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Development and Assessment of the Stability of Hospital Derivation of Tacrolimus

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Tacrolimus is an immunosuppressive drug widely used in hospitals for the treatment of transplant patients. Often, hospitalized patients undergo the administration of tacrolimus in liquid pharmaceutical forms. However, this drug has been marketed only in solid form for oral use. This work aimed to develop and evaluate the stability of tacrolimus in a simple liquid formulation with comparable efficacy, safety, and stability. The analyzes were developed according to the USP method (2018), being co-validated, guaranteeing its accuracy, precision, and specificity. Based on the analyses, the derivation of tacrolimus in suspension was determined, as well as the wetting agent in its ideal proportion to obtain drug content in HPLC within acceptable limits. The formulation was observed for the evaluation of physical-chemical parameters, being viscosity, pH, sedimentation volume, and organoleptic characteristics. Dosing was carried out for 30 days at room temperature and a temperature of 40°C. The formulation maintained its integrity during the 30 days of storage at room temperature with content between 90 – 110%, growth above 200 cP, neutral pH, and sedimentation volume below 3 mL. However, tacrolimus is controlled sensitively to heat, observing degradation in the first ten days of analysis at 40°C, with possible formation of degradation products. Based on this work, the tacrolimus formulation developed in 8% polyethylene glycol 400 has adequate dosage and homogeneity for 30 days and should be kept away from light and at room temperature.

Keywords: Tacrolimus, Suspensions, Dosage Forms, Validation Study, Drug Stability

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Introduction

Tacrolimus (Figure 1) is an immunosuppressive drug, being administered in hospitals to transplant patients to avoid rejection of a new organ, usually the liver, kidney, and heart (1).

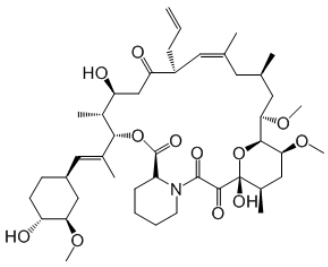

Figure 1. Chemical Structure of Tacrolimus (4)

Its mechanism of action is to induce immune tolerance, reducing the action and activation of lymphocytes and cytokines (2). Another therapeutic application of tacrolimus is related to the non-hospital use for atopic dermatitis in cases of ineffective treatment of first choice, being applied topically (3). Currently, a liquid commercial form of tacrolimus is fundamental in hospitals to allow adequate treatment for transplanted children. Considering that tacrolimus is a lipophilic molecule, it becomes impracticable to carry out a formulation only with simple syrup (4,5). Thus, it is necessary to derive this drug in the form of suspension, as well as guarantee its stability during a certain period of use. Derivation of drugs allows treatment in an appropriate way for each specific case, regarding dosage and application. For patients with difficulty or inability to swallow, such as those that require receiving treatment by nasogastric tube or gastrostomy, administering oral solutions becomes essential for treatment (6,7). As for children, there are two main benefits, greater adherence to treatment due to the palatability and ease of diluting or fractioning the drug for the necessary dosage (8). The objective of this study was to develop a liquid formulation of tacrolimus by derivation with less complexity and

equivalent or better effectiveness, without compromising the safety of the treatment in hospital use, evaluating its stability during a limited period (7,9) through a validated chromatographic method (10).

Experimental

Materials

Tacrolimus Raw Material was acquired from local supplier. Tacrolimus 5 mg capsules (Tarfic ®) was supplied by Hospital de Clínicas de Porto Alegre. Acetonitrile HPLC grade was purchased from Vetec (São Paulo, Brazil) Purified water was prepared using Milli-Q Plus® (Millipore, Bedford, USA).

Methods

Preparation of reference standard solution

The reference standard solution was prepared by weighing 10 mg of the reference standard and diluting it in acetonitrile until reaching a concentration of 1mg/mL.

Development of Hospital Derivation of Tacrolimus

Tacrolimus oral liquid preparations were developed by using the information in the literature about the most used wetting agents, proportions and about formulation already developed with other lipophilic molecules, besides tacrolimus (6, 10, 11). There are different wetting agents available with less cost and equivalent effect as stability agent suspension. Some adjuvants with different proportions were tested: glycerin in 2-7%, Polyethylene Glycol (PEG 400) in (4%, 6%, 8%), Polyoxyethylene Sorbitan Fatty Acid Esters (Tween 80) in 3% and Propylene Glycol in 4%, 10%, and 25%. The suspension was prepared using just one method for all of them. First, the powder of the tacrolimus capsules (5 mg) was crushed for thirty seconds. After this, the wetting agent was added and homogenized. Finally, the vehicle (syrup) was used to complete the end volume of suspension, until reaching the concentration of 1mg.mL^{-1}. The formulation was stocked in an amber PET bottle, protected from moisture and light. The HPLC analysis was developed with these formulations diluted until 50 µg/mL, as the standard. Also, it was developed formulations without the wetting agent, changing only by the presence or absence of powder grinding, but using the same general preparation method (1). All liquid preparations containing tacrolimus were carried out in accordance with personal safety standard.

High-performance liquid chromatography (HPLC)

Liquid chromatographic analysis was conducted in a Shimadzu LC-10A system (Kyoto, Japan) equipped with an LC-20AT pump, SPD-20AV UV-VIS variable wavelength detector, DGU-20A5 degasser, CBM-20A controller system, and SIL-20A injection valve with 100 µL loop. Luna Phenomenex® C18 column (250 x 4.6mm; 5µm) was kept at temperature of 70°C. The mobile phase used was a mixture of acetonitrile and water (65:35, v/v) at a flow rate of 1.7 mL/min and an injection volume of 50 µL. UV detection was carried out at 214 nm using a photodiode-array detector. All tests were performed using a concentration of 50 µg.mL^{-1}. The chromatographic method was previously developed according by US Pharmacopeia (USP) (10) and it was revalidated to confirm its selectivity, accuracy, and precision (12,13).

Selectivity

The selectivity was developed by comparing the analysis of the chromatograms of reference standard (RS) and sample solutions containing only the excipients and the formulation vehicle. The placebo solution was developed using the mixed with all the excipients of the capsules, being hypromellose, sodium croscarmellose, monohydrate lactose, and magnesium stearate, and of the vehicle syrup simple with potable water, sucrose, and parabens (PS).

Precision

The precision was evaluated in two days and different analysts. It was used the sample and the stock solution of standard, diluted until 50 µg.mL^{-1}. Both were filtered and analyzed on HPLC six times for the sample and three times for the standard. It was prepared one stock standard for each analysis.

Accuracy

Accuracy was developed using the standard addition model. The 1mg/mL sample was diluted in the mobile phase to 60 µg/mL. Afterward, an aliquot of this diluted sample was removed and added to a volumetric flask to reach a concentration of 30 µg.mL^{-1}. Simultaneously, the 1mg/mL standard stock solution was prepared. In volumetric flasks containing equivalent to 30 µg/mL of tacrolimus, from the sample, known amounts of the standard were added, starting from the stock solution, to reach three final concentrations, low, medium, and high, being 40 µg.mL^{-1}, 50 µg.mL^{-1}, and 60 µg.mL^{-1}, respectively. HPLC analyses were performed in triplicate. The standard and sample solutions were also evaluated at 50 µg.mL^{-1} (working concentration).

Stability of Hospital Derivation of Tacrolimus

The stability of tacrolimus suspension was evaluated for sixty days. It was determined the organoleptic characteristics, drug content, viscosity, pH, sedimentation volume, and microbiology contamination. To evaluate the dosage of the formulation was used the chromatographic method already described. In addition, the dosage was evaluated at room temperature (25 °C) and temperature of

40 °C. Analyzes at higher temperatures, such as 40 °C, are used to predict situations that may occur in hospital routine. The collections were performed every ten days, starting from time zero to the sixtieth day. The samples which were stocked at 40°C were collected on the tenth, twentieth, and thirtieth days. The viscosity was measured with a Brookfield viscometer (model DV - I+) and the pH values were a calibrated potenciometer (Hanna Instruments®). Sedimentation volume was determined by observing the volume of the precipitated solid in a beaker. Microbiological evaluation experiments were performed according to the Brazilian Pharmacopeia. The total aerobic bacterial and combined yeasts and molds were counted by the surface-spread plate method, and the specific pathogens (*Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella sp*) were measured by the pour plate method. The bottles employed in the formulation's storage were previously cleaned with 70% alcohol, dried in an oven and sealed with lids until use.

Results and Discussion

High-performance liquid chromatography (HPLC)

The chromatographic method was tested by determining the selectivity, precision, and accuracy for covalidation. There is no interference that elute in the same drug retention time, characterizing the method as selective (Figure 2).

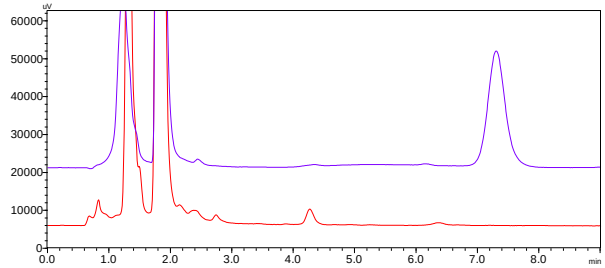

Figure 2. Chromatograms of placebo (red) and Tacrolimus sample (purple) solutions. Chromatographic conditions: Column C18 (Phenomenex); Mobile phase (acetonitrile and water (65:35, v/v); UV Detection (214 nm).

Precision

The results are described in the table 1below which shows the method proved to be precise, with a mean drug close to 100% and a relative standard deviation of less than 5%.

Table 1. Method precision results.

Repeatability	Mean (%)	*RSD (%)
Day 1	106.02	1.09
Day 2	101.14	0.79
Analyst 2	96.39	0.23
Inter-day analysis	101.19	4.07

*RSD: relative standard deviation

Accuracy

The accuracy was intended to assess the chromatographic method's ability to recover the drug. The results showed that the method was accurate with mean recovery values close to 100% in all three evaluated concentrations (Table 2).

Table 2. Method accuracy results.

Level (µg/mL)	Mean (%)	RSD (%)
40	102.56	0.63
50	102.21	0.54
60	100.12	0.43

*RSD: relative standard deviation

Development of Hospital Derivation of Tacrolimus

According to JACOBSON, et al., 1997, and the method described at USP (10), there is a suspension of tacrolimus with Ora-Plus® that have accurate stability of 56 days and it is safe. However, Ora-Plus® is very expensive to acquire in many hospitals. Other authors show the tacrolimus suspension with other vehicles, for example, hydroxymethyl cellulose or methylcellulose (11, 14). Liquid pharmaceutical preparations containing Tacrolimus are required in hospital routine considering the administration to pediatric and elderly patients, whose palatability and swallowing represent a challenging task (15). For comparative purposes, formulations without wetting agents, changing only by the presence or absence of powder grinding, and the formulations with wetting agents in different proportions of them were evaluated. Powder grinding is essential to increase the contact of the powder with the aqueous vehicle, mainly considering its lipophilicity. However, it was not enough to reduce the particle size to generate the necessary interaction and promote the stability of the formulation, observing just an increase in recovery and a slight reduction in the RSD of the amount of tacrolimus in formulation with grinding compared to that performed without grinding the powder. Tacrolimus is a lipophilic molecule, and it does not distribute homogeneously in aqueous vehicles, such as simple syrup, requiring the addition of adjuvants to stabilize the suspension, such as wetting agents. These excipients are very important and must be chosen carefully because there are possible incompatibilities and some of them are toxic or there is not much information in the literature (15, 16) The glycerin and propylene glycol were strong candidates because they are very used in manipulation pharmacies with the same function and because there is information about their security. Both excipients had results favorable in different proportions until reached a plateau, where adding more of them did not increase drug recovery. The point of saturation of glycerin was 4%, reaching 90% of the content. Propylene glycol shows better results, with recovery tacrolimus almost 93%, but this wetting agent showed to interfere with the chromatographic analysis, making tacrolimus

area values unreliable. Another wetting agent tested, the polyoxyethylene sorbitan fatty acid esters (Tween 80®), is toxic and should be not used more than 3% in an oral formulation. Because of this, it was used in the maximum proportion allowed keeping the action as wetting with security. The results showed that this adjuvant interfered in the chromatographic analysis, co-eluting with the drug and therefore making the values found unreliable. In addition, this wetting agent did not get significant results in recovery and RSD. The results demonstrated that polyethylene glycol (PEG 400) at 8% was the better wetting agent, reaching drug content very close to 100%, as required by the Pharmacopeia, and demonstrated dose homogeneity by the low RSD (< 5). In addition, the others wetting agents had drug recovery lower than required and high values of RSD or showed impairments in the chromatographic analysis, despite presenting better results than the formulations without the adjuvants. With all these facts, the chosen formulation used simple syrup and PEG 400 in 8% (PEG 3) as a wetting agent, with high recovery (close to 100%) and slow RSD in comparison with other formulations. The Table 3 shows the mean drug content in the different situations of preparation.

Table 3. Results of tacrolimus recovery in different pharmaceutical formulations (PhF)

PhF	TR (%)	RSD
G1	80.98	16.64
G2	87.55	7.50
G3	90.05	2.71
G4	81.22	8.02
G5	84.86	3.09
G6	78.42	14.97
PPG 1	86.81	3.41
PPG 2	92.68	2.36
PPG 3	92.39	5.06
PEG 1	86.69	2.79
PEG 2	86.78	0.42
PEG 3	97.04	2.07
Tween 80®	88.28	3.49

G1: Glycerin 2%; G2: Glycerin 3%; G3: Glycerin 4%; G4: Glycerin 5%; G5: Glycerin 6%; G6: Glycerin 7%; PPG 1: Propylene glycol 4%; PPG 2: Propylene glycol 10%; PPG 3: Propylene glycol 25%; PEG 1: Polyethylene Glycol 400: 4%; PEG 2: Polyethylene Glycol 400: 6%; PEG 3: Polyethylene Glycol 400 8%; TR (%): Tacrolimus recovery (%)

Stability of suspension

Polyethylene terephthalate (PET) amber bottle was choosed because tacrolimus is photosensitive, and it was not described any incompatibles between the components of the formulation and PET. Still, glass bottle is more expensive than PET and can cause accidents if it is broken. Table 4 shows the recovery of tacrolimus during all analysis days. The drug kept the content close to that recommended (90 to 110%) for 40 days when the formulation was stored at room temperature. On the fifth day, the drug content reduced to the lowest value, below the recommended limit. On the last day of analysis, the recovery increased, but the deviation was high and therefore the formulation did not maintain dose homogeneity due to some change in physical or chemical stability. It means that the formulation stored at room temperature for 50 days or more cannot be used because the dosage will not be correct for the treatment.

Table 4. Results of tacrolimus content in after storage in amber PET bottles at room temperature (25 ºC), for 60 days.

Time (days)	Tacrolimus (%)				
	1	2	3	Mean	RSD
0	99.93	102.61	101.71	101.27	2.19
10	96.34	99.27	95.62	97.61	3.41
20	95.37	89.60	96.61	94.40	4.38
30	98.89	96.99	90.82	96.16	4.81
40	94.12	104.74	98.58	99.55	4.80
50	90.33	86.14	84.90	87.12	3.10
60	97.29	97.04	87.55	93.96	5.98

In addition, the analysis at 40°C showed possible formed degradation products, already on the tenth day. Then, the formulation is temperature sensitive and, because of this, storage at adequate temperature is essential to keep the characteristics of the formulation, without prejudice to the quality, safety, and effectiveness (Table 5). Therefore, this formulation is not safe if it is kept at high temperatures, causing an eventual change in dose homogeneity.

Table 5 Results of tacrolimus content in after storage in amber PET bottles at 40 ºC temperature, for 30 days.

Time (days)	Tacrolimus (%)				
	1	2	3	Mean	RSD
0	99.93	102.61	101.71	101.27	2.19
10	89.29	99.58	92.42	96.04	5.40
20	90.36	87.21	84.31	87.91	5.03
30	91.33	90.33	93.51	91.33	5.96

Figure 3 show the results of pH of the formulation stocked at room temperature. The formulation kept a pH between 5 and 7, which is the stable pH of tacrolimus, following recommendations in the literature for liquid preparations containing Tacrolimus.

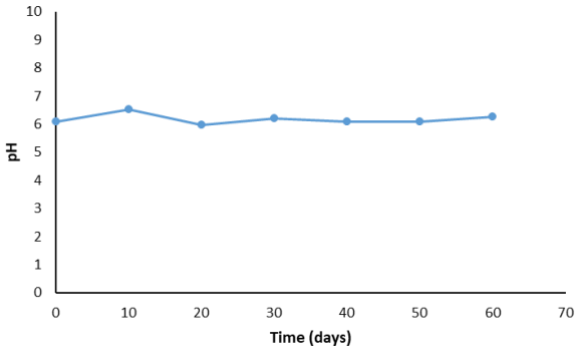

Figure 3. The pH profile of PEG 3 Tacrolimus during the stability study

Tacrolimus maintained its organoleptic characteristics throughout the study, characterized as a whitish and viscous suspension with small-suspended solid particles. After the fiftieth day, the viscosity demonstrated a slight decrease, being below 200 cP. The resulting viscosity is presented in Table (6).

Table 6. Viscosity profile at room temperature of PEG 3 containing Tacrolimus.

Time (days)	Viscosity (cP)		
	1	2	3
0	266.60	282.20	284.20
10	277.00	26500	262.60
20	255.80	253.00	259.80
30	224.20	220.20	215.40
40	254.00	223.80	213.00
50	198.60	199.40	190.00
60	207.40	235.80	228.20

A hypothesis for this is that the formulation developed crystals, possibly of sucrose, reducing the consistency of the formulation (Figure 4).

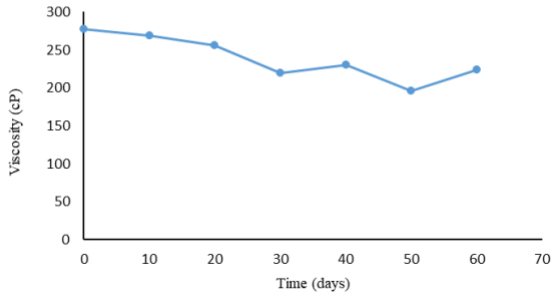

Figure 4. The viscosity profile of PEG 3 Tacrolimus during the stability study

Considering that sucrose is in saturation concentration to form the simple syrup, it might have an interaction between the compounds of the formulation reducing the sucrose solubility. The crystals were responsible for the sedimentation volume, which remained at 3 mL during the first 30 days and stabilized at 5 mL from day 50 onwards. Another hypothesis is that the formulation had a decrease in volume, which could change the sensitivity of the equipment. Microbiological evaluation experiments showed that microbiological contamination was not detected during the study of stability. The values found were in accordance with the recommendations by the Brazilian pharmacopoeia, within the limits for the presence of bacteria and fungi in the formulation during 30 days of storage (<10 CFU/mL) and absence of the pathogens studied (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*; Salmonella sp.).

Conclusions

Through this work, it can be concluded that tacrolimus in suspension kept the dosage between the required limits during 40 days of analysis at room temperature. The formulation shows physical-chemical and microbiologic stability during all the analyses with pH neutral, values of viscosity close to each other, despite having decayed on the fiftieth day, formation of crystals, possibly of sucrose, at the bottom of the bottle and pathogens within the recommended limits. This article shows an option for treatment with tacrolimus using a dosage liquid form which can be stored for forty days keeping the physical-chemical characteristics and the correct dosage. Therefore, suspension of tacrolimus using simple syrup and a wetter agent, polyethylene glycol 400 in 8% should be used only when no alternative to oral administration is available because no tests were performed to determine bioavailability.

Conflict of interest

The authors declare no conflicts of interest.

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