DRUG ANALYTICAL RESEARCH
ISSN: 2527-2616 *www.seer.ufrgs.br/dar*

Lipid core nanocapsules-loaded tacrolimus: Development and evaluation of quality parameters

Graziela Scheuer Gomes[a], Luiza Abrahão Frank[a*], Adriana Raffin Pohlmann[a], Silvia Stanisçuaski Guterres[a]

[a] *Universidade Federal do Rio Grande do Sul, Programa de Pós-graduação em Ciências Farmacêuticas*
Av. Ipiranga 2752, CEP 90610-000, Porto Alegre -RS, Brasil
**Corresponding author: luiza.frank@ufrgs.br*

This study aimed to revalidate an HPLC-based analytical methodology to determine tacrolimus within lipid-core nanocapsules and to evaluate the quality of such nanosystems. Chromatographic separation was achieved by employing a C18 column as a stationary phase and a ternary mixture of acetonitrile: water: phosphoric acid (700:299:1 v/v) as the mobile phase. The revalidated method proved to be linear in the range of 1-60 µg.mL^{-1} for tacrolimus (r^2 >0.998). Detection and quantification limits were 45.38 ng.mL^{-1} and 137.51 ng.mL^{-1}, respectively, which assures the methodology sensitivity. The method was also precise (RSD = 1.78% between samples). Besides, the methodology demonstrated accuracy and robustness. The lipid-core nanocapsules-loaded tacrolimus showed exclusively nanosized particles (±190 nm and polydispersity index of ≤v0.2), negative zeta potential (-13.67±1.16), and slightly acidic pH (5.58 ± 0.06), with a content of 98.90 ± 2.32% and encapsulation rate of 99.23±0.32%. Tacrolimus-loaded in lipid-core nanocapsules-loaded tacrolimus showed stability for at least 30 days at room temperature and a sustained release profile compared to the drug in solution.

Keywords: Tacrolimus; HPLC-UV; nanotechnology; lipid-core nanocapsules

Article received at 13/06/2022 and accepted at 07/07/2022.
https://doi.org/10.22456/2527-2616.125229

Introduction

Tacrolimus (TAC), a macrolide isolated from Streptomyces with immunosuppressive activity, is used in autoimmune diseases, as psoriasis (1). Its mechanism of action is based mainly on its high ability to inhibit the activation of T lymphocytes. This drug also acts on other cells that play a role in psoriasis, such as mast cells, Langerhans cells, and B lymphocytes (2). TAC has high hydrophobicity, and high molecular weight; those characteristics are the limiting factor for its topical use (3). Due to the low penetration in the cutaneous route, different strategies have been proposed to circumvent this limitation instead of nanotechnology.

The employment of nanotechnology results in specific characteristics for the drugs that are well known (4). For example, improved penetration and permeation through the skin (5), low toxicity, controlled release of the drug (6), and protection of labile substances from degradation (7,8). Recent research on pharmaceutical formulations containing TAC has focused on increasing TAC solubility in water (9,10), controlling its release (11), and improving problems related to the adverse effects to achieve optimal therapeutic benefits in patients (9-16). Some examples of strategies using nanotechnology to load TAC into the topical treatment of psoriasis are lipospheres (9), liquid crystalline nanoparticles (10,11), solid lipid nanoparticles (11), nanostructured lipid carrier (11,12,13), liposomes (11), polymeric micelles (14), nanoemulsion (15) and polymeric nanocarriers (16).

In this way, the lipid-core nanocapsules (LNC), developed by our research group has demonstrated to be promising for the topical application (17,18). LNC increased the deposition of capsaicinoids in the stratum corneum and epidermis, acting as a reservoir system in the skin and promoted their permeation without systemic absorption (19), and providing a controlled release profile. The LNC structures are formed by a polymeric wall arranged around an organogel as core formed by the mixture of medium-chain triglycerides and sorbitan monostearate (20). Poly(ε-caprolactone) (PCL) (Mn 45, 50, and 80 kg.mol^{-1}) is the polymer used to formulate these nanostructures (18) and has characteristics of biocompatibility and biodegradability. Calgaroto and coworkers (2018) demonstrated the PCL Mn 80 kg.mol^{-1} has greater stability when compared with PCL of less molecular mass and the ability to protect encapsulated drugs (21). Also, it presents a slower degradation process compared to PCL with lower molecular weight (Mn 10kg.mol^{-1} and the mixture of Mn 80kg.mol^{-1} and 10kg.mol^{-1}). These characteristics are due to its lower crystallinity and longer chains, which decrease the polymer chains' mobility, making the degradation slower and controlling the drug release.

A recent study had developed TAC-loaded LNC, using PCL Mn 80 kg.mol^{-1} to treat autoimmune diseases where the potential of drug release control (22,23). An improvement in the pharmacodynamic effect due to TAC nanoencapsulation after oral administration (6 and 10 mg.kg^{-1}/day) in an animal model (CF1 albino mice) was observed (22). In another study (23), the TAC-loaded LNC was used to treat an autoimmune as rheumatoid arthritis disease in Wistar rats (2.0 mg.kg^{-1}/day, intraperitoneal route for eight days). So, nanoencapsulation is a promising approach to treat diseases that deregulate the immune system.

Considering that psoriasis is an autoimmune disease that affects the skin, the promising results of the dermal applications of LNC, and the importance of a validate method, this study aimed to demonstrate a complete analytical revalidation, important for a complete study of quality parameters (25). In this way, this study demonstrated the analytical revalidation of chromatographic method to quantify the drug tacrolimus, on time this method is not present in the literature. The method revalidation was performed by determination of the linearity, specificity, precision, accuracy, robustness parameters and limit of detection (LD) and quantification (LQ), in agreement with international guidelines (25,26). Besides that, it aimed to develop and characterize LNC-loaded TAC (TAC-LNC) and prepared with PCL Mn 80kg.mol^{-1} to provide sustained release of the drug.

Experimental Section

Materials

Tacrolimus was purchased from Fagron (São Paulo, Brazil), Poly(ε-caprolactone) (Mn = 80,000 Da) was kindly provided from Perstorp UK limited (Warrington, England). Caprylic/capric triglyceride and polysorbate 80 were delivered by Delaware (Porto Alegre, Brazil). Sorbitan monostearate (Span 60®) were acquired from Sigma-Aldrich (São Paulo, Brazil). All other chemicals and solvents were of analytical or pharmaceutical grade and were used as received.

Development of analytical method

Chromatographic analyses were performed using Perkin Elmer S-200 equipment (HPLC-UV, Series 2000, PerkinElmer, Waltham, MA, USA). Data were obtained and processed using the LC Solution software. Chromatographic separation was performed using a Spherisorb ODS2 (4.6×250 mm, 5 µm) Waters® column.

The HPLC method developed was based on Friedrich and coworkers (2014) (22). The mobile phase was composed of acetonitrile: acid water (with phosphoric acid (70:30v/v) and filtered in a 0.45mm membrane filter (Millipore, Bedford, USA) and degassed using an ultrasound bath for 30 min. The flow was 1.5 mL min $^{-1}$, the temperature was maintained at 50°C. TAC was detected at 210 nm after 100 µL of samples injection.

Preparation of samples and standard solution

The standard solution of TAC was prepared from weighing 5 mg of drug in a 5 mL volumetric flask supplemented with water: ethanol (1:1v/v), which was maintained in an ultrasound bath for 30 min, resulting in a solution of 1 mg.mL^{-1}. From this standard solution, dilutions were performed to evaluate the analytical parameters. TAC-LNC sample was prepared to dissolve the formulation (0.25 mL) in 10 mL of acetonitrile, which was maintained in an ultrasound bath for 45 min, resulting in a solution of 20 µg. mL^{-1}.

Analytical method revalidation

The parameters evaluated were linearity, accuracy, specificity, intra- and inter-day precision, robustness, detection limit (LD), and quantification (LQ). According to the Brazilian Health Regulatory Agency (25) and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (26) and all parameters were developed and adequately validated.

Linearity

Eight different concentrations were used: 1, 3, 5, 10, 15, 20, 40 and 60 µg.mL^{-1}. All analyses were performed in triplicate, and the mean of three independent curves was used. The correlation and determination coefficients were considered to analyze the linearity of the method. The linearity results were analyzed by one-way ANOVA to significance.

Specificity

The specificity was evaluated by the absence of interferents in the same retention time of TAC and the peak purity index. A lipid-core nanocapsules without drug (LNC) formulation prepared the same way described in section "*Development of characterization of the lipid-core nanocapsules"* was used as control.

Precision

Intra-day precision was evaluated by preparing a solution (20 µg.mL^{-1} of TAC), prepared on the same day (n = 3). Inter-day precision was performed by repeating the same procedure on three different days (n = 9 replicates). The results were expressed as percentage of the drugs content (%) and by the relative standard deviation (RSD).

Accuracy

Accuracy was determined by calculating the percentage of drug recovery in three concentration levels (10, 20, and 40 µg.mL^{-1}). In this case, the matrix was the LNC. This procedure was performed in triplicate (n=3) and the results were expressed as percentage of the drugs content (%) and by the relative standard deviation (RSD).

Robustness

In this study, the chromatographic parameters were evaluated by small changes (n=3) in phase flow (±0.1 mL min^{-1}), in temperature of column (50±2^{o}C), and the composition of mobile phase (710:290 v/v ACN:H_2O and 690:310 v/v ACN:H_2O). Based on recommendations, these parameters were defined (25,26). The analysis used a standard solution of TAC and TAC-LNC, both with a concentration of 20 µg. mL^{-1} of the drug. The values obtained were calculated by the mean recovery compared with the proposed method conditions and analyzed by one-way ANOVA to significance.

Limit of detection (LD) and quantification (LQ)

LD and LQ were determined based on calibration curve parameters using these equations (25,26):

$$LD = 3.3\frac{\sigma}{IC}$$

Eq. (1)

$$LQ = 10\frac{\sigma}{IC}$$

Eq. (2)

Where IC is the calibration curve inclination; σ is the standard deviation obtained from the residual standard deviation of the regression line.

Development and characterization of the lipid-core nanocapsules

TAC-LNC was prepared by self-assembling using the solvent displacement method (22). In summary, the organic phase constituted by PCL (Mn 80 kg.mol^{-1}) (0.1g), Span 60® (0.04g), caprylic/apric triglyceride mixture (160 µL) and TAC (0.008g) dissolved in acetone at 40°C was injected under moderate magnetic stirring into an aqueous solution containing polysorbate 80 (0.08g). The turbid solution was stirred for 10 min. Then, acetone was removed, and the suspension was concentrated under reduced pressure to 10 mL. The final concentration of TAC-LNC was 0.80 mg.mL^{-1}. All formulations were prepared in triplicate (n = 3).

Determination of particle size distribution

The particle size distribution was determined by laser diffraction (LD) (Mastersizer 2000®, Malvern Instruments, UK). The formulations (200 µL) were added to the equipment sampling apparatus containing distilled water until the laser obscuration of 2% was achieved. The diameter was expressed as volume-weighted mean diameter ($d_{4,3}$), and the volume-weighted particle size distribution was used to calculate the polydispersity (SPAN) using Equation 3:

$$Span = \frac{(d_{0.9} - d_{0.1})}{d_{0.5}}$$

Eq. (3)

Where $d_{0.1}$, $d_{0.5}$, and $d_{0.9}$ correspond to the 10th, 50th, and 90th percentiles of the cumulative size distribution, respectively.

TAC-LNC and LNC were also characterized by photon correlation spectroscopy (PCS) using a Zetasizer® Nano-ZS ZEN 3600 model (Malvern Instruments Ltd., UK). The *z*-average diameters and polydispersity index (PdI) were determined after dilution (1:500 v/v) of the samples (20µL) in ultrapure water (10mL).

Nanoparticle tracking analysis (NTA) was also performed (NanoSight LM 10 & NTA 2.2 Analytical Software, NanoSight Ltd., Amesbury, UK). TAC-LNC and LNC were previously diluted in ultrapure water 10,000 times and injected into the sample chamber cell. The CCD camera was coupled to an optical microscope, and a laser light (635 nm) was focused on the sample. Images were registered for 60 s. Data were collected and analyzed using software to identify and track the light individually scattered by the nanoparticles under Brownian motion. This movement's speed is related to the hydrodynamic radius of a sphere calculated using the Stokes-Einstein equation [Eq. (4)]:

$$\overline{(x,y)^2} = \frac{2k_B T}{3r_h \pi \eta}$$

Eq. (4)

Where k_B is the Boltzmann constant and $\overline{(x,y)^2}$ is the mean-squared speed of a particle at a temperature (T), in a medium of viscosity (η), with a hydrodynamic radius of (r_h).

The results correspond to the arithmetic average of the calculated sizes of all the particles analyzed. All measurements were carried out at 25 °C and all samples were analyzed in triplicate batches (*n* = 3).

Zeta potential

The zeta potential was measured by electrophoretic mobility using a Zetasizer Nano-ZS ZEN 3600 model (Malvern Instruments Ltd., UK). The measurements were performed at 25°C after diluting the samples (1:500 v/v) in 10 mM NaCl aqueous solution. All the samples were performed in triplicate batches (*n* = 3).

Determination of pH

After preparation, the pH values were measured using a calibrated potentiometer directly in the aqueous suspensions without previous treatment. All measurements were performed in triplicate batches (n = 3).

Determination of drug content and encapsulation efficiency

The total drug content in TAC-LNC was measured by reverse-phase liquid chromatography after preparation described in section "*Development of analytical method"*. The ultrafiltration-centrifugation technique was used to quantify non-encapsulated TAC. This technique allows the separation of the drug in the ultrafiltrate from the LNC in the colloidal turbid solution using Ultracel YM-100. The solutions were centrifuged at 3.600 rpm for 15 min. The encapsulation efficiency (EE%) was calculated as the difference between the total and non-encapsulated drug concentration [Eq. (5)]. All measurements were performed in triplicate batches (n = 3).

$$\%EE\% = \frac{(Ct - Cf)}{Ct} x100$$

Eq. (5)

Where Ct is the total concentration of drug in the nanoformulation and Cf is the concentration of drug in the aqueous phase of the suspension.

Stability study

The formulations stored in flask glass and under room temperature (25 ± 2 °C) protected from light were monitored following 30 days determining drug content, pH, SPAN, PdI, and particle size as analytical parameters. The analyses were performed in triplicate batches (n = 3).

Evaluation of the release profile

The *in vitro* drug release of TAC-LNC was conducted by the dialysis bag method (n = 3). The diffusion of TAC

from its solution of ethanol/water (50:50 v/v) was also evaluated. The hydroalcoholic solution was named TACfree. Under sink conditions, water/ethanol (70:30 v/v) was used as a medium at 37°C. The dialysis bag (25 × 16 mm, cut off 12,000 to 14,000 Da, Sigma Aldrich) containing 1 mL of each formulation (0.8 mg mL^{-1} of TAC) was placed into 100 mL of dialysis medium under constant moderate stirring. A volume of 1 mL of the external medium was withdrawn from the system at a predetermined time interval, replaced by an equal fresh medium volume. The samples were filtered through a 0.45µm membrane, and TAC was assayed using the HPLC methods previously described. The profiles were modeled by first-order monoexponential and first order biexponential models using Scientist software (MicroMath Scientist®, USA). The best correlation coefficient and the best model selection criterion (MSC) [Eq. (6)] were selected. The Model Selection Criterion (MSC) provided by the Scientist Software (MicroMath, Saint Louis, Missouri USA) is a criterion for comparing models.

$$MSC = \ln\left(\frac{\Sigma_i w_i [y(t_i) - \bar{y}\,(t_i)].^2}{\Sigma_i w_i [y(t_i) - \bar{\hat{y}}\,(t_i)].^2}\right) - 2p/n \qquad \text{Eq. (6)}$$

Where n is the number of independent measurements considered in the fit; p is the number of fitted parameters and w_i is the weight applied to the residual of acquisition.

Statistical analyses

The present study results were statistically analyzed using Excel Software and one-way analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

Results and Discussion

Revalidation of the analytical methodology

The linearity of the analytical method was evaluated by plotting calibration curves for the drug, which showed to be linear in this range (Figure 1) and was expressed as a correlation coefficient (R> 0.999) and coefficients of determination (R^2= 0,998). The equation that describes the straight line of the drug was obtained (y= 47557x - 1961). For the establishment of linearity, a minimum of five concentrations is recommended (26), and in our study eight points were evaluated (1 to 60 µg.mL^{-1} for TAC). The results demonstrated that the equipment response is indeed proportional to the drug concentrations in the samples (25). Statistical analyzes of these results were performed using ANOVA ($p < 0.05$), indicating a linear relationship between drug concentration and analytical signal.

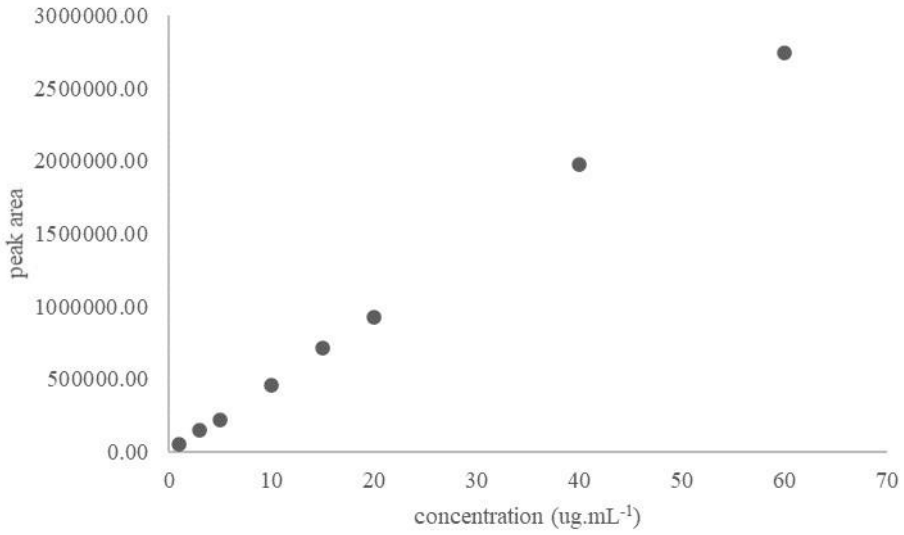

Figure 1. Mean calibration curve obtained for tacrolimus using working standard solutions (1 mg.mL^{-1}) at the concentration range from 1.0 to 60.0 µg.mL^{-1}. Results (n=3) analysed by the test one-way ANOVA using the significance level of α=0.05 (95% of confidence interval), p= 6.7e^{-7}.

The specificity was evaluated by determining the capacity of the method to measure TAC in a mixture with the formulation excipients. TAC-LNC and LNC chromatograms (Figure 2) demonstrated no interferent signals at the same retention time as the peaks of interest. There were no interfering materials from the mobile phases and the formulation, that elute before 7.78min, that correspond to the TAC. The method showed selectivity from an analytical point of view. It was able to identify and quantify the analyte of interest unequivocally in the presence of components present in the sample, such as impurities and matrix components following the regulatory agencies' rules (25,26).

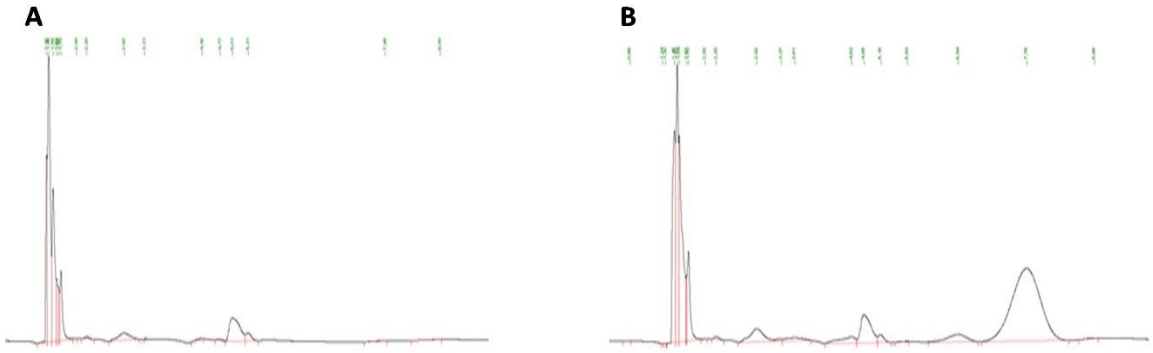

Figure 2. Representative HPLC chromatograms obtained from LNC (A) and TAC-LNC (B). Mobile phase: ACN: acidified water (70:30 v/v); flow rate: 1.5 mL.min^{-1}; detection wavelength: 210 nm; retention time=7.78 min column temperature: 50±2 °C; and injection volume: 100 µL.

The method revalidated in this work showed adequate precision and accuracy. Repeatability and intermediate precision results were expressed as percentages of the drug content and RSD (Table 1). Repeatability assessment was conducted by analyzing different samples (20 µg.mL^{-1}) on the same day and the same experimental conditions. The RSD values were 1.67%. A total of nine analyses evaluated intermediate precision for three days, where RSD values were 1.78% for TAC in all assays. All RSD values were lower than 2% within the established limits (25,26).

Table 1. Content values for TAC, expressed as % and RSD, representing repeatability (n=3) and intermediate precision (n=3).

	Concentration (µg/mL)	Drug content (%)	Repeatability (%)	RSD (%)
	20.59	102.96		
Day 1	20.44	102.22	101.32	1.67
	19.75	98.77		
	19.91	99.55		
Day 2	19.89	99.46	99.99	0.64
	20.19	100.94		
	19.57	97.86		
Day 3	19.16	95.81	96.68	0.81
	19.28	96.39		
			Intermediate precision (%)	**RSD (%)**
			99.33	1.78

RSD= relative standard deviation

This method also demonstrated adequate accuracy, with a recovery percentage close to 100% and RSD lower than 2% in the three evaluated concentrations, as shown in Table 2. Thus, the method proved to agree with the requirements of the regulatory agencies (25,26). The repeatability and intermediate precision (Table 1) are close to that demonstrated by previous studies, with similar analytical conditions, which obtained 100% and RSD less than 2% (24).

Accuracy must be verified from at least nine determinations, considering the linear range of the analytical method: three concentrations (low, medium, and high) with three repetitions at each level (25,26). The results obtained from the three concentration levels analysis must be close to the theoretical values for the method to be certified as accurate (25,26). Therefore, this method is necessary since the drug's recovery rate is close to the theoretical value (100%), and the data showed a small RSD (≤ 1.68%). Also, this method showed greater precision and accuracy than the similar method but used methanol: water (30:70) as a mobile phase that obtained results around 80% (13) that means that the method is suitable for quantifying TAC from a complex matrix, despite changes in formulation.

Results to robustness are shown in Table 3. The F (calculated) value was lower than the F (tabulated) among the groups, indicating no significant difference between the robustness values compared to the changed conditions and the normal condition. The method is considered robust when the results showed less than 5% RSD with

the changes (26). Besides that, all results showed % RSD ≤2. Changes in flow (± 0.1 mL.min^{-1}), temperature (± 5°C), or mobile phase composition (ACN: water 71:29 or 69:31) were within the range of variation of precision and accuracy.

The parameters of the limit of detection (LD) and the limit of quantification (LQ), correspond to the lesser amount of analyte that could be detected in the analysis, and the lesser amount of analyte can be quantified, respectively. Our results (LD=45.38 ng.mL^{-1} and LQ= 137.51 ng.mL^{-1}) demonstrated that the method was sensible enough to determine TAC in LNC. Results similar were obtained by Gabriel and coworkers (2016) that revalidated a method to quantify TAC using different mobile phases (water and 1% of TFA) and demonstrated LD and LQ of 38 ng.mL^{-1} and 115 ng.mL^{-1}, respectively. However, other methodologies to quantify TAC with a similar methodology and same mobile phase (22), or with methanol: water (30:70) (13) as mobile phase obtained considerably higher results, LD=300 ng.mL^{-1}, LQ = 920 ng.mL^{-1} and LD=200 ng.mL^{-1}, LQ =500 ng.mL^{-1} respectively. Finally, LD and LQ values are important parameters when there are low concentrations of compounds in the matrices, such as nanoparticles, *in vitro* assays, and *in vivo* assays. The results obtained for theoretical limits demonstrate that the method is sensitive enough to determine TAC in a small concentration.

Table 2. Values of accuracy of the method

Theorical concentration (µg/mL)	Concentration (µg/mL)	Recuperation (%)	RSD (%)
10	9.83	98.36	1.68
20	19.52	97.61	1.34
40	39.96	99.90	0.60

Percentage (%) content values and RSD (%) for TAC recovery (n=3). RSD = relative standard deviation

Table 3. Analysis of variance (ANOVA) for robustness, considering temperature (50±5°C), flow (1.5 ±0,1 mL.min^{-1}) and mobile phase (710:290 (ACN: H_2O) and 690:310 v/v (ACN: H_2O)).

	Parameter	Degrees of freedom	Sum of squares	Mean square	F calculated	P-value*	F tabulated
Standard solution (TAC)	Temperature (±5°C)	2	0.67458	0.337329	0.486227	0.637231	5.143253
	Flow (±0,1 mL.min^{-1})	2	12.21736	6.108682	3.375787	0.104175	5.143253
	Mobile Phase	2	9.465896	4.732948	3.504655	0.098105	5.143253
TAC-LNC	Temperature (±5°C)	2	0.619935	0.309967	0.505414	0.626824	5.143253
	Flow (±0,1 mL.min^{-1})	2	0.363723	0.181862	2.36559	0.174987	5.143253
	Mobile Phase	2	0.758314	0.379157	3.639727	0.92239	5.143253

(*) p>0.05

Physico-chemical characterization of LNC and TAC-LNC

LNC and TAC-LNC suspensions presented an opalescent bluish aspect, typical of the Tyndall effect in nanoformulations (24). The formulations (LNC and TAC-LNC presented a unimodal distribution with a SPAN below 2.00 (1.78±0.02 and 1.55±0.18 respectively). The analysis of laser diffraction showed unimodal distribution in the nanometer range, the LNC presented d4,3 around 202.33 ±2.3nm and TAC-LNC presented $d_{4,3}$ values of 184 ±18 nm. The volume-weighted mean diameters ($_{D4,3}$) and the diameters at percentiles 10 ($D_{0.1}$), 50 ($D_{0.5}$), and 90 ($D_{0.9}$) under the size distribution curves by volume and by the number of LNC and TAC-LNC were plotted in a

radar chart (Figure 3). The shape of the radar chart curves is the fingerprint characteristic of unimodal particle size distribution, as previously determined for different polymeric nanocapsules (27). The diameter results showed in the radar chart indicated that the nanocapsules had no size changes with the incorporation of the drug, considering the results were overlapping in the chart.

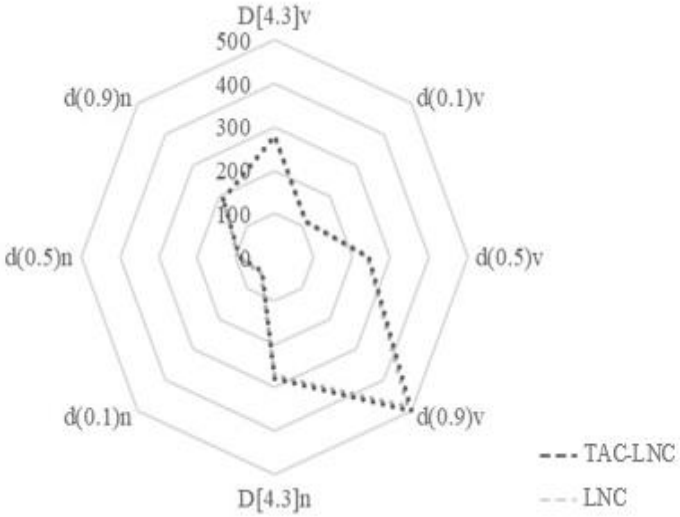

Figure 3. Radar chart presenting the volume-weighted mean diameters ($D_{4,3}$) and the diameters at $D_{0.1}$, $D_{0.5}$, $D_{0.9}$, under the size distribution curves by volume and by number of particle (LNC and TAC-LNC).

Considering that the profiles obtained by laser diffraction showed no micrometric populations for both nanoformulations, measures of z-average diameters by PCS were applicable. The diameter distributions were unimodal, with z-average diameters of 195 ± 4 nm and 199 ± 13 nm for LNC and TAC-LNC, respectively. The polydispersity index for LNC was 0.100 ± 0.02 and TAC-LNC 0.101 ± 0.04. Previous study that analyzed nanostructured lipid transporter TAC-loaded by DLS showed results around 220 nm (13) and 212 nm (11).

The zeta potential was negative for all formulations with or without TAC (-13.67 ± 1.16 and -15.60 ± 2.84, respectively). The results of negative zeta potential agree with other works in which were developed PCL nanocapsules (22,29) that is attributed to the chemical structure of PCL (Mn 80 $kg.mol^{-1}$), which has in its structure a negative density of charge of the carboxylate functions in water presence (24). The pH value directly determined by potentiometry without any dilution showed to be slightly acid for all formulations (LNC=5.46±0.14 and TAC-LNC = 5.58 ± 0.06). Overall, LNC and TAC-LNC had no statistical difference, which shows the properties of size and pH of nanoparticles were not affected by the drug. The zeta potential reflects the surface charge of the nanocapsules, being influenced by the characteristics of the formulation excipients. The slightly acid pH value obtained can also be related to the characteristics of the polymer, as the ionization of the functional groups present in the polymer chains with terminal carboxyl groups reduces the pH (24,30). Previous works (31,32) reported slight acid pH values (6.56±0.09 and 6.01±0.04, respectively) utilizing the same synthesizing technique nanocapsules and similar components of nanoformulation. These pH values are compatible with the cutaneous application. Moreover, the obtained values of zeta and pH align with those normally found in the literature to these systems, prepared by the nanoprecipitation method.

The data determined by NTA showed a narrow diameter distribution for LNC and TAC-LNC with a diameter of about 219 ±7.4 nm and 222.60 ±9.5 nm and respectively, and a concentration of $2.60333e^{13}$ $particles.mL^{-1}$ (TAC-LNC). The distribution profiles were similar between nanoparticles with and without the drug (Figure 4). The NTA analyses showed an increase in diameter in our results when compared with DLS and Master technique, the distribution profiles were similar between nanocapsules with and without the drug (219 ±7.4 nm and 222.60 ±9.5 nm, respectively). LNC and TAC-LNC showed the mean diameter determined by NTA significantly bigger ($p \leq 0.05$) when compared with the laser diffraction techniques. NTA technique is based on tracking single particles while the laser difraction is based on measuring a bulk of particles (28). Besides that, the intense light scattering by large particles makes the small particles more difficult to be detected and prevents some of them from being tracked by the NTA software (28). This phenomenon could explain the data obtained for NTA analyses. It was described the use of NTA as a valuable tool to detect drug-nanocrystals as contaminants in drug-loaded lipid core nanocapsules (28). The main aspect for detecting the drug crystals in the samples is the broad distribution by intensity. For the LNC and TAC-LNC, the NTA analyzes showed narrow distribution by

intensity, indicating that probably no drug crystals coexist in the samples.

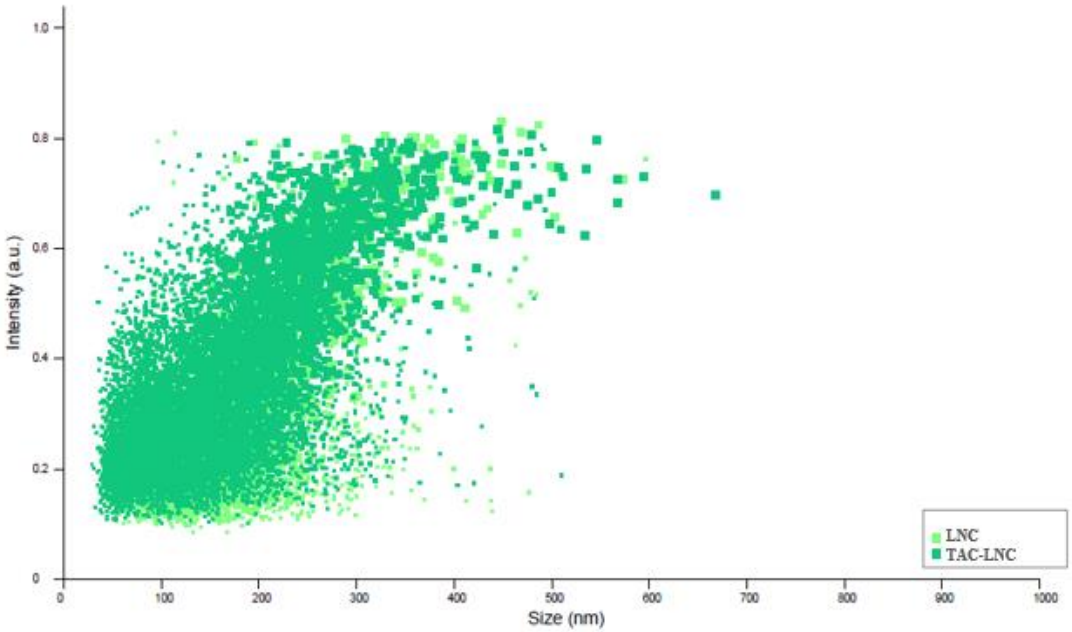

Figure 4. LNC and TAC-LNC 2D distributions by nanoparticle tracking analysis (NTA): intensity (y-axis) *versus* particle size (x-axis)

Finally, the determination of drug content and encapsulation efficiency were close to 100%, 98.90±2.32% (concentration of 0.08 $mg.mL^{-1}$) and 99.23±0.32%, respectively, close to the expected theoretical values. The encapsulation efficiency found was like that obtained by Friedrich and co-workers (2014) (99.49±0.01%) for the NC-TAC with PCL (Mn 50$kg.mol^{-1}$) (22). High encapsulation efficiency was also demonstrated with other TAC-loaded nanostructures, a highly lipophilic drug, as liquid crystalline nanoparticle (± 99%) (10), and nanostructured lipid carrier by hot homogenization technique (± 94%) (13). However, in the other investigation that studies TAC-loaded in nanostructured lipid carrier produced by hot homogenization, the %EE was 50% (12). The encapsulation rate is an important parameter because it can influence the *in vitro* and *in vivo* results. For the particles with low encapsulation rate, the drug release will not be controlled entirely. The high encapsulation efficiency is characteristic of lipophilic drugs that present a high logD value, in LNC obtained by interfacial deposition method of preformed polymers. Our research group proposed an algorithm that classifies six drug distribution models in LNC formulations (34). Using this algorithm, it was possible to correlate the logarithm of the distribution coefficient D (logD) to the mechanism of drug distribution in the formulations. In this case, the prediction of the mechanism of drug distribution can be achieved by calculating the drug's logD. High values of logD reflect a higher drug affinity for the lipophilic phase compared to an aqueous phase. Considering the LogD of TAC (3.96) (22), the high encapsulation efficiency (>99%) and the absence of microcrystals in TAC-LNC formulation, TAC is more concentrated at the inner pseudo-phase in LNC, corresponding to the type VI mechanism of drug distribution. In this work, we produced TAC-LNC with adequate nanometric characteristics demonstrated by several analytical techniques that use different methodologies to carry out the analyzes.

Stability study

The TAC-LNC presented satisfactory results in terms of stability during storage at room temperature (25 ± 2 °C), for 30 days (table 4). The nanometric size maintained their initial characteristics ($p = 0.9789$) for 30 days. Similar result was observed for the zeta potential ($p=0.8628$), drug content ($p=0.9950$), PdI ($p=0.0807$) and SPAN ($p= 0.4476$). The particle diameter and the SPAN are important parameters to indicate the physical stability and the physicochemical behavior of encapsulated compounds. The system's stability can be attributed to surfactant polysorbate 80, which prevents formation of agglomerates by steric stabilization. These results agree with previous studies that studied the stability at room temperature of LNC with PCL and polysorbate in its composition loading different drug as dithranol (34) and lycopene (32). The pH values of the formulations decreased after 30 days. Similar result was also observed after 28 days in other studies that used PCL as polymer (32,35). This reduction may be due to the partial degradation of the polymeric wall and the consequent release of the polyester monomer during PCL hydrolysis (24, 36,37). Another hypothesis that can explain this decrease is the hydrolysis of polysorbate 80 that results in oleic acid which can reduce pH values in nanoformulation (38,39).

Table 4. Stability data of TAC-LNC for 30 days

	Drug content (%)	size (nm)	PdI	SPAN	Zeta	pH
Day 0	104.92±0.44	199.60±13	0.101±0.04	1.658±0.144	-15.73±0.31	5.74±0.04
Day 7	104.45±2.94	200.63±12	0.121±0.04	1.721±0.231	-18.50±5.86	5.53±0.37
Day 30	105.38±2.59	182.60±18	0.128±0.3	1.555±0.184	-16.97±1.86	5.00±0.08*

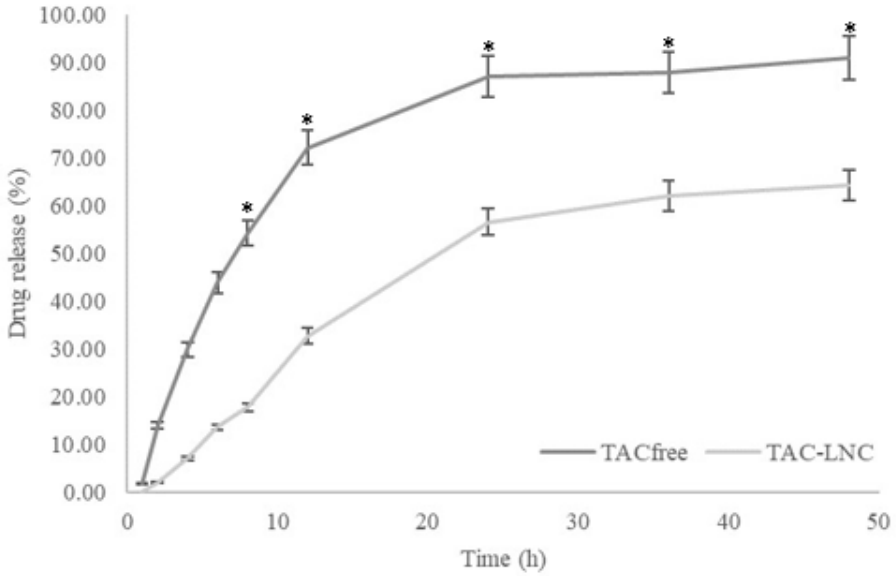

Figure 5. *In vitro* release profiles of tacrolimus from the dialysis of TACfree and TAC-LNC (n = 3). The lines correspond to the fitting to the biexponential equation. (*) Significant differences observed ($p<0.05$).

Release profile for the nanocapsules

The *in vitro* drug release study was conducted for the TACfree and TAC-LNC at the same concentration (0.80 mg/mL). The release experiment showed different profiles (p=0.0009) between both formulations (Figure 5). In the case of the TACfree, about 87% of the drug was released after 24 h of experiment, while 64% of TAC was released from TAC-LNC after 48 h. For the biexponential model the MSC and correlation coefficient for TACfree and TAC-LNC were 5.56 ± 0.77 and 0.99, 4.94 ± 1.77 and 0.99 respectively. For the monoexponential model the results were MSC 2.28±0.03 and 3.47±1.68 and correlation coefficient of 0.91 and 0.96 for TACfree and TAC-LNC respectively. So, considering the release profiles, the mathematical modeling showed that TACfree and TAC-LNC fitted the biexponential modelThe release profiles were plotted as a percentage of drug dialyzed as a function of time. The TAC solution demonstrated fast release in 24 h, while TAC-LNC showed controlled release during 48h. In this context, the LNC was able to delay TAC release, indicating the interaction of the drug with the formulation components. Also, according to the algorithm proposed by Oliveira and coworkers (2013), the drug is fully encapsulated and interacting with the core components, which was reflected by the high % EE and sustained drug release. The best model to describe the release profile of TAC from solution and LNC was the biexponential model. The first order biexponential behavior corresponds to a biphasic release profile, consisting of a rapid release phase (burst) and a second phase involving sustained release. Casarini and coworkers (2021) also found results in this way using PCL Mn 80 $kg.mol^{-1}$ for encapsulated phloretin, a lipophilic drug (33). Interestingly, Friedrich and coworkers (2014) showed that TAC was dialyzed approximately 20% after 24h from LNC (PCL Mn 50$kg.mol^{-1}$), while in our study, the drug was dialyzed about 56%. However, in our work, this profile was sustained by 48 h, while in the other work (22), after 48 h, the release increased to around 40%.

The polymeric wall constitution can explain the difference in the drug release profile from complex polymeric matrices. PCL Mn 80 $kg.mol^{-1}$ shows less crystallinity than the PCL Mn 50 $kg.mol^{-1}$ (21). PCL with less molar weight present a lower polymer degradation rate in the first stages of release because the crystalline domains are less permeable (21). However, in

the following stages, the high crystallinity leads to a greater loss of the polymeric wall due to the shorter polymeric chains, which favors hydrolysis. Thus, polymers of greater molecular mass and, consequently, less crystallinity influence the hydrolytic degradation of the polymeric wall. In this way, the drug will be released slower from the polymeric wall when this shows more crystallinity, and the drug will be released faster when the polymer used shows greater crystallinity (21). These PCL characteristics with a higher molar weight, as PCL Mn 80 $kg.mol^{-1}$, can maintain the release of the drug sustained for a longer time.

The controlled release profile of TACfree shown (Fig. 5) can be explained because the polysorbate 80 was used to disperse TAC due to its physical-chemical characteristics and low solubility. The addition of polysorbate 80 can affect the molecular release since it can be trapped in the micelles of surfactant and not pass freely through the membrane (40,41). However, it important to note that a more pronounced controlled release for TAC-LNC was observed in comparison with TACfree. When the nanoparticle can ensure a drug sustained release into the skin, this feature is advantageous because it might help reduce the irritation and toxic effects while maintaining the concentration gradient across the skin. For instance, solid lipid nanoparticles and nanostructured lipid carrier with TAC showed similar release profile with initial burst release followed by sustained release (11).

Conclusion

The present study revalidated an isocratic, fast, and sensitive HPLC method, allowing TAC determination in a complex polymeric matrix following international and national guidelines. TAC-LNC has been produced and extensively characterized by several analytical techniques, and all their nanotechnological quality attributes have been proven. Our results demonstrated that TAC-LNC using PCL (Mn 80 $kg.mol^{-1}$) shows a sustainable release profile following the biexponential release model. Furthermore, TAC-LNC showed to be a highly promising drug-delivery system for the topical treatment of autoimmune diseases.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments.

The authors would like to thank the Research Support Foundation of Rio de Grande do Sul State (FAPERGS - Brazil; PRONEX FAPERGS/CNPq 12/2014 - Grant 16/2551-0000467-6), National Council for Scientific and Technological Development (CNPq - Brazil; Grant #465687/2014-8) and National Institute of Pharmaceutical Nanotechnology (INCT-NANOFARMA - Brazil; Grant #2014/50928-2) for financial support.

References

1- Zhai J, Gu J, Yuan J, Chen J. Tacrolimus in the treatment of ocular diseases. BioDrugs. 2011 Apr 1;25(2):89-103. doi: 10.2165/11587010-000000000-00000. PMID: 21443273.

2- Gao L, Liu J, Zhang Y, Chen X, Gao L, Zhang C, Liu Y, Kong P, Zhong J, Sun A, Du X, Su Y, Li H, Liu H, Peng X, Zhang X. Low incidence of acute graft-versus-host disease with short-term tacrolimus in haploidentical hematopoietic stem cell transplantation. Leuk Res. 2017 Jun;57:27-36. doi: 10.1016/j.leukres.2017.02.006. Epub 2017 Feb 24. PMID: 28273549.

3- Ueyama A, Yamamoto M, Tsujii K, Furue Y, Imura C, Shichijo M, Yasui K. Mechanism of pathogenesis of imiquimod-induced skin inflammation in the mouse: a role for interferon-alpha in dendritic cell activation by imiquimod. J Dermatol. 2014 Feb;41(2):135-43. doi: 10.1111/1346-8138.12367. Epub 2014 Jan 3. PMID: 24387343.

4- Frank LA, Chaves PS, D'Amore CM, Contri RV, Frank AG, Beck RC, Pohlmann AR, Buffon A, Guterres SS. The use of chitosan as cationic coating or gel vehicle for polymeric nanocapsules: Increasing penetration and adhesion of imiquimod in vaginal tissue. Eur J Pharm Biopharm. 2017 May;114:202-212. doi: 10.1016/j.ejpb.2017.01.021. Epub 2017 Feb 1. PMID: 28161547.

5- de Brum TL, Fiel LA, Contri RV, Guterres SS, Pohlmann AR. Polymeric Nanocapsules and Lipid-Core Nanocapsules Have Diverse Skin Penetration. J Nanosci Nanotechnol. 2015 Jan;15(1):773-80. doi: 10.1166/jnn.2015.9185. PMID: 26328441.

6- Mora-Huertas CE, Fessi H., & Elaissari A. Polymer-based nanocapsules for drug delivery. International Journal of Pharmaceutics, 2010; 385(1-2), 113–142. doi:10.1016/j.ijpharm.2009.10.018.

7- Guterres SS, Alves MP, Pohlmann AR. Polymeric nanoparticles, nanospheres and nanocapsules, for cutaneous applications. Drug Target Insights. 2007;2:147-57. Epub 2007 Jul 11. PMID: 21901071; PMCID: PMC3155227.

8- Pivetta TP, Simões S, Araújo MM, Carvalho T, Arruda C, Marcato PD. Development of nanoparticles from natural lipids for topical delivery of thymol: Investigation of its anti-inflammatory properties. Colloids Surf B Biointerfaces. 2018 Apr 1;164:281-290. doi: 10.1016/j.colsurfb.2018.01.053. Epub 2018 Jan 31. PMID: 29413607.

9- Jain A, Doppalapudi S, Domb AJ, Khan W. Tacrolimus and curcumin co-loaded liposphere gel: Synergistic combination towards management of psoriasis. J Control Release. 2016;243:132-145. doi:10.1016/j.jconrel.2016.10.004

10- Thapa RK, Yoo BK. Evaluation of the effect of tacrolimus-loaded liquid crystalline nanoparticles on psoriasis-like skin inflammation. J Dermatolog Treat. 2014;25(1):22-25. doi:10.3109/09546634.2012.755250

11- Jain S, Addan R, Kushwah V, Harde H, Mahajan RR. Comparative assessment of efficacy and safety

potential of multifarious lipid based Tacrolimus loaded nanoformulations. Int J Pharm. 2019;562:96-104. doi:10.1016/j.ijpharm.2019.03.042

12- Nam H, Ji XY, Park JS. Investigation of tacrolimus loaded nanostructured lipid carriers for topical drug delivery. Bulletin of the Korean Chemical Society v.32, p.956–960, 2011.

13- Viegas JSR, Praça FG, Caron AL, et al. Nanostructured lipid carrier co-delivering tacrolimus and TNF-α siRNA as an innovate approach to psoriasis. Drug Deliv Transl Res. 2020;10(3):646-660. doi:10.1007/s13346-020-00723-6.

14- Lapteva M, Mondon K, Möller M, Gurny R, Kalia YN. Polymeric micelle nanocarriers for the cutaneous delivery of tacrolimus: a targeted approach for the treatment of psoriasis. Mol Pharm. 2014;11(9):2989-3001. doi:10.1021/mp400639e.

15- Sahu S, Katiyar SS, Kushwah V, Jain S. Active natural oil-based nanoemulsion containing tacrolimus for synergistic antipsoriatic efficacy. Nanomedicine (Lond). 2018;13(16):1985-1998. doi:10.2217/nnm-2018-0135

16- Gabriel D, Mugnier T, Courthion H, et al. Improved topical delivery of tacrolimus: A novel composite hydrogel formulation for the treatment of psoriasis. J Control Release. 2016;242:16-24. doi:10.1016/j.jconrel.2016.09.007

17- Jäger E, Venturini CG, Poletto FS, et al. Sustained release from lipid-core nanocapsules by varying the core viscosity and the particle surface area. J Biomed Nanotechnol. 2009;5(1):130-140. doi:10.1166/jbn.2009.1004.

18- Pohlmann AR, Fonseca FN, Paese K, et al. Poly(ϵ-caprolactone) microcapsules and nanocapsules in drug delivery. Expert Opin Drug Deliv. 2013;10(5):623-638. doi:10.1517/17425247.2013.769956

19- Venturini CG, Bruinsmann FA, Contri RV, et al. Co-encapsulation of imiquimod and copaiba oil in novel nanostructured systems: promising formulations against skin carcinoma. Eur J Pharm Sci. 2015;79:36-43. doi:10.1016/j.ejps.2015.08.016

20- Fiel LA, Rebêlo L M., de Melo Santiago T, Adorne MD, Guterres SS, Soares de Sousa J, & Pohlmann A R.. Diverse deformation properties of polymeric nanocapsules and lipid-core nanocapsules. Soft Matter, 2011; 7(16), 7240. doi:10.1039/c1sm05508a.

21- Calgaroto S, Fauri LE, Frank LA, Paese K, Guterres SS, Pohlmann AR. Data of characterization and related assays of lipid-core nanocapsule formulations and their hydrolysis mechanism. Data Brief. 2018;21:918-933. Published 2018 Oct 12. doi:10.1016/j.dib.2018.10.027

22- Friedrich RB, Dimer FA, Guterres SS, Beck RC, Pohlmann AR. Nanoencapsulation of tacrolimus in lipid-core nanocapsules showed similar immunosuppressive activity after oral and intraperitoneal administrations. J Biomed Nanotechnol. 2014;10(8):1599-1609. doi:10.1166/jbn.2014.1842.

23- Friedrich RB, Coradini K, Fonseca FN, Guterres SS, Beck RC, Pohlmann AR. Lipid-Core Nanocapsules Improved Antiedematogenic Activity of Tacrolimus in Adjuvant-Induced Arthritis Model. J Nanosci Nanotechnol. 2016;16(2):1265-1274. doi:10.1166/jnn.2016.11673.

24- Schaffazick SR, Guterres SS, Freitas L de L, & Pohlmann AR. Caracterização e estabilidade físico-química de sistemas poliméricos nanoparticulados para administração de fármacos. Química Nova, 2003; 26(5), 726–737. doi:10.1590/s0100-40422003000500017.

25- Brazil. Resolução RDC no 166 de 24 de julho de 2017- Critérios para a validação de métodos analíticos. *Órgão emissor: ANVISA – Agência Nacional de Vigilância Sanitária.*

26- International Conference On Harmonisation. Validation of analytical procedures: Text and methodology.Q2(R1). London: ICH, 2005 (ICH Harmonised Tripartite Guideline)

27- Bianchin MD, Külkamp-Guerreiro IC, De Oliveira CP, Contri RV, Guterres SS, Pohlmann A R. Radar charts based on particle sizing as an approach to establish the fingerprints of polymeric nanoparticles in aqueous formulations. Journal of Drug Delivery Science and Technology, v.30, p.180–189, 2015.

28- Filipe V, Hawe A, Jiskoot W. Critical evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates. Pharm Res. 2010;27(5):796-810. doi:10.1007/s11095-010-0073-2.

29- Colomé LM, Freitas GM, Bastiani JM, Pereira TCB, Bajerski L, Bender EA, Haas SE. Validation of analytical method by HPLC for determination of dapsone in polymeric nanocapsules based on crude rice brain oil. Journal Of Applied Pharmaceutical Science v.7, p.230–233, 2017.

30- Gomes GS, Maciel TR, Piegas EM, et al. Optimization of Curcuma Oil/Quinine-Loaded Nanocapsules for Malaria Treatment. AAPS PharmSciTech. 2018;19(2):551-564. doi:10.1208/s12249-017-0854-6.

31- Paese K, Jäger A, Poletto FS, et al. Semisolid formulation containing a nanoencapsulated sunscreen: effectiveness, in vitro photostability and immune response. J Biomed Nanotechnol. 2009;5(3):240-246. doi:10.1166/jbn.2009.1028.

32- Dos Santos PP, Paese K, Guterres SS, Pohlmann AR, Costa TH, Jablonski A, Rios A Development of lycopene-loaded lipid-core nanocapsules: physicochemical characterization and stability study. Journal of Nanoparticle Research, v.17(2), p.2917-5, 2015.

33- Oliveira CP, Venturini CG, Donida B, Poletto FS, Guterres SS, Pohlmann A R. An algorithm to determine the mechanism of drug distribution in lipid-core nanocapsule formulations. Soft Matter, v. 9, n. 4, p. 1141-1150, 2013.

34- Savian AL, Rodrigues D, Weber J, et al. Dithranol-loaded lipid-core nanocapsules improve the

photostability and reduce the in vitro irritation potential of this drug. Mater Sci Eng C Mater Biol Appl. 2015;46:69-76. doi: 10.1016/j.msec.2014.10.011.

35- Wu L, Zhang J, Watanabe W. Physical and chemical stability of drug nanoparticles. Adv Drug Deliv Rev. 2011;63(6):456-469. doi:10.1016/j.addr.2011.02.001.

36- Da Silva, M. M; Nora, L.; Flores Cantillano, R. F.; Paese, K.; Guterres, S. S.; Pohlmann AR., Costa TMH., Rios A De O. The Production, Characterization, and the Stability of Carotenoids Loaded in Lipid-Core Nanocapsules. Food and Bioprocess Technology**.** v.9, p.1148–1158, 2016.

37- Kishore RSK, Kiese S, Fischer S, Pappenberger A, Grauschopf U, Mahler H. The Degradation of Polysorbates 20 and 80 and its Potential Impact on the Stability of Biotherapeutics. Pharmaceutical Research v.28, p.1194–1210, 2011.

38- Can E, Udenir G, Kanneci AI, Kose G, Bucak S. Investigation of PLLA/PCL blends and paclitaxel release profiles. AAPS PharmSciTech. 2011;12(4):1442-1453. doi:10.1208/s12249-011-9714-y.

39- Larson NR, Wei Y, Prajapati I, et al. Comparison of Polysorbate 80 Hydrolysis and Oxidation on the Aggregation of a Monoclonal Antibody. J Pharm Sci. 2020;109(1):633-639. doi:10.1016/j.xphs.2019.10.069.

40- Casarini TPA, Frank LA, Benin T, Onzi G, Pohlmann AR, Guterres SS. Innovative hydrogel containing polymeric nanocapsules loaded with phloretin: Enhanced skin penetration and adhesion. Mater Sci Eng C Mater Biol Appl. 2021;120:111681. doi:10.1016/j.msec.2020.111681.

41- Abouelmagd SA, Sun B, Chang AC, Ku YJ, Yeo Y. Release kinetics study of poorly water-soluble drugs from nanoparticles: are we doing it right?. Mol Pharm. 2015;12(3):997-1003. doi:10.1021/mp500817h.