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Article

Determination of amphotericin B in PLA-PEG blend nanoparticles by HPLC-PDA

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In this work, we developed and validated an effective reversed-phase HPLC method with photodiode array (PDA) detection for the quantitative analysis of amphotericin B (AmB) in poly(lactide)-poly(ethylene glycol) (PLA-PEG) blend nanoparticles. Chromatographic runs were performed on a reverse phase C18 column using a mobile phase comprising a 9% acetic acid and acetonitrile mixture (40:60, v/v) under isocratic elution with a flow rate of 1 mL/min. AmB was detected at a wavelength of 408 nm. The validation process was performed considering the selectivity, linearity, precision, accuracy, robustness, limit of detection (LOD) and limit of quantitation (LOQ) of the method. A concentration range of 1-20 µg/mL was used to construct a linear calibration curve. The LOQ and LOD were 55 and 18 ng/mL, respectively. The mean recovery of AmB from the samples was 99.92% (relative standard deviation (RSD) = 0.34%, n=9), and the method was robust for changes in the flow rate of the mobile phase (maximum RSD=4.82%). The intra- and inter-assay coefficients of variation were less than 0.59%. The method was successfully used to determine the entrapment efficiency of AmB in PLA-PEG blend nanoparticles.

Uniterms: Amphotericin B/determination. Amphotericin B/encapsulation. High performance liquid chromatography/reverse phase/quantitative analysis. Nanoparticles.

Neste trabalho desenvolveu-se e validou-se um efetivo método por cromatografia líquida de alta eficiência (CLAE) em fase reversa com detecção por fotodiodos para a análise quantitativa de anfotericina B (AmB) em nanopartículas compostas por blendas de poli(ácido lático)-polietilenoglicol (PLA-PEG). Corridas cromatográficas foram realizadas sob coluna C18 de fase reversa com fase móvel consistindo de ácido acético 9% e acetonitrila (40:60, v/v), em eluição isocrática com fluxo de 1 mL/min. A AmB foi detectada no comprimento de onda de 408 nm. O processo de validação foi realizado considerando a seletividade, linearidade, precisão, exatidão, robustez, limite de detecção (LD) e limite de quantificação (LQ) do método. Uma faixa de concentração entre 1-20 µg/mL foi usada para obter a curva-padrão linear. Os valores de LD e LQ foram 55 e 18 ng/mL, respectivamente. A recuperação média da AmB a partir das amostras foi de 99,92% (desvio padrão relativo = 0,34%, n=9) e o método foi robusto, considerando alterações no fluxo da fase móvel (desvio padrão relativo máximo=4,82%). Os coeficientes de variação intra e inter dia foram inferiores a 0,59%. O método foi utilizado com sucesso para a determinação da eficiência de encapsulação da AmB em nanopartículas de PLA-PEG.

Unitermos: Anfotericina B/determinação. Anfotericina B/encapsulação em nanopartículas. Cromatografia líquida de alta eficiência/fase reversa. Nanopartículas.

INTRODUCTION

Amphotericin B (AmB) is the most widely used antifungal agent for the treatment of systemic fungal infections and is also applied in visceral leishmaniasis (Kullberg, Pauw, 1999; Sundar, Chakravarty, 2013). It is a polyene macrolide antibiotic produced by an actinomycete, *Streptomyces nodosus*, first isolated in 1955, and was the first antifungal to be approved by the U.S. Food and Drug Administration (FDA), in 1965 (Wu, 1994). The AmB chemical structure has an amphipathic nature, which compromises its water solubility. Thus, in the presence of water, AmB has a tendency to self-aggregate and produce

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dimers and oligomers, which is responsible for its affinity to ergosterol, a component of fungi cell membranes, and also cholesterol, a component of human cell membranes, which leads to its toxicity. The aggregated state of the drug is the cause of its serious side effects, such as nephrotoxicity, hepatotoxicity, hematotoxicity, and other complications related to AmB treatment (Espada *et al*., 2008a; Espada *et al*., 2008b; Legrand *et al*., 1992; Adams, Kwon, 2003).

Many AmB formulations have been tested with the aim of maintaining the drug in the monomeric state and decreasing its toxicity. Some of them based on nanotechnology are already commercially available, such as the liposomal product Ambisome®, the lipid complex Abelcet® and the colloidal dispersion Amphocil®. These formulations, particularly the liposomal, increase the therapeutic index of the drug by reducing its toxicity but have the disadvantage of requiring parenteral infusion administration, requiring patient hospitalization, decreasing patient compliance and causing variability in pharmacokinetics (Vyas, Gupta, 2006; Adler-Moore, Proffitt, 2008; Barratt, Bretagne, 2007; Nahar *et al*., 2008). An alternative delivery system for AmB could circumvent these drawbacks by providing a safe and effective alternate route of administration, such as oral.

Polymeric nanoparticles may be important tools for addressing AmB solubility and pharmacokinetic drawbacks in order to reduce its toxicity. Polymeric nanoparticles present valuable physicochemical properties that improve the biological effects of the carried drug. By tailoring the particle size, surface charge, polymer composition and molecular weight, nanoparticles can be manipulated for a particular purpose. Nanoparticles optimize therapeutic regimens, decreasing the dose needed for treatment by prolonging drug release, which contributes to reducing drug toxicity (Kumari, Yadav, Yadav, 2010; Pinto Reis *et al*., 2006). Poly(ethylene glycol) (PEG) has been used to coat nanoparticles and prevent premature opsonization and phagocytosis, extending the half-life of nanoparticles and prolonging the drug release and its effects (Essa, Rabanel, Hildgen, 2010; Nguyen *et al*., 2003).

A suitable drug delivery system must present high drug encapsulation efficiencies in order to reach its therapeutic goal. With the purpose of defining the drug content of nanoparticles, a quantitation method must be appropriately validated. Various methods have been described in the literature for determining the AmB content in samples, such as the use of biological matrices, high performance liquid chromatography (HPLC) (Italia, Singh, Kumar, 2009; Espada *et al*., 2008; Eldem, Arican-Cellat, 2001), second-derivative spectrophotometry (Ganière Monteil *et al*., 1998), and liquid chromatography tandem mass spectrometry (LC–MS/MS) (Deshpande *et al*., 2010). In lipid formulations, HPLC (Manosroi, Kongkaneramit, Manosroi, 2004a; Manosroi, Kongkaneramit, Manosroi, 2004b; Eldem, Arican-Cellat, 2000) and UV/Vis-spectrophotometry (Legrand *et al*., 1997; Larabi *et al*., 2004; Jung *et al*., 2009) have been reported. For polymeric nanoparticles, most studies have achieved the analytical determination of AmB by UV-Vis spectrophotometry (Vyas, Gupta, 2006; Van de Ven *et al*., 2012; Xu *et al*., 2011, Shim *et al*., 2011; Falamarzian, Lavasanifar, 2010). In the context of HPLC methods to quantify AmB in polymeric nanoparticles, the study of Nahar *et al*. (2008) cites an HPLC method, but details such as the peak characteristics and validation data were not described. Therefore, the aim of this work was to develop and validate a simple and rapid reverse phase HPLC method with PDA detection to determine the encapsulation efficiency of AmB in poly(lactide)-poly(ethylene glycol) (PLA-PEG) blend nanoparticles.

MATERIAL AND METHODS

Material

Amphotericin B (AmB), poly(lactide) (PLA) (MW 85-160 kDa), poly(ethylene glycol) (PEG) (MW 10 kDa) and polyvinyl alcohol (PVA, 31 KDa, 88% hydrolyzed) were purchased from Sigma-Aldrich (USA). Chloroform and dimethyl sulfoxide (DMSO) were purchased from Biotec® (Brazil), and dichloromethane was obtained from FMaia® (Brazil). HPLC-grade solvents, such as methanol, acetonitrile and acetic acid, were purchased from JTBaker® (USA). Water was purified using a Milli-Q Plus system (Millipore®).

Instrumentation

A Waters 2695 Alliance HPLC system (Milford, MA, USA) was used for method development. The HPLC system was equipped with a column compartment with temperature control, an on-line degasser, a quaternary pump, an auto sampler and a photodiode array (PDA) wavelength detector (Waters 2998). Data acquisition, analysis, and reporting were performed using Empower chromatography software (Milford, MA, USA). HPLC analysis was conducted using a RP C18 column (Xterra Waters®) with a 5 µm particle size, a 4.6 mm internal diameter and a 250 mm length.

Chromatographic conditions

The mobile phase comprised a 9% acetic acid and acetonitrile mixture (40:60, v/v) under isocratic elution with a flow rate of 1 mL/min. The sample injection volume was 20 µL, and the PDA detector was set at a wavelength of 408 nm. The analysis was performed at a temperature of 25 °C and the method run time was 6 min.

Preparation of standards and samples

A stock standard solution of 1 mg/mL of AmB was prepared in DMSO, and subsequent dilutions in methanol were performed to obtain seven standard solutions (1, 2, 3, 4, 5, 10 and 20 µg/mL). Similarly, seven standard solutions in methanol were obtained (0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0 µg/mL) to determine the limit of detection (LOD) and limit of quantitation (LOQ) of this method. The samples were appropriately diluted in methanol. The standards and samples had previously been filtered through a 0.22 µm pore size filter (Millipore, Bedford, USA) prior to injection.

System suitability of the developed method

The system suitability parameters were studied to verify the system performance. Six replicate standards containing AmB (10 µg/mL) were analyzed using the developed method. Factors such as the theoretical plate count, the tailing factor and the capacity factor were taken into consideration for testing the system suitability.

Method validation

The HPLC method was validated according to the International Conference on Harmonization (ICH) guidelines (2005). The following characteristics were considered for validation: specificity, linearity, robustness, precision, accuracy, range, LOD and LOQ. The specificity was evaluated by comparing representative chromatograms from samples containing possible interfering substances and samples containing AmB. Additionally, the specificity was demonstrated by performing stress studies (i.e., pH and temperature variation, oxidation and light stability).

The linearity was determined by calculating a regression line from the plot of peak area vs. concentration for the seven standard solutions in methanol (i.e., 1, 2, 3, 4, 5, 10 and 20 µg/mL) using the linear least squares methodology.

Analysis of three different AmB standards (1, 10 and 20 µg/mL) three times each on the same day was carried out to evaluate the repeatability or intra-day precision. The intermediate precision was determined by analyzing the three standard solutions on three different days. The precision results were reported as the standard deviation (SD) and the relative standard deviation (RSD).

The accuracy was determined by calculating the percent recovery of the mean concentration of AmB at three different concentrations (1, 10 and 20 µg/mL), and the RSD was determined. The mean concentration value obtained for each level was compared to the theoretical value, which was considered to be 100%.

The robustness was evaluated by deliberately varying the flow rate (0.9 and 1.1 mL/min) of the mobile phase.

The LOD and LOQ were determined from the specific calibration curve obtained using seven standard solutions (0.2, 0.4, 0.6, 0.8, 1.0, 1.5 and 2.0 µg/mL) that were closest to the LOQ. The following equations (1 and 2) were used according to ICH (2005):

$$LOD = 3.3\sigma/S \quad \text{Eq. 1}$$

$$LOQ = 10\sigma/S \quad \text{Eq. 2}$$

where σ is the standard deviation of the response, and S is the slope of the calibration curve. All samples were analyzed in triplicate.

Method applicability

Preparation of AmB-loaded PLA-PEG blend nanoparticles

PLA-PEG blend nanoparticles containing AmB were prepared using an oil-in-water (O/W) emulsification/solvent evaporation technique. First, PLA and PEG at a PLA:PEG ratio of 5:1 were dissolved in dichloromethane. They were then added to an organic solution (DMSO and chloroform) containing AmB, which was emulsified into a PVA aqueous solution (1%, m/v) and sonicated for 5 min to produce an O/W emulsion. The emulsion was subjected to evaporation under vacuum with continuous stirring at 37°C. The nanoparticles were isolated from the non-encapsulated drug by ultracentrifugation (19,975 × *g*, 30 min, 4 °C) and washed twice with ultrapure water. The precipitate was suspended in 5% sucrose and freeze-dried. The resultant supernatants were collected for further analyses. Additional details regarding the methods used in this study are in the deposited patent in the *Instituto Nacional de Propriedade Industrial* (INPI) in Brazil (PI#1107205-9 A2) (Mainardes *et al*., 2011) and are protected according to the Brazilian regulatory agency.

The mean nanoparticle size and size distribution were analyzed using dynamic light scattering (BIC 90 plus, Brookhaven Instruments Corp.). The measurements were

performed at a scattering angle of 90° at 25 °C. For each sample, the mean particle diameter, polydispersity and standard deviation for ten determinations were calculated.

Determination of AmB encapsulation efficiency

To determine the encapsulation efficiency (EE), an indirect analysis was performed. The supernatant-containing free AmB obtained from the ultracentrifugation of nanoparticles was appropriately diluted in methanol, and the samples were analyzed by the HPLC method described in this work. The measurements were performed in triplicate. The amount of AmB loaded in the nanoparticles was calculated by subtracting the quantity in the supernatant from the total used initially using the following equation (eq. 3):

% EE = theoretical amount – analytical amount/ theoretical amount x 100 Eq. 3

RESULTS AND DISCUSSION

Method development

Initially, the analyses were performed using acetonitrile and water in variable proportions to the mobile phase. Although the retention time was less than 4 min, the peaks presented no symmetry. Alternatively, a mobile phase consisting of acetonitrile and sodium acetate buffer was tested, but there were no peaks after a run of 40 min. When methanol and water were tested as eluents in a proportion of 75:25 (v/v), a peak was observed after 11 min but presented an irregular shape. Methanol and sodium acetate buffer (25:75, v/v) were tested, and despite presenting a short retention time of approximately 3 min, a noticeable tailing of the AmB peak was observed. Lastly, a mobile phase consisting of acetonitrile and 9% acetic acid was used. After some modifications to the proportions of the eluents, a regular and symmetric peak was detected at approximately 4.5 min (Figure 1) using acetonitrile and 9% acetic acid (60:40, v/v) with a flow rate of 1 mL/min.

System Suitability

The system suitability of this method was evaluated by analyzing the capacity factor, the peak symmetry and the theoretical plates of the column during the run of the AmB methanol solution over six repetitions. The developed method produced a theoretical plate number of over 2000, with a tailing factor less than 1.5 and a capacity factor less than 2, which ensures the suitability of the developed method. The system suitability results are summarized in Table I; it can be observed that the

TABLE I – System suitability of the HPLC method

Chromatographic Parameter	Result*	Acceptance Criteria
Capacity factor (*K'*)	1.25 ± 0.2	*K'* < 2
Tailing factor (*T*)	1.084 ± 0.057	*T* < 2
Theoretical plates (*N*)	2600.72 ± 46.87	*N* > 2000

* Presented as mean value ± standard deviation (n = 3)

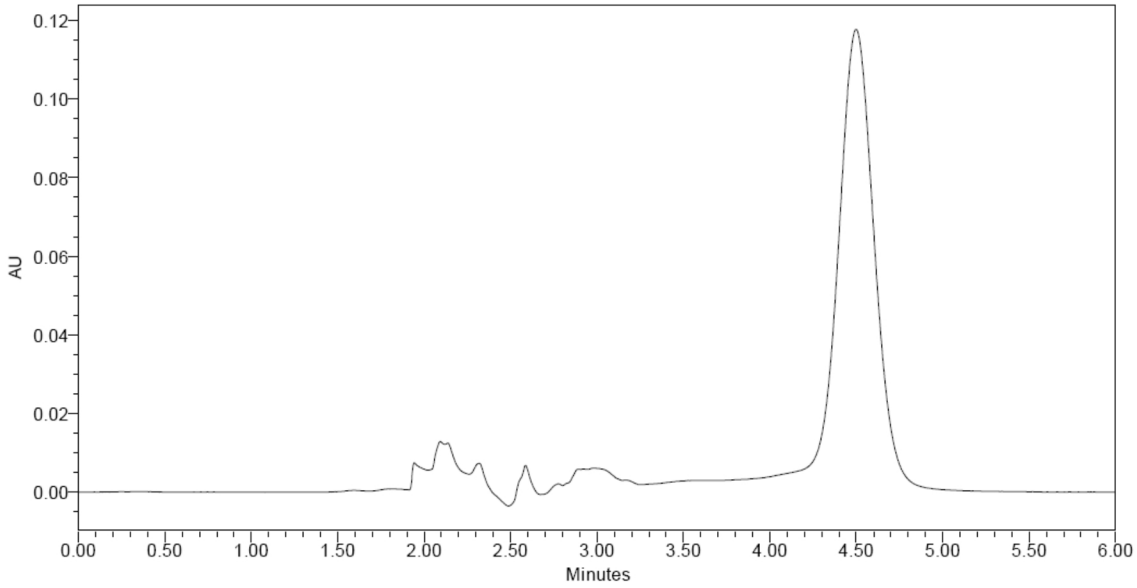

FIGURE 1 - Representative HPLC chromatogram of a 20 µg/mL AmB standard solution. Conditions: mobile phase, 9% acetic acid:acetonitrile (40:60, v/v); flow rate, 1 mL/min; PDA detector, 408 nm; column temperature, 25 °C; injection volume, 20 µL.

parameters analyzed were in accordance with acceptance criteria (Sutariya, Wehrung, Geldenhuys, 2012; Hussen, Shenoy, Krishna, 2013).

Method validation

Specificity

The specificity of the method was evaluated by comparing the chromatograms of both the AmB standards and the samples to those of potential interfering formulation components. For this study, blank nanoparticles (without AmB) were prepared as described previously, and the supernatant obtained after their ultracentrifugation was diluted with methanol and analyzed by the described HPLC method. The representative chromatogram of the AmB sample (Figure 2A) showed an AmB peak at

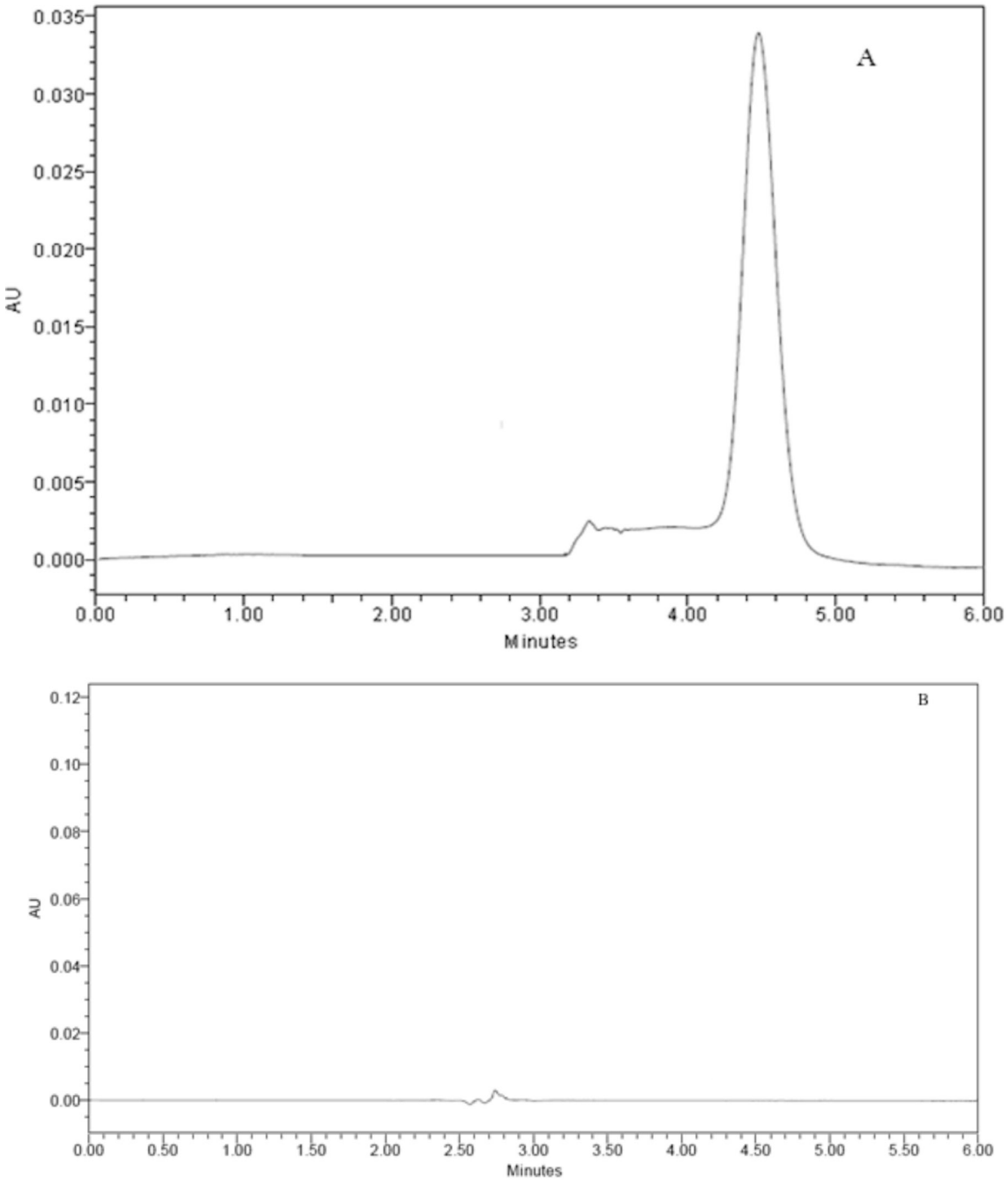

FIGURE 2 - Representative HPLC chromatograms of the AmB sample (AmB in supernatant from the nanoparticles) (A) and of the supernatant from the blank nanoparticles (B). Conditions: mobile phase, 9% acetic acid:acetonitrile (40:60, v/v); flow rate, 1 mL/min; PDA detector, 408 nm; column temperature, 25 °C; injection volume, 20 µL.

approximately 4.5 min, which was in agreement with that obtained for the AmB standard (Figure 1). No peaks at this retention time were observed in the chromatogram of the supernatant from the blank nanoparticles (Figure 2B), which indicates there was no interference in the quantitative determination of AmB from the formulation components.

Additionally, the specificity of the method was assessed by submitting AmB to stress conditions (i.e., temperature, visible light, pH and oxidation) to detect the occurrence of possible interfering peaks at 408 nm resulting from the degradation of AmB. These tests are regarded as helpful tools in establishing degradation pathways and the inherent stability of the molecule and help validate the ability of the method to study drug stability (Das Neves *et al*., 2010). The results are presented in Table II. The percent recovery under stress conditions revealed that AmB was affected by visible light, oxidation and pH variations, although there were no degradation peaks. The acid pH did not result in an AmB peak most likely because the acid conditions were very severe. Ideally, the study would be initiated with 0.1 M HCL (Silva *et al*., 2009). This method can be considered specific due to the absence of interfering peaks in the AmB retention times.

Linearity

The linearity was evaluated at seven concentrations ranging from 1 to 20 µg/mL by calculating the regression equation (Eq. 4) and the correlation coefficient (r) using the method of least squares:

$$Y = 9.86.10^4 \times A - 8.64.10^3 \qquad \text{Eq. 4}$$
$$r = 0.9998$$

where Y is the peak area and A is the standard solution concentration in µg/mL. The r-value near 1 indicates linearity in the proposed range.

The validity of the assay was confirmed by an analysis of variance, which showed that the linear regression was significant and the deviation from linearity was not significant ($p<0.01$).

Precision

Precision is a measure of the relative error for the method and is expressed as the RSD of the repeatability and intermediate precision. Three concentrations of AmB (1, 10 and 20 µg/mL) were prepared in triplicate and analyzed over either one or three different days to evaluate the intra- and inter-day variations, respectively. The RSD of the responses were calculated for each case and are shown in Table III, presenting a maximal RSD of 0.59%, which indicates precision.

Accuracy

The accuracy was assessed by calculating the percent recovery and the RSD of the mean concentration of the analyte at three different concentrations (1, 10 and 20 µg/mL). The detailed results are presented in Table IV. The mean percent recovery of AmB from the samples was 99.92% (RSD = 0.34%, n=9), which indicates agreement between the experimental and theoretical values.

Robustness

A robustness assay is used to verify the influence of small changes in the analytical procedures/parameters on the response. The evaluation of robustness was based on the percent recovery and RSD values obtained using different parameters for the flow rate of the mobile phase. The method was robust concerning these alterations in chromatographic parameters (Table V). The maximum RSD obtained was 4.82%.

Limit of quantitation and limit of detection

The lowest concentration at which the analyte could

TABLE II - Specificity to AmB under stress conditions

Condition	Percentage of Recovery (%) ± RSD[a] (n=3)			
	1 µg/mL	10 µg/mL	20 µg/mL	Mean
Reference (none)	99.66 ± 0.57	100.03 ± 0.11	99.83 ± 0.29	99.84 ± 0.33
Visible light (24 h)	98.33 ± 1.52	94.47 ± 2.54	90.00 ± 3.90	94.27 ± 2.66
Freeze: -18° C (24 h)	100.66 ± 1.53	96.33 ± 2.31	97.16 ± 0.57	98.05 ± 1.47
Oxidation: H_2O_2 (2 h)	24.33 ± 7.76	14.00 ± 2.20	21.10 ± 5.14	19.81 ± 5.03
pH variation (2 h)				
Basic: NaOH (1 M)	16.33 ± 0.57	23.00 ± 2.64	1.48 ± 0.10	13.60 ± 1.11
Acid: HCl (1 M)	-	-	-	-

[a]RSD = relative standard deviation

be detected (LOD) or quantified (LOQ) with acceptable precision and accuracy was calculated from the SD of the response and the slope obtained from the linear regression of the specific calibration curve (0.2-2.0 µg/mL) in the low-end region of the proposed range. The method was linear in this range because the r-value was 0.998. The LOD and LOQ were found to be 18.0 and 55.0 ng/mL, respectively.

TABLE III - Precision results for the different levels of AmB in the standard solutions

Standard solution (µg/mL)*	Measured concentration ± SD[a] (µg/mL)	RSD[b] (%)
Analysis repeatability (n=3)		
1	0.99 ± 0.006	0.58
10	10.00 ± 0.04	0.44
20	20.00 ± 0.04	0.21
Intermediate precision (n=3)		
Day 1		
1	0.99 ± 0.005	0.58
10	10.00 ± 0.01	0.12
20	19.96 ± 0.06	0.29
Day 2		
1	0.99 ± 0.005	0.58
10	10.03 ± 0.015	0.12
20	20.02 ± 0.05	0.26
Day 3		
1	0.98 ± 0.006	0.59
10	10.02 ± 0.015	0.15
20	20.03 ± 0.03	0.13

*n=3; [a]SD = Standard deviation; [b]RSD = relative standard deviation

TABLE IV - Accuracy results for the AmB concentrations in the standard solutions

Standard solution (µg/mL)*	Recovery (%)	RSD[a] (%)
1	99.66	0.58
10	100.23	0.23
20	99.88	0.21

*n=3; [a]RSD = relative standard deviation

Range

The working range of the method, defined as the range that exhibited the required linearity, accuracy and precision, was between the LOQ and 20 µg/mL. Consequently, samples at these concentrations can be assayed using the proposed HPLC method.

Method applicability

The proposed analytical method was performed to evaluate the encapsulation efficiency of AmB in PLA-PEG blend nanoparticles. The indirect method was chosen, in which the supernatant containing free AmB was analyzed using HPLC. Through the specificity test, it was demonstrated that no interfering or unusual peaks were observed in the chromatograms during drug quantitation.

The nanoparticles containing AmB were successfully obtained by the emulsion-solvent evaporation method. The mean diameter of the nanoparticles was 223 ± 25 nm (n=3), but a bimodal size distribution profile was obtained, as can be observed in Figure 3. The encapsulation efficiency of AmB in the PLA-PEG blend nanoparticles was 68.9 ± 4.5% (n=3). This value is considered high considering the amphiphilic characteristic of AmB. The work of Verma, Pandya and Mishra (2011) describes a drug entrapment efficiency of 42.5 ± 6.41% for AmB in poly(lactide-co-glycolide) nanoparticles. Therefore, the PLA-PEG nanoparticles developed in this work are potential carriers for AmB, and its effectiveness and toxicity are under investigation.

One of the characterization steps for polymeric nanoparticles is the determination of their drug content. This parameter must be properly verified because the drug must be efficiently loaded into the nanoparticles to achieve the desired therapeutic efficacy. Therefore, a suitable and

TABLE V - Robustness results for the different flow rates

Changes to original method*	Percentage of Recovery (%) ± RSD[a] (n=3)			
	1 µg/mL	10 µg/mL	20 µg/mL	Mean
None	99.66 ± 0.57	100.27 ± 0.15	100.08± 0.26	100.00 ± 0.32
0.9 mL/min	100.00 ± 2.64	99.00 ± 2.00	100.83± 0.57	99.94 ± 1.06
1.1 mL/min	96.00 ± 2.65	94.00 ± 2.00	93.66 ± 4.82	94.55 ± 3.15

*1 mL/min. [a]RSD = relative standard deviation

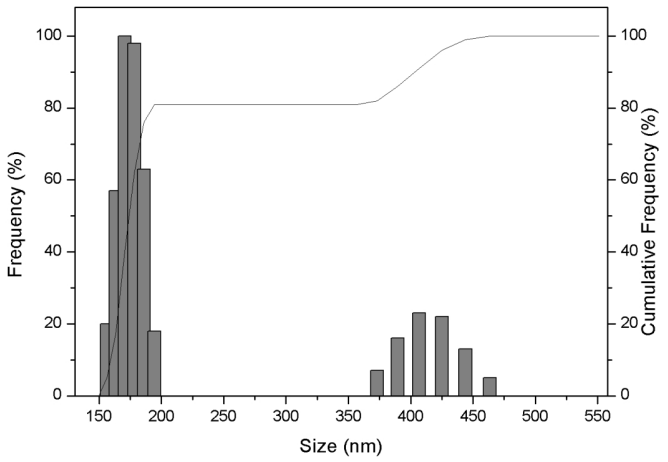

FIGURE 3 - Size distribution profile of the PLA-PEG nanoparticles containing AmB determined by dynamic light scattering.

validated quantitation method is required to assess this parameter (Do Nascimento *et al*., 2012).

In this work, we developed a simple, fast and effective HPLC method to quantitatively analyze AmB in PLA-PEG blend nanoparticles. The literature describes mainly spectrophotometric methods for AmB quantitation in nanoformulations (Vyas, Gupta, 2006; Van de Ven *et al*., 2012; Xu *et al*., 2011, Shim *et al*., 2011; Falamarzian, Lavasanifar, 2010), but these methods are not as convenient as HPLC methods in terms of sensitivity. One of the few studies using HPLC-UV/Vis was proposed by Nahar *et al.,* (2008) and used a mobile phase composed of acetonitrile:1% acetic acid:water (41:43:16, v/v/v) at a flow rate of 1.5 mL/min and achieved an AmB retention time of 4.3 min. Despite the high flow rate and increased solvent consumption, the method seems appropriate; however, the authors only cited the chromatographic conditions and did not provide any information about the peak characteristics or method validation.

The HPLC method developed and validated in this work represents an alternative to other methodologies and provides detailed data for the analysis of AmB in nanoparticles via HPLC-PDA detection. The short retention time of AmB allowed the analysis of a large number of samples in a short period of time with reduced solvent costs.

CONCLUSION

The HPLC method using PDA detection for determining the encapsulation efficiency of AmB in PLA-PEG nanoparticles fulfilled all of the requirements to be considered a reliable and feasible method of analysis and could also be applied for other assays involving AmB-loaded nanoparticles, such as in vitro AmB release profile and stability studies.

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