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Efavirenz-loaded polymeric nanocapsules: Formulation, development, and validation of an RP-UHPLC-DAD method for drug quantification, determination of encapsulation efficiency, stability study, and dissolution profile

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ABSTRACT

This study aims at preparing efavirenz (EFV)-loaded polymeric nanocapsules and carrying out the analytical method development and validation in order to provide a suitable tool for evaluating these formulations in terms of encapsulation efficiency, stability, and dissolution profile. The nanoformulations were obtained by the interfacial deposition of preformed polymer(s). The analytical method was specific, linear (r^2 = 0.9990), precise, accurate, and robust from 1.0 to 50.0 µg/ml and demonstrated a drug retention time of 1.6 minutes. The mean encapsulated drug content was higher than 99.0%. All formulations showed stability problems at room temperature since the values of pH, particle size, and polydispersity index increased, while the zeta potential intensified its negative value after 60 days of storage. However, the storage in the refrigerator was able to prevent this process in most of the investigated formulations. Concerning the drug loading, all EFV-loaded nanocapsules based on poly(ε-caprolactone) and [poly(ethylene glycol) 6000] were statistically stable after 60 days of storage. The nanoencapsulation was responsible for prolonging the drug release for both EFV-loaded formulations by anomalous transport.

INTRODUCTION

Polymeric nanocapsules have been recognized as promising drug carrier systems and the use of efavirenz (EFV)-loaded delivery systems is a suitable strategy for the treatment of acquired immunodeficiency syndrome, as these formulations can improve the dissolution profile, prolong the pharmacological effect, lead to a decrease in dosage, and, consequently, reduce side effects (Dimer *et al*., 2020; Lyra *et al*., 2017; Patel *et al*., 2013; Varshosaz *et al*., 2018). Nevertheless, the development of the nanodrug delivery systems requires previous and mandatory validation of an analytical method in order to provide an accurate determination of the drug loaded into the nanoformulation during the different stages of the pharmaceutical R&D process.

This validation process, according to international standards (ICH, 2005), involves several parameters for providing documented evidence that the method does what it is intended to do. In that sense, the advanced search carried out in indexed databases (Science Direct and Google Scholar) on May 10, 2020, using English language and title mode, was not reported considering the keywords "validation" and Ultra High-Performance Liquid Chromatography "UHPLC" (or as Waters calls it, "ultra performance liquid chromatography") and "EFV" and "nanoparticles". Concerning other pharmaceutical dosage forms, the literature shows only restricted data regarding the validation of UHPLC methods for quantifying EFV.

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Induri *et al*. (2016) validated a UHPLC-photodiode array detector (DAD) method for simultaneous quantification of EFV and lamivudine in tablets using a mobile phase containing 10 mM phosphate buffer (pH = 4.0) and 10.0% acetonitrile in methanol. Sravanthi and Madhavi (2020) provided an analytical study concerning the validation of stability indicating the UHPLC-DAD procedure to determine emtricitabine, tenofovir, and EFV in tablets by using a mobile phase containing 0.01 N potassium dihydrogen phosphate buffer (pH = 4.5) and acetonitrile. Dos Santos Martins *et al*. (2020) reported a validation method for EFV determination in plasma by UHPLC-DAD. This method involved the addition of the plasma precipitation using acetonitrile and solvent evaporation. Methanol, acetonitrile, and 0.1 M formic acid were used as the mobile phase. Huang *et al*. (2011) used a UHPLC-mean squares (MS)/MS method for the determination of ritonavir, indinavir, atazanavir, and EFV in serum and tissues of mice. The formulations were obtained by wet-milling. The method was carried out using a gradient mobile phase containing 5.0% acetonitrile in methanol and 7.5 mM ammonium acetate (pH = 4.0). Acetonitrile was used as part of the mobile phase in these three first studies and this solvent has a higher financial cost than methanol (Gomes *et al*., 2015). In addition, the last paper employed tandem mass spectrometry for drug quantification, which is less available in routine analysis. Therefore, no paper is provided by literature regarding the validation of a UHPLC method to determine EFV in polymeric nanocapsules by a fast and simple UHPLC method.

Moreover, the method validation is essential to provide a suitable tool for further analyses concerning the encapsulation efficiency (EE) determination, the drug quantification during stability testing, and the dissolution studies. These R&D stages can be only carried out if a validated method is available (Camargo *et al*., 2020). Furthermore, the literature shows that the Brownian movement of the nanoparticles avoids sedimentation and flotation process (Sari *et al*., 2017). However, the colloidal suspensions can demonstrate nanoparticle aggregation over time (Carletto *et al*., 2016). Consequently, the physicochemical stability studies are also critical for expanding the knowledge about nanosystem stability (Schaffazick *et al*., 2003). In that sense, to the best of our knowledge, this is the first report about the stability of polymeric nanoformulations containing EFV.Taking all these into account, this study aimed to obtain EFV-loaded nanocapsules based on poly(ε-caprolactone) (PCL) and [poly(ethylene glycol) 6000] (PEG) and to describe the validation of a rapid UHPLC assay for the EFV determination in these formulations. The proposed method was developed to serve different purposes as EE determination, stability testing, and dissolution study in order to facilitate the pharmaceutical R&D routine.

MATERIALS AND METHODS

Materials

Efavirenz (EFV, 98.0% pure), PEG (M_w = 5,400–6,600 g/mol), and PCL (M_w =10,000–14,000 g/mol) were purchased from Sigma-Aldrich (St. Louis, MO). high performance liquid chromatography (HPLC)-grade methanol was provided by ApplyChem Panreac Química (Barcelona, Spain). Milli-Q Plus water purification system (Millipore, Bedford, MA) was used for collecting purified water. The other reagents and solvents were of analytical grade.

Equipment

Experiments were carried out in a Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan), equipped with degasser (DGU-20A5RA), quaternary pump (LC-30AD), thermostatic column compartment (CTO-20AC), automatic sampler (SIL-30 AC), and a photodiode array detector (DAD) (SPD-M20A). The acquisition and processing of the data were obtained with the LabSolutions® Software version 5.73 (Shimadzu, Kyoto, Japan).

Preparation of stock and sample solutions

Stock solution containing EFV was obtained in methanol:water acidified with 0.15% acetic acid (87:13, *v/v*) at 500.0 µg/ml. Dilutions were carried out in order to obtain sample solutions with a concentration between 1.0 and 50.0 µg/ml, where the concentration of 15.0 µg/ml was defined as 100.0%. The sample solutions were filtered before injection using a polytetrafluoroethylene filter (Cromafil® Xtra, 0.2 µm × 13 mm, Macherey-Nagel, Düren, Germany).

Preparation of the polymeric nanocapsules

Nanocapsule suspensions were prepared in triplicate from PCL or PCL-PEG blend (3:1) by the interfacial deposition procedure using preformed polymer(s) as previously reported (Fessi *et al*., 1989; Rudnik *et al*., 2020) and depicted in Table 1. Briefly, the polymer or polymeric blend was solvated in acetone with sorbitan monooleate 80 (Span® 80), EFV, and triglycerides of capric/caprylic acids [middle chain triglycerides (MCT)]. This phase was dripped into the aqueous medium containing polysorbate 80 (Tween® 80) and water under vigorous magnetic stirring (Fisaton, 754A model, São Paulo, Brazil) at 40°C. The magnetic stirring was kept for 10 minutes and then the rotary evaporation (Fisaton, 803 model, São Paulo, Brazil) was carried out in order to obtain a final volume of 10 ml and a theoretical drug loading of 5 mg/ml. PCL-0 and PCL-PEG-0, suspension of nanoparticles with no EFV, were also obtained.

Table 1. Composition of polymeric nanocapsules.

Formulation	Organic phase						Aqueous phase	
	EFV (mg)	PCL (mg)	PEG (mg)	MCT (mg)	Acetone (ml)	Span® 80 (mg)	Tween® 80 (mg)	Water (ml)
PCL-0	–	100	0	300	27	77	77	53
PCL-EFV	50	100	0	300	27	77	77	53
PCL-PEG-0	–	75	25	300	27	77	77	53
PCL-PEG-EFV	50	75	25	300	27	77	77	53

UHPLC-DAD method development

Several chromatographic conditions were tested and defined by the authors during the method development. Different compositions, pH, and flow rates of the mobile phase were tested, as well as column temperature and detection wavelength.

UHPLC-DAD method validation

A C18 Shim-pack XR-ODS III reverse phase column (200 mm × 2 mm with a particle size of 2.2 µm, Shimadzu, Kyoto, Japan) coupled to the precolumn (C18 Shim-pack GVP-ODS, 10 mm × 4.6 mm, Shimadzu, Kyoto, Japan) was used for the method validation procedure. The injection volume was 5 μl and the elution was carried out in isocratic mode using methanol:water acidified with 0.15% acetic acid (87:13, *v/v*) as mobile phase with a flow of 0.500 ml/minutes at 40°C. The running time was 2 minutes and the wavelength chosen for EFV determination was 247 nm.

The analytical validation was carried out considering the guidelines published by the ICH (2005). Specificity, linearity, precision, accuracy, detection and quantification limits, and robustness were the assessed parameters.

The chromatograms of the EFV-loaded nanocapsules were compared to those obtained for formulations with no drug in order to investigate the method's specificity. This analysis is critical to confirm that no component of the formulation interferes with the quantification of EFV.

The linearity was assessed by obtaining and analyzing three analytical curves. For this, seven concentrations levels of EFV were obtained from dilutions of the standard solution in the mobile phase (1.0, 5.0, 10.0, 15.0, 20.0, 25.0, and 50.0 µg.ml^{-1}). The analysis of linear regression was carried out using the least squares method and the slope was tested by analysis of variance (ANOVA) at a significance level of 0.05.

The slope (S) and the standard deviation (SD) of the response of three analytical curves were used for determining the limits of detection (LOD) and quantification (LOQ) according to equations (1) and (2), respectively:

$$LOD = \left(\frac{3.3 \times SD}{S}\right), \quad \text{(Eq. 1)}$$

$$LOQ = \left(\frac{10 \times SD}{S}\right), \quad \text{(Eq. 2)}$$

Repeatability and intermediate precision were used for testing the method precision. Repeatability was achieved at the concentration theoretically defined as 100.0% (15 µg/ml) in sextuplicate. Intermediate precision was investigated in triplicate for three different concentrations at 3.0, 17.0, and 35.0 µg/ml. Repeatability and intermediate precision were assessed by SD and relative standard deviation (RSD) and analyzed intraday, inter-day, and with different analysts.

The recovery analysis in triplicate was used for determining the method's accuracy. The amount of 10 µg was added to the sample solutions containing 3.0, 17.0, and 35.0 µg/ml. The accuracy (%) was calculated using:

$$\%\ Accuracy = \left(\frac{CFS - CNFS}{TCA}\right) \times 100, \quad \text{(Eq. 3)}$$

where CFS denotes the concentration of the experimentally fortified sample; CNFS denotes the concentration of the experimentally non-fortified sample; and TCA denotes the theoretical concentration added.

The robustness was determined in the samples at 15.0 µg/ml by providing variations in the flow rate (0.495 and 0.505 ml/minutes) and mobile phase [methanol:water acidified with 0.15% acetic acid 86:14 (*v/v*) and 88:12 (*v/v*)]. RSD values were used to study the effect of these changes on the standard chromatographic conditions.

Method applicability

Determination of encapsulation efficiency

The loaded drug in PCL-EFV and PCL-PEG-EFV nanocapsules was indirectly determined in triplicate. Each nanosuspension (500 µl) was submitted to ultrafiltration in an Amicon® device (M_w cutoff = 10,000 g.mol^{-1}, Merck Millipore, Bedford, MA). EFV was quantified in ultrafiltrate by the aforementioned UHPLC method. Considering the EFV loading, the EE was obtained by.

$$EE(\%) = \frac{theoretical\ drug\ loading - free\ drug\ content}{theoretical\ drug\ loading} \times 100. \quad \text{(Eq. 4)}$$

Stability testing

The nanosuspensions were maintained at 25°C ± 2°C (room temperature) and 6°C ± 2°C in amber glass bottles protected from light. The nanoformulations were checked at 30 and 60 days of storage. A digital potentiometer (Hanna, HI 2221 model, São Paulo, Brazil) was used for pH determination after the previous calibration. Analyses on the equipment Zetasizer Nanoseries (Malvern Instruments, NANO ZS 90 model, Malvern, UK) were carried out for the particle size determination, the polydispersity index (PDI) evaluation, and the zeta potential measurement. These assays were carried out after dilution at 1:500 using ultrapure water. The drug loading was investigated by the aforementioned UHPLC-DAD method to obtain the EE within 30 and 60 days of preparation. The samples were assayed in triplicate.

In vitro dissolution experiments

Free EFV and nanoformulations PCL-EFV and PCL-PEG-EFV were submitted to the dialysis method in triplicate for investigating the drug release (Gaur *et al*., 2014; Gomes *et al*., 2019). The bag was firstly soaked in purified water before use for 12 hours. Each sample (2 ml) was inserted into a dialysis bag (Spectra/Por® molecular porous membrane tubing, molecular weight cut-off 10,000, Spectrum Laboratories, Rancho Dominguez, CA) and the dissolution experiments were carried out in phosphate-buffered saline (PBS, 400 ml, 50 mmol/l) containing 0.1% (*w/v*) Tween® 80 at pH 7.4. This apparatus was kept at 37°C and stirred at 50 rpm for 72 hours. At predetermined time intervals, aliquots of 2 ml were collected and immediately replaced by PBS. EFV concentration was quantified using the previously validated UHPLC-DAD method. The dissolution profiles were fitted to the Korsmeyer-Peppas model for obtaining insights about the drug release mechanism (Gomes *et al*., 2019; Korsmeyer and Peppas, 1983).

Statistical analysis

Data were presented as mean ± SD. The simple linear regression was used for evaluating the linearity data. RSD was reported as mandatory. The Student's *t*-test or ANOVA with Tukey's *post-hoc* test was used for experiments involving validation, physicochemical, and dissolution data. Statistical power at a significance level of 5.0% (α = 0.05) was previously defined. Statistical analysis was carried out using the GraphPad Prism software version 5.03 (San Diego, CA). The MicroMath Scientist™ 2.01 software (Salt Lake City, UT) was used for studying the Korsmeyer-Peppas data.

RESULTS

Preparation of polymeric nanocapsules

The polymeric nanocapsules in suspension presented a milky and opalescent aspect with a bluish reflection related to the Brownian movement of the colloidal structures (Tyndall effect) as previously described (Schaffazick *et al*., 2003; Mora-Huertas *et al*., 2010).

UHPLC-DAD method development

The UHPLC-DAD was proposed for EFV quantification; due to this, the separation method is increasing compared with the HPLC. In general, UHPLC reduces the sample volume and the particle size (< 2 µm), shows faster analysis time, increases separation efficiency due to the high pressure used (> 350 bar), and decreases the waste amount (Mukherjee, 2019).

After the run using an exploratory gradient, the methanol:water acidified with 0.15% acetic acid in the ratio 87:13 (*v/v*) as mobile phase, the flow rate of 0.500 ml/minutes at 40°C was chosen as the standard conditions for the proposed method. The maximum absorption wavelength was set by the DAD detector at 247 nm. The running time and retention time for EFV were 2.0 and 1.6 minutes, respectively. This condition is suitable for different stages of the pharmaceutical R&D process (Nadal *et al*., 2015).

The method was developed using methanol (87.0%), which is a cheaper solvent than acetonitrile and more available (Lyra *et al*., 2017). The short analysis time (2.0 minutes) and retention time (1.6 minutes) were very suitable for the pharmaceutical industry routine.

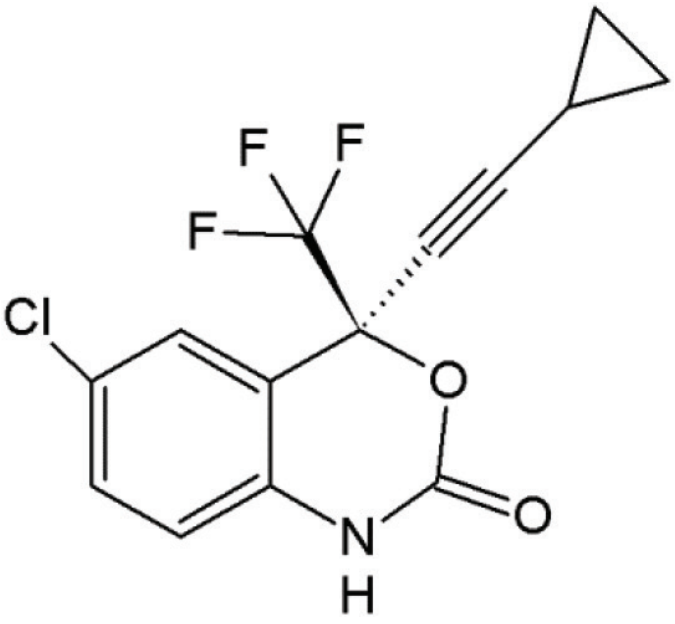

Figure 1. Chemical structure of efavirenz (C14H9ClF3NO2, MW = 315.675 g/mol).

UHPLC-DAD method validation

Considering the previously described conditions, the UHPLC-DAD procedure was then validated by evaluating the performance features according to ICH guidelines (ICH, 2005).

Specificity

Specificity was confirmed when the chromatograms of EFV-loaded and nonloaded formulations were compared (Fig. 2). In this regard, no interference at the area under the curve of EFV and the retention time from the other components used in nanoformulation preparation (Rudnik *et al*., 2020), as well as possible impurities present in EFV raw material (Gaspar *et al*., 2020), was observed.

Linearity

The linearity of the UHPLC-DAD method is shown in Figure 3. Peak area and concentration of EFV revealed that a linear relationship at seven concentration levels from 1.0 to 50.0 µg/ml was recorded. The least-square procedure resulted in the following linear equation: *y* (peak area) = 34,345 × (concentration at µg/ml) + 16,836 (*n* = 3). An appropriate correlation coefficient (*r* = 0.9995) was obtained. The literature reports that linearity is usually obtained when *r* is near 1 (Gomes *et al*., 2015). However, a more rigorous test must be used in order to avoid that the results were obtained due to random chance and to confirm that there was no error due to the lack of fit (Nadal *et al*., 2015). Hence, the linearity data were submitted to the ANOVA and the data are shown in Table 2. *F*-value was lower than *F*-tabulated for the lack of fit at 95.0% confidence interval (α = 0.05). Consequently, no lacking of fit was demonstrated and the linear regression was established.

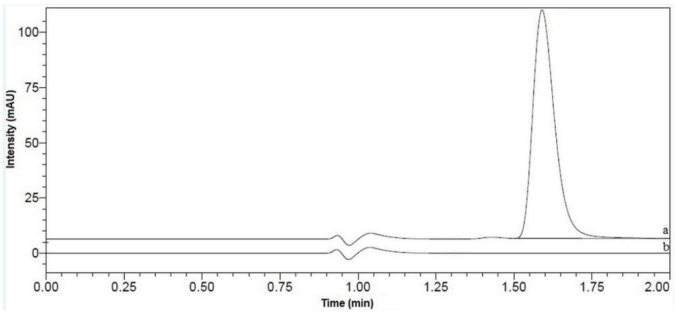

Figure 2. Representative UHPLC chromatograms obtained from unloaded and EFV-loaded nanocapsules: (a) standard EFV solution at 15.0 µg/ml, and (b) non-loaded (PCL-0). Chromatographic conditions = mobile phase: methanol:water acidified with 0.15% acetic acid; flow rate: 0.500 ml/minute; UV detection wavelength = 247 nm; column temperature = 40°C ± 2°C; and run time = 2 minutes.

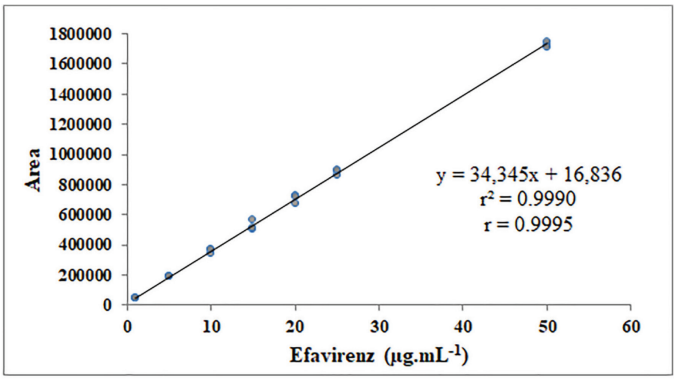

Figure 3. Mean calibration curve obtained for EFV using working standard solutions in the concentration range of 1.0–50.0 µg/ml ($n = 3$) ($\lambda = 247$ nm).

Table 2. ANOVA results for linearity test.

EFV	SS	Df	MS	*F*	*F*tab
Model	5.6462×10^{12}	1	5.6462×10^{12}	15,019.53	2.990
Residual	7.1426×10^{9}	19	3.7593×10^{8}	Linear	
Lack of fit	488,388,051	5	97,677,610	0.2055	2.307
Pure error	6.6542×10^{9}	14	475,301,957	No lack of fit	

SS = sums of squares; Df = degrees of freedom; MS = mean squares; *F* = *F*-value of the test; *F*tab = fixed *F*-value.

Limits of detection and quantification

LOD is the lowest point on the calibration curve that can be detected, while the LOQ is the lowest point that can be accurately and reproducibly quantified (Almeida *et al*., 2018). The LOD and LOQ values were 0.30 and 0.90 µg/ml, respectively. Therefore, an appropriate sensitivity was achieved since these parameters were lower than 1.0 µg/ml, which was the EFV minimum concentration of the calibration curve.

Precision

The precision represents the proximity of individual measurements of a substance when the technique is used repeatedly to multiple samples (ICH, 2005). Repeatability depicts the precision under the same operating conditions over a short period of time and intermediate precision represents inter-day variations which with different analysts were evaluated. The results of repeatability and intermediate precision are described in Table 3. All results were lower than 5.0%, which denote that the analytical method reached the precision requirements (Lopes *et al*., 2017).

Accuracy

The recovery test was used for studying the accuracy requirements. According to Table 4, the mean recoveries were near 100.0% and showed an RSD lower than 5.0%. These data were in accordance with the limits recommended by ICH (2005).

Robustness

The robustness represents the effect that small changes in the analytical parameters can provide on the method's reliability (Lyra *et al*., 2017). No significant difference ($p > 0.05$) was achieved for the retention time of EFV and the area under curve after variations in flow rate and mobile phase composition. The flow rate was changed to 0.495 and 0.505 ml/minutes and resulted in RSD of 1.98% and 2.22%, respectively. The mobile phase proportion was altered to 86:14 and 88:12 and led to RSD values of 2.09% and 2.25%, respectively. In that sense, the RSD data after robustness experiments were not above 5.0%. Thus, the developed UHPLC method can be considered robust according to the ICH guidelines (Cartagena-Molina *et al*., 2016).

Table 3. Repeatability and intermediate precision data for efavirenz analysis using loaded nanocapsules.

	Theoretical concentration (µg/ml)	Experimental concentration (µg/ml, Mean ± SD[a])	RSD[b] (%)
Repeatability			
n = 6	15	15.04 ± 0.57	3.81
n = 3	3	3.13 ± 0.02	0.70
n = 3	17	16.99 ± 0.72	4.28
n = 3	35	34.16 ± 0.37	1.07
Intermediate precision			
Intraday			
n = 6	15	15.14 ± 0.34	2.22
n = 3	3	3.13 ± 0.02	0.48
n = 3	17	17.08 ± 0.64	3.74
n = 3	35	34.64 ± 1.37	3.96
Inter-day			
n = 6	15	15.06 ± 0.68	3.00
n = 3	3	3.11 ± 0.04	1.18
n = 3	17	16.91 ± 0.66	3.91
n = 3	35	34.13 ± 0.36	1.06
Different analysts			
n = 6	15	14.73 ± 0.31	2.16
n = 3	3	3.12 ± 0.08	2.63
n = 3	17	17.13 ± 0.54	3.16
n = 3	35	34.53 ± 1.32	3.83

[a]SD = standard deviation.
[b]RSD = relative standard deviation.

Table 4. Accuracy analysis carried out by the recovery method at three different concentration levels.

Final theoretical concentration (µg/ml)	Accuracy (% ± SD[a])	RSD[b] (%)
13	100.44 ± 1.17	1.17
27	98.64 ± 0.84	0.85
45	98.32 ± 4.22	4.29

[a]SD = standard deviation.
[b]RSD = relative standard deviation.

Method applicability

Evaluation of encapsulation efficiency

The aforementioned UHPLC-DAD analytical method was applied for quantifying the EFV loading and for determining the EE of EFV in PCL and PEG-PCL nanocapsules. The EE of these nanocapsules was carried out by the validated method and the achieved data are indicated in Table 5. The formulations showed suitable EE values near 100.0% with low SD and RSD

Table 5. Efavirenz-loaded and EE for nanocapsules suspensions (n = 3).

Formulation (5,000 µg/ml)	Efavirenz-loaded (µg/ml; mean ± SD[a])	EE	
		% ± SD[a]	RSD[b]
PCL-EFV	4,969.50 ± 0.46	99.39 ± 0.01	0.01
PCL-PEG-EFV	4,970.47 ± 0.04	99.41 ± 0.01	0.01

[a]SD = standard deviation.
[b]RSD = relative standard deviation.

Table 6. Physicochemical properties of the colloidal suspensions of non-loaded and loaded nanocapsules immediately after preparation.

Formulation	pH	Particle size (nm)	PDI	Zeta potential (mV)
PCL-0	6.20 ± 0.06	245.17 ± 9.21	0.28 ± 0.01	−37.70 ± 2.35
PCL-EFV	6.00 ± 0.06	241.93 ± 9.23	0.27 ± 0.02	−37.97 ± 2.20
PCL-PEG-0	6.10 ± 0.10	271.40 ± 4.60	0.20 ± 0.04	−34.53 ± 2.70
PCL-PEG-EFV	6.10 ± 0.12	282.60 ± 4.57	0.27 ± 0.04	−36.57 ± 1.78

Legend: mean (n = 3) ± standard deviation.

values. Regarding these results, a low drug amount was lost during nanoencapsulation since encapsulation efficiencies very close to 100% were verified. These findings may be related to the low solubility of EFV in water (4 µg/ml at 20°C) (Kamble *et al*., 2016; Makoni *et al*., 2019), which resulted in low drug partition to the external water phase.

Considering the EE, similar results were obtained comparing to other studies for EFV. EFV loaded in PCL nanoparticles produced by double emulsion/spray-drying showed an EE higher than 86.0% (Tshweu *et al*., 2014). EE values superior to 94.0% were obtained for submicron particles composed of PCL, Eudragit® RS100, and blends prepared by two techniques: emulsion/solvent diffusion/evaporation and nanoprecipitation (Seremeta *et al*., 2013). Therefore, the results of this study are consistent with literature data, and the validated method was successfully used for the investigation of drug loading and EE of nanoformulations containing EFV.

Stability testing

The formulations were submitted to the stability testing in amber flasks, protected from light, and during the 60-day period at 6°C ± 2°C and 25°C ± 2°C. At the end of the experiment, all samples maintained their initial appearance, with no color change, signs of aggregation, or phase separation (sedimentation/flotation). Table 6 summarizes the data obtained for the pH determination, the particle size measurement, the PDI analysis, and the zeta potential quantification immediately after the nanoencapsulation procedure.

The chosen method for preparing polymeric nanocapsules typically provides particle diameters from 200 to 300 nm and PDI values between 0.2 and 0.3 (Ferreira *et al*., 2018). However, the presence of PEG can lead to higher diameters for these nanocapsules (Aditya *et al*., 2014). Besides, negative zeta potential values are usually observed for nanosystems based on PCL because this polyester shows carboxylic acid groups of anionic nature (Schaffazick *et al*., 2003). The PEG chains can be also responsible for reducing the electrical potential of such pegylated formulations. Taking all these considerations into account, the investigated physicochemical parameters were in agreement with those previously reported for polymeric nanocapsules (Gomes *et al*., 2019).

However, pH, mean diameter, and zeta potential values were statistically similar between the nonloaded and EFV-loaded polymeric nanocapsules at the initial time ($p > 0.05$). A statistically significant difference was only observed for PDI values when PLC-0 and PCL-PEG-0 were compared immediately after preparation. The literature demonstrated that PEG increases the viscosity of the organic phase and restricts its dispersion in the aqueous medium during stirring and leads to broader polydispersity (Aditya *et al*., 2014).

Figure 4 shows the results verified for pH, particle size, PDI, and zeta potential during the stability testing. These data were obtained at different moments in time: 0, 30, and 60 days of storage. At the end of the experiments, all formulations showed a statistically significant decrease in the pH values at room temperature. On the other hand, the storage in the refrigerator was able to prevent this process in most of the evaluated formulations.

The pH reduction during the stability testing can be associated with three different reasons: (a) ester hydrolysis of Tween® 80 and oleic acid release (Larson *et al*., 2020); (b) PCL hydrolysis and carboxylic acid group release from PCL oligomers or monomers (Zanetti *et al*., 2019); and (c) MCT release from the oily core and its hydrolysis to obtain free fat acids (Külkamp *et al*., 2009). Considering the storage in the refrigerator, the low temperatures decreased the kinetics of these hydrolysis reactions.

Concerning the particle size and the PDI, most of the samples were statistically stable after 30 days of storage. However, all samples showed a statistical increase in these parameters after 60 days when stored at room temperature. These results confirm that aggregation can occur over time, which affects the physical stability of nanosuspensions containing EFV. In addition, the refrigerator storage was not consistent in preventing these physical changes. In that sense, EFV-loaded nanocapsule suspensions are recommended for extemporaneous use since they demonstrated no long-term physical stability.

The zeta potential was significantly intensified for nanoformulations after 60 days at room temperature. As aforementioned, PCL presents carboxylic acid functional groups in its polymeric chain, which provide a negative surface potential to the polymeric nanocapsules (Schaffazick *et al*., 2003).

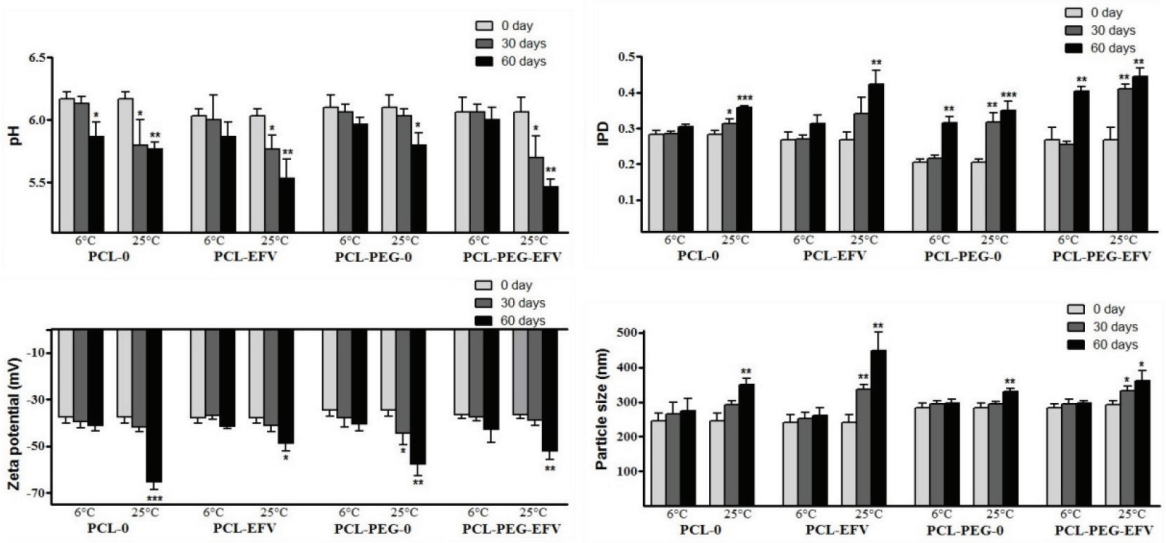

Figure 4. Particle size, pH, PDI, and zeta potential of non-loaded (PCL-0) and EFV-loaded nanocapsules (PCL-EFV and PCL-PEG-EFV), immediately after preparation and after 60 days of storage. The symbol ** represents a significant difference in relation to the initial time obtained by the Student's *t*-test with Tukey's *post-hoc* test (** $p < 0.01$).

Table 7. Concentration of efavirenz-loaded and EE ($n = 3$) of nanoformulations submitted to the stability test, after 60 days.

Formulation (5,000 µg/ml)		Efavirenz-loaded (µg/ml; Mean ± SD[a]) % ± SD[a]	EE	
			RSD[b]	
PCL-EFV	8°C	4,968.44 ± 0.75	99.37 ± 0.02	0.02
	25°C	4,961.46 ± 0.99	99.23 ± 0.02	0.02
PCL-PEG-EFV	8°C	4,969.97 ± 0.63	99.40 ± 0.01	0.01
	25°C	4,959.83 ± 1.48	99.20 ± 0.03	0.03

[a]SD = standard deviation.
[b]RSD = relative standard deviation.

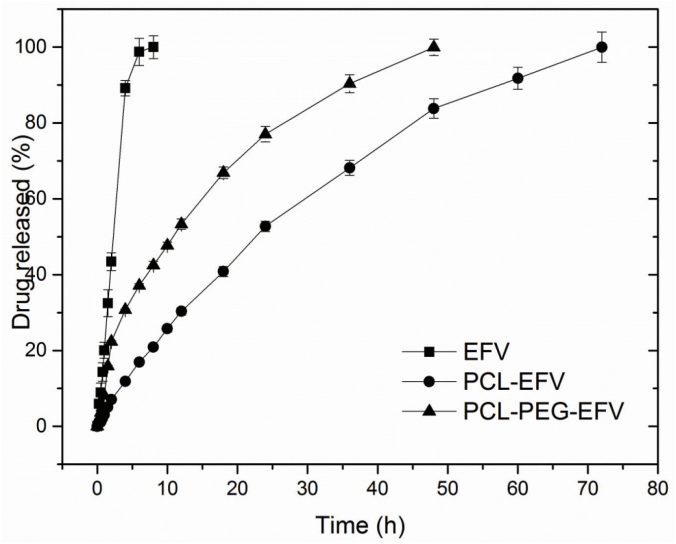

Figure 5. *In vitro* release profiles for free drug and EFV-loaded polymeric nanocapsules based on PCL and PCL-PEG.

However, hydrolysis reactions occurred during the storage time mainly at room temperature as demonstrated for pH. Therefore, it is appropriate to affirm that the zeta potential value should also enhance since the number of functional groups available was increased. This hypothesis was experimentally confirmed and the hydrolysis reactions had an impact on both pH and zeta potential values.

Regarding the drug loading and EE, Table 7 summarizes the drug quantification values obtained after 60 days of storage. These data were very similar to those achieved immediately after preparation. These data demonstrate that nanocapsules containing an oil core represent a suitable reservoir system for EFV. In spite of the aforementioned degradation of the polymeric shell by hydrolysis, the oil core was able to maintain the loaded drug. Concerning the method applicability, the validated UHPLC-DAD method was a suitable analytical tool to quantify EFV in nanocapsule suspensions even after 60 days of storage.

In vitro *dissolution experiments*

The dissolution profiles for free drug (EFV) and polymeric nanocapsule suspensions (PCL-EFV and PCL-PEG-EFV) obtained by dialysis are shown in Figure 5.

Free EFV showed a faster drug release pattern in PBS and demonstrated a mean drug-release value of 80.0% at 218 minutes (3.6 hours). PCL-PEG-EFV released 80.0% of EFV at 1,680 minutes (28 hours). PCL-EFV released 80% of EFV at 2,700 minutes (45 hours). Therefore, both loaded nanocapsules

had prolonged dissolution without burst effect compared to EFV. PEGylation provides an amphiphilic shell when associated with usual polyester (Deng *et al*., 2020). This amphiphilic character of the PCL-PEG shell may be responsible for improving the dissolution properties of EFV from these polymeric nanocapsules since it may increase the water penetration and hence the dissolution and the diffusion (Fattahi *et al*., 2018). A prolonged EFV release was also reported in the literature using solid lipid nanoparticles, in which 60.61% to 98.22% drug release was achieved in 24 hours by dialysis method (Gaur *et al*., 2014).

The Korsmeyer-Peppas model was used for investigating the drug release mechanism. Free EFV and EFV-loaded polymeric nanocapsules (PCL-EFV and PCL-PEG-EFV) presented *n* values of 1.10, 0.79, and 0.59, respectively. The free EFV showed a drug release mechanism based on the super case-II transport, which resulted from the entrance of the dissolution medium and its interaction with the drug crystals. This solvent caused the dissolution of drug molecules from the crystal surface and promoted the erosion of its crystalline structure. However, the polymeric nanocapsules showed *n* values between 0.43 and 0.85, which represents a drug release mechanism governed by anomalous transport. In this situation, the mechanism is associated with the superposition of the Fickian diffusion and the nanocapsule erosion by polymer(s) relaxation/degradation (Farago *et al*., 2008).

The current method validation represents an alternative method in the laboratory routine of pharmaceutical industries, particularly those devoted to the development and production of antiretroviral nanoformulations. Further studies can be carried out to investigate the pharmacokinetics of EFV-loaded polymeric nanocapsules.

CONCLUSION

The interfacial deposition of the preformed polymer(s) was suitably used for obtaining polymeric nanocapsules containing EFV. The UHPLC-DAD method was then validated for determining EFV in the PCL and PCL-PEG nanoformulations. This method proved to be simple, specific, linear, sensitive, precise, accurate, and robust, with well-defined drug peaks and resolutions. EE close to 100.0% was verified for both EFV-loaded formulations based on PCL and PCL-PEG. The storage in the refrigerator was responsible for preventing the physicochemical instability in most of the evaluated nanoformulations. The drug-loading remained unchanged after 60 days of storage. The nanoencapsulation prolonged the drug release for these nanocapsules by anomalous transport.

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AUTHORS' CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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This study does not involve experiments on animals or human subjects.

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