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Article

Development, physicochemical evaluation, and *in vivo* permeation studies of topical formulations containing 0.1% tacrolimus

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The objective of this paper was to develop and evaluate two semi-solid pharmaceutical forms containing 0.1% tacrolimus: cream (CRT01) and gel (GLT01). For the evaluation of physicochemical stability, at times 0, 30, 60 and 90 days, at 23°C and at 40°C, High Performance Liquid Chromatography coupled with a Diode Array Detector (HPLC-DAD) was employed. This method was developed and validated for tacrolimus quantification. The occlusivity test and skin permeation assay were also performed, using an animal model (Wistar rats), and the CRT01 and GLT01 were compared to the 0.1% tacrolimus ointment (PFU01) obtained from the University Pharmacy, Federal University of Rio de Janeiro, Brazil. CRT01 and GLT01 presented a homogeneous aspect and consistency adequate for topical products, along with sensory characteristics above PFU01. They also presented adequate physicochemical stability for 90 days and a lower occlusive effect than PFU01 ($p<0.05$). CRT01 showed greater affinity for the skin when compared to PFU01 and GLT01, with low systemic absorption. The CRT01 semi-solid formulation was considered the most adequate one to treat patients with atopic dermatitis or other dermatologic inflammatory diseases, promoting rational use of tacrolimus.

Key words: Semi-solid pharmaceutical forms**.** Analytical methodology. Occlusivity test. *In vivo* permeation. Tacrolimus.

INTRODUCTION

A wide range of studies have been developing medications that can treat or reduce disorders caused by several chronic skin pathologies, such as eczematous dermatitis, seborrheic dermatitis, lichen, psoriasis, vitiligo, gangrenous pyoderma, Behçet disease and lupus erythematosus, among others (Gontijo *et al*., 2008; Sánchez-Pérez, 2008).

Tacrolimus is an immunosuppressive agent that belongs to the group of hydrophobic macrolides and was originally isolated from bacterium *Streptomyces tsukubaensis* in Japan, in the 1980s. Initially, it was approved for systemic use, orally, in the prevention of graft rejection in patients subjected to organ transplants (Macleod, Thomson, 1991). Its topical use was approved for the treatment of atopic dermatitis in Japan (1999), in

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the United States of America (2000), in the European Union (2002), and in Brazil (2005) (Gontijo *et al*., 2008; Sánchez-Pérez, 2008; Baldo *et al*., 2009).

Topical use of tacrolimus is indicated as a second-line treatment for atopic dermatitis in the concentrations of 0.03% and 0.1%. It is employed in adult patients and children older than 2 years of age, who are not immunocompromised and who cannot undergo conventional therapies due to potential risks (Sánchez-Pérez, 2008). Despite this restriction, tacrolimus has been employed in other types of dermatologic inflammatory diseases due to its immunosuppressive effect and to the lower number of adverse effects observed, when compared to topical corticosteroids (Alomar Corella, García-Navarro, 2008).

Tacrolimus is a hydrophobic molecule that has a molar mass of 822.03 $g.mol^{-1}$ in its monohydrate form ($C_{44}H_{69}NO_{12}.H_2O$) and a LogP value of 3.3. It is soluble in methanol, ethanol, acetone, ethyl acetate, chloroform and diethyl ether; sparingly soluble in hexane and petroleum ether; and insoluble in water. Drugs with high molecular weights (above 500 Daltons) have more difficult penetrating the epidermis, thus reducing the risks of systemic exposure and adverse effects associated with their use (Ansel, Popovich, Allen, 2007).

The main action site of tacrolimus is the dermis, where T lymphocytes are located. The drug needs to penetrate the *stratum corneum* and reach the dermis in sufficient concentrations to exert its activity (Goebel, Neubert, Wohlrab, 2011). The addition of solvents, such as propylene glycol and ethanol, traditional emulsifiers or other promoting agents for permeation, can increase cutaneous permeability of the formulation (Martins, Veiga, 2002).

Treatment adherence is an important factor for effective disease control, especially in chronic diseases. The high cost of commercial tacrolimus ointments and its sensory aspect are the main factors of non-adherence to the treatment by patients. Consequently, it is necessary to develop other pharmaceutical formulations, such as cream and gel, that provide better spread, absorption and removal, and that have the same stability and bioavailability of the drug (Ortonne *et al*., 2006; Vissers *et al*., 2008).

Currently, the lipophilic ointment of tacrolimus is manipulated at the Pharmacy University (FU) of the Federal University of Rio de Janeiro (*Universidade Federal do Rio de Janeiro*, UFRJ). The main objective is to offer the topical medication with an affordable price to patients at the Clementino Fraga Filho University Hospital (*Hospital Universitário Clementino Fraga Filho*, HUCFF). However, this pharmaceutical form has characteristics that can reduce adherence to the treatment because, in addition to not being sensorially attractive, it is not indicated for certain skin lesions, such as exudative ones, and presents difficult removal by washing. Commonly, the FU also receives medical prescriptions containing tacrolimus in cream and gel, but it cannot meet the patients' demands due to the lack of studies proving the efficacy and safety of these formulations.

Thus, the objective of this paper was to develop two semi-solid formulations, 0.1% tacrolimus cream (CRT01) and 0.1% tacrolimus gel (GLT01), to be employed in the treatment of atopic dermatitis and other pathologies, to evaluate their physicochemical stability, at times 0, 30, 60 and 90 days, at 23°C and at 40°C, using High Performance Liquid Chromatography coupled with a Diode Array Detector (HPLC-DAD), as well as to evaluate occlusivity and skin permeation of the CRT01 and GLT01 formulations, as compared to the 0.1% tacrolimus ointment (PFU01) obtained from the University Pharmacy, Federal University of Rio de Janeiro, Brazil.

MATERIAL AND METHODS

Material

Tacrolimus was obtained from the American Pharmacopeia (Rockwille, USA) and was employed as a reference chemical substance (RCS). The drug used to prepare the cream and gel was purchased from Pharma Nostra (Campinas, BR) in monohydrate form and with 99.2% purity. The excipients used in the preparation of the semi-solid formulations were as follows: ceteareth-20, cetearyl alcohol, octyl stearate and glyceryl stearate, purchased from Farmos (Rio de Janeiro, BR); propylparaben and methylparaben, from Pharma Nostra (Campinas, BR); propylene glycol, from Galena (Campinas, BR); petrolatum, from Fagron (São

Paulo, BR); ammonium acryloyldimethyltaurate/VP copolymer (Aristoflex AVC®), from Pharma Special (Rio de Janeiro, BR); and distilled water. Methanol (METOH) and acetonitrile (CAN) with chromatographic grade, purchased from Tédia (Rio de Janeiro, BR), and ultrapure water were used. The components of the 0.1% tacrolimus ointment are as follows: tacrolimus, purchased from Pharma Nostra (Campinas, BR); petrolatum, purchased from Fagron (São Paulo, BR); and anhydrous lanolin, from Galena (Campinas, BR).

Methods

Preparation of semi-solid formulations

Tacrolimus cream 0.1 wt%

The CRT01 formulation was prepared by means of the single phase process. The components and their percentages (%w/w) employed in the formulation were weighed individually, transferred to a stainless steel container under constant stirring, and heated to 75°C: 2.5 wt% of ceteareth-20; 10.0 wt% of cetearyl alcohol; 3.0 wt% of octyl stearate; 5.0 wt% of glyceryl stearate; 5.0 wt% of propylene glycol; 2.0 wt% of petrolatum; 0.1 wt% of propylparaben; 0.1 wt% of methylparaben and 24.0 wt% of distilled water. The heating was then turned off and an amount of distilled water was gradually added to the container under moderate and constant stirring until achieving room temperature. Tacrolimus was solubilized in 20.0 wt% of propylene glycol and dispersed in the cream (Melo *et al*., 2016).

Tacrolimus gel 0.1 wt%

To develop the GLT01 formulation, the methylparaben (0.1 wt%) was solubilized in an amount of distilled water, in a stainless steel container, under constant stirring, and heated to 75°C. After solubilization, the heating was turned off and propylene glycol was added. Aristoflex® (3.0 wt%) was then added gradually and dispersed under agitation, until a gel had been developed. Tacrolimus was solubilized in 20.0 wt% of propylene glycol and incorporated in the gel (Melo *et al*., 2016).

Tacrolimus ointment 0.1 wt%

The PFU01 formulation, obtained at the Pharmacy University, consists of 0.1 wt% in a simple ointment vehicle: petrolatum (70.0 wt%), and anhydrous lanolin (30.0 wt%) (Melo *et al*., 2016).

Stability study

The CRT01 and GLT01 formulations were submitted to the accelerated stability test, for up to 90 days, at room temperature, at 23 ± 2°C, and at 40 ± 2°C (ANVISA, 2005). The samples were evaluated in triplicate (n=3) for organoleptic characteristics, pH and density, as well as to determine the tacrolimus content (Brazil, 2010).

Organoleptic characteristics

The macroscopic characteristics of the formulations, including appearance, color change and odor, were evaluated.

Determination of pH

The pH of the formulations was determined by means of a potentiometric method using a digital potentiometer (Bante Instruments, model 922) equipped with an SC06 model electrode (Sensoglass). The mean and standard deviation (SD) were calculated.

Density

The density of the formulations was evaluated according to the methodology described in the Brazilian Pharmacopoeia 5th ed. (Brazil, 2010). Density was calculated according to Equation 1:

$$\rho_t = d_{(water)} \times d_t^t + 0.0012 \qquad \text{(Equation 1)}$$

where ρ_t is the mass density of a substance at temperature *(t)*; $d_{(water)}$ is the density of water at the same temperature *t*, and d_t^t is the relative density of the substance. d_t^t is the ratio between its mass and the mass of a similar volume

of water, both at the same temperature *t*. Mean ± SD was assessed.

Determination of the tacrolimus content

The tacrolimus content in the developed formulations was determined. Two semi-solid formulations were developed without tacrolimus: cream (CRST) and gel (GLST), which were evaluated under the same conditions of the formulations with tacrolimus, CRT01 and GLT01. This comparison was made to ensure that no interference from excipient degradation of the formulations could be detected at the same retention time of tacrolimus.

The analysis of the tacrolimus content was performed according to a previously developed and validated analytical methodology by Guiling Li et al (2012), with adaptations and validated according to ANVISA (2003).

Development and validation of the analytical method for tacrolimus analysis by HPLC-DAD

Equipment and chromatographic conditions

The study used HPLC, Agilent Technologies, model 1260 Infinity, equipped with a DAD, G4212B model; a G1311B model quaternary pump; a G1329B model automatic sampler; a G1322A model degassing unit; a G1316A model thermostatic column compartment, connected directly to a computer equipped with Agilent Chemstation for the LC 3D systems G2170BA software.

Tacrolimus was quantified at 60°C, using an HPLC Kromasil 100 C_{18}- column (AkzoNobel), with dimensions of 4.6 mm × 250 mm, and 5 µm particle diameter. The mobile phase was ACN:water (70:30, v/v), isocratic mode at a 1.0 mL/min flow rate. The injected sample volume was 40 µL. The detector wavelength was set at 210 nm, and the run time was 15 minutes.

Preparation of the standard solution

Three milligrams of the tacrolimus RCS were weighed in an analytical scale (Shimadzu, AUW220D model) and transferred to a 10 mL volumetric flask. Approximately 5 mL of METOH were added. After tacrolimus solubilization, the volume of the volumetric flask was completed with METOH. Subsequently, 1.0 mL of this solution were transferred to a 10 mL volumetric flask, and the volume was completed with the same solvent at a final concentration of 30 µg/mL.

Extraction of tacrolimus from semi-solid formulations

The 300 mg CRT01 formulation was weighed in an amber glass bottle, and 9 mL of METOH were added. The bottle was then vigorously shaken. The suspension remained in the magnetic stirrer for 24 hours, at room temperature. After that period, it was transferred to a 10 mL volumetric flask, and the volume was adjusted with METOH and homogenized. This suspension was centrifuged at 6,400 rpm for 20 minutes. The resulting supernatant was filtered through a polyvinylidene fluoride (PVDF) membrane with 0.2 µm porosity. The final solution had a theoretical concentration of 30 µg/mL. The same procedure was used to extract the GLT01 formulation. The CRST and GLST formulations were also analyzed for comparison purposes.

Validation of the analytical method

The parameters of the validated analytical method were as follows: selectivity, linearity, repeatability (intra-assay precision), intermediate precision (inter-assay precision), accuracy, robustness, and limits of detection (LD) and of quantification (LQ) (ANVISA, 2003).

Selectivity. Selectivity was evaluated employing four solutions: (a) tacrolimus standard solution at 30 µg/mL; (b) solution obtained after extraction procedure in the CRST and GLST formulations; (c) solution obtained after extraction procedure in the CRT01 and GLT01 formulations; (d) solutions obtained after extraction procedure in CRST and GLST formulations contaminated with the drug, obtaining a final concentration of 30 µg/mL.

Linearity. Linearity was determined by constructing three standard tacrolimus curves (n=3), with five concentration levels each, between 10 and 36 µg/mL, in HPLC-DAD. The linear regression analysis was applied in the results and the correlation coefficient (r)

was obtained, using Excel®. The acceptable minimum criterion for the correlation coefficient was 0.99.

Limits of detection and quantification. The LD and the LQ were calculated (Equations 2 and 3) using the data from the three standard curves.

$$LD = \frac{SD \times 3}{CS} \quad \text{(Equation 2)}$$

$$LQ = \frac{SD \times 10}{CS} \quad \text{(Equation 3)}$$

where SD is the standard deviation of the interception with the y-axis and CS is the mean of the curve slope of three standard curves.

Repeatability (intra-assay precision) and intermediate precision (inter-assay precision). To determine repeatability, the CRT01 and GLT01 formulations were used. The tacrolimus solutions were obtained at 30 µg/mL, using the aforementioned drug extraction methodology. Six measurements (n=6) were performed at 100% of a 30 µg/mL concentration, on the same day.

Intermediate precision was evaluated under the same conditions of the repeatability analysis. On each analysis day, six measurements were performed (n=6), totalizing 12 determinations in two days.

Precision was evaluated employing the RSD values, and those below 5.0% were admitted.

Accuracy. Accuracy was determined by recovery of the drug from the cream and gel formulations in the following concentrations: 0.08 wt%, 0.10 wt% and 0.12 wt% of tacrolimus. The same extraction methodology mentioned above was followed. The study was performed in triplicate for each concentration level (n=9).

Accuracy was expressed by the relationship between the average concentration determined experimentally and the corresponding theoretical concentration (Equation 4):

$$AR\% = \left(\frac{AEC}{TC}\right) \times 100 \quad \text{(Equation 4)}$$

where AR% is the accuracy based on drug recovery, AEC is the average experimental concentration, and TC is the theoretical concentration.

Robustness. A solution of tacrolimus RCS in methanol was prepared at a concentration of 30 µg/mL. The changes in the analytical conditions were as follows: temperature at 55°C, flow at 1.2 mL/min, wavelength at 214 nm, and the proportion of the ACN:water mobile phase at 80:20.

Occlusivity test

The occlusion factor (F) of the CRT01 and GLT01 formulations was compared to the PFU01 formulation. The system consists of a glass container, with a capacity of 40 mL and 4.6 cm in diameter, and a filter paper (cellulose filter, 90 mm; Whatman number 6; cutoff size: 3 µm, USA). The filter paper was previously molded to cover the glass container perfectly (Wissing, Müller, 2002; Teeranachaideekul *et al.*, 2008).

Distilled water (30 g) was placed in each container and approximately 220 mg of each formulation were weighed on the filter paper. The formulations were spread homogenously, covering the area of the filter paper (13.3 mg/cm^2). Each system was then weighed and kept at 40°C for up to 48 hours. The amount of residual water in each glass container was analyzed after 6, 24 and 48 hours by the weight of the systems. A system without formulation was used as reference (Teixeira *et al.*, 2019).

All experiments were performed in triplicate (n = 3), using the average of the values to determine F, calculated according to Equation 5.

$$F = [(A - B/A)] \times 100 \quad \text{(Equation 5)}$$

where A stands for the water loss in the container without sample (reference) and B is the water loss in the container with sample. The occlusion factor can vary from 0 to 100: zero means non-occlusive effect and 100 is maximum occlusion (Wissing, Müller, 2002; Teeranachaideekul *et al.*, 2008; Teixeira *et al.*, 2019).

Skin permeation assay

The assay was carried from the Nuclear Energy Research Institute (*Instituto de Pesquisa em Energía Nuclear*, IPEN), using the methodology described by Cerqueira-Coutinho *et al.* (2015), Pople and Singh (2012) and Marques *et al.* (2018). The study (logged under protocol number 23076.020528/2013-27) was approved by the Animal Ethics Committee of the Biological Sciences Center belonging to the Federal University of Pernambuco, Brazil. Skin permeation of the tacrolimus present in the CRT01 and GLT01 formulations was evaluated and compared to the PFU01 formulation.

The formulations were radioactively marked with technetium-99 m and then applied to the previously trichotomized dorsal skin of healthy Wistar rats, weighing approximately 300 g, in triplicate (n = 3).

The rats were sedated and an area of 1 cm^2 of their back was removed with a chemical depilatory (Veet®). After 15 minutes, 150 µL of the sample solution marked with technetium-99 m was applied over the rats' skin with the aid of an automatic pipette. After 120 minutes, the animals were sacrificed by CO_2 asphyxia, their organs were removed and weighed, and the radioactivity uptake was counted in an automatic gamma counter (2470 Wizard model, Perkin-Elmer, USA). To assess sample retention on the skin, the application area was removed and weighed.

The mean and standard deviation of the radiation dose in the skin and blood were calculated for each sample. The results were expressed as a percentage of the radioactivity dose per gram of tissue (skin, brain, liver, kidneys, intestines, lungs, stomach, spleen, heart and blood).

Statistical analysis

The experimental results obtained were expressed as mean ± SD or RSD using the GraphPad Prism® software, version 5.0 for Windows. To compare the groups, this study employed Analysis of variance (ANOVA) and the Tukey test at a 95% confidence interval ($p<0.05$ was considered significant).

RESULTS AND DISCUSSION

Developed formulations

The CRT01 and GLT01 formulations showed a stable and homogeneous appearance. As tacrolimus is a hydrophobic molecule and insoluble in water, it was solubilized with propylene glycol, which acts as a co-solvent (Panchagnula *et al.*, 2004; Simonsen *et al.*, 2004).

Physicochemical stability

According to the American Pharmacopoeia (USP 31), chemical stability assesses whether or not a drug maintains its chemical integrity and initial potency within the specified limits. Physical stability assesses whether or not the original physical properties are maintained.

Organoleptic characteristics

During the 90 days of study, at 23 ± 2°C and at 40 ± 2°C, CRT01 and GLT01 kept their organoleptic characteristics: the cream remained white and the gel colorless and translucent, both with unchanged odor and adequate appearance.

Evaluating the organoleptic characteristics is important, as chemical degradation can be accompanied by changes in physical appearance of the product. Some of these changes can modify the color and odor of formulations, and may show a problem related to stability (USP 31; Lachman, Lieberman, Kanig, 2001).

pH

According to Table I, the pH values of the CRT01 formulation varied between 5.5 and 5.9, showing a small increase during the stability study. The pH of the cream can be considered slightly acid and compatible with the pH of the skin, which presents its surface covered by a protective hydro-lipid film and pH ranging between 4 and 6 (Segger *et al.*, 2008; Duncan *et al.*, 2013). The pH values obtained in the GLT01 formulation were also acid, varying between 4.5 and 4.7. pH is also compatible with the skin's pH.

TABLE I - pH values of the CRT01 and GLT01 formulations, during the 90 days of study, at 23 ± 2ºC and at 40 ± 2ºC

Time (days)	pH[a]			
	23 ± 2ºC		40 ± 2ºC	
	CRT01	GLT01	CRT01	GLT01
0	5.501 ± 0.03	4.664 ± 0.02	-	-
30	5.611 ± 0.01	4.712 ± 0.006	5.521 ± 0.001	4.648 ± 0.02
60	5.704 ± 0.01	4.664 ± 0.009	5.615 ± 0.03	4.508 ± 0.005
90	5.933 ± 0.03	4.753 ± 0.01	5.904 ± 0.04	4.662 ± 0.02

[a]**Mean ± SD of 3 determinations.**

It is recommended that the pH of a topical product should range between 5 and 6, which was observed for the developed formulations. Consequently, the formulations that present pH values outside this range can cause redness and skin irritation (Segger *et al.*, 2008; Duncan *et al.*, 2013). In addition, several enzymes involved in the synthesis and maintenance of skin barrier characteristics are impacted by pH, which can be altered by many dermatoses. A number of studies showed that children with atopic dermatitis had a significant increase in skin pH. This high pH value in the affected area was also associated with more intense itching and dryness (Sparavigna, Setaro, Gualandri, 2006; Ali, Yosipovitch, 2013).

The pH of the formulation can also influence the degradation of drugs, as pH decreases or increases; therefore, this process can increase or decrease the drug degradation rate (USP 31). The results observed for the CRT01 and GLT01 formulations suggest that the drug remained stable and possibly without degradation during the study, as tacrolimus presents greater stability in the pH range between 2 and 6, with a substantial increase in its decomposition rate at high pH values (Trissel, 2012).

Density

Table II shows the density values of the developed formulations.

TABLE II - Density (g/mL) values of the CRT01 and GLT01 formulations, during 90 days, at 23 ± 2ºC and at 40 ± 2ºC

Time (days)	Density[a]			
	23 ± 2ºC		40 ± 2ºC	
	CRT01	GLT01	CRT01	GLT01
0	0.8926 ± 0.01	0.9948 ± 0.003	-	-
30	0.8957 ± 0.004	0.9959 ± 0.0001	0.9006 ± 0.02	1.0138 ± 0.005
60	0.8961 ± 0.01	1.0126 ± 0.002	0.9287 ± 0.01	± 0.002
90	0.8959 ± 0.02	1.0146 ± 0.001	0.9398 ± 0.01	1.0164 ± 0.0003

[a]**Mean ± SD of 3 determinations.**

There were no significant differences ($p>0.05$) in the density values of the CRT01 formulation at 23 ± 2ºC during the study. The CRT01 formulation at 40ºC ± 2ºC showed a significant difference in the density values, according to time ($p<0.05$). This can be explained by evaporation of the water from the cream, with a consequent decrease in its mass. There was also a significant difference between the density values of the cream formulation maintained at 23 ± 2ºC and at 40ºC ± 2ºC ($p<0.05$), possibly for the same reason.

The GLT01 formulation showed a significant difference between the density values at 23 ± 2ºC over time ($p<0.05$). The density values of the gel formulations maintained at 40ºC showed no statistical difference ($p>0.05$) over time. There was also a significant difference between the density values of the gel formulation kept at 23 ± 2ºC and at 40ºC ± 2ºC ($p<0.05$), at all times. The difference in the density values observed during the time of the formulation kept at 23ºC and between the formulations kept at 23ºC and 40ºC was possibly due to water loss by evaporation.

Determination of the tacrolimus content

The drug content in the CRT01 and GLT01 formulations was evaluated at time zero (T0), and after 30, 60 and 90 days, at 23 ± 2ºC and at 40 ± 2ºC, by HPLC-DAD.

In both formulations, CRT01 (Table III) and GLT01 (Table IV), the tacrolimus concentrations showed a small increase during the study, but with no statistically significant difference ($p>0.05$). Both formulations, CRT01 and GLT01, showed no statistically significant difference ($p>0.05$) between the content values at 23 ± 2ºC and at 40 ± 2ºC. The tacrolimus content remained in the range between 90% and 110%, which is adequate for semi-solid formulations, indicating chemical stability during the study (USP 31). The RSD values remained adequate, below 5%, in the CRT01 and GLT01 formulations. The CRST and GLST formulations were evaluated under the same conditions as the CRT01 and GLT01 formulations, respectively. These formulations showed no interfering agent in the same tacrolimus retention time, showing selectivity of the method during the 90-day period.

TABLE III - Tacrolimus content (%) and RSD (%) in the CRT01 formulation, during 90 days of study, at 23 ± 2ºC and at 40 ± 2ºC

Time (days)	Content (%)[a]		RSD (%)[b]	
	23 ± 2º	40 ± 2ºC	23 ± 2º	40 ± 2ºC
0	99.83		1.52	
30	101.08	104.51	0.04	1.45
60	102.52	104.22	2.79	0.86
90	103.89	104.76	0.89	0.72

[a] **Mean of 3 determinations;** [b] **RSD of 3 determinations.**

TABLE IV - Tacrolimus content (%) and RSD (%) in the GLT01 formulation, during 90 days of study, at 23 ± 2ºC and at 40 ± 2ºC

Time (days)	Content (%)[a]		RSD (%)[b]	
	23 ± 2ºC	40 ± 2ºC	23 ± 2ºC	40 ± 2ºC
0	100.11		1.49	
30	100.17	103.15	1.63	1.36
60	101.09	102.41	0.49	1.88
90	101.12	104.78	2.46	3.69

[a] **Mean of 3 determinations;** [b] **RSD of 3 determinations.**

Validation of the analytical method

Selectivity of a method is defined as the ability to evaluate the substances under analysis in the presence of others that may interfere in their determination in a given sample. To verify similarity, spectral scans were performed at the beginning, middle, and end of the peaks. The spectra of the tacrolimus RCS and samples (CRT01 and CRST) were compared, and the similarity index was evaluated. The analysis of spectral purity demonstrated a rectangular "ratiogram", showing that the ratio was constant and next to 1, proving selectivity of the method.

Tacrolimus has a retention time of approximately 12.6 minutes. The chromatograms obtained from the CRST formulation, tacrolimus RCS, and CRST formulation contaminated with tacrolimus showed absence of interfering agents in detection of the drug, such as degradation products or excipients from semi-solid formulations. No unknown peak was identified at the same tacrolimus retention time in the chromatogram of the solution obtained from the CRST formulation. The same result was observed in the analysis of the gel formulation. Therefore, presence of substances that could interfere in the analysis was not observed.

A linearity analysis provides information on the ability of the analytical method to demonstrate that the obtained results are directly proportional to the concentration of the drug in the sample, within a specified range. The correlation coefficient (r) determined in this study was 0.9999, which corresponds to the minimum acceptable criterion (ANVISA, 2003).

The determination of the detection and quantification limits can prove that it is possible to detect and quantify a drug in the variation of the chosen concentration (ANVISA, 2003). LD and LQ values of 1.408 and 4.694 µg/mL, respectively, were calculated, showing that sensitivity of the method is adequate for the proposed objective, as the drug concentration after extraction corresponds to 30 µg/mL.

Precision proves the ability of the method to provide the same result for a same sample at different time intervals. In the repeatability evaluation (intra-assay precision), the CRT01 and GLT01 formulations had average contents of 102.83% ± 2.04% and 101.86% ± 3.09%, respectively; the DPR values of CRT01 and GLT01 were 1.98% and 3.04%, respectively. In the evaluation of intermediate precision (inter-assay precision), the CRT01 and GLT01 formulations had an average content of 98.75% ± 0.71% and 99.27% ± 3.48%, respectively; the DPR values of CRT01 and GLT01 were 0.72% and 3.50%, respectively. In both formulations, the relative standard deviation was considered satisfactory, as the DPR results were below 5.0%, in accordance with the recommendation set forth in resolution number 899/2003 (ANVISA, 2003).

Accuracy proves the effectiveness of the method in quantifying an analyte in a sample. Values between 95.0% and 105.0% were considered satisfactory. The results of the recovery test of the samples analyzed, in the three different concentration levels (80%, 100% and 120%) for the CRT01 formulation, were 97.47% ± 3.59%, 101,00% ± 1.83% and 101.02% ± 1.23%, respectively; for the GLT01 formulation, they were 99.97 ± 2.10%, 102.32% ± 2.13% and 102.32 ± 1.84%, respectively. The results were satisfactory, with RSD values below 5.0% (ANVISA, 2003).

Robustness evaluates the method's ability to resist the small and deliberate variations of the analytical parameters and indicates its confidence during normal use. The variation, in percentage, between the absolute value of the area obtained in the solution analyzed by the proposed method and by the method submitted to chromatographic changes was as follows: 1.90% for temperature; 19.15% for the mobile phase flow; 25.55% for wavelength; and 1.64% for the ACN:H_2O mobile phase ratio. Therefore, the method is not robust when the mobile phase flow and the wavelengths are changed.

Occlusivity test

The occlusion factor of the CRT01 and GLT01 formulations was determined and compared to PFU01. It was observed that all formulations (CRT01, GLT01 and PFU01) were able to form a homogeneous layer on the filter paper, during the spreading process, covering the area completely. After 48 hours, the mean occlusion factor of the PFU01, CRT01 and GLT01 formulations were 78.37% ± 3.64; 38.13% ± 4.26; and 6.98% ± 5.15, respectively.

Figure 1 shows the occlusion factor values for the CRT01, GLT01 and PFU01 formulations. PFU01

presented a significantly higher occlusion factor than CRT01 and GLT01 at times 6, 24 and 48 hours ($p<0.05$). The CRT01 formulation presented a significantly higher occlusion factor than GLT01 after 48 hours of study ($p<0.05$). These results were already expected, as the ointments are semi-solid formulations historically known for their occlusive properties (Mumford, 1941).

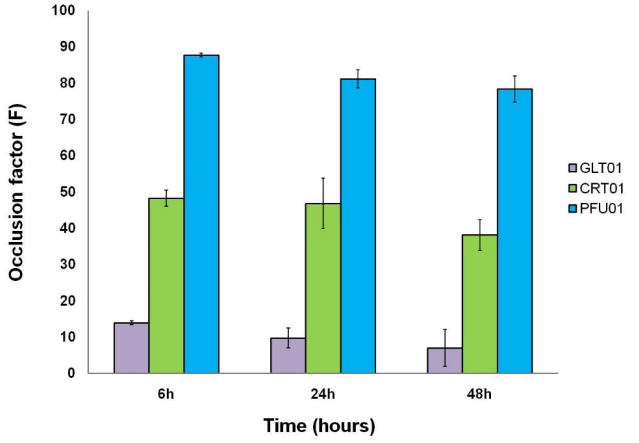

FIGURE 1 - Graphical representation of the mean occlusion factor (F) of the GLT01, CRT01 and PFU01 formulations, at 6, 24 and 48 hours. The error bars indicate the SD for the triplicate.

The occlusion factor is a parameter that evaluates the ability of the formulation to maintain skin hydration and is directly related to sample volume, particle size, crystallinity, lipid concentration and type of colloidal system (Wissing, Müller, 2002; Teeranachaideekul *et al.*, 2008). In relation to its pharmaceutical form, the occlusive power of fatty substances increases skin hydration and substance absorption. Skin occlusion can promote absorption, as formulations with occlusive capacity result in the formation of a film on the skin, reducing water loss to the environment. Some substances can be used for this purpose, but it is necessary to evaluate the final aspect of the product, as sensorial quality is one of the determining factors for its acceptance (Wissing, Müller, 2002; Björklund *et al.*, 2010).

According to Fernándes-Montes (2005), the addition of wetting polyols in emulsions, such as propylene glycol, reduces the loss of evaporated water and increases the occlusion factor. However, the fatty substances and the propylene glycol present in the CRT01 formulation were not able to cause an occlusive effect similar to the one produced by the PFU01 formulation.

According to Pople and Singh (2012), the ointment is very occlusive due to the nature of the vehicle's components, and this can result in esthetic attraction loss. In these cases, choosing a cream, which presents an intermediate occlusion factor but sensory properties superior to the ointment, can contribute to increase inpatient adherence to the treatment, as the sensory aspect and the hydration level on the skin are factors of considerable importance in the use of a topical medication. However, as the high skin hydration level is associated with an increase in the skin's substance absorption, it is necessary to pay attention to the drug penetration ability, as permeation is not desirable due to possible systemic side effects.

Skin permeation assay

The developed formulations, CRT01 and GLT01, were compared to the PFU01 formulation. In Figure 2, it is possible to observe that the CRT01 formulation was the most retained on the skin, while PFU01 showed intermediate retention and GLT01 was the least retained. In the case of tacrolimus, the formulation must remain on the skin and only reach the epidermis and dermis, as its action targets are the T lymphocytes. Tacrolimus must not be absorbed in such a way as to reach the bloodstream and other organs.

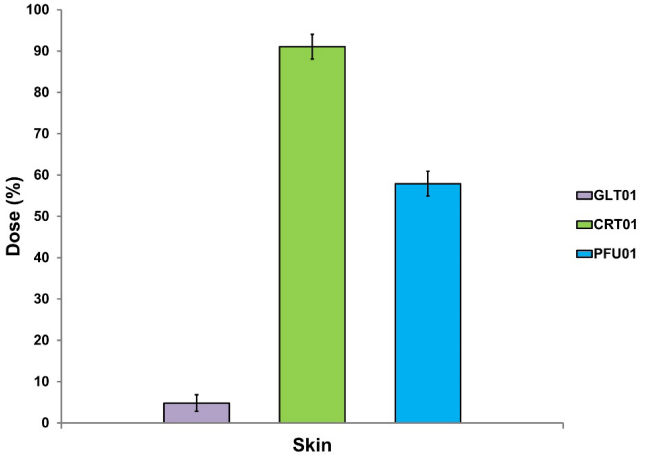

FIGURE 2 - Retention of the GLT01, CRT01 and PFU01 formulations with technetium-99 m on the skin.

The CRT01 formulation was found in greater amount on the skin (91.05%), but it was also observed in the gastrointestinal tract (8.70%) and the bladder (0.23%). This higher concentration of the drug on the skin was most likely due to the characteristics of the formulation components, including fatty substances and surfactants, which provide emollience and emulsifying action to the formulation. Use of these substances on the skin can cause a decrease in interfacial tension and changes in the protein conformation of the *stratum corneum*, allowing for the penetration of drugs. Therefore, it is particularly interesting for the treatment of atopic dermatitis and other dermatological diseases (Herkenne *et al.*, 2008). Some studies report that non-ionic surfactants can increase the penetration of drugs that are practically insoluble in water, and these play an important role in the apparent solubility of the drug (Nokhodchia *et al.*, 2002; Femenía-Font *et al.*, 2005).

The interactions of the vehicle with the skin are important factors in percutaneous absorption, as excipients can modify the skin condition and permeability. Safe and non-toxic penetration promoters can also be used to increase penetration of drugs (Femenía-Font *et al.*, 2005; Dragicevic, Maibach, 2017). Some components present in the composition of creams, lotions and gels can act as permeation enhancers, promoting greater penetration of active substances by the *stratum corneum* through reversible removal of the skin barrier (Dragicevic, Maibach, 2017).

Propylene glycol is a small molecule, able to pass through layers of the skin, changing its solubility characteristic and promoting an increase in the absorption speed of several active substances. In addition, it acts as a co-solvent, altering the thermodynamic activity of the drug by increasing its solubility in the vehicle. (Panchagnula *et al.*, 2004; Simonsen *et al.*, 2004). On the other hand, tacrolimus is a hydrophobic molecule insoluble in water. Therefore, its solubilization in propylene glycol can contribute to drug permeation.

The PFU01 formulation both permeated and was partially retained on the skin, as 57.9% was found in this organ. Another 41.0% was also found in the gastrointestinal tract, which suggests the occurrence of cutaneous absorption followed by fecal excretion, while 0.94% was found in the kidneys and less than 0.05% in the heart and liver. No significant amounts were identified in other organs or in blood. PFU01 basically consists of petrolatum and lanolin, which are lipophilic substances. Petrolatum has high occlusion capacity, protecting the skin from excessive water evaporation and preventing its dehydration (Ansel, Popovich, Allen, 2007). Lanolin is an emollient agent that can optimize skin permeation and facilitate the absorption of active ingredients (Femenía-Font *et al.*, 2005; Dragicevic, Maibach, 2017). Thus, occlusive vehicles such as ointments can alter skin hydration and reduce water loss, promoting penetration of substances. Probably, the absence of propylene glycol in the PFU01 formulation compromised drug penetration into the skin when compared to the cream.

The GLT01 formulation was detected in a small amount on the skin (4.8%) and with a high concentration in the gastrointestinal tract (73.5%). It was also detected in the bladder (0.35%) and in the brain (0.04%). These data suggest that there was permeation of the formulation, as well as cutaneous absorption, followed by fecal excretion. The characteristics of the gel components did not contribute to its retention in the skin because the gel is a dispersed system constituted by a high percentage of water and polymer, such as Aristoflex AVC®; therefore, it does not present an occlusive character. Probably, the propylene glycol added was responsible for penetration of the drug, minimizing its concentration at the site of action. In a study by Gon Lee *et al.* (Sang *et al.*, 2016), the *in vitro* permeation and skin retention of a tacrolimus hydrogel, using transcutol as a co-solvent, was higher when compared to a cream formulation with Protopic® (Medication Guide, 2011), which is considered a candidate with therapeutic potential in the treatment of atopic dermatitis.

In a study conducted with orally administered radiolabelled tacrolimus, it was observed that this type of tacrolimus presents extensive fecal elimination, approximately 90%, and reduced urinary elimination, approximately 2%. The tacrolimus available in the blood is mainly metabolized in the liver by CYP3A4. In topical applications, its metabolism was not detected on the skin. The lowest blood concentration of tacrolimus in which systemic effects are observed is unknown (Möller *et al.*, 1999; Medication Guide, 2011).

CONCLUSION

The organoleptic characteristics of the CRT01 and GLT01 formulations remained unchanged during the 90 days of the stability study. Both formulations developed showed adequate aspect, good consistency and sensory characteristics that were superior to PFU01. The pH and density values of both formulations remained within the appropriate range. The drug content in the CRT01 and GLT01 formulations remained within the acceptable limits of the pharmacopeia during the study period and conditions, in the range between 90% and 110%.

The occlusion factor of the PFU01 formulation was the highest, CRT01 had an intermediate value, and GLT01 was the least occlusive.

In the *in vivo* permeation test, CRT01 showed greater retention in the skin, indicating greater affinity for this organ when compared to PFU01 and GLT01. The lower permeation of the drug suggests that the CRT01 formulation is safe for topical use.

Thus, it is possible to assert that, when compared to the gel and the ointment, the cream formulation (CRT01) is a candidate for greater acceptance by the patient, which may well increase adherence to the treatment and promote rational use of the medication.

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DISCLOSURE STATEMENT

The authors declare that they have no conflict of interest.

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