibition of calcineurin (Figure 1).[9]

Tacrolimus is a white crystalline powder practically insoluble in water ($\log P = 2.7$) and has a low bioavailability.[10] Tacrolimus possesses a narrow therapeutic index. Therefore, it needs therapeutic drug monitoring (TDM) for the prevention of transplant rejection. The extent and rate of absorption significantly are diminished by food. Cytochrome CYP3A enzymes metabolize tacrolimus in the small intestine.[11]

Its available dosage forms are immediate-release capsules (Prograf 0.5, 1 and 5 mg), extended-release capsules (0.5, 1, 3 and 5 mg), extended-release tablets (0.75, 1 and 4 mg), injectable solution (5 mg/mL), oral suspension (0.2 and 1 mg/packet) and ointment (0.03 and 0.1%).[12] The recommended shelf life of Prograf injectable solution and capsules are two and three years, respectively.[13]

The quality of immunosuppressants affects their efficacy and safety.[14] The stability of pharmaceutical products is mainly affiliated with quality evaluation.[15] Chemical, physical, microbiological, therapeutic, and toxicological studies are considered in stability investigations. The findings from stability studies have a key role in determining the shelf life of drugs. Having proper information about the stability of drugs, make it possible to design suitable packaging for pharmaceutical formulations. For example, secondary packaging can be used for light-sensitive drugs, or hygroscopic materials can be applied in the packaging of drugs that are sensitive to hydrolysis. Also, the results of drug stability studies provide good information on storage conditions for drug products. Stability studies ensure the purity of active pharmaceutical ingredients and the quality of the final product. In these studies, the impurities of pharmaceutical formulations are carefully analyzed which prevents exceeding the amount of the defined permissible range.[16] Tacrolimus, a macrolide lactam with three double-bonds, has three carbonyls and various functional groups including hydroxyl, amide, ester, and ether in the structure (Figure 2). Therefore, different degradation pathways such as dehydration, isomerization of double bonds, hydrolysis, rearrangement, and epimerization can be done on it.[17] Rotation of amide bond consequences an

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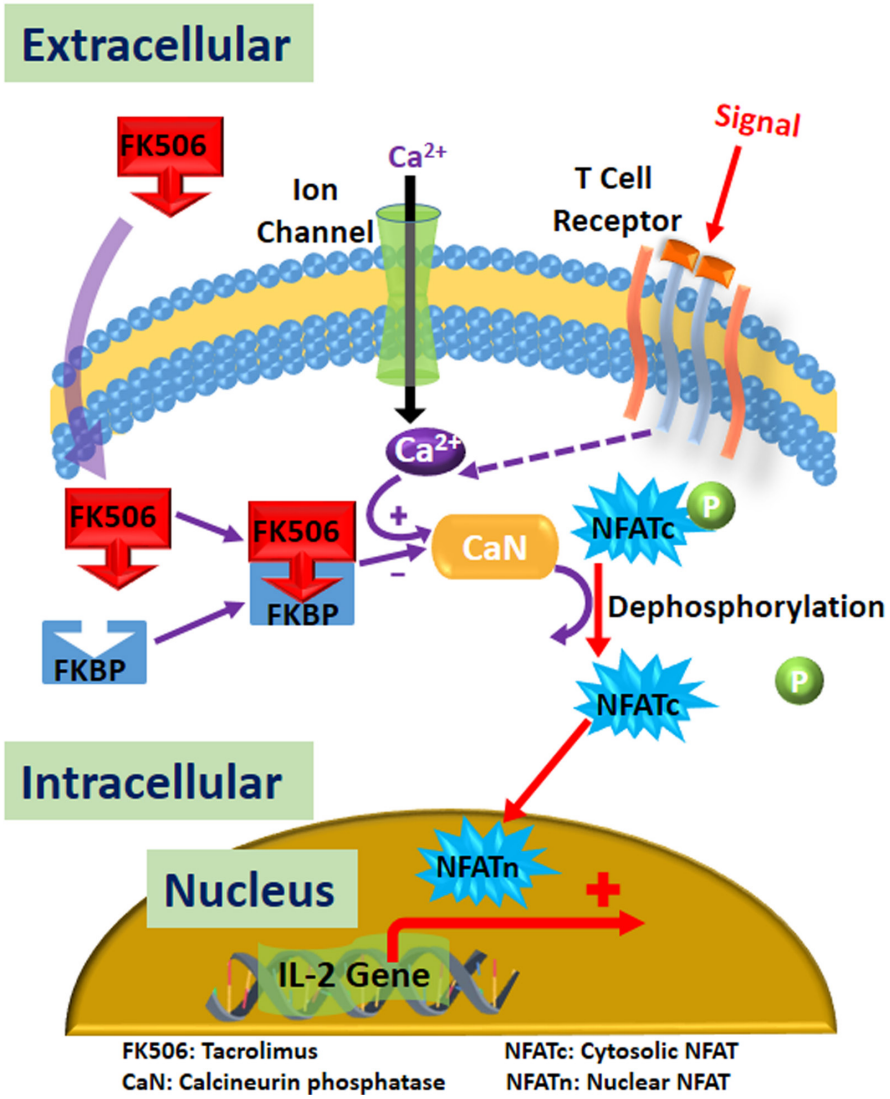

Figure 1. Mechanism of action of tacrolimus. Tacrolimus decreases the production of IL2 and other inflammatory cytokines by inhibition of calcineurin.

equilibrium between *trans* and *cis* rotamers of tacrolimus in the pipecolic acid moiety.[18] Pipecolic acid moiety is often considered a vital factor for the biological activities of some natural pharmaceutical products from microbial sources.[19] Different studies announced that tacrolimus has two tautomeric forms. Akashi et al. analyzed tautomeric forms of tacrolimus quantitatively and concluded that by increasing the content of water, the equilibrium shifts to tautomer I (Figure 2).[20] Namiki et al observed a tautomeric phenomenon in the ethanol solution of tacrolimus by the high-performance liquid chromatography (HPLC) method. However, the IR spectrum of tautomer I was similar to the tacrolimus spectrum. Nuclear magnetic resonance (NMR) spectrum of tautomer I had a new signal affiliated with the OH group and there was a significant change at C- 9 and C- 10 positions. Proton nuclear magnetic resonance (^{1}H NMR) and IR spectrum of tautomer II were matched with tacrolimus spectrum and it was identified as tacrolimus 10-epimer.[21,22] Subasranjan et al developed the Ultra-high-performance liquid chromatography (UHPLC) method for the separation of the impurities and tautomeric forms of tacrolimus.[23] The comparison of HPLC methods utilized to determine tautomeric forms is shown in Table 1.

Forced degradation studies

Stability guidelines for drug substances or products are prepared by the International Conference on Harmonization (ICH) as Q1A (R2) guidelines. Forced degradation or stress tests suggested by ICH guidelines mainly consist of acid/base, temperature, photolysis, humidity, and oxidation tests.[24] The objective of stress tests is the providing information about degradation products and the mechanisms that they were produced.[25] The five major stress tests have been done on tacrolimus as an active pharmaceutical ingredient which is reviewed in the following.

Acidic hydrolysis

Skytte et al studied some transformations of tacrolimus in acidic conditions. In the research, tacrolimus was dissolved in toluene and heated for one hour. Afterward, it was washed with water and then with a highly concentrated sodium chloride solution and dried over sodium sulphate.

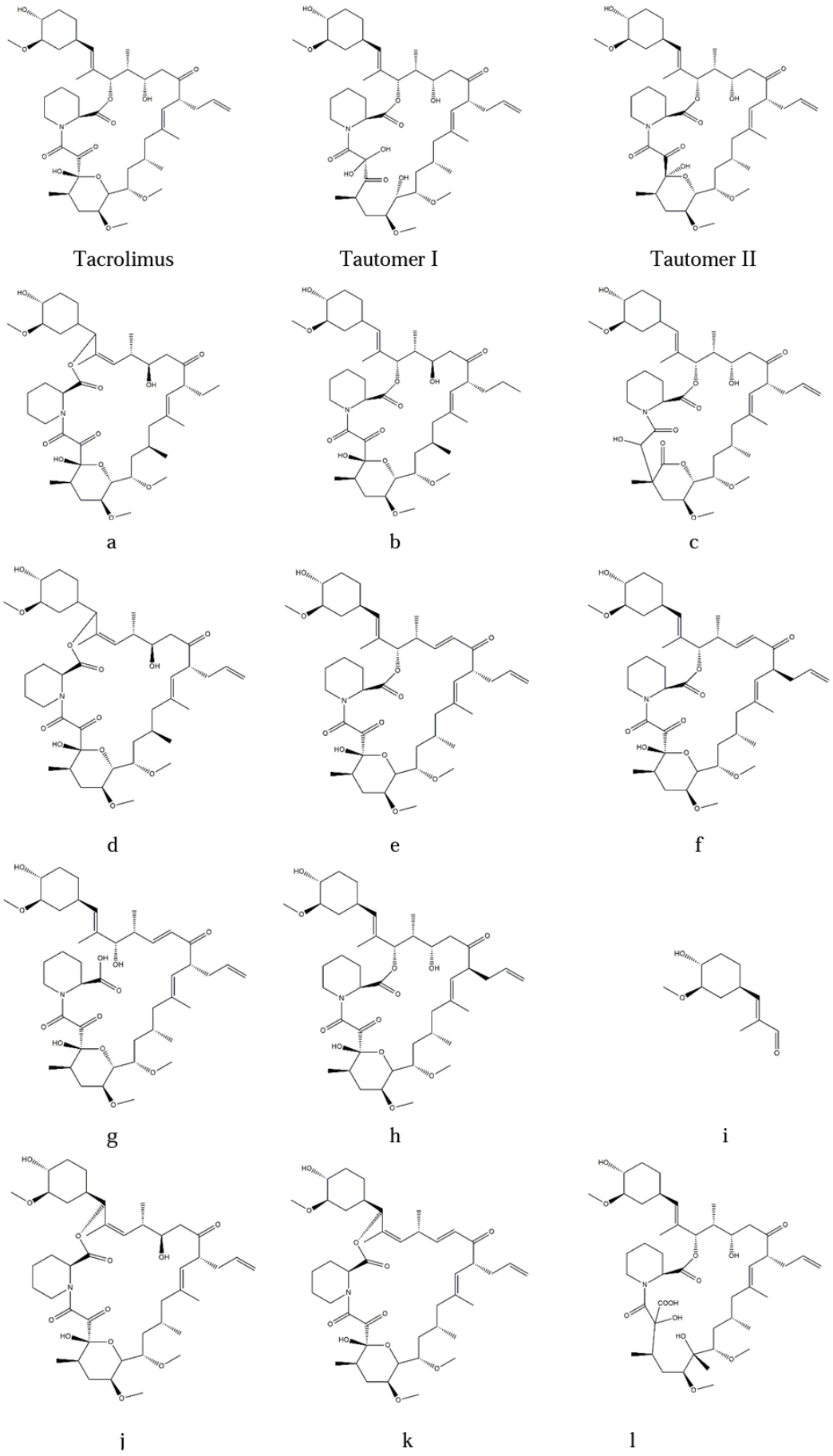

Figure 2. The structures of tacrolimus, its tautomeric forms, and impurities (a-d), degradation products of acidic hydrolysis (e,f),degradation products of weak alkaline hydrolysis (g,h), degradation product of strong alkaline hydrolysis (i), thermal degradation products (j,k), and oxidative degradation product (l).

Table 1. HPLC-UV determinations of tacrolimus tautomeric forms

Detector wavelength (nm)	Column	Column temp	Elution mode	Mobile phase	Flow rate	Injection volume	Impurities	Ref.
215	RP-18	50°C	Isocratic	Acetonitrile-water-phosphoric acid (600:400:1)	2 mL/min	20 µL	Tautomer I and II	[20]
220	TSK OH-120	25°C	Isocratic	Hexane, ethylene dichloride, and acetonitrile (6: 3: 1)	1 mL/min	5 µL	Tautomer I and II	[21]
220	C_{18}	50°C	Isocratic	Water- isopropyl alcohol - tetrahydrofuran (5:2:2, v/v)	1 mL/min	10 µL	Tautomer I and II	[22]
210	C_8	50°C	Gradient A–B 55: 45 (V/V); then 45: 55 (V/V); within 20 min. 55:45; 21 to 25 min.	Mobile phase A (90: 10 v/v of 0.1% v/v trifluoroacetic acid solution and acetonitrile) mobile phase B (90: 10 v/v acetonitrile and water)	0.6 mL/min	5 µL	Tautomer I and II, immunomycin (impurity-A), propyl analogue (impurity-B), delta lactone (impurity-C), regioisomer (impurity-D) (Figure 2 (a-d))	[23]

At last, HPLC was applied for analyzing the produced products. In this process, the degradation products of tacrolimus were colorless crystals and white amorphous solids which are demonstrated in Figure 2(e, f), respectively.[26] Peterka et al evaluated acidic hydrolysis of tacrolimus solution at hydrochloric acid solutions (0.01 and 0.1 mol/L) at room temperature for 24 hours. The data showed that tacrolimus was stable at pH 3-5.[27]

Alkaline hydrolysis

Tacrolimus is susceptible to hydrolyzing from the lactone group and dehydrates at C-5 in weak basic conditions like 1,5-diazabicyclo [4.3.0] nonene (DBN) solution. The structures of degradation products are illustrated in Figure 2 (g, h). 8-Epitacrolimus is a crystalline product of C-8 epimerization of tacrolimus via transition of the carbonyl group. At first, it was isolated with liquid chromatography-mass spectrophotometry (LC-MS) and ^{1}H NMR and carbon-13 nuclear magnetic resonance (^{13}C NMR) described by Skytte et al. Single-crystal X-ray diffractometry confirmed this crystalline material. The formation of 8-epitacrolimus takes place under acid with the catalysis of free radicals and under mild alkaline conditions.[28] Degradation of tacrolimus in treatment with sodium t-butoxide and 0.1 mol/L sodium hydroxide as strong bases resulted in the formation of an aldehyde that is indicated in Figure 2i.[26,29]

Thermal

Thermolysis of tacrolimus causes rearrangement of allylic ester moiety and production of some degradation materials. Metal catalysts facilitate the degradation pathway and the production of degradants can take place in mild conditions. Dehydration of β-hydroxyketone moiety (Figure 2j) occurs after the rearrangement (Figure 2k). These alterations in the structures were identified by ^{1}H NMR and ^{13}C NMR.[30] Skytte et al analyzed the thermal degradation of tacrolimus by the HPLC method shown in Table 2.[26] The structures of degradation products are displayed in Figure 2(g, h). Rozman Peterka et al studied tacrolimus solution (2 mL of 30 mg/mL dissolved in acetonitrile mixed with 1 mL deionized water) at 60°C for 24 hours. Elevation in tacrolimus regioisomer was observed.[17] Peterka et al designed and implemented a study to evaluate degradation products of amorphous tacrolimus in solid form at high temperature (50°C) under various humidity conditions (30, 50, and 75%) for 1 month. The main products were tacrolimus regioisomer and tacrolimus epimer as can be seen in Figure (2h, j). All impurities of amorphous material increased with high moisture content. Also, a thermal test was done in solution at 60°C for a day. A known degradation product of thermal conditions in the aqueous solution was tacrolimus regioisomer.[27]

Oxidative

Treatment of tacrolimus with free radicals like iodine in boiling toluene results in the same products formed upon the weak acidic conditions as demonstrated in Figure 2(e, f).[26] The impurities of amorphous tacrolimus in a solid form significantly increased under oxygen-induced degradation at 50°C for 1 month.[27] Tacrolimus aqueous solution was approximately stable after treatment with 3% hydrogen peroxide at room temperature for 24 hours.[27] Tacrolimus epimer was a main degradation product of tacrolimus in the presence of radical initiator 4,4′-azobis (4-cyanovaleric acid) abbreviated as ACVA at 60°C during 24 hours. Fe^{3+} ions catalyzed this process and tacrolimus alpha-hydroxy acid, tacrolimus epimer, and tacrolimus diene were formed (Figure 2(e, l).[27]

Photolytic

The photostability of tacrolimus was analyzed both in a solid state and solution. Tacrolimus samples were dissolved in acetonitrile-water (70:30) to obtain 3 mg/mL of tacrolimus. Then, the solution was exposed to different conditions: artificial sunlight (250 W/m^2; 300-800 nm) for 2 hours and a fluorescent lamp (cool white; 2000 lux) for 22 hours. Tacrolimus epimer was the known degradation product of tacrolimus in solution after exposure to artificial sunlight but no degradation occurred under a fluorescent lamp.

Table 2. Various stress tests were studied for the analysis of degradation products and impurity profiling of tacrolimus

Stress test	Test condition	Sample	Instrument used	Mobile phase	Elution mode	Site of cleavage	Degradant(s) profiling	Ref.
Acidic	p-Toluenesulphonic acid	Tacrolimus	RP-HPLC X-ray crystallography MS ^{1}H and ^{13}C NMR	Acetonitrile-water (65:35)	Isocratic mode	Dehydration of β-hydroxyketone epimerization	5-deoxy-$\Delta^{5,6}$-tacrolimus 5-deoxy-$\Delta^{5,6}$-8-epitacrolimus	26
	p-Toluenesulphonic acid	8-Epitacrolimus	RP-HPLC X-ray crystallography ^{1}H and ^{13}C NMR	Acetonitrile-water (65:35)	Isocratic mode	Dehydration of β-hydroxyketone	5-deoxy-$\Delta^{5,6}$-8-epitacrolimus	26
	p-Toluenesulphonic acid	Tacrolimus	HPLC	0.1% acetic acid- acetonitrile-tetrahydrofuran (58:12:27)	Isocratic mode	Dehydration of β-hydroxyketone epimerization	5-deoxy-$\Delta^{5,6}$-tacrolimus	31
Basic	1,5-Diazabicyclo [4.3.0] nonene	Tacrolimus	RP-HPLC ^{1}H and ^{13}C NMR	Acetonitrile-water (60:40)	Isocratic mode	Epimerization Hydrolysis of lactone group Dehydration at C-5	8-Epitacrolimus Open-chain acid of 5-deoxy-$\Delta^{5,6}$-tacrolimus	26
	Sodium t-butoxide	Tacrolimus	-	-	-	Allylic ester	An aldehyde	26
Thermal	-	Tacrolimus	RP-HPLC ^{1}H and ^{13}C NMR	Acetonitrile-water (65:35)	Isocratic mode	Rearrangement of the allylic ester Dehydration of β-hydroxyketone	Tacrolimus regioisomer An enone	26
	Xylene	Tacrolimus	^{1}H and ^{13}C NMR	-	-	Rearrangement of the allylic ester Dehydration of β-hydroxyketone	Tacrolimus regioisomer An enone	30
	50°C/75% RH	Amorphous tacrolimus	UHPLC	A: 0.1% phosphoric acid B: 850 mL of ACN and 80 mL of MTBE	Gradient mode A: B/time; 63:37/1.0 min, 52:48/9.0 min, 30:70/11.0 min, 30:70/13.5 min, 63:37/14.0	Rearrangement of the allylic ester	Tacrolimus regioisomer Tacrolimus epimer	27
	60°C for 24 h	Tacrolimus aqueous solution	UHPLC	A: 0.1% phosphoric acid B: 850 mL of ACN and 80 mL of MTBE	Gradient mode A: B/time; 63:37/1.0 min 52:48/9.0 min, 30:70/11.0 min, 30:70/13.5 min, 63:37/14.0	Rearrangement of the allylic ester	Tacrolimus regioisomer	27
Free radicals	Iodine in boiling toluene	Tacrolimus	RP-HPLC ^{1}H and ^{13}C NMR	Acetonitrile-water (65:35)	Isocratic mode	Epimerization Dehydration of β-hydroxyketone	8-Epitacrolimus 5-deoxy-$\Delta^{5,6}$-tacrolimus 5-deoxy-$\Delta^{5,6}$-8-epitacrolimus	26
Oxygen	O_2	Amorphous tacrolimus	UHPLC	A: 0.1% phosphoric acid B: 850 mL of ACN and 80 mL of MTBE	Gradient mode A: B/time; 63:37/1.0 min 52:48/9.0 min, 30:70/11.0 min, 30:70/13.5 min, 63:37/14.0	All reactions can be possible.	Increase in total impurities	27

In solid-state, tacrolimus was exposed to artificial sunlight (250 W/m^2; 300-800 nm) for 16 hours and a fluorescent lamp (cool white; 2000 lux) for 22 hours.[27] As presented in Table 2 tacrolimus epimer and other unknown degradation products appeared after both manners of photolytic tests in the solid state of tacrolimus. Photolytic tests the drug was placed under artificial sunlight for a predetermined time. In this approach, the energy is supplied in the form of light. The level of these degradation products was comparable with dark control samples.

Analytical methods

Different analytical methods used to analyze tacrolimus and its impurities or degradation products in the formulations are classified into three sections below:

Ultraviolet (UV) spectrometry

Khan et al developed solid lipid nanoparticles (SLN) of tacrolimus in gel form and evaluated several

physicochemical properties like polydispersity index (PDI), viscosity, surface tension, and entrapment efficacy (EE%). The formulation was diluted in phosphate buffer and EE% was determined by UV spectrometry at 294 nm. They concluded that, by enhancement in surfactant amount as the main component of SLN, EE% was increased.[31] Mittal et al applied eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and linseed oil to formulate a nanoemulsion gel of tacrolimus. EPA and DHA showed inhibitory effects against IL-6 and TNF-α production, respectively. Linseed oil possesses various fatty acids that convert to EPA and DHA and exhibit anti-inflammatory effects. The uniformity of drug content was determined by a UV spectrophotometer at 205 nm after dilution in methanol. In this investigation, physical stability consists of centrifugation (5000 rpm for 30 minutes), freeze-thaw (-20°C for 24 hours), and heating-cooling studies (4 and 40°C; 6 cycles). Nanoemulsions showed appropriate physical stability without creaming, cracking, precipitation, etc.[32]

Al-Tamimi and Hussein developed new drug delivery named self-microemulsifying to intensify oral bioavailability. In physical stability tests, centrifugation (3500 rpm for 30 minutes), heating-cooling (4 and 45°C; 6 cycles), and freeze-thaw tests (-21 and +25°C; 3 cycles) were done and phase separation and precipitation were also analyzed. The concentration of tacrolimus solution in methanol was determined by a UV spectrophotometer at 213 nm. All prepared formulations were accepted in stability tests.[33] An outline of the UV spectrophotometer analyzing method of tacrolimus formulations is described in Table 3. It should be noted that UV spectrometry is a quick and easy method to analyze drugs. However, lack of sensitivity and selectivity is one of its main disadvantages. Also, interference with the matrix makes the measurement difficult.

Liquid chromatography (HPLC, UHPLC, and LC) coupled with various detectors

Castro et al constructed a cationic polymer containing tacrolimus and overcame poor penetration of tacrolimus to corneocytes. In this study, the LC method was used (with a diode array detector at 210 nm) to quantify the amount of tacrolimus in the ocular formulation. Tacrolimus concentration in aqueous humor samples (transparent medium between the lens and the cornea) was determined by LC-tandem mass spectrophotometry (LC-MS/MS). Tacrolimus-nanoparticles indicated suitable physicochemical properties for this application and its long-term stability showed no meaningful change in pH, PDI, EE%, and zeta potential.[34]

HPLC method is utilized in various studies to quantify the concentration of tacrolimus. Hirasawa et al developed a new multi-unit formulation of tacrolimus with a vehicle named universal orbicular (UniORV) that was produced by the dropwise spheroidizing method. To evaluate the stability of this formulation, they studied thermal and photostability studies, and the concentration of tacrolimus was analyzed by HPLC as described in Table 4.[35]

The results of this study demonstrated that UniORV formulation possessed appropriate stability.[35] TNF-α, an inflammatory cytokine has a key role in psoriasis outbreaks. In a study, Viegas et al invented a nanostructure lipid carrier (NLC) for delivering tacrolimus and TNF-α siRNA concurrently. In this research, tacrolimus was quantified by the HPLC method as can be seen in Table 5. The physical stability of this formulation consisted of the evaluation of the average size and PDI in diverse storage temperatures (4, 25, and 37°C) for 30 days. Results of this study demonstrated that it was stable at 25 and 37°C but unstable at 4°C during 1 month.[36] In another study, lipid nanoparticles were used as a topical formulation of tacrolimus to increase its dermal penetration. The concentration of tacrolimus in the mixture was evaluated by HPLC. The particulars of this method are described in Table 5. To evaluate the chemical stability of this formulation, tacrolimus was dissolved in heated lipid (75°C). The results of this study revealed that the content of the drug was consistent and the formulation had sufficient chemical stability.[37] Rebibo et al created a new ophthalmic drop containing tacrolimus nanocapsules. Drug concentration and EE% were analyzed by the HPLC

Table 3. UV spectrometer analyzing method of tacrolimus formulations

Formulation	Diluent	λ_{max} (nm)	Ref.
Tacrolimus-loaded solid lipid nanoparticle gel	Phosphate buffer pH = 7.4	294	[31]
Tacrolimus-loaded nanoemulsion gel	Methanol	205	[32]
Self-microemulsifying drug delivery system of tacrolimus	Methanol	213	[33]

Table 4. Stability tests on the UniORV/ tacrolimus formulation

Sample	Condition	Time (wk)	Decrease percentage (%)
Sealed with aluminum laminated polyethylene layer	25 ± 2°C/60 ± 5%	12	2.6
Sealed	40 ± 2°C/75 ± 5% RH	24	2.7
Sealed	50 ± 2°C	8	4.9
Open	40 ± 2°C/75 ± 5% RH	4	0.83
Open	1.2 million lux hours	-	21

Table 5. HPLC analyzing method of tacrolimus formulations

Formulation	Column (column oven temperature)	Mobile phase	Elution mode	Flow rate (mL/min)	Injection volume (µL)	Detector	Ref.
UNiORV Multi-unit dosage form	C_{18} (50 °C)	Water: 2–propanol: tetrahydrofuran (5:2:2)	Isocratic	0.7	-	UV at 220 nm	35
Co-delivering tacrolimus and TNF-α siRNA	C_{18}	Methanol: water (30:70, v/v)	Isocratic	1	30	UV at 210 nm	36
Tacrolimus-loaded nanostructured lipid carrier	C_{18} (50 °C)	Acetonitrile: water: isopropanol (70:28:2)	Isocratic	1	20	UV at 210 nm	37
Tacrolimus nanocapsules eye drop	C_{18} (60 °C)	Acetonitrile: water (95:5 v/v)	Isocratic	0.5	5	UV at 213 nm	38
Disintegrating tacrolimus tablets	C_{18} (40 °C)	Methanol: acetonitrile: water (45:45:10)	Isocratic	1.5	10	UV at 214 nm	39
Tacrolimus loaded nanocarrier	C_{18} (40 °C)	Acetonitrile: 0.02 M phosphoric acid (70:30 v/v)	Isocratic	1	-	Fluorescence	40
Inhalable tacrolimus dry powder formulation	C_{18} (50 °C)	A: 0.2% phosphoric acid in water B: 100% acetonitrile	Gradient: A:B/time; 52:48/7 min 30:70/10 min 30:70/12 min 52:48/12.5 min 52:48/15 min	1.5	50	UV at 215 nm	41
Tacrolimus/ hydroxypropyl-β-cyclodextrin eye drop	C_{18} (60 °C)	Acetonitrile: water (65:35 v/v)	Isocratic	1.5	10	Diode array detector	42,43
Tacrolimus eye drop	C_{18} (670 °C)	A: aqueous ortho-phosphoric acid solution at 85% (0.1 %, v/v). B: 500 mL of acetonitrile and 47 mL of MTBE	Gradient: A:B/time 63:37/1 min 60:40/12 min 45:55/17 min 10:90/19 min 10:90/22.5 min 63:37/23 min 36:37/27 min	1	-	Diode array detector	44
Tacrolimus loaded PEG-cholecalciferol based micelles	C_{18}	Acetonitrile: water (95:5 v/v)	Isocratic	1	-	Diode array detector	45
Cyclosporine A and tacrolimus eye drop	C_{18}	A: 20 mM aqueous ammonium formate B: methanol	Start with 30 % B 0-5min: 30-95 %B 5-10min: 95 % B	0.3	20	Mass spectrometry	46

method. Three storage conditions were chosen in stability tests: 4°C, 25°C/60% RH, and 40°C/75 % RH. Lyophilized powder of tacrolimus was stable at 4°C, 25°C, and 40°C and no ascomycin and tacrolimus 8-epimer as impurity and degradation product were detected but reconstituted product was stable for 2 and 4 weeks at 25°C and 4°C, respectively.[38] Ponnammal et al formulated amorphous oral tablets of tacrolimus prepared by hot melt extrusion with polyvinylpyrrolidone vinyl acetate, Soluplus, and hydroxypropyl cellulose to improve the dissolution of tacrolimus. Stability tests of drug products were studied under accelerated conditions (40°C/75% RH) for 3 months. Drug concentration was analyzed by HPLC. Results of differential scanning calorimetry and X-ray powder diffraction revealed an amorphous form of tacrolimus during storage conditions that could increase the solubility of tacrolimus and improve the dissolution profile of new tablets.[39] Liu et al provided a biodegradable nanocarrier of tacrolimus to enhance its penetration to eye tissue. ^{1}H NMR, transmission electron microscope, size, and zeta potential were applied to study the characterization of tacrolimus in this formulation. Stability investigations were done under various conditions: 4°C/ 75% RH and 25°C/ 60% RH for 1 month and drug content was determined by the HPLC method. PDI and zeta potential were increased at 25°C during 4 weeks due to nanocarrier degradation. However, it was stable at 4°C.[40] A new inhalation using thin film freezing with elevated drug loading was prepared for lung transplant patients. The stability test of this formulation was studied under 40°C/75% RH and 25°C/60% RH for 6 months and then physical properties, physicochemical stability, and the performance of aerosol were evaluated. Results of particle size, specific surface area, and moisture content as physical stability parameters and concentration of tacrolimus that was quantified with HPLC, demonstrated good stability of this formulation during 6 months. Also, studies about the effect of packaging on the physical stability of drug products showed that encapsulation of powder with desiccant is the best package that absorbed less moisture and had a lesser amount of decrease in particle size and specific surface area.[41] García-Otero et al formulated tacrolimus eye drops containing hydroxypropyl beta-cyclodextrin. The stability of unopened drops was determined for 4 months at three different temperatures (4°C, 25°C, and 40°C). Osmolality, pH, and microbial growth were evaluated for this matter. Also, macroscopic changes such as color, turbidity, and sediment were

examined visually. The concentration of tacrolimus was determined by the HPLC method as shown in Table 5. Tacrolimus concentration did not decrease at 4°C during 4 months. Likewise, osmolality, pH, and macroscopic change did not alter during the study and no microorganism was found in the samples.[42] Similar to this research, Luaces-Rodríguez et al investigated the pre-clinical and clinical characteristics of tacrolimus ophthalmic drops which were prepared by adding Prograf ampoules into various vehicles containing a salt solution, Liquifilm®, and hyaluronic acid that were named TBS, TLI, and THA, correspondingly. They researched the stability of these formulations in three conditions: Room (18 to 22°C), refrigerated (2 to 8°C), and frozen (-15 to -20°C) temperatures for 90 days. Similar to the previously mentioned study, osmolality, pH, microbial growth, visual color change, turbidity, and precipitation were analyzed and the concentration of tacrolimus was also determined by the HPLC method. The expiration date of the formulation was chosen when the drug concentration was 90% of its initial concentration. The formulation demonstrated first-order kinetics. Pharmacokinetic parameters illustrated a meaningful increase in the retention time of THA and TLI. TLI was stable in the refrigerator and freezer, TBS was only stable at frozen temperature and THA was unstable in all storage conditions.[43] The physicochemical stability of the new ophthalmic formulation of tacrolimus was investigated by Barrieu et al. Unopened ophthalmic drops were analyzed in terms of visual parameters (like cloudiness, gas production, and visible particles), pH, viscosity, osmolality, turbidity, chromaticity, micelle size (by dynamic light scattering method), and sterility assay (soya trypcase and thioglycolate were culture media) for 9 months. The results of these tests demonstrated that physical parameters did not vary significantly. The concentration of opened drops was analyzed twice a day (morning and evening) for one month. In this study, two different concentrations of tacrolimus (0.2 and 1 mg/mL) were analyzed for 9 months at three different temperatures (5°C, 25°C, and 35°C). The concentration of tacrolimus was decreased and multiple degradation products appeared. Degradation products of formulations stored at 5°C were compared with the injectable form of Prograf. The amount of impurity A was like Prograf sample, tacrolimus alpha hydroxy acid was less than Prograf, and tacrolimus regioisomer was not found in Prograf. Additionally, to estimate the effect of temperature on drug degradation, the procedure kinetics was studied. In conclusion, these eyedrops possessed sufficient physicochemical stability for nine months at 5°C.[44] Kutlehria et al prepared an ocular formulation of tacrolimus containing polyethylene glycol 2000 in conjugation with a vitamin D analog named cholecalciferol. A new formulation of tacrolimus entrapped in micelles that were integrin targeted was developed. Tacrolimus can treat ophthalmic inflammatory diseases without inducing glaucoma. The advantage of this formulation is the p-glycoprotein (p-gp) inhibitory effect of cholecalciferol. P-gp is an efflux protein highly expressed in epithelial cells that outflow lipophilic drugs. The content of the drug in this formulation was determined with the HPLC method (Table 5).[45] Ghiglioni et al formulated an ophthalmic drop containing tacrolimus and cyclosporine A for the treatment of ocular inflammatory disease. The stability of opened drops was tested at 5 and 25°C/60% RH for 3 months and the stress tests of unopened drops were done at 40°C. The amount of both drugs was evaluated by HPLC-MS as described below. The amount of decrease in tacrolimus and its reference standard (Prograf) was 10%-20% at 5 and 25°C. Even though the degradation rate of cyclosporine A was increased at 25°C but cyclosporine A was more than 80 % for 1 month.[46] These studies are justified briefly in Table 5.Drug-excipient interactions can affect the final product stability in the formulation. Peterka et al. evaluated this compatibility by mixing magnesium stearate with tacrolimus in the ratio of 1:1. UHPLC (with BEH C_{18} column kept in 65°C) method and photodiode array detector was performed to analyze the degradation products. The mobile phase composition was a mixture of 0.01% phosphoric acid solution and a mixture of acetonitrile: methyl tert-butyl ether at a flow rate of 0.75 mL/min. Magnesium stearate can reduce the stability of tacrolimus in solid dispersion formulation which resulted in tacrolimus alpha-hydroxy acid elevation. However, this degradation product did not increase in the presence of stearic acid. It is deduced that magnesium played a key role in this degradation pathway. Magnesium increased diketoamide against cyclic hemiketal form. Also, the existence of magnesium salts results in the basicity of media, and basic condition facilitated the formation of this degradation product.[17] As previously mentioned, tacrolimus is sensitive to dehydration, hydrolysis, and epimerization in aqueous solutions. Hydrolysis was proposed as the main reaction for the instability of tacrolimus in the aqueous solution. To prevent this, Prajapati et al used cyclodextrin for ocular delivery.[47] In addition, it could increase its solubility in the aqueous moiety. The stability of tacrolimus formulations containing cyclodextrin by UHPLC was evaluated as elucidated in Table 6. The degradation kinetic of tacrolimus was investigated by LC-MS/MS as clarified in Table 7.

The results of research done by Prajapati et al illustrated that tacrolimus kinetic in the complex of cyclodextrin at constant temperature and pH was pseudo-first order. Also, the effect of cyclodextrin concentration, several cyclodextrins, and pH on the degradation of tacrolimus was evaluated. The results of these studies proved that boosting the cyclodextrin concentration hindered the degradation of tacrolimus in aqueous solutions and 2-hydroxy-β-cyclodextrin was the greatest stabilizer. In this study, tacrolimus was unionized at various pH quantities and 4-6 was reported as the stable pH range of this formulation.[47] Savić et al made a tacrolimus nanoemulsion with lecithin and monocaprylated propylene glycol to enhance the

Table 6. UPLC analyzing method of tacrolimus formulations

Formulation	Column (column oven temperature)	Mobile phase	Elution mode	Flow rate (mL/min)	Injection volume (µL)	Detector	Ref.
Tacrolimus in cyclodextrin solution	C_{18} (50°C)	Acetonitrile and water containing 0.1 % v/v trifluoroacetic acid (60:40)	Isocratic	0.4	10	UV	47
Leciten-based tacrolimus formulation	C_{18} (50°C)	0.1 % formic acid in a mixture of acetonitrile and water (65:35, v/v)	Isocratic	0.25	-	MS	48
Tacrolimus loaded chitosan nanoparticles	C_{18} (60°C)	100% Acetonitrile	Isocratic	0.6	10	MS	49

Table 7. LC-MS/MS analyzing method of tacrolimus formulations

Formulation	Sample	Column (Column oven temperature)	Mobile phase	Elution mode	Flow rate (mL/min)	Injection volume (µL)	Ionizer	Ref.
Polymeric aqueous tacrolimus formulation	Eye tissue	C_8 (50 °C)	A: 0.1 % v/v formic acid in water B: 0.1 % v/v formic acid in acetonitrile	Gradient mode A:B/time 60:40/0.5 min 0:100/1 min 0:100/3 min 60:40/4 min	0.5	10	Electrospray ionization	50
Tacrolimus in a dried matrix on paper discs	Blood	C_{18} (50 °C)	A: 2 mmol/L ammonium acetate in water, 0.1 % formic acid B: 2 mmol/L ammonium acetate in methanol, 0.1 % formic acid	Gradient A:B/time 50:50/0.6 min 0:100/1.2 min 50:50/2 min	0.5	25	Electrospray ionization	51
Tacrolimus self-microemulsifying drug delivery system	Blood	C_{18} (50 °C)	Acetonitrile: water (90:10 v/v)	Isocratic	0.3	2	Electrospray ionization	52
Tacrolimus in cyclodextrin solution	The drug in thebuffer solution	C_{18}	A: 10 mM ammonium acetate in water (pH=5.5) B: 10 mM ammonium acetate in water (pH=5.5)	Gradient A:B/time 60:40/0.1 min 0:100/5 min 0:100/5.5 min 60:40/5.6 min	0.5	4	Electrospray ionization	47

dermal delivery amount of tacrolimus. Particle size, PDI, zeta potential, pH, rheological properties, EE%, and conductivity of tacrolimus nanoemulsion formulation was characterized to estimate the stability of the formulations at 20 ± 2°C storage temperature during 6 months. Likewise, the separation of phase and new formations appearance were probed. This formulation was stable during the defined time, particularly attributable to the absence of tacrolimus from the aqueous phase.[48] Fereig et al formulated chitosan-based hydrogel for dermal delivery of tacrolimus in psoriasis treatment. UHPLC was applied for drug content analysis in the hydrogel. The formulations were kept at 4°C for 3 months and then PDI, particle size, zeta potential, and EE% were determined. At the end of the study, particle size and zeta potential did not change significantly. PDI was decreased due to some large structures. The EE% was diminished because of the lipophilicity of tacrolimus. Generally, it possessed suitable stability for dermal purposes and ultimately, boosted the rate of hair growth in animal studies that verified the recovery of the skin.[49]

The polymeric ophthalmic formulation of tacrolimus was developed by Badr et al. Chitosan-based amphiphile trapped encapsulated tacrolimus that was the fabrication of a thin-film hydration procedure. To analyze the content of tacrolimus in made formulation, HPLC with UV detector at 218 nm was used (C_{18} column at 50°C-0.1% phosphoric acid and acetonitrile were utilized in gradient mode as mobile phase A and B, respectively). Some physical properties like particle size, zeta potential, osmolarity, and viscosity were evaluated. Stability tests were done in various conditions: 4°C, 25 °C/60% RH and 40°C/75 % RH for 30 days and then physicochemical properties were investigated. In vitro studies examined the concentration of tacrolimus in the eye tissue of rabbits by LC-MS/MS as mentioned in Table 5. The new formulation was stable at 4 °C for 30 days but it was unstable at 40 °C as a result of returning to crystalline form.[50] Bressán et al in 2021 applied LC-MS/MS for TDM of tacrolimus. The samples were collected by using a strategy denominated as a dried matrix on paper discs (DMPD). The concentration of tacrolimus in DMPD with whole blood was compared by utilizing LC-MS/MS method as explained in Table 7. By validation of this method, it was illustrated that, a fast and simple method for TDM of immunosuppressants.[51] Tao et al studied the stability and absorption of self-microemulsified drug delivery of tacrolimus formulation. In this research, the stress test was performed in three different conditions (high temperature (40°C), high relative humidity (90%), and light radiation up to 4500 lux). Drug concentration was determined by HPLC in three days including 0, 5, and 10. Citric acid was used to

adjust the pH. The addition of 0.25% citric acid decreased drug degradation, especially in the high humidity and thermal studies. However, high concentrations of citric acid (for example 0.3 %) promoted drug degradation and reduced drug concentration. The result obtained from this research indicated that tacrolimus degradation was dependent on pH and the drug was stable in a mild acid (pH = 5) but it was unstable in highly acidic and basic conditions. In addition, tacrolimus degradation is less affected by temperature. Furthermore, to determine the blood concentration in the pharmacokinetic study, LC-MS/MS was used as explained in Table 7.[52] Even though the methods based on LC are costly, they are highly sensitive and time-saving methods. LC-MS/MS is an important tool in TDM as it offers high specificity and selectivity.

Other methods

Modi et al developed an ophthalmic hydrogel of tacrolimus that consisted of chitosan and pluronic F127. The hydrogel was in liquid form at a lower temperature. However, altered to gel form at body temperature that sustained the drug release. Human tacrolimus, the enzyme-linked immunosorbent assay (ELISA) kit, and the ELISA reader at 450 nm were used to quantify drug content in tacrolimus gel. The concentration of the drug was measured from the equation obtained from the standard curve.[53] Laboratory powder diffraction and synchrotron powder diffraction were utilized to identify the crystallinity of tacrolimus.[54] Thermogravimetric analysis and differential scanning calorimetry are mainly applied to investigate polymorphism, pseudo-polymorphism, stability, and purity of tacrolimus in pharmaceutical products.[39] Fourier transform infrared (FT-IR) spectrometry is a fast and sensitive method to study the structure of tacrolimus.[39] It should be noted that most of these methods are rapid and easy to use. FT-IR, a convenient technique has high accuracy and precision. Thermal analysis is simple, fast, and cost-effective but sensitive to any changes.

Conclusions

In the present review, tacrolimus as a known calcineurin inhibitor was comprehensively introduced and the developed degradation tests, degradation pathways, and degradation products were summarized. According to the literature, degradation products of acidic hydrolysis were 5-deoxy-$\Delta^{5,6}$-tacrolimus and 5-deoxy-$\Delta^{5,6}$-epitacrolimus. They were formed by dehydration of β-hydroxyketone and epimerization. Tacrolimus was stable at a pH of 3-5. Degradation products of tacrolimus in mild alkaline conditions were 8-epitacrolimus and open chain acid of 5-deoxy-$\Delta^{5,6}$-tacrolimus. However, tacrolimus was cleaved from allylic ester moiety in treatment with an alkaline solution and an aldehyde was formed. Thermolysis of tacrolimus caused rearrangement of allylic ester moiety and production of tacrolimus regioisomer as a degradation product. Then, by dehydration of the β-hydroxyketone moiety of tacrolimus regioisomer, a new degradation product was formed. All impurities of solid-state tacrolimus were increased when it was exposed to oxygen. Epi-tacrolimus was produced from tacrolimus in treatment with ACVA at 60°C but tacrolimus was stable in treatment with H_2O_2 at room temperature. The results of photolytic tests on amorphous tacrolimus displayed the appearance of epi-tacrolimus. New formulations were developed for solubility enhancement of tacrolimus like applying the amorphous form of tacrolimus in disintegrating tablets and cyclodextrin complexation in ocular formulations. Nanoencapsulation of tacrolimus, chitosan-based hydrogels, and biodegradable nanocarriers were developed to increase the retention time in eye tissue. In an ophthalmic formulation, using cholecalciferol inhibited p-gp efflux. In conclusion, this research could give valuable insight to researchers in the field of immunosuppressant drugs, especially tacrolimus.

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Authors' Contribution

Sara Sajjadi: Writing the manuscript and preparing the original draft; Mohammadreza Siahi-Shadbad: Supervision, project administration, and contributing to funding acquisition; Mohammad Reza Afshar Moghaddam: Editing the article. All authors have read and agreed to the published version of the manuscript.

Competing Interests

The author(s) declare that they have no competing interests.

Ethical Approval

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