Biotechnological Processes Development Laboratory[1], School of Chemical Engineering, State University of Campinas (UNICAMP), Campinas, São Paulo; Laboratory of Nanotechnology and Nanomedicine (LNMed)[2], University of Tiradentes (Unit) and Institute of Technology and Research (ITP), Aracaju, Brazil; Department of Biological and Environmental Sciences[3], University of Messina, Messina, Italy; Laboratory of Biomaterials and Nanotechnology for the Development and Evaluation of Bioactive Substances[4], University of Sorocaba, São Paulo, Brazil; Department of Pharmaceutical Technology[5], Faculty of Pharmacy; Center for Neuroscience and Cell Biology & Institute for Biomedical Imaging and Life Sciences (CNC-IBILI)[6], University of Coimbra, Coimbra, Portugal

Validation of an UV spectrophotometric assay for the quantification of polymyxin B in solid lipid nanoparticles

P. Severino[1,2], E. F. Silveira[2], M. Vazzana[3], M. V. Chaud[4], M. H. A. Santana[1], E. B. Souto[5,6]

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Patricia Severino, Biotechnological Processes Development Laboratory, School of Chemical Engineering, State University of Campinas, Campinas, São Paulo, Brazil
pattypharma@gmail.com
Eliana B. Souto, Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra (FFUC), Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal
ebsouto@ff.uc.pt

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Polymyxins are efficient antibiotic drugs used for the treatment of Gram-negative bacterial infections. These compounds are not absorbed in the gastrointestinal tract and are responsible for serious toxicological effects. In order to enhance their therapeutic effectiveness, decrease the adverse/toxic side effects and promote a sustained release profile, a derivative – polymyxin B sulphate – has been formulated in solid lipid nanoparticles (SLNs) intended for buccal administration. To quantify polymyxin B in the formulation, UV spectrophotometry analysis was applied, validating the analytical methodology by assessing the selectivity, accuracy, precision, linearity, and repeatability. Analyses were performed at 210 nm keeping the samples at 25 °C. Results showed that lipid composition of SLNs did not interfere with the polymyxin B spectra. The linearity showed a correlation coefficient of 0.9977 in the range of 5-90 μg/mL. Quantification of polymyxin B by UV spectrophotometry, at 210 nm in SLN formulations, was approved in all analyzed parameters, validating the methodology proposed in this work.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that causes serious infections in compromised patients (Valot et al. 2015). Bacteria create a biofilm in the infected tissue, causing inflammatory responses and drug bacterial resistance (Alipour et al. 2009). The increased morbidity and mortality rate from *Pseudomonas aeruginosa* infections is therefore a serious public health problem (Cruz 2014). Recent studies show that *Pseudomonas aeruginosa* bacteremia can cause mortality rates from 33 to 61% among all patients and approximately 50% of patients die in the first 24-72 h.

Polymyxins have been used for the treatment of diseases caused by opportunist pathogens. They have the advantage of not being absorbed in the gastrointestinal tract because of their low bioavailability (Alipour et al. 2008), but impair severe adverse side effects, such as neurotoxicity (Akajagbor et al. 2013) and nephrotoxicity (Nandha et al. 2013). Therefore, the clinical use of polymyxin B is limited and confined in part to the intestinal sterility and topical treatment of infections caused by susceptible microorganisms.

In order to enhance its therapeutic effectiveness, decrease toxic effects and promote a sustained release profile, polymyxin B has been formulated as liposomes (Wang et al. 2009; He et al. 2013), nanoemulsions or nanoparticles (Pattani et al. 2006; Severino et al. 2015). Other possible types of nanocarriers include solid lipid nanoparticles (SLNs) useful to load a wide variety of hydrophilic (Severino et al. 2011; Severino et al. 2014; Severino et al. 2014, Severino et al. 2015) and lipophilic (Singh, Dobhal et al. 2010, Severino, Andreani et al. 2015) drugs, including thermolabile compounds, such as peptides and proteins (Almeida and Souto 2007; Severino et al. 2013). SLNs are composed of biodegradable lipids, recognized as physiological and biocompatible carriers (Doktorovova et al. 2014), produced by techniques which are easily to scale up (Severino et al. 2012).

Quantification of drugs in formulations is of utmost importance for quality assurance, so that the analytical procedure should be reproducible, combining the good manufacturing practices. Moreover, the validation can be used for subsequent stability studies, quality control and clearance. For validation purposes, it is necessary to determine the analytical parameters, i.e. specificity, linearity, range, precision, limit of quantification, sensitivity and accuracy (Cielecka-Piontek et al. 2015; Muralidharan et al. 2015).

The Brazilian Pharmacopoeia does not describe an analytical assay for polymyxin B, thus the aim of this study was to inves-

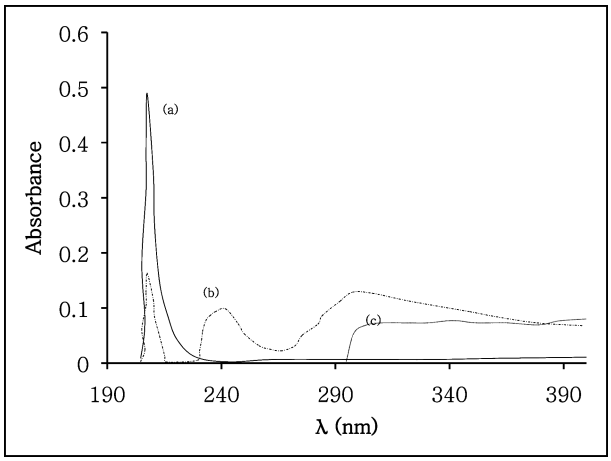

Fig. 1: Absorption spectra of (a) Polymyxin B solution (b) Polymyxin B-loaded SLN and (c) empty SLN.

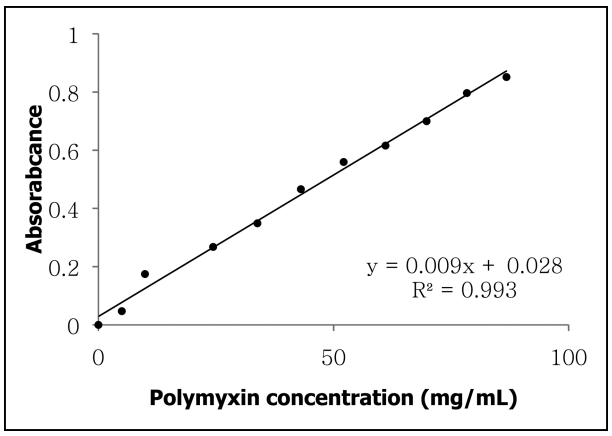

Fig. 2: Analytical curve of polymyxin B by spectrophotometry, λ=210 nm.

tigate the effect of the SLNs formulation components in the quantitative analysis of polymyxin B when loaded in the lipid matrix composed of cetyl alcohol. In addition, we planned to validate the analytical methodology by UV spectrophotometry, according to the Brazil National Agency for Sanitary Surveillance (ANVISA) Guide for Validation of Bioanalytical and Analytical Methods (RDC 899), the International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH, 2005) and USP XXVI analyzing selectivity, linearity, repeatability and accuracy (de Siqueira-Mour et al. 2008).

2. Investigations, results and discussion

To record the wavelength of the largest absorption of polymyxin B sulphate in water, a drug solution of 10 μg/mL was analyzed within the scanning range of 190-400 nm (Fig. 1a). The optimized concentration of 10 μg/mL has been selected after reaching the best absorption peak between 0.1 and 1 (Lambert-Beer Law). From the spectrum obtained, the wavelength of maximum absorption chosen for quantitative analysis was 210 nm. In order to verify the specificity and selectivity of the method, a solution of 20 μg/mL polymyxin B sulphate was analyzed in the presence of the emulsified system (i.e. without drug) under the same analytical conditions.

The EE of SLNs loading polymyxin B was 37.36 ± 2.25%. SLNs have been commonly used to load lipophilic drugs reaching high EE due to the high affinity of these compounds to the lipid matrix. For hydrophilic drugs, the EE is comparatively lower. To improve EE, *in situ* formation of an ion pairing between drug and lipid is recommended to enhance the encapsulation of hydrophilic drugs. The low EE obtained for polymyxin B sulphate in SLN was attributed to the hydrophilic character of the drug (water solubility 50 mg/ml). Figure 2 shows the calibration curve of polymyxin B. According to RDC 899, the minimum acceptable criterion of correlation coefficient of the calibration curve is 0.99.

Table 1 shows the individual values of intercept, slope and correlation coefficient of the calibration curves of polymyxin B.

Table 2 shows the results of the repeatability of the method, which has been verified by analysis of three concentrations in triplicate (n = 3) at each concentration at two different days.

The results of intra-day repeatability obtained for each concentration investigated in both assays were analyzed using t-Student test to determine statistical differences. No statistical difference was seen ($p < 0.05$) between the values recorded for the concentrations of 4.9 μg/mL, 43.0 μg/mL, 78.0 μg/mL, with the p values of 0.074, 0.071, 0.26, respectively. The result of the statistical analysis confirms the accuracy of the method for different assays.

Table 1: Individual values of intercept, slope and correlation coefficient of the calibration curves of polymyxin B obtained in the validation of the analytical method sulfate

Day	Curve	Intercept	Slope	Correlation Coefficient
1	1	0.0060	0.0099	0.9990
	2	0.0060	0.0099	0.9990
	3	0.0029	0.0100	0.9990
2	1	0.0268	0.0098	0.9913
	2	0.0097	0.0097	0.9917
	3	0.0097	0.0097	0.9934
	Average	0.0101	0.0098	0.9955
	*SD	0.0085	0.0001	
	**RSD		1.23	

*SD: Standard deviation; **RSD: Relative standard deviation.

Table 3 shows the results of intermediate precision of the method, which has been verified by the analysis of three concentrations (1, 50, 100 μg/mL) (n = 3) in triplicate for each concentration at two different days.

The relative standard deviation (RSD) obtained in the evaluation of the accuracy of the method was shown appropriate (less than 5.0%). The analytical method may assign high degree of agreement between the results when conducted under the same conditions carried out in this study.

Table 4 shows the results of the recovery method, which was verified by the analysis of three concentrations in triplicate (n = 3) of each concentration. The accuracy obtained in the evaluation of the method was appropriate. The analytical method may assign high degree of agreement between the results when conducted under the same conditions.

The calculated values for the limit of detection (LD) and limit of quantification (LQ) sensitivity and specificity of the method for the determination of polymyxin B, were 5.37 mg/mL and 17.92 g/ml respectively (Table 5).

The robustness was verified by the analysis of a solution of polymyxin B in a concentration of 43 μg/ml in triplicate (n = 3), and the results are shown in Table 6.

The variations were found to be among the values allowed by the ANVISA, which is ± 10% for accuracy and 5% for RSD. Therefore, the UV spectrophotometive method can be considered robust for the quantification of polymyxin B.

Table 2: Results of the evaluation of the accuracy for repeatability (intra-day)

Theoretical Concentration	1° day			2° day		
	4.9 μg/mL	*43.0 μg/mL*	*78.0 μg/mL*	*4.9 μg/mL*	*43.0 μg/mL*	*78.0 μg/mL*
n = 1	4.50	44.70	77.00	4.60	45.80	81.70
n = 2	4.50	44.70	77.00	4.80	45.80	77.20
n = 3	4.60	45.80	77.00	4.80	48.20	80.00
Average	4.52	45.06	77.15	4.73	46.60	79.63
SD	0.05	0.51	0.30	0.09	1.13	1.85
RSD	1.10	1.15	0.38	1.99	2.42	2.32

*SD: Standard deviation; **RSD: Relative standard deviation.

Table 3: Results of the evaluation of intermediate precision (inter-run)

	Concentration (μg/mL)		
	4.9 μg/mL	*43.0 μg/mL*	*78.0 μg/mL*
Average Day 1	4.52	45.06	77.15
Average Day 2	4.73	46.60	79.63
Average	4.62	45.83	78.39
SD	0.14	1.08	1.75
RSD	3.21	2.37	2.23

*SD: Standard deviation; **RSD: Relative standard deviation.

Table 4: Results of the evaluation of the accuracy

	Concentration (μg/mL)		
	4.9	43.0	78.0
Day 1	4.53	45.00	77.60
Day 2	4.73	46.60	79.63
Average	4.63	45.83	78.61
SD	0.14	1.03	1.43
RSD	3.05	2.36	1.82
Accuracy			
Average	96.76	108.72	102.86
SD	2.94	2.68	1.87

*Equation for accuracy y = 0.0098x + 0.0168.
*SD: Standard deviation; **RSD: Relative standard deviation.

The excipients contained in formulation demonstrated not to absorb in the wavelength of the highest absorption of polymyxin B, thus they do not interfere with the analysis and quantification of the drug. The validated method showed selectivity, accuracy, linearity and repeatability in the range of 5 – 90 μg/mL. This method is fast and of low cost, and has demonstrated to be suitable for the determination of polymyxin B sulphate incorporated in the lipid nanoparticles.

3. Experimental

3.1. Materials

Polymyxin B sulphate was kindly donated by Cristália (Itapira, Brazil). Soybean phospholipid (Lipoid S75®) was donated by Lipoid (Ludwigshafen am Rhein, Germany). Block copolymers based on ethylene oxide (Pluronic F68®) was purchased from Sigma-Aldrich (Munich, Germany). Cetyl Alcohol (Crodacol C90®) was donated by Croda (Campinas, Brazil). Ethanol was bought by Synth (Diadema, Brazil). Double distilled water was used after filtration in a Millipore system (home supplied).

3.2. Methods

3.2.1. Preparation of sample solutions and validation parameters

Analyses were carried out by UV spectrophotometry (Thermo Electron Corporation, Genesys 6, USA). To prepare the stock solution, 50 mg of polymyxin B were weighed and dissolved in 50 mL Milli®-Q water. The stock solution was then diluted with water to achieve concentrations of 5, 10, 25, 30, 40, 50, 60, 80, and 90 μg/mL and quality controls were 4.9, 43.0, and 78.0 μg/mL. According to the Brazilian Pharmacopoeia for validation of standard procedures, six calibration curves were prepared from different stock solutions and analyzed for linearity and quality parameters. Quality controls for each curve were different from those used to prepare

Table 5: Results of limit of detection (LD) and limit of quantification (LQ) polymyxin B

	Line equation	Slope (a)	Intercept (b)
Day 1	y = 0.009x + 0.005	0.0090	0.0050
Day 2	y = 0.0097x + 0.0287	0.0097	0.0287
Average		0.0093	0.0168
SD		0.0004	0.016758431
	Limit of		
detection	Limit of quantification		
	5.377 μg/mL	17.92 μg/mL	

Table 6: Results of the study of robustness for quantification of polymyxin B by spectrophotometry

Parameters		Concentration (μg/mL)	Accuracy	RSD
Sample temperature	40 °C	44.22 ± 0.007	102.83 ± 0.617	0.525
	10 °C	42.24 ± 0.009	98.23 ± 0.598	0.636

*Equation for robustness y = 0.0098x + 0.0168.

the standard curve. Samples of SLN, with or without drug, were dispersed in ethanol, the solution was filtered through a 0.45 mm membrane. Appropriate aliquots of the filtrate were transferred and completed to the volume with water. For validation of the UV spectrophotometry, the following analytical parameters were considered.

3.2.1.1. Linearity. Linearity is the ability of the analytical method to demonstrate that the results obtained are directly proportional to the concentration of compound in the sample. Six calibration curves were built with solutions of polymyxin B in water at different concentrations (5 – 90 μg/mL). The concentration curve of polymyxin B as a function of absorbance was built and linearity of the method in the range of tested concentrations was evaluated by linear regression fit of the data using the method of least squares.

3.2.1.2. Precision. Precision was evaluated by testing instrumental accuracy, repeatability and intermediate precision. In the assay, three consecutive analyses of the standard solution of polymyxin B were carried out at three different concentrations (4.9, 43.0, and 78.0 μg/mL), followed by the calculation of the average, the standard deviation and the relative standard deviation. The repeatability was evaluated by analysis, on the same day, of three consecutive samples of the standard solution of polymyxin B at three different concentrations. The intermediate precision was then determined on two different days by three consecutive analyses of a standard solution of polymyxin in three different concentrations. According to RDC 899, the standard deviation was calculated by Eq. (1)

$$\mathrm{RSD} = \frac{\mathrm{SD} \times 100}{\mathrm{DAC}} \quad (1)$$

where RSD stands for the relative standard deviation, SD for the standard deviation and DAC for the determined average concentration.

3.2.1.3. Accuracy. The accuracy of an analytical method is the closeness of the results obtained from the study in relation to the true value. The accuracy should be verified from at least nine determinations covering the linear range of the procedure, i.e. three concentrations (low, medium and high), with three replications each. Accuracy is expressed as the ratio between the average concentration determined experimentally, and the corresponding theoretical concentration, and was calculated by Eq. (2) (Sanitária 2003).

$$\mathrm{Accuracy} = \left(\frac{\mathrm{EAC}}{\mathrm{TC}}\right) \times 100 \quad (2)$$

where EAC is the experimental average concentration and TC is the theoretical concentration. According to the regulations, the results should not exceed 5%.

3.2.1.4. Limit of detection and limit of quantification. The limits of detection (LD) and quantification (LQ) were calculated using values of the intercept with the axis and the slope of the analytical curves. The theoretical LD and LQ were calculated by Eqs. (3) and (4), respectively.

$$\mathrm{LD} = \frac{(\mathrm{IA} \times 3.3)}{\mathrm{SC}} \quad (3)$$

$$\mathrm{LQ} = \frac{(\mathrm{IA} \times 10)}{\mathrm{SC}} \quad (4)$$

where IA is the intercept with the axis Y and SC is the slope of the calibration curve.

3.2.1.5. Robustness. The robustness of an analytical method is a measure of its ability to resist against small and intentional variations of the analytical parameters. In the present work, robustness was checked, stressing the samples at 40 °C and at 10 °C.

3.2.2. Statistical analysis

The results obtained from the tests for intra accuracy and inter-runs reproducibility were submitted to Student's t test to determine the statistical differences (confidence level 95%).

3.2.3. Preparation of polymyxin B sulphate SLN

The SLN were prepared by solvent emulsification/evaporation method described previously with slight modifications. Briefly, 0.1 g lipid, and 0.05 g phospholipid were dissolved in 5 mL ethanol, and polymyxin B sulphate was dissolved in water (1 mg/mL) separately; drug and lipid solutions were mixed together under mechanical stirring (Ultra-turrax®, IKA T25, Germany). The organic phase was dispersed in Pluronic® solution (1.5 mg/mL) and mixed together with mechanical stirring. Finally, the formulation was homogenized (Microfluidizer, Germany) applying 500 bar of pressure and 1 cycle. The organic solvent was completely evaporated with mixed under magnetic stirring for 24 h. Non-encapsulated drug was removed by centrifugation (60,000 rpm for 2 h at 4 °C). The amount of polymyxin B in the supernatant was determined at 210 nm using UV-Vis spectrophotometer after appropriate dilution, and the encapsulation efficiency (EE) was determined using the Eq. 6.

$$\%\mathrm{EE} = \left(\frac{\text{Total drug content} - \text{Free drug}}{\text{Total drug content}}\right) \times 100 \quad (6)$$

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