Stability of tacrolimus ophthalmic solution

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Purpose. The stability of 0.3-mg/mL tacrolimus ophthalmic solution at different storage temperatures was studied.

Methods. A sterile ophthalmic solution of 0.3 mg/mL tacrolimus was prepared in triplicate under aseptic conditions by diluting tacrolimus in eye drops. Three aliquots of this solution were transferred into polypropylene bottles and stored at 25, 2–8, or –15 to –25 °C. Samples were collected immediately after preparation and at selected time points and assayed in triplicate using high-performance liquid chromatography (HPLC). Samples were also visually examined for macroscopic changes. The 0.3-mg/mL tacrolimus solution was also exposed to acidic treatment and heat to force its degradation and to evaluate the selectivity of the analytic method. The tacrolimus ophthalmic solution was considered stable if at least 90% of the mean initial concentration remained when analyzed by HPLC.

Results. When stored at 2–8 °C and between –15 and –25 °C, at least 90% of the initial tacrolimus concentration remained throughout the 85-day study period. There were no significant differences in tacrolimus concentrations between the starting and ending points (p > 0.05). However, when tacrolimus solution was stored at 25 °C, the percentage of the initial tacrolimus concentration remaining had decreased to less than 90% on day 28.

Conclusion. Tacrolimus diluted to 0.3 mg/mL in eye drop solution was stable for 20 days when stored at 25 °C and for at least 85 days when stored at 2–8 °C or between –15 and –25 °C in polypropylene bottles and protected from light.

Keywords: drug stability, ophthalmic solutions, tacrolimus

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Therapeutic indications for systemic tacrolimus include prophylactic rejection treatment in liver, kidney, or heart allograft transplant recipients and treatment of allograft rejection resistant to other immunosuppressive drugs.1-3 Off-label clinical indications for tacrolimus include the treatment of autoimmune diseases,4,5 atopic dermatitis, eczema, psoriasis, and vitiligo.6 It is also being evaluated as an active agent in the treatment of rheumatoid arthritis.7 In addition, some immunosuppressive agents such as cyclosporine and tacrolimus have been successfully used for dry eye treatment in graft-versus-host disease (GVHD).8,9

Tacrolimus is a very potent immunosuppressive drug; at the molecular level its effects appear to be mediated by binding to a cytosolic protein (FKBP12), resulting in its intracellular accumulation.10-12 The FKBP12–tacrolimus complex binds to and inhibits calcineurin, leading to calcium-dependent inhibition of T-cell signal transduction pathways, thereby preventing transcription of a discrete set of lymphokine genes. In particular, tacrolimus inhibits the formation of cytotoxic lymphocytes that are primarily responsible for graft rejection; it suppresses T-cell activation and T-helper cell–dependent B-cell proliferation, as well as the formation
of lymphokines (such as interleukin-2, interleukin-3, and γ-interferon and expression of the interleukin-2 receptor.

Administration of systemic immunosuppressive agents is associated with an increased risk of infection and systemic toxicity.\textsuperscript{13–15} However, topical 0.05% cyclosporine and 0.03% tacrolimus ointment has been successfully used as a prophylactic, thus avoiding systemic immunosuppressant administration in GVHD patient ocular pathologies.\textsuperscript{8,9} Although beneficial effects of topical tacrolimus eye drops alone have not yet been demonstrated in GVHD patients, they have proven to be effective in preventing corneal rejection,\textsuperscript{16} severe allergic conjunctivitis, and systemic cyclosporine-intolerant keratoconjunctivitis.\textsuperscript{17,18}

No ophthalmic dosage formulations of tacrolimus are commercially available. Ohashi and colleagues\textsuperscript{19} reported the preparation of an extramoraneous 0.1% tacrolimus ophthalmic suspension using polyvinyl alcohol as an eye lubricant and benzalkonium chloride as a preservative, though no stability data are available for this formulation.

Different methods have been used to analyze tacrolimus and related substances.\textsuperscript{19,20} High-performance liquid chromatography (HPLC) is the gold standard for stability studies because it is highly selective and sensitive.\textsuperscript{21–26} In addition, the most prestigious guidelines for drug stability studies recommend forced-degradation studies to provide information about the intrinsic chemical stability of drugs.\textsuperscript{27,28}

The objective of this study was to determine the stability of extramoraneous 0.03% tacrolimus eye drops.

**Methods**

**Reagents and solutions.** I.V. tacrolimus ampules (5 mg/mL)\textsuperscript{a} and 14 mg/mL Liquifilm\textsuperscript{b} sterile ophthalmic eye drops were purchased from a commercial source. The acetonitrile\textsuperscript{c} and methanol\textsuperscript{d} used were HPLC-grade; water was also HPLC-grade and purified using a flex system\textsuperscript{e} immediately before use. Analytic-grade glacial acetic acid\textsuperscript{g} and sulfuric acid\textsuperscript{i} were used for aqueous mobile-phase acidification and acid-accelerated tacrolimus degradation, respectively.

**Instrumentation.** A glass-calomel electrode pH meter\textsuperscript{h} was used to adjust pH; all solutions and mobile phases were filtered through 0.45-μm nylon membrane filters.\textsuperscript{r} Sample cleanup before direct injection in the chromatographic system included filtration through a 0.22-μm polytetrafluoroethylene membrane filter.\textsuperscript{j} Later, the sample was placed in a centrifuge\textsuperscript{m} and shaken vigorously in a Vortex mixer.\textsuperscript{l} A digital heat block incubator\textsuperscript{n} with a ±0.1 °C temperature control was used to incubate the samples in the forced-degradation experiments.

**HPLC method.** The HPLC equipment was a Waters integrated modular system\textsuperscript{o} comprising a separation module based on a quaternary pump, online degasser system, automatic injection system, column oven, and dual wavelength ultraviolet–visible light detector.\textsuperscript{r}

Chromatographic separation was performed at a controlled tempera-
of Liquifilm eye drops under aseptic conditions, prepared in triplicate. Three 15-mL aliquots were taken from each of these stock solutions, and 2 of these were transferred into 2 50-mL polypropylene bottles. One aliquot was stored at room temperature (24 ± 2 °C), the second was re-refrigerated (2–8 °C), and the third was frozen (−15 to −25 °C). All 3 aliquots were protected from light. The frozen sample was separated into 11 1-mL aliquots in polypropylene tubes (1 for each sampling time) to avoid repeated freeze–thaw cycles. The accuracy and precision of the assay were tested with tacrolimus solutions of 100, 150, and 300 μg/mL.

Sampling procedure. A sample of 150 μL was withdrawn from each of the aliquots with a micropipette immediately after preparation, at 4 and 12 hours, and on days 1, 2, 3, 7, 14, 28, 42, 55, and 85. Frozen samples were thawed for 10 minutes at room temperature. Each sample was assayed in triplicate by HPLC. In total, 9 tacrolimus concentrations were analyzed for each time point.

Because polyvinyl alcohol is insoluble in the elution mobile phase, we added cold acetonitrile to all samples to prevent the precipitation of polyvinyl alcohol in the HPLC system. Samples were then mixed in a Vortex mixer for 20 seconds and centrifuged at 10,800 rpm for 5 minutes. After adding filtered supernatant, 20 μL of each sample was injected into the HPLC system.

Samples were also visually inspected for any macroscopic changes (e.g., color, turbidity, precipitation). Microbiological testing was not performed because each commercial vehicle contained the preservative benzalkonium chloride.

Forced degradation. We conducted forced-degradation studies using a solution of tacrolimus 300 μg/mL prepared by diluting the stock solution with eye drop solution. Tacrolimus decomposition under acidic conditions (sulfuric acid 1N) was induced at 75 °C for 3 hours. Samples were prepared and analyzed in triplicate. The samples were neutralized and filtered before injecting them into the HPLC system.

Preparation of standard solutions and calibration curves. A 1-mL stock solution of tacrolimus was prepared on each sample analysis day. Standard solutions of tacrolimus (100, 150, 190, 230, 270, 300, and 330 μg/mL) were prepared by diluting the stock solution with eye drop solution, and 300- and 150-μg/mL solutions were prepared as analytic-method controls. A standard curve was constructed by linear regression of the peak tacrolimus areas against their respective concentrations.

Data analysis. Stability was defined as the time at which 90% of the initial concentration of tacrolimus remained (t90); the initial concentration (time point 0) was considered to be 100%. Tacrolimus concentration was expressed as the percentage of the initial concentration and was estimated by semilogarithmic regression of the percentage of the remaining tacrolimus concentration versus time, assuming first-order degradation kinetics. The t90 value was the lower limit of the 95% confidence interval (CI). We performed statistical evaluation of the data with OpenOffice Calc (Apache OpenOffice 4, version 4.1.2).

The a priori level of significance was 0.05. For linear regression analysis, IBM Statistics for windows, version 22.0. (IBM Corp., Armonk, NY) was used.

Results

HPLC method. The retention time for tacrolimus was approximately 7.8 minutes. Linearity was determined by plotting tacrolimus concentrations against peak tacrolimus chromatogram areas and characterized by a straight regression line with an intercept of −9.76E+03 (95% CI, −3.04E+04 to 1.09E+04) and a slope of 1.15E+04 (95% CI, 1.07E+04–1.24E+04); the coefficient of determination was greater than 0.996 within the concentration range of 0.3–100 μg/mL.
range tested. The interday and intraday coefficients of variation for the tacrolimus assay were 3.58% and 4.33%, respectively. The limit of quantification was 100 µg/mL.

**Stability study.** Table 1 displays the mean percentage of tacrolimus remaining in the eye drop solution when stored at various temperature conditions. When stored at 2–8 °C and between –15 and –25 °C, at least 90% of the initial tacrolimus concentration remained throughout the 85-day study period. There were no significant differences in tacrolimus concentrations between the starting and ending points (p > 0.05). However, when tacrolimus solution was stored at 25 °C, the percentage of the initial tacrolimus concentration remaining had decreased to less than 90% on day 28. The $t_{50}$ values and corresponding 95% CIs were obtained by interpolation of the calculated regression line (percentage of remaining tacrolimus concentration versus time). The $t_{50}$ for tacrolimus was 28 days, but the lower 95% CI limit was 20 days (95% CI, 20–36 days).

**Forced degradation.** The addition of acidic treatment followed by drastic heating conditions caused an 80% decrease of the initial tacrolimus peak area. The retention times of the forced-degradation product peaks were lower than those observed for tacrolimus and demonstrated no chromatographic coelution. These degradation products were observed but not measured or quantified. No interfering peaks were observed because no decomposition products eluted at or near the tacrolimus retention time.

No physical changes (precipitates, color variations, solid particles, gas, turbidity, or bacterial growth) were observed during the study period.

**Discussion**

The analytic method validated in this study meets all of the requirements for robustness, specificity, precision, and accuracy for it to be used as a quality control for stability studies. This study found that tacrolimus, formulated as an extemporaneous 0.3-mg/mL ophthalmic solution made from a commercially available drug product in sterile ophthalmic eye drops as a vehicle, is stable for at least 85 days when refrigerated (2–8 °C) or frozen (–15 to –25 °C); its stability for longer time periods is unknown. This study also confirmed that tacrolimus starts to degrade at room temperature and loses more than 30% of its initial drug concentration at day 85.

The forced-degradation study confirmed the absence of products generated by tacrolimus degradation that can potentially interfere with HPLC analysis; therefore, the chromatographic method used was adequately selective. This study’s stability results are similar to those published by Gauthier and colleagues, who reported that a 0.06% tacrolimus eye drop formulation, made with tacrolimus monohydrate powder and virgin castor oil under similar conditions to those reported herein, is stable for 28 days.

Given the physical properties of tacrolimus, it is also important to consider how compounded tacrolimus ophthalmic solution is stored. Tacrolimus is stable as an admixture if kept in polypropylene plastic syringes; therefore, plastic bottles manufactured from a polypropylene resin were used in this study. Given that tacrolimus is highly lipid soluble, with an n-octanol–water partition coefficient of more than 1,000, these container helps to avoid possible drug loss. Therefore, the aforementioned decrease in tacrolimus concentration in plastic bottles was likely due to chemical degradation rather than adsorption onto the plastic surface.

The results of this study suggest that tacrolimus eye drops can be successfully stored at 2–8 °C for at least 85 days. This lengthy storage duration has substantial advantages in healthcare settings, allowing more efficient work organization and planning. In addition, these results allow health professionals to give information about the optimal storage conditions of this drug when dispensed to ambulatory care patients.

**Conclusion**

Tacrolimus diluted to 0.3 mg/mL in eye drop solution was stable for 20 days when stored at 25 °C and for at least 85 days when stored at 2–8 °C or between –15 and –25 °C in polypropylene bottles and protected from light.

**Disclosures**

The authors have declared no potential conflicts of interest.

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1. Prograf, Astellas Ireland Co. Limited, Killorgin, County Kerry, Ireland, lot 5A32862.
2. Liquifilm, Allergan Pharmaceuticals Ireland, Westport, Ireland, lot E76022.
3. Acetonitrile HPLC grade, multisolvent, Scharlab, S.L., Sentmenat, Barcelona, Spain.
4. Methanol HPLC grade, multisolvent, Scharlab, S.L.
5. ELGA-PURELAB flex system, ELGA laboratory water, Rivas VaciaMadrid, Madrid, Spain.
7. Sulfuric acid (purity ≥98.0%), Panreac Quimica S.A.U., Castellar del Vallés.
8. Model 2489, Waters Corp., Ann Arbor, MI.
9. Dual wavelength UV/vis detector, model e2695, Waters Corp., Milford, MA.
10. Dual wavelength UV/vis detector, model 2489, Waters Corp.
12. 0.45-µm nylon membrane filters, Pall Corp., Ann Arbor, MI.
13. 0.22-µm polytetrafluoroethylene membrane filters, GE Healthcare Europe GMBH, Roselló i Porcel, Barcelona, Spain.
14. Eppendorf tube centrifuge, Abbott Laboratories, North Chicago, IL.
15. Vortex mixer, model SA3, Stuart Scientific, Staffordshire, United Kingdom.
17. Separation module, model e2695, Waters Corp., Milford, MA.
18. Dual wavelength UV/vis detector, model 2489, Waters Corp.
19. XBridge column, 5-µm packing, 150 x 4.6 i.d. mm, Waters Corp.

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