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Validated spectrophotometric and spectrofluorimetric methods for determination of chloroaluminum phthalocyanine in nanocarriers

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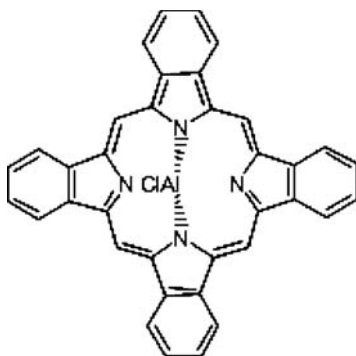
UV-VIS-Spectrophotometric and spectrofluorimetric methods have been developed and validated allowing the quantification of chloroaluminum phthalocyanine (CIAIPc) in nanocarriers. In order to validate the methods, the linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and selectivity were examined according to USP 30 and ICH guidelines. Linearities range were found between 0.50–3.00 $\mu\text{g}\cdot\text{mL}^{-1}$ ($Y = 0.3829 X [\text{CIAIPc}, \mu\text{g}\cdot\text{mL}^{-1}] + 0.0126$; $r = 0.9992$) for spectrophotometry, and 0.05–1.00 $\mu\text{g}\cdot\text{mL}^{-1}$ ($Y = 2.24 \times 10^6 X [\text{CIAIPc}, \mu\text{g}\cdot\text{mL}^{-1}] + 9.74 \times 10^4$; $r = 0.9978$) for spectrofluorimetry. In addition, ANOVA and Lack-of-fit tests demonstrated that the regression equations were statistically significant ($p < 0.05$), and the resulting linear model is fully adequate for both analytical methods. The LOD values were 0.09 and 0.01 $\mu\text{g}\cdot\text{mL}^{-1}$, while the LOQ were 0.27 and 0.04 $\mu\text{g}\cdot\text{mL}^{-1}$ for spectrophotometric and spectrofluorimetric methods, respectively. Repeatability and intermediate precision for proposed methods showed relative standard deviation (RSD) between 0.58% to 4.80%. The percent recovery ranged from 98.9% to 102.7% for spectrophotometric analyses and from 94.2% to 101.2% for spectrofluorimetry. No interferences from common excipients were detected and both methods were considered specific. Therefore, the methods are accurate, precise, specific, and reproducible and hence can be applied for quantification of CIAIPc in nanoemulsions (NE) and nanocapsules (NC).

1. Introduction

Chloroaluminum phthalocyanine (CIAIPc) [chloro(29H,31H-phthalocyaninato)aluminum] is a tetrapyrrolic macrocycle that has nitrogen atoms linking the individual pyrrole units and a central metal ion (Al III) (Sharman et al. 1999). It belongs to the second generation of sensitizer molecules used in the photodynamic therapy (PDT) and other photodynamic processes (PDP). This therapy has been used for treatment of several oncological, dermatological (MacCormack 2008), and ophthalmic diseases. PDT is based on activation of the photosensitizer drug by visible light at a specific wavelength in the presence of

molecular oxygen (Dougherty et al. 1998; Nunes et al. 2004; Ficheux 2009).

The photodynamic reactions generate cytotoxic species such as the reactive oxygen species (ROS) $^1\text{O}_2$ (singlet oxygen), O_2^- , OH, H_2O_2 , as well as other reactive species, for example, CH_3 , NO_2 , $\text{R}\cdot\text{CO}_2$. All these cytotoxic species can cause cellular death by apoptosis and/or necrosis, leading to destruction or regression of tumor tissues (Ochsner 1997; Kessel 2006). PDP is an emergent field, in this area when the same principles of PDT were applied to bacteria, fungi, virus, and many others no-oncological diseases in dentistry, veterinary as well as environment research field. CIAIPc present the most favorable photophysical properties for application in PDT and PDP, i.e., relatively long-lived excited singlet and triplet states that are produced in high quantum yields allowing a complex pathway of energy transfer an electron to induce ROS production. In spite of this, the poor aqueous solubility of CIAIPc is the major obstacle for the assessment of pharmacological properties of this photosensitizer drug and for its use in therapy (Nunes et al. 2004). An approach for overcoming such inconvenience is the incorporation into colloidal drug carriers, such as nanoemulsions (NE) and nanocapsules (NC) (Konan et al. 2002; Chatterjee et al. 2008). NE consist of fine oil-in-water dispersions while NC are characterized by a central oily core surrounded by a thin polymeric wall, both having droplets or particles in the 100–600 nm range. In these nanocarriers, the poorly water-soluble drugs can be dissolved in the oil phase and/or adsorbed on the oil-water



CIAIPc

interface of the emulsion, or still adsorbed on polymeric membrane of the NC. Moreover, such a nanocarrier could change pharmacokinetic profile of drugs decreasing their toxicity and side effects (Soppimath et al. 2001; Bouchemal et al. 2004). NE and NC have been proposed for several administration routes, for instance, parenteral, oral, and topical route. Concerning topical application, these nanometric systems ensure close contact with stratum corneum due to their small size enhancing the penetration of drug through skin surface. In addition, nanocarriers are able to delay the release of their content, and therefore they allow to supply the skin with drug over a long period of time (Chen et al. 2006; Alves et al. 2007; Primo et al. 2008). In this context, the development of NE and NC containing CIAIPc has been studied by our research group for topical application against skin cancer, likewise in others areas as dentistry and tissue engineering (cicatrisation process).

A survey of the literature has not revealed any analytical method for determination of CIAIPc in pharmaceutical formulation, dissolution media or biological fluids. Therefore, the objective of the present study was to develop two simple, precise, accurate, validated, economic analytical methods, in accordance with International Conference on Harmonisation (ICH) and USP 30, for quantification of CIAIPc in NE and NC.

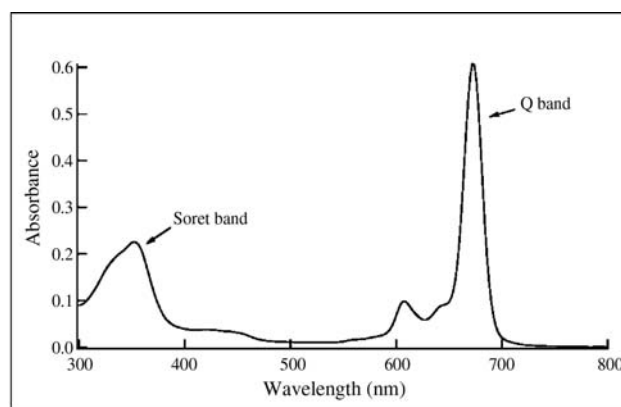
2. Investigations, results and discussion

2.1. Physicochemical characterization of nanocarriers

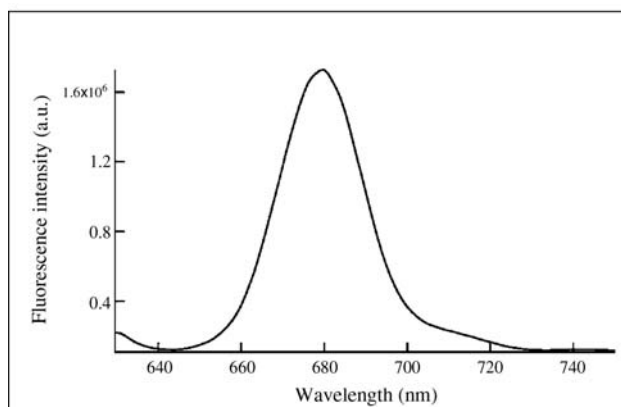
Physicochemical properties of colloidal suspensions were assessed immediately after preparation. The production methods used were able to produce NE and NC with mean size of 239 nm (± 8.7) and 219 nm (± 6.1), respectively. It was also observed that the drug encapsulation did not affect the mean size of droplet or particles since empty formulations exhibited a mean diameter of 200 nm (± 9.9) and 227 nm (± 9.0) for NE and NC, respectively. NE demonstrated a narrow PdI of 0.280 (± 0.060) and NC with a PdI of 0.248 (± 0.006), these results reveal the formation of monodisperse and homogeneous colloidal dispersions. Concerning zeta potential, NE and NC presented negative values ($-40.2 \text{ mV} \pm 4.06$ and $-46.9 \text{ mV} \pm 2.60$, respectively) due to negatively charged phospholipids as well as the carboxyl groups present at the end of PLGA chain of the NC (Mosqueira et al. 2000).

2.2. UV-VIS absorption and fluorescence emission

The solvent which demonstrated the best characteristics for this method was acetonitrile since it is able to dissolve all formulation components especially PLGA polymer as well as it did not present any interference on maximum absorption or fluorescence intensity of CIAIPc. The phthalocyanines absorption spectrum is formed by two major bands which are in the violet or ultraviolet region (300–350 nm) and in the visible light region (600–700 nm), and such bands are known as B-band (or Soret band) and Q-band, respectively (Tedesco et al. 2003). The macrocycle displays intense visible region absorption (the Q-band) and, with appropriate metals in the central cavity, good fluorescence emission as well. As it can be seen from Fig. 1a, the absorption spectrum of CIAIPc in acetonitrile shows the typical Soret and Q-bands characteristics of metallophthalocyanine with λ_{max} at 674 nm which was chosen as maximum absorption of analyte. Besides that, the fluorescence spectrum also demonstrated high fluorescence intensity with excitation wavelength (λ_{ex}) at 615 nm, and emission (λ_{em}) at 674 nm (Fig. 1b), as described in the literature due to high fluorescence quantum yields of phthalocyanine (Tedesco et al. 2003; Nunes et al. 2004).



(a)



(b)

Fig. 1: UV-VIS absorption (a) and Fluorescence (b) spectra of CIAIPc solutions in acetonitrile (concentrations: $1.50 \mu\text{g}\cdot\text{mL}^{-1}$ and $0.50 \mu\text{g}\cdot\text{mL}^{-1}$ for absorption and fluorescence, respectively)

2.3. Validation study

The linearity of an analytical method can be defined as the ability to obtain test results which are directly proportional to the concentration of the analyte. Linear regression analyses were carried out by plotting absorbance or relative fluorescence intensity versus CIAIPc concentration ($\mu\text{g}\cdot\text{mL}^{-1}$). Satisfactory linearity was detected for spectrophotometric and spectrofluorimetric methods in the $0.50\text{--}3.00 \mu\text{g}\cdot\text{mL}^{-1}$ (Fig. 2) and $0.05\text{--}1.00 \mu\text{g}\cdot\text{mL}^{-1}$ range (Fig. 3), respectively. Least square regression for spectrophotometric method showed excellent correlation coefficient ($r=0.9992$) and the straight-line equation was: $\text{absorbance} = 0.3829 \times [\text{CIAIPc, concentration at } \mu\text{g}\cdot\text{mL}^{-1}] + 0.0126$; while for spectrofluorimetry the correlation coefficient was of 0.9978, and linear regression equation: $\text{relative fluorescence intensity} = 2.24 \times 10^6 \times [\text{CIAIPc, concentration at } \mu\text{g}\cdot\text{mL}^{-1}] + 0.0126$.

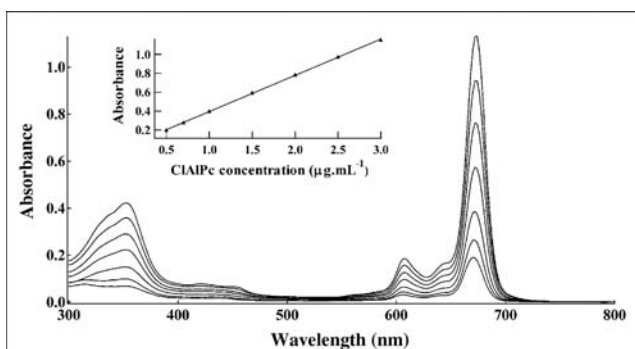


Fig. 2: Absorption spectra of CIAIPc at different concentrations ($0.50\text{--}3.00 \mu\text{g}\cdot\text{mL}^{-1}$). Inset: plot of CIAIPc concentration vs. absorbance

Table 1: Results of one-way ANOVA for the linear model of proposed spectrophotometric^a and spectrofluorimetric^b methods

Source	Sum of squares	Degrees of freedom ^{a,b} (df)	Mean square	F-ratio
Linear model	3.89989 ^a 2.152×10^{13b} 0.00753 ^a	1	3.89989 ^a 2.152×10^{13b} 0.00023 ^a	17,087.76 ^a (4.15) ^c 6,064.15 ^b (4.15) ^c
Residual	1.171×10^{11b} 0.00113 ^a 2.478×10^{10b}	33	3.549×10^{9b} 0.00023 ^a 4.956×10^{9b}	0.989 ^a (2.56) ^d 1.503 ^b (2.56) ^d
Lack-of-fit	0.00640 ^a 9.232×10^{10b} 3.90742 ^a	5	0.00023 ^a 3.297×10^{9b}	
Pure error		28		
Total	2.164×10^{13b}	34		

^{c,d}Theoretical values of $F(1,33)$ and $F(5,28)$, respectively, based on one-way ANOVA test at $p=0.05$ level of significance

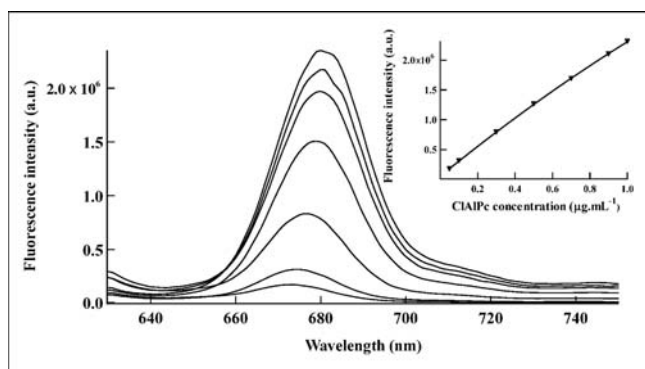


Fig. 3: Fluorescence spectra of CIAIPc at different concentrations (0.05–1.00 $\mu\text{g}\cdot\text{mL}^{-1}$). Inset: plot of CIAIPc concentration vs. fluorescence intensity

concentration at $\mu\text{g}\cdot\text{mL}^{-1}] + 9.74 \times 10^4$. However, a value of correlation coefficient very close to unity (for instance, $r > 0.99$) is not necessarily the outcome of a linear relationship and in consequence a Lack-of-fit test should be checked (AMC 1994; González et al. 2006). The testing for lack-of-fit is carried out with the F -ratio of mean squares for lack-of-fit (the sum of squares of lack-of-fit divided by its number of the degrees of freedom) and mean squares for pure error (the sum of squares of pure error divided by its number of the degrees of freedom) as it can be seen in the Table 1 (Xu et al. 2003; Deschepper et al. 2006).

Table 1 lists the analysis of variance (one-way ANOVA) results. For both methods, the calculated F -values (17,087.76 and 6,064.15) for linear model were larger (several hundreds of time) than the critical F -value 4.15 (Table 1), indicating that the regression equations are statistically significant within the 95% confidence interval. Furthermore, the goodness of fit of regression equations was supported by calculated F -values (0.989 and 1.503) of lack-of-fit test which were lower than the tabulated F -value 2.56 (Table 1) confirming that the resulting linear model

is fully adequate, that is, there is no a significant lack of fit ($p > 0.05$). Therefore, the calibration model is significantly linear and consequently the linear model fully describes the data.

The limit of detection (LOD) of an analytical method is the lowest amount of analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions while the limit of quantification (LOQ) is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy (ICH 2005; USP 2007). Spectrophotometric LOD and LOQ were found to be 0.09 and 0.27 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively. Regarding the spectrofluorimetry, LOD and LOQ values were of 0.01 and 0.04 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively. The spectrofluorimetric method showed the best sensitivity, better than spectrophotometry.

According to ICH guidelines the precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions (ICH 2005). Precision was determined by studying at two levels: repeatability (intra-day analysis) and intermediate precision (inter-day analysis). For spectrophotometric method, intra-day analysis (RSD%) ranged from 0.58% to 2.30% while spectrofluorimetric method ranged from 0.16% to 3.23% (Table 2). Intermediate precision was expressed within-laboratory variation on three different days and with two different analysts (A and B). In both methods, obtained RSD% values were found to be less than 5.00% for different days and analysts which indicating reasonable results (Table 2). Relative standard deviation ranged from 1.32% to 2.63% and 1.27% to 4.02% on different days for spectrophotometry and spectrofluorimetry, respectively. Analyses between analysts revealed RSD values of 0.32% to 1.80% for spectrophotometric determination and RSD of 1.18% to 3.61% for fluorescence emission. Precision results indicated the closeness of agreement between the several measurements of analyte (CIAIPc) carried out under the same operating conditions over a short interval of time as well as over a long period of time and with different analysts.

Table 2: Precision of proposed spectrophotometric and spectrofluorimetric methods

Concentration ($\mu\text{g}\cdot\text{mL}^{-1}$)	Intra-day analysis RSD (%)			Inter-day analysis RSD (%)		Inter-analyst analysis RSD (%)	
	Day 1	Day 2	Day 3			A	B
Spectrophotometry	0.50	1.93	2.30	1.13	2.63	1.80	1.28
	1.50	2.03	0.62	0.58	2.10	0.44	0.49
	3.00	1.40	0.94	0.98	1.32	0.32	1.16
Spectrofluorimetry	0.10	3.23	3.81	0.16	4.02	3.50	3.61
	0.50	1.38	1.05	0.98	1.27	3.55	1.57
	0.90	1.12	0.73	1.36	3.69	3.05	1.18

Table 3: Results from the determination of the accuracy for spectrophotometric method

Formulations	CIAIPc in pre-analyzed formulation*	CIAIPc added*	Total CIAIPc found**	Recovery (%)***	RSD (%)
Nanoemulsion	0.57	0.55	1.136 (± 0.0092)	102.7 (± 1.97)	1.9
	0.57	1.05	1.634 (± 0.0282)	101.2 (± 2.12)	2.1
	0.57	1.55	2.130 (± 0.0392)	100.6 (± 2.26)	2.2
Nanocapsule	0.56	0.50	1.074 (± 0.0197)	102.6 (± 4.56)	4.4
	0.56	1.00	1.549 (± 0.0331)	98.8 (± 3.47)	3.5
	0.56	1.50	2.073 (± 0.0396)	100.8 (± 2.51)	2.5

*Concentrations at $\mu\text{g}\cdot\text{mL}^{-1}$ **Data are average concentrations (\pm SD) for three different samples***Data are averages (\pm SD) for three different samples**Table 4: Results from the determination of the accuracy for spectrofluorimetric method**

Formulations	CIAIPc in pre-analyzed formulation*	CIAIPc added*	Total CIAIPc found**	Recovery (%)***	RSD (%)
Nanoemulsion	0.25	0.26	0.507 (± 0.0082)	100.0 (± 3.85)	3.8
	0.25	0.36	0.612 (± 0.0032)	100.9 (± 1.60)	1.6
	0.25	0.46	0.684 (± 0.0022)	94.2 (± 1.25)	1.3
Nanocapsule	0.30	0.18	0.478 (± 0.0073)	96.3 (± 3.21)	3.3
	0.30	0.28	0.583 (± 0.0019)	101.2 (± 2.06)	2.0
	0.30	0.38	0.664 (± 0.0133)	95.6 (± 3.01)	3.1

*Concentrations at $\mu\text{g}\cdot\text{mL}^{-1}$ **Data are average concentrations (\pm SD) for three different samples***Data are averages (\pm SD) for three different samples

The accuracy of an analytical procedure is the closeness of test results obtained by that procedure to the true value (USP 2007). Accuracy of the analytical methods was determined by spiking known amounts of CIAIPc to samples of pre-analyzed loaded NE or NC. The obtained results are summarized in the Tables 3 and 4, and they are expressed as the percentage of recovery and relative standard deviation (RSD%). The mean percent recoveries between 94.2% and 102.7% were reached in all cases as well as RSD values below of 5.00%. These results demonstrated that any small change in the CIAIPc concentration in the solutions could be accurately determined by the intended analytical methods indicating a good agreement between amounts added and found.

The selectivity is defined as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, for instance, matrix components (ICH 2005). In order to assess the possible analytical application of the proposed methods the effect of common excipients of formulations was studied. Selectivity of the methods was demonstrated after observing that the excipients did not interfere in the absorption, and fluorescence spectra of CIAIPc at 674 nm. The spectra of CIAIPc were not changed in the presence of common excipients used in the formulation of NE and NC. The calculated *t*-values (Tables 5 and 6) were found to be less than that of the critical *t*-value (tabulated value of 2.776, for 4 degrees of freedom and a confidence limit of 95%), such as results indicate that statistically there was no significant difference between the mean of CIAIPc standard solutions spiked with unloaded nanocarriers

and correspondent CIAIPc standard solutions alone ($p > 0.05$). Therefore, intended methods are able to quantify CIAIPc specifically.

In summary, the intended analytical methods were simple, accurate, precise, efficient, specific, and rapid. Hence, they are reliably able to determine CIAIPc content in nanoemulsions and nanocapsules. In general, the spectrofluorimetric method demonstrated to be more sensitive due to the lowest LOD and LOQ and can be used for dissolution, release, and biological studies which require analytical methods quite sensitive. On the other hand, the spectrophotometric method is suitable for the routine analysis of CIAIPc in such nanocarriers. Therefore, both validated methods can be used for main analyses of drug quality control.

2.4. Application of the validated methods

CIAIPc content in NE and NC has been determined by the present validated methods. The association/encapsulation efficiency of CIAIPc in NE and NC, respectively, ranged from 63% to 71%, these findings could be related with the low water solubility of CIAIPc leading to entrapment of this drug into oil core of the nanocarriers. In general, there was no statistically significant difference between the means of spectrophotometric and fluorimetric methods as well as between NE and NC ($p > 0.05$), since the spectrophotometric analyses demonstrated for NE and NC an incorporation efficiency of 66.7% (± 7.43) and 71.0% (± 4.82), respectively; while the fluorimetry revealed an

Table 5: Selectivity study for the spectrophotometric method

Theoretical concentrations*	Actual concentrations**	Concentrations** of standard solutions spiked with unloaded NE ^a or NC ^b			<i>t</i> -calculated
0.50	0.494 (±0.0153)	0.496 ^a (±0.0189)	0.511 ^b (±0.0174)	0.142 ^a	1.271 ^b
1.50	1.497 (±0.0109)	1.458 ^a (±0.0363)	1.471 ^b (±0.0713)	1.782 ^a	0.624 ^b
3.00	2.934 (±0.0232)	2.878 ^a (±0.0399)	2.871 ^b (±0.0368)	2.101 ^a	2.508 ^b

*Concentrations of CIAIPc at $\mu\text{g}\cdot\text{mL}^{-1}$ **Data are average concentrations of CIAIPc solutions (\pm SD) for three different samples

Table 6: Selectivity study for the spectrofluorimetric method

Theoretical concentrations*	Actual concentrations**	Concentrations** of standard solutions spiked with unloaded NE ^a or NC ^b			t-calculated
0.22	0.215 (±0.0034)	0.211 ^a (±0.0091)	0.209 ^b (±0.0028)	0.713 ^a	2.359 ^b
0.31	0.314 (±0.0045)	0.307 ^a (±0.0044)	0.316 ^b (±0.0100)	1.926 ^a	0.316 ^b
0.40	0.395 (±0.0027)	0.386 ^a (±0.0059)	0.394 ^b (±0.0085)	2.402 ^a	0.194 ^b

*Concentrations of CIAIPc at $\mu\text{g}\cdot\text{mL}^{-1}$

**Data are average concentrations of CIAIPc solutions (±SD) for three different samples

efficiency for NE and NC of 63.7% (±2.87) and 63.2% (±3.26), respectively.

3. Experimental

3.1. Materials

Aluminum phthalocyanine chloride (85%), poly(D,L-lactide-co-glycolide) (PLGA) 50:50 polymer, Poloxamer 188, soybean oil, and acetonitrile were purchased from Sigma (Sigma-Aldrich co., St. Louis, MO, USA). Soy phosphatidylcholine was obtained from Lipoid GmbH (Ludwigshafen, Germany). Dimethyl-sulphoxide (DMSO) and acetone were purchased from J.T.Baker (Mallinckrodt Baker, Phillipsburg, USA), and Miglyol 812 was obtained from Huls (Puteaux, France). All solvents used were of analytical grade. Ultrapure water was obtained from E-pure apparatus (Barnstead, Iowa, USA).

3.2. Instruments

A double-beam Hitachi U-3000 Spectrophotometer (Tokyo, Japan) and a Horiba Jobin Ivo-Spex Fluorolog-3 Spectrofluorimeter (New Jersey, USA) were used. The spectrophotometric analyses were done with quartz cells of 10 mm path length and the spectra were recorded using 2 nm slit and 1200 nm·min⁻¹ scanning speed. Absorbance was measured in the spectral range 300–800 nm. The spectrofluorimeter was set with excitation and emission slits at 5 and 10 nm, respectively. Fluorescence emission was recorded in the 630–750 nm range, with excitation fixed at 615 nm. For all spectrofluorimetric analyses were also used four face quartz cells (10 mm).

3.3. Preparation and characterization of CIAIPc loaded-nanoemulsions and nanocapsules

NE were obtained by spontaneous emulsification process describes by Tabosa do Egito et al. (1994), and NC were prepared by interfacial deposition of a preformed polymer on the interface oil-in-water emulsion according to Fessi et al. (1989) both with some modifications. Briefly, the organic phase (acetone) was prepared containing oil, soy phospholipid, CIAIPc (NE) and polymer (for NC) at 40 °C. Subsequently, this organic solution was added into the aqueous phase containing Poloxamer 188 under magnetic stirring. Organic solvent was removed by evaporation under reduced pressure at 40 °C. Finally, the formulations were concentrated to a final volume of 10 mL. The formulations without the drug were prepared, under the same conditions, to be used as reference compounds in the spectroscopic analyses. All formulations were characterized by their mean size, polydispersity index (PDI), and zeta potential. The mean size and PDI of colloidal dispersions were determined at 25 °C by laser light scattering at angle of 173°, and zeta potential was measured by electrophoretic mobility both using a Zetasizer® (Nano ZS, Malvern PCS Instruments, UK). Data are the mean (±SD) of three different batches.

3.4. Analytical methods validation

The validation study was performed according to the ICH guidelines: *Validation of Analytical Procedures: Text and Methodology Q2(R1)* (ICH 2005), which are similar to those established by USP 30 (USP 2007).

3.4.1. Linearity, limit of detection and limit of quantification

To establish linearity of the proposed methods five calibration curves were constructed at seven concentrations levels within the range of 0.50–3.00 $\mu\text{g}\cdot\text{mL}^{-1}$ (n=35) for the spectrophotometric method and 0.05–1.00 $\mu\text{g}\cdot\text{mL}^{-1}$ (n=35) for the spectrofluorimetric method. In the spectrophotometric analyses the Beer's law was obeyed. Least square regression analysis was done for the data. One-way analysis of variance (ANOVA) and a Lack-of-fit test ($p=0.05$) were used to determine whether the linear model adequately explains obtained data (AMC 1994; González et al. 2006). The limits of detection (LOD) and limits of quantification (LOQ) of CIAIPc

by the intended methods were estimated from the calibration curve of the analyte. LOD and LQD were calculated as follow $3.3(\text{SD}/b)$ and $10(\text{SD}/b)$, respectively, where SD is the standard deviation of y-intercept of regression equation and b is the slope of the calibration curve.

3.4.2. Precision

Precision was evaluated at the repeatability (intra-day precision) and intermediate precision levels. Repeatability was studied using three different concentration levels of drug: low, medium and high, corresponding, respectively, to 0.50, 1.50, and 3.00 $\mu\text{g}\cdot\text{mL}^{-1}$ (n=9) for spectrophotometry and 0.10, 0.50, and 0.90 $\mu\text{g}\cdot\text{mL}^{-1}$ (n=9) for spectrofluorimetry. The intra-day precision values were obtained preparing CIAIPc solutions two different times during the same day, under the same experimental conditions. Intermediate precision was determined for three different days and analysts with same concentration levels taken in repeatability. During these three days, intermediate precision was evaluated by analysis of the standard solutions prepared each different day (n=27). Both precision were evaluated by percent relative standard deviation (RSD%).

3.4.3. Accuracy

The accuracy of the analytical methods was assessed by measuring the drug recoveries by the standard addition method. In this study, different concentrations of CIAIPc were added to a known pre-analyzed loaded NE (n=9) or NC (n=9) samples and the total concentration was determined using the proposed methods. The percent of recovery was calculated from following Eq. (1):

$$\text{Recovery}(\%) = \frac{C_{\text{TF}} - C_{\text{FO}}}{C_{\text{AF}}} \times 100 \quad (1)$$

Where C_{TF} is the total CIAIPc concentration found after standard addition; C_{FO} , CIAIPc concentration in the formulation; C_{AF} , CIAIPc concentration added to formulation.

3.4.4. Selectivity

In order to evaluate the possible interference of common excipients used in nanocarriers formulations were prepared CIAIPc standard solutions spiked with empty NE or NC (100 μL). Standard solutions containing 0.50, 1.50, and 3.00 $\mu\text{g}\cdot\text{mL}^{-1}$ (n=9), and 0.22, 0.31, and 0.40 $\mu\text{g}\cdot\text{mL}^{-1}$ (n=9) of CIAIPc were prepared for spectrophotometric and spectrofluorimetric methods, respectively. A comparison between the results obtained from standard solution spiked with empty formulations and standard solution alone was carried out. Data analyses were done using Student's t test ($p=0.05$).

3.5. Preparation of samples solution for determination of CIAIPc in nanocarriers

The determination of the total CIAIPc content in NE and NC was done using the intended methods. Briefly, aliquots (60 μL) of NE or NC containing CIAIPc were appropriately dissolved in DMSO (1:1, v/v) and sonicated for 5 min in order to release all content of CIAIPc. After that, the samples were diluted to the final volume (3 mL) with acetonitrile and filtered through a 0.45 μm membrane. Besides that, the free CIAIPc content in the formulations was determined by measuring the non-incorporated drug present in a clear ultrafiltrate obtained through separation of aqueous phase using an ultrafiltration/ultracentrifugation procedure (Microcon Ultracel YM-100, Millipore, Ireland) at $12857 \times g$ for 1 h at 4 °C (Eppendorf, Centrifuge 5810 R, Hamburg, Germany) (Teixeira et al. 2004). The association/encapsulation efficiency of CIAIPc into NE or NC was determined from the following Eq. (2):

$$\text{Efficiency}(\%) = \frac{C_{\text{T}} - C_{\text{F}}}{C_{\text{TH}}} \times 100 \quad (2)$$

Where C_{T} is the total CIAIPc concentration; C_{F} , the free CIAIPc concentration; C_{TH} , is the theoretical concentration of CIAIPc. The sample solutions

were assayed by absorbance measurement at 674 nm, which corresponds to the maximum absorption wavelength (λ_{max}) of CIAIPc while the relative fluorescence intensity was measured spectrofluorimetrically at 615 nm (λ_{ex}) and 674 nm (λ_{em}). The analyses were performed in triplicate and the mean results (\pm SD) are reported. All results obtained were compared using a Tukey post test, and statistical significance was considered at $p < 0.05$. All statistic analyses were performed with STATISTICA version 8.0 (Stat Soft, Inc. 2007, Tulsa, OK, USA).

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