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Development of derivative spectrophotometric method for simultaneous determination of pyrazinamide and rifampicin in cubosome formulation

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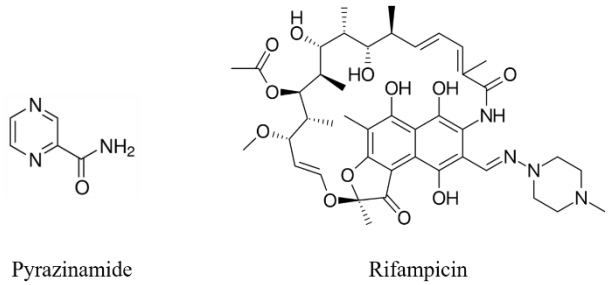
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The ultraviolet spectrophotometry analysis for quantitative assay of drugs is a method accurate, sensitive, selective and reproductive with the advantage of being a simple and less expensive method. In this study, a derivative ultraviolet spectrophotometric method was developed for simultaneous determination of pyrazinamide (PYZ) and rifampicin (RIF). The spectrophotometric method was evaluated according to validation guidelines for specificity, linearity, limits of detection and quantification, precision, accuracy and robustness. The first-derivative spectra were obtained and by the zero-crossing point, the wavelength 247 nm and 365 nm were selected for PYZ and RIF quantification, respectively. No interference from cubosome excipients was detected in the proposed method. The results demonstrated linearity in a range of 4.0 – 12.0 µg/mL with an adequate correlation coefficient for both drugs. The intra and inter-day precision results (RSD < 5%) indicated the reproducibility of the method. The accuracy data showed satisfactory results (RSD < 5%) from recovery test. In addition, the robustness results showed that the PYZ and RIF content were unaffected by the solvent alteration of methanol to methanol:water (99:1, v/v). The derivative ultraviolet spectrophotometric method proved to be an excellent strategy for simultaneous determination of PYZ and RIF.

Keywords: Pyrazinamide, Rifampicin, UV spectrophotometry, derivative spectrophotometry.
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Introduction

World Health Organization recognizes four drugs for tuberculosis diseases: pyrazinamide (PYZ), rifampicin (RIF), isoniazid and ethambutol (1). Pyrazinamide (pyrazine-2-carboxamide; Figure 1) is classified as a crystalline white or almost white powder, slightly soluble in water and ethanol (2). This drug is an analog of nicotinamide with bactericidal property (3). The mechanism of action of PYZ is unknown and experimental evidence suggests that bactericidal action is dependent of the pyrazinamidase enzyme from *Mycobacterium tuberculosis* (4). Once PYZ is converted into pyrazinoic acid by bacterial pyrazinamidase, the high concentrations of pyrazinoic acid in the bacterial cytoplasm decrease the intracellular pH causing inactivation of vital enzymes, resulting in the death of the bacillus (5).



Pyrazinamide Rifampicin

Figure 1. Chemical structure of pyrazinamide and rifampicin.

RIF ((7S,9E,11S,12R,13S,14R,15R,16R,17S,18S,19E,21Z)-2,15,17,27,29-pentahydroxy-11-methoxy-3,7,12,14,16,18,22-heptamethyl-26-[(1E)-[(4-methylpiperazin-1-yl)imino]methyl]-6,23-dioxo-8,30-dioxa-24-azatetracyclo[23.3.1.1⁴,⁷.0⁵,²⁸]triaconta-1(29),2,4,9,19,21,25,27-octaen-13-yl acetate; Figure 1) is a red to orange odorless powder, very slightly soluble in water and ethanol; and soluble in methanol (2). RIF is a semi-synthetic antibiotic derived from rifamycin B, produced by strains of *Amycolatopsis rifamycinica* (6). It presents a broad spectrum bactericidal action, inhibiting the activity of the DNA-dependent RNA polymerase enzyme, which prevents the synthesis of messenger RNA (mRNA) and protein by the bacillus, causing cell death (7).

In order to prevent the emergence of bacterial resistance, PYZ and RIF are used in combination (1,3). In addition to the pharmacotechnical challenges for the preparation of formulations containing this association, it is necessary to develop new analytical methods for the simultaneous determination of drugs. Several published studies used different analytical techniques for the determination of antituberculosis drugs alone and in combination, including High Performance Liquid Chromatography (HPLC) with UV detector (8,9), High Performance Thin Layer Chromatography (HPTLC) (10), Ultra-high Performance Liquid Chromatography (UHPLC) with UV detector (11,12), Fourier Transform Infrared (FTIR) spectroscopy in combination with multivariate calibration of partial least square (13) and UV spectrophotometry (14). Due to the absence of an analytical method for the simultaneous

determination of PYZ and RIF by derivative spectrophotometric method and considering the importance of analytical methods for the simultaneous determination of drugs, this study aims to develop and validate a simple and rapid derivative spectrophotometric method for the determination of PYZ and RIF in cubosome formulation.

Experimental section

Materials

The PYZ and RIF (pharmaceutical grade) were gently donated by Fundação para o Remédio Popular (Brazil) and Farmanguinhos (Brazil), respectively. The phytantriol (3,7,11,15-tetramethyl-1,2,3-hexadecanetriol, 96.9%) and poloxamer 407 (PEO98-PPO67-PEO98, MW 12,500 g/mol) were purchased from Alianza (Brazil) and Via Farma (Brazil), respectively. Methanol analytical grade was supplied from J.T. Barker and was used to prepare all solutions for analysis.

Preliminary tests

The validation of the PYZ and RIF quantification by ultraviolet spectrophotometry method started with preliminary tests (data not showed). Different solvents were tested, such as acetonitrile, phosphate buffer, water, ethanol and methanol. The methanol was selected since it had a great capability to dissolve the phytantriol, the main excipient of the cubosomes.

Instrumental

The PYZ and RIF were quantified by spectrophotometric method carried out on LAMBDA 265 UV/Vis Spectrophotometer (PerkinElmer, USA) connected to UV LAB 4.0.0 software. The absorbance was determined within the range of 200 to 400 nm using matched 1 cm quartz cells. Measurements were performed using a standard solution of PYZ and RIF in methanol in order to obtain first-derivative spectra using the zero crossing point.

Method validation

The spectrophotometric method for simultaneous determination of PYZ and RIF was validated for specificity, linearity, limits of detection (LOD) and quantification (LOQ), precision, accuracy and robustness according to the International Conference on Harmonization (ICH) guidelines Q2 (R1) for the validation of analytical methods (15) and to RDC nº 166 of July 24, 2017 (16).

Preparation of standard solutions

For the preparation of the standard solution, PYZ (5 mg) and RIF (5 mg) were dissolved, separately, in methanol (10 mL) and sonicated for 5 minutes.

Preparation of unloaded cubosomes

Cubosomes were prepared as described previously (17) with some modifications. Phytantriol (750 mg), poloxamer 407 (187.5 mg) and deionized water (30 mL) were homogenized for 25 min by a 500 W ultrasonic processor (Ecosonics, Brazil) at 99% of amplitude and with a 13 mm diameter probe. The final volume was corrected to 30 mL with deionized water.

Specificity

Specificity was evaluated by analyzing solutions of unloaded cubosomes. The system response was determined through the presence of overlaps of unloaded cubosomes with the PYZ and RIF response.

Linearity

The linearity experiments were conducted from a standard solution of PYZ and RIF. Aliquots of standard solution were diluted in methanol to obtain the final concentrations of 4.0, 6.0, 8.0, 10.0 and 12.0 µg/mL of PYZ and RIF. All solutions were prepared in triplicate and analyzed on three different days. Calibration curves (concentration *vs* absorbance) were plotted, and the equation of the line was determined through the linear regression, using the method of least squares. The analysis of variance (ANOVA) was also calculated for the statistical parameters. The linearity was expressed as the correlation coefficients.

Limits of detection and quantitation

The LOD and LOQ were estimated using the standard deviation of the value of the intercept with the Y axis and slope of three calibration curves. The LOD and LOQ were evaluated based on the previously constructed calibration curve.

Precision

For repeatability (intra-day precision) experiment, six solutions containing a combination of PYZ and RIF at central point of the standard curves (concentration of 8.0 µg/mL of each drug) were evaluated on the same day under the same experimental conditions. The intermediate precision (inter-day precision) of the method was assessed with PYZ (8.0 µg/mL) and RIF (8.0 µg/mL) solution and performed on three different days. The experimental results were expressed as the relative standard deviations (RSD).

Accuracy

The accuracy experiments were determined by applying the method to quantify PYZ and RIF in the presence of the unloaded cubosome. Unloaded cubosomes were spiked with amount of PYZ and RIF at concentrations of 4.0, 8.0 and 12.0 µg/mL of each drug. The analyses were performed in triplicates. Accuracy was expressed as percentage of

PYZ and RIF recovered in the lowest, intermediate and highest concentrations.

Robustness

The robustness was determined by small variation in the established analytical condition. Samples of PYZ (8.0 µg/mL) and RIF (8.0 µg/mL) prepared with methanol:water (99:1, v/v) were utilized to conducted the test. The experimental results were expressed as the RSD values.

Results and Discussion

Validation of the quantification method

The spectrophotometry analysis for determination of drugs is a method accurate, sensitive, selective, reproductive and proved to be a less expensive alternative when compared to the HPLC method. However, analysis of drug mixtures by ultraviolet-visible spectrophotometry is often compromised due to the overlap of the electronic transition bands. As seen in Figure 2, the zero-order spectra of PYZ and RIF showed an overlapping, making the detection and quantification of drugs unfeasible. The zero-order spectra of PYZ and RIF were processed to obtain first-derivative spectra. By overlapping spectra of first-derivatives, it is possible to individualize the constituents and even eliminate the interference of one component on the other. The spectra of the first-derivative of PYZ and RIF were superimposed and showed that at wavelength 247 nm there is the annulment of the spectrum of the drug RIF as well as at wavelength 365 nm, the annulment of PYZ occurs (Figure 3).

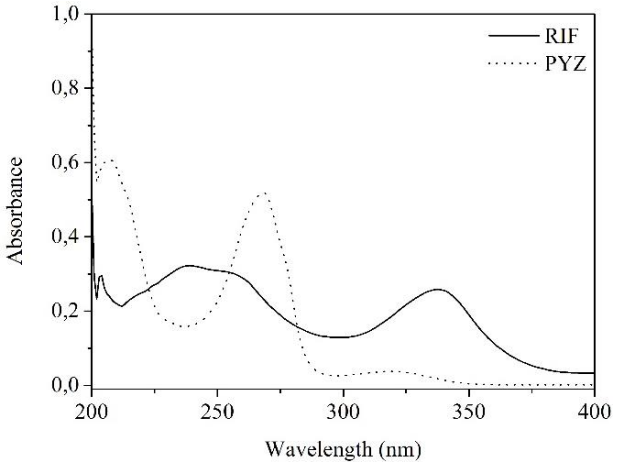


Figure 2. Zero-order absorption spectra of PYZ (8.0 µg/mL) and RIF (8.0 µg/mL) obtained in methanol.

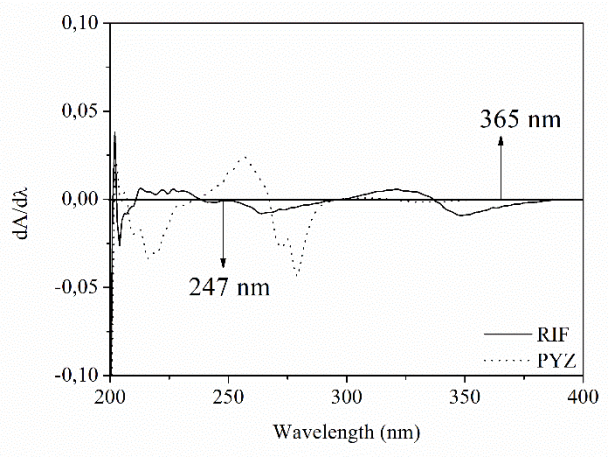


Figure 3. Absorption spectra of the first-derivative spectra of PYZ (8.0 µg/mL) and RIF (8.0 µg/mL) obtained in methanol.

Specificity

The interference of the unloaded cubosome at the PYZ and RIF measurement was demonstrated in the specificity test. As clearly seen in the first-derivative spectrum of unloaded cubosome (Figure 4), a minimum absorption of 0.0002 at 247 nm and 0.0001 at 365 nm was observed. These results are in agreement with previous studies (18,19), which observed that the first-derivative spectrophotometric method was able to eliminate the interference from excipients. The proposed analytical method is able to quantify these specific drugs in the presence of a complex matrix, such cubosomes, that contain the inputs phytantriol and poloxamer 407.

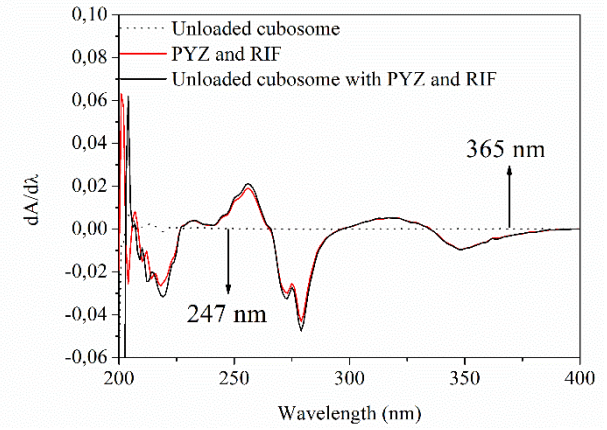


Figure 4. Absorption spectra of the first-derivative of unloaded cubosome, standard solution of PYZ and RIF and unloaded cubosome with standard solution of PYZ and RIF.

Linearity

The results from linearity test are summarized in Table 1. The least square regression showed excellent correlation between the PYZ concentration and the peak amplitude (247 nm) in the range of 4.0 and 12.0 µg/mL with r value of 0.999. Similarly correlation was observed for the RIF concentration and peak amplitude at 365 nm (r=0.998). Statistical analysis by ANOVA confirmed a linear

regression for PYZ ($F_{calculated}$ = 608.19 > $F_{tabulated}$ = 4.96; p=0.05) and RIF ($F_{calculated}$ = 573.97 > $F_{tabulated}$ = 4.96; p=0.05) and also shows that there is no deviation from linearity for PYZ ($F_{calculated}$ = 0.20 < $F_{tabulated}$ = 3.71; p=0.05) and RIF ($F_{calculated}$ = 3.65 < $F_{tabulated}$ = 3.71; p=0.05).

Table 1. Linearity results obtained from standard curve of first-derivative spectrophotometric method for pyrazinamide and rifampicin quantification.

Parameter	Results	
	PYZ	RIF
Linear range	4.0 – 12.0 µg/mL	4.0 – 12.0 µg/mL
Regression equation	y = 0.001x – 0.0007	y = -0.0006x + 0.0001
Correlation coefficient (r)	0.999	0.998

Limits of detection and quantitation

The calculated LOD for PYZ and RIF was 0.87 µg/mL and 0.82 µg/mL, respectively, which demonstrated a great sensitivity to detect the drugs. The LOQ was estimated at 2.89 µg/mL for PYZ and 2.73 µg/mL for RIF. The results show that the proposed method allowed detection and quantification of low drug concentration, indicating that the method is sensitive enough for both drugs.

Precision

The repeatability (intra-day precision) and intermediate precision (inter-day precision) data expressed as RSD are showed in Table 2. The first-derivative spectrophotometric method for simultaneous quantification of PYZ and RIF demonstrated an adequate repeatability. The RSD for both drugs were less than 5.0%, which are in accordance with the guidelines. Similar results were observed in intermediate precision (inter-day precision) with acceptable RSD range (≤ 5.0%) which represents an agreement between the results obtained when performing the analysis in the same laboratory but on different days.

Table 2. Repeatability and intermediate precision values of first-derivative spectrophotometric method for pyrazinamide and rifampicin quantification.

Parameter	PYZ ± SD (µg/mL)	RSD (%)	RIF ± SD (µg/mL)	RSD (%)
Repeatability[a]	7.86 ± 0.18	2.25	7.67 ± 0.15	2.00
Intermediate precision				
Day 1[b]	8.012 ± 0.17	2.09	7.09 ± 0.23	3.21
Day 2[b]	7.79 ± 0.17	2.19	7.69 ± 0.27	3.48
Day 3[b]	7.80 ± 0.13	1.70	7.38 ± 0.23	3.17

[a]Mean of six determinations; [b]Mean of three determinations.

Accuracy

The method accuracy was studied at three different levels (low, medium and high concentration) and by adding unloaded cubosomes. The drugs recovery experiments were carried, and the results are shown at Table 3.

Table 3. Recovery results of pyrazinamide and rifampicin in the presence of unloaded cubosomes analyzed by derivative spectrophotometric method.

Level	Drugs	Concentration (µg/mL)			
		Amount added	Amount found	Recovery (%)	RSD (%)
1	PYZ[a]	4.00	4.67	116.67	2.47
	RIF[a]	4.00	3.78	94.44	2.55
2	PYZ[a]	8.00	8.21	102.63	3.40
	RIF[a]	8.00	7.72	96.53	2.49
3	PYZ[a]	12.00	12.40	103.33	1.40
	RIF[a]	12.00	11.73	97.73	4.45

[a]Mean of three determinations.

The derivative spectrophotometric method was considered accurate for the proposed drugs, presenting a satisfactory RSD value between 1.40% and 4.45%.

Robustness

The results of the robustness are summarized in Table 4. The PYZ and RIF content were unaffected by the alteration of the methanol concentration used in the sample preparation, with RSD values lower than 5% for both drugs. This results demonstrate that little variation in the proportion of methanol:water used in the preparation of the samples did not influence the drug recovery.

Table 4. Effects of the variation of analytical parameters in the pyrazinamide and rifampicin content for the robustness test.

Drugs	Mean concentration ± SD (µg/mL)	Mean recover (%)	RSD (%)
PYZ[a]	8.12 ± 0.11	101.52	1.35
RIF[a]	7.75 ± 0.17	96.84	2.21

[a]Mean of six determinations.

Conclusions

A first-derivative spectrometric method was developed and validated for simultaneous determination of PYZ and RIF in cubosome formulation. The results of validation showed that the analytical method demonstrating to be simple, rapid, sensitive, precise and accurate.

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Conflict of interest

The authors declare no conflicts of interest.

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