ECLÉTICA QUÍMICA

Eclet. Quim. 51 | e-1643, 2026

Original Articles in Chemistry and Related Areas

https://doi.org/10.26850/1678-4618.eq.v51.2026.e1643

Development and validation of a UV spectrophotometric method for measuring ciprofloxacin hydrochloride in chitosan films used for orthopedic implants coatings

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Abstract

A simple, precise, and accurate ultraviolet-visible (UV-Vis) spectrophotometric method was developed and validated to quantify ciprofloxacin hydrochloride (CIP.HCl) in chitosan films for coating orthopedic implants. Methods were developed to enable quantification in both acidic and basic conditions. The validation parameters were established according to the Resolution of the Collegiate Board (RDC) No. 166, of July 24, 2017, of the Brazilian National Health Regulatory Agency (ANVISA). Samples with CIP.HCl exhibited maximum absorption at 277 nm in acidic medium and 271 nm in basic medium. Linearity was achieved in the concentration range of 2–10 μg mL^{-1} with a correlation coefficient 0.999 and statistical significance. The relative standard deviation for precision was ≤ 2%. Recovery percentages of the analyte in the films exceeded 98.0%. No matrix effect was identified, and the method demonstrated robustness. The detection limit was 0.11 μg mL^{-1} for acidic and 0.21 μg mL^{-1} for basic conditions, while the quantification limit was 0.34 μg mL^{-1} for acidic and 0.64 μg mL^{-1} for basic pH.

Article History

Received March 06, 2025
Accepted May 15, 2025
Published July 22, 2026

Keywords

1. chitosan
2. osteomyelitis
3. antimicrobials
4. UV spectrophotometry
5. analytical validation

Section Editors

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Highlights

- The method showed selectivity for acidic and basic pH.
- The method showed linearity with statistical validity in the 2–10 μg mL^{-1} range.
- Precise and accurate with low standard deviation (≤ 2%).
- Robust method without matrix effect.
- Suitable for dosing ciprofloxacin hydrochloride in chitosan films.

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1. Introduction

Osteomyelitis is an acute or chronic infection and inflammation that affects bone structures. They can be caused by several microorganisms, including fungi and bacteria, and diseases caused by the latter are the most difficult to treat, in part due to antimicrobial resistance to the prominent etiological agent, the gram-positive bacterium *Staphylococcus aureus* (Urish and Cassat, 2020). One of the aggravating factors of the disease is that it can progress to bone necrosis. The persistence of necrotic tissue causes the condition to evolve from an acute to a chronic state, which requires complex treatment due to the low availability of antimicrobials in bone tissue (Viana *et al.*, 2023).

Conventional clinical treatment consists of the use of systemic antimicrobials administered orally and parenterally, but it has low efficacy and adverse effects due to high doses and long treatment periods. The high failure rate is associated with low local blood supply and low solubility/permeability of antimicrobials in bone tissue. Another aggravating factor is that prolonged use of these drugs has led to recurrent cases of microbial resistance. The low effectiveness of systemic treatment makes it necessary to perform multiple debridement sessions (surgical approach) and fix orthopedic metal implants due to bone loss. However, there are reports that these can induce osteomyelitis in patients by facilitating the adhesion of microorganisms. (Zhong *et al.*, 2023).

Epidemiological studies have shown that approximately 31.1% of osteomyelitis cases occurred after surgery, 26.2% after trauma and 23% through the hematogenous route. In 29.6% of cases, the predominant microorganism was methicillin-sensitive S. aureus, and the prominent bones affected were the tibia (24.1%) and femur (21.8%). Of 344 patients studied, approximately 234 (68.0%) underwent surgery, 113 (32.8%) underwent debridement, 97 (27.4%) underwent debridement and bone reconstruction, and 24 (7%) underwent amputation (García Del Pozo *et al.*, 2018). Other studies have shown that approximately half of the cases of osteomyelitis in adults may be associated with the use of implants. Approximately 33% of cases are associated with S. aureus and 32% with coagulase-negative staphylococci (Hirsiger *et al.*, 2017).

Using local treatment by inserting devices containing antimicrobial drugs has proven to be a viable alternative, as it allows better local distribution of the active ingredient and greater treatment efficiency. Another advantage is that these devices can be made in different formats, such as films, pellets, bone cements and scaffolds that can promote a large contact surface with bone tissue and present a significant rate of infection eradication (Kuang *et al.*, 2021; Smith *et al.*, 2022).

Among the most promising devices, those designed based on polymeric systems are the first choice due to their versatility, efficiency and large contact surface (Mckee *et al.*, 2010). Devices containing chitosan in their composition have gained a lot of attention for this purpose, because, due to its availability, non-toxicity and biocompatibility, this natural polymer has been widely studied for medical applications (Zhong *et al.*, 2023). The potential of chitosan for the production of medicines containing antimicrobial drugs for the treatment of osteomyelitis was demonstrated in an animal model, where the group of animals treated with chitosan films incorporated with ciprofloxacin presented a significantly lower development of the disease than those that received the film without the drug (Costa *et al.*, 2016).

The use of CIP.HCl gains importance due to its broad spectrum of action (Uncu *et al.*, 2019). Fluoroquinolones such as ciprofloxacin are the most studied antimicrobials for treating chronic osteomyelitis. Cure percentages between 60 and 80% and low failure rates against *S. aureus* are reported. However, high doses and treatment periods have been used, and ciprofloxacin has been the focus of most of these studies (Spellberg and Lipsky, 2012).

The literature reports chitosan-based polymeric systems impregnated with antimicrobials. These systems have poor mechanical properties, making their application as coatings impracticable (Leceta *et al.*, 2013). This characteristic is intrinsic to polysaccharides, so modifying these systems with additives such as plasticizers can improve these physicochemical properties and, in addition, ensure better drug release, increasing its availability at the site of action (El-Gendy, 2012; Tao *et al.*, 2021).

Therefore, the development of chitosan films impregnated with CIP.HCl with better physicochemical properties for coating bone inserts is necessary, as they have great potential in the treatment and/or prevention of osteomyelitis. Dosing a drug in pharmaceutical form can certify its efficacy and safety (Uncu *et al.*, 2019). Among the possible methods, Brazilian pharmacopoeia suggests using analytical methods such as UV-Vis spectrophotometry to dose the drug in solid pharmaceutical forms, such as tablets (Brazil, 2024).

UV-vis spectroscopy is a simple, versatile, non-destructive, cost-effective analytical technique suitable for a broad spectrum of chemical compounds (Khalid *et al.*, 2024). Therefore, developing a simple and accurate UV-Vis spectrophotometric method may provide a valuable alternative for the routine analysis of CIP.HCl in bone inserts.

Thus, this work aims to develop and validate an analytical methodology using UV-Vis spectrophotometers for the identification and dosage of the drug CIP.HCl is used in chitosan films used to coat bone inserts developed for the treatment of osteomyelitis.

2. Experimental

The method development and validation procedures were prepared and validated as provided in the RDC of the ANVISA and complementary documents, such as the Guide for Statistical Treatment of Analytical Validation of ANVISA, guide for Validation and Analytical Quality Control of Pharmaceuticals in Products for Animal Feed and Veterinary Medicines of the Ministry of Agriculture, Livestock and Food Supply (MAPA), ICH Q2 (R2) Validation of analytical procedures – scientific guideline and scientific articles.

2.1. Reagents and solutions

The CIP.HCl standard was purchased from Sigma-Aldrich (Brazil), and glacial acetic acid was purchased from FMAIA (Brazil). In the production of basic Phosphate Buffer Saline (PBS), the salts dibasic sodium phosphate and monobasic potassium phosphate were purchased from VETEC (Brazil), and potassium chloride and sodium chloride were supplied by Synth (Brazil). All experiments used water treated by reverse osmosis in a Milli-Q water apparatus (Millipore brand, Direct-Q model, Morshem, France), Shimadzu analytical balance, and Thermo Fischer Scientific spectrophotometer model Evolution 201. Other reagents and equipment are part of the Federal University of Minas Gerais Pharmaceutical Technology Laboratory and are of analytical grade, used without further purification.

2.2. Methods

2.2.1. Preparation of standard ciprofloxacin hydrochloride stock solution

Stock solution A was prepared by dissolving 109.8 mg of the standard (equivalent to 100 mg of ciprofloxacin) in 100 mL of 2% (v/v) acetic acid.

Stock solution B, containing the standard in a physiological buffer, was prepared by dissolving 4.4 mg of the standard (equivalent to 4.0 mg of ciprofloxacin) in 100 mL of phosphate-buffered saline pH 7.4 (PBS 7.4).

2.2.2. Preparation of calibration standard solutions

The standard solutions were prepared by diluting stock solution A with 2% (v/v) acetic acid, and five solutions were prepared with 2, 4, 6, 8, and 10 μg mL^{-1} concentrations. Calibration standards in physiological medium were prepared at identical concentrations by diluting stock solution B with PBS 7.4.

2.2.3. Preparation of solutions of the white matrix fortified with the standard

Blank films (without the drug) were cut into 4.5 mm diameter disks and placed in 50 mL Falcon tubes. 20 mL of 2% (v/v) acetic acid was added to each tube. Volumes of 50, 100, 150, 200, and 250 μL of the standard stock solution A were added to the tube and left in an ultrasound for 1 h and stirred for 24 h, at room temperature, in a benchtop shaker, IKA, model KS 4000. The resulting solution was quantitatively transferred to a 25.00 mL volumetric flask, and the volume was completed with 2% (v/v) acetic acid solution, resulting in a solution with 2, 4, 6, 8 and 10 μg mL^{-1}. Unfortified solutions were prepared similarly, but without adding the standard stock solution.

2.2.4. Preparation of sample solutions of chitosan films containing the drug

The film containing the drug was cut into 4.5 mm diameter discs, weighed and placed in 50 mL Falcon tubes. 20 mL of 2% (v/v) acetic acid was added to the tubes, left in an ultrasound for 1 h, and under agitation for 24 h in a benchtop shaker, IKA, model KS 4000 at 25 °C. The resulting solution was quantitatively transferred to a 25.00 mL volumetric flask, and the volume was completed with 2% (v/v) acetic acid solution. Film samples in basic medium were prepared in the same way. However, the films were placed inside dialysis membranes, and the ends were sealed to prevent the passage of the polymer.

2.2.5. Determination of the maximum absorption wavelength

The maximum absorption wavelength was determined by measuring the absorbance of a 6 μg mL^{-1} solution, prepared as described in **2.2.2**, over the 200 to 400nm wavelength range. The procedure was performed similarly in the basic medium, except that stock solution B was diluted with PBS 7.4.

2.2.6. Selectivity

To determine selectivity in acid medium, the spectrum of the standard solutions in solvent at a concentration of 6 μg mL^{-1} prepared as described in **2.2.2**, of the blank matrix solution (without the standard) and the matrix solution fortified with the standard at a concentration of 6 μg mL^{-1} prepared as per **2.2.3** and the film solution containing the drug prepared as per **2.2.4** were determined in the wavelength range of 200 to 400 nm.

2.2.7. Linearity

The analytical curve was constructed by measuring the absorbance of solutions prepared in triplicate, as described in section **2.2.2**, at a wavelength of 277 nm for the acidic medium and 271 nm for the basic medium. The analytical curve and statistical evaluation were performed using Excel software. The homoscedasticity was evaluated using the Cochran test, and the assessment of normality was conducted using the Shapiro-Wilk test.

2.2.8. Matrix effect

The effect of the matrix in an acidic medium was evaluated by constructing a calibration curve with the blank sample matrix fortified with the drug standard prepared according to item **2.2.3** and read at 277 nm.

2.2.9. Intraday (repeatability) and interday (intermediate) precision

To assess repeatability in an acidic medium, sextuplicate of the white matrix solutions fortified with standard concentrations of 2, 6 and 10 μg mL^{-1} were read in a spectrophotometer set at a wavelength of 277 nm immediately after preparation. To assess interday precision, solutions prepared similarly were stored for 24 h at room temperature and read by a different analyst. The same procedure was performed for samples prepared in PBS 7.4.

2.2.10. Accuracy

To assess accuracy in acidic medium, sextuplicate of the blank matrix solutions fortified with standard concentrations of 2, 6 and 10 μg mL^{-1} were measured in a spectrophotometer set at a wavelength of 277 nm. Samples in PBS 7.4 buffer were prepared at the same concentrations and measured at a wavelength of 271 nm.

2.2.11. Detection limit (LD)

The detection limit was obtained from the standard deviation of the response and the slope of the analytical curve and expressed according to the **Eq. 1**.

$$LD = 3.3\sigma S^{-1} \quad (1)$$

where: σ = the standard deviation of the response obtained from the standard error of the regression and S = the slope of the calibration curve.

2.2.12. Limit of quantification (LQ)

The limit of quantification was obtained from the standard deviation of the response and the slope of the analytical curve and expressed according to the **Eq. 2** (ICH, 2023):

$$LQ = 10\sigma S^{-1} \quad (2)$$

where: σ = the standard deviation of the response obtained from the standard error of the regression and S = the slope of the calibration curve.

2.2.13. Robustness

The robustness of the method developed at both pH conditions was assessed by analyzing the parallelism of the linear regression lines obtained from calibration curves obtained by measuring the absorbance values of blank matrix

https://doi.org/10.26850/1678-4618.eq.v51.2026.e1643

solutions fortified with standard at concentrations of 2, 4, 6, 8 and 10 µg mL^{-1} at wavelengths of 274 nm and 280 nm for the acidic conditions, and at 268 e 274 nm for PBS 7.4.

To investigate the method's robustness in PBS 7.4 solvent, the standards at concentrations of 2, 4, 6, 8, and 10 µg mL^{-1} prepared according to item **2.2.2** were read at 268 and 274 nm wavelengths.

2.2.14. Statistical analysis

The data obtained were entered into Microsoft Excel software for statistical analysis. The values were expressed as means ± standard deviation. The relationships between the signals and the concentrations were evaluated by linear regression analysis. The quality of the model was verified using the coefficient of determination (R^2), correlation coefficient (R) and the ANOVA. A statistically significant difference was considered when the *p*-value was less than 0.05.

3. Results and discussion

3.1. Maximum absorption wavelength

Determining the maximum absorption of an analyte is essential for accurate quantitative analysis. This is because selecting a wavelength within a region of the absorption spectrum where the analyte molar absorptivity remains relatively constant helps minimize deviations from the Lambert-Beer law. It can be observed in **Fig. 1** that the standard in an acidic and physiological solvent presented maximum absorption at 277 and 271 nm, respectively. These wavelengths found are consistent with previous results for CIP.HCl in 0.1 mol/L HCl solution and PBS 7.4 (Makwana *et al.*, 2016; Prasad and Ratna, 2018).

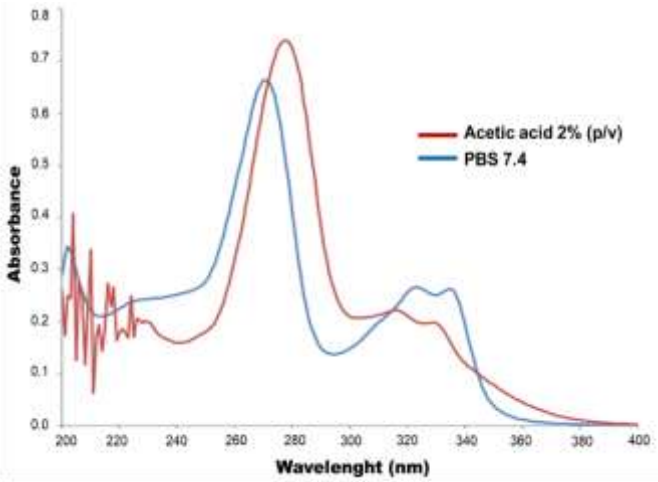

Figure 1. Spectrum of ciprofloxacin standards in acetic acid 2% (v/v) and PBS 7.4.
Source: Elaborated by the authors.

Figure 1 shows a hypsochromic shift of the maximum absorption band to the spectrum obtained in 2% acetic acid (v/v). This shift is characteristic of drugs such as CIP.HCl that have an amphoteric structure. A hypochromic effect accompanies this hypochromic shift: a decrease in absorption intensity. Similar effects are reported in the literature (Czyrski, 2022; Drakopoulos and Ioannou, 1997; Huang *et al.*, 1997).

Furthermore, these pH values were chosen so that it would be possible to quickly measure the drug present in the device, since chitosan has high solubility in acidic conditions. pH 7.4 was chosen so that it would be possible to measure the drug under conditions like physiological conditions, as is done in in vitro release studies.

3.2. Selectivity

Selectivity is an essential parameter for the development of the analytical method. It evaluates the method's capacity to provide signals without interference from other substances in the sample (Vessman *et al.*, 2001). As observed in **Fig. 2**, the unfortified white matrix solution - Placebo (purple) does not show absorption at the selected wavelength. However, the white matrix solution added with the standard and the standard solution in solvent (red), chitosan film with plasticizer and ciprofloxacin hydrochloride (blue) showed maximum absorption at 277 nm. The curves of the standard samples in solvent and the white matrix fortified with standard overlapped. Similar selectivity results were demonstrated for ciprofloxacin hydrochloride in the literature (Uncu *et al.*, 2019).

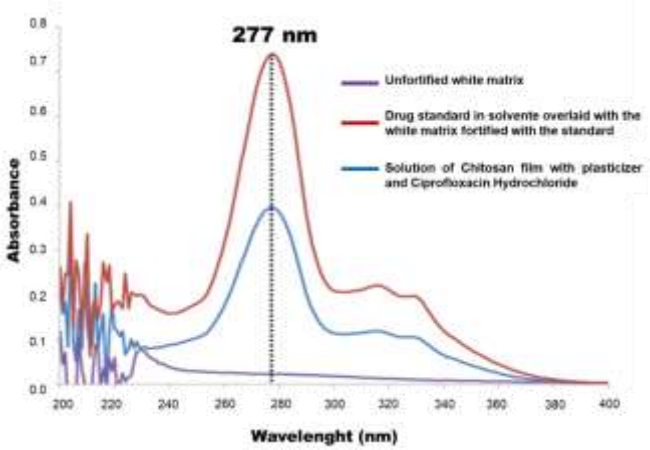

Figure 2. Result for the selectivity test of the method in acetic acid 2% (v/v).
Source: Elaborated by the authors.

To evaluate the method's selectivity in a basic medium, the solvent PBS 7.4, CIP.HCl standard in the solvent and the sample of the release test were read in the wavelength range of 200 nm to 400 nm, and the results are shown in **Fig. 3**. The 6 µg mL^{-1} standard and the release test sample showed a maximum absorption at 271 nm. No absorption was observed for the PBS 7.4 solvent at the same wavelength as the samples with the CIP.HCl standard and active pharmaceutical ingredient (IFA). The blank solution consisted only of solvents since the dialysis membrane capable of retaining the polymer was used in the release test.

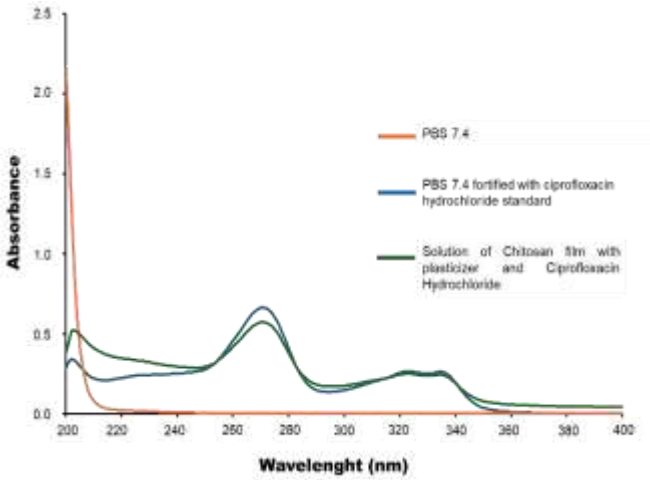

Figure 3. Selectivity result in PBS 7.4.
Source: Elaborated by the authors.

3.3. Linearity

The linearity expressed the capability to obtain analytical responses that are directly proportional to the concentration of an analyte in a sample (Brazil, 2024). The linear relationship between concentration and absorbance was initially evaluated using standard solutions of CIP.HCl in the 5 to 50 µg mL^{-1} concentration range at a wavelength of 277 nm for the acidic medium and 271 nm for the PBS 7.4 solvent. However, linearity was only observed within the range of 5 to 15 µg·mL^{-1}. A subsequent literature review was conducted to identify the linear working range reported in previously validated methods (Cazedey and Salgado, 2012; Uncu *et al.*, 2019). Based on these findings, the working range was established as 2 to 10 µg·mL^{-1}.

This range was selected to ensure that absorbance values remained within the optimal interval of 0.1 to 1.0, thereby minimizing potential deviations from the Lambert-Beer law typically associated with absorbance values outside this range (Skoog *et al.*, 2008).

Absorbance values for the prepared standard solutions are shown in **Table 1**. The values for the acidic medium are compatible with those reported in the literature for validated methods for determining ciprofloxacin in optic drops (Uncu *et al.*, 2019). However, they were nearly twice the values reported for curves constructed in PBS 7.4 for determining ciprofloxacin hydrochloride in ophthalmic gel, which can be explained by the hypsochromic and hypochromic effect (Ipte *et al*, 2019; Makwana *et al.*, 2016; Mangalgiri *et al*, 2022; Skoog *et al.*, 2008).

Table 1. Absorbance results of reading working standards in 2% (v/v) acetic acid and PBS 7.4.

Level (µg mL^{-1})	Acetic acid 2% (p/v)		PBS 7.4	
	Average absorbance n = 3	Relative standard deviation (%)	Average absorbance n = 3	Relative standard deviation (%)
2	0.243	1.66	0.209	4.57
4	0.490	0.54	0.421	0.41
6	0.740	0.34	0.631	0.81
8	0.983	0.27	0.839	0.68
10	1.222	0.37	1.036	0.20

Note: n = number of replicates; p/v = weight to volume concentration.
Source: Elaborated by the authors.

In addition, based on these values, a graph was constructed representing the absorbance response (y-axis) as a function of concentration (x-axis) and constituting the analytical curve of the method. The obtained analytical curve exhibits a visually linear relationship with a correlation and determination coefficient greater than 0.990. These results follow the literature (Brazil, 2024), as shown in **Fig. 4**.

To evaluate the regression quality, absorbance values must demonstrate homoscedasticity (Brazil, 2024), that is, homogeneity of variances (Riffenburgh, 2012). Cochran's test is recommended to assess homoscedasticity, and for data to be considered homoscedastic, the calculated test value must be lower than the critical value (Barbosa *et al.*, 2017; Brazil, 2017b).

As shown in **Table 2**, the results demonstrated homoscedasticity since the Cochran test value was lower than the critical value. In addition to homoscedasticity, the Guide for Statistical Treatment of Analytical Validation published by ANVISA defines that those residuals, calculated as the difference between expected concentration and observed concentration using absorbance values and the regression equation, should follow a normal distribution, and the Shapiro-Wilk test must be applied (Brazil, 2017b). For the Shapiro-Wilk test, the calculated value must be above the critical value (Lopes *et al.*, 2013; Ronowicz *et al.*, 2014).

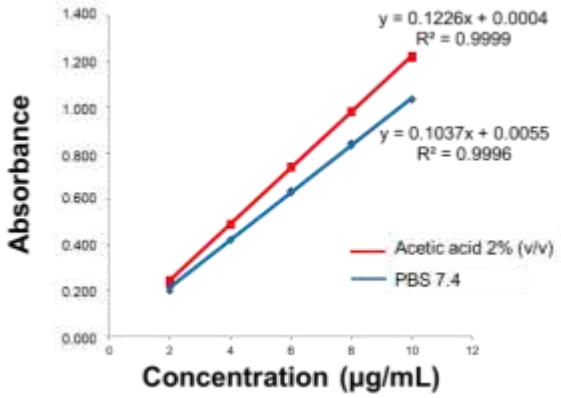

Figure 4. Calibration curves of ciprofloxacin in acetic acid 2% (v/v) (red) and PBS 7.4 (blue).
Note: v/v = volume to volume concentration.
Source: Elaborated by the authors.

Table 2. Results of Cochran and Shapiro-Wilk tests in acetic acid 2% (v/v) and PBS 7.4.

Test	Reference value (95% significance)	Value calculated for 2% acetic acid condition	Value calculated for PBS 7.4 condition
Cochran	0.684	0.357	0.580
Shapiro Wilk	0.881	0.959	0.952

Source: Elaborated by the authors.

The results also showed a normal distribution since the calculated Shapiro-Wilk test value was higher than the critical value. The normal distribution was further confirmed by obtaining the residual distribution graph, which exhibited a random distribution, as shown in **Fig. 5**. This random distribution is characteristic of homoscedastic and normalized data (Brazil, 2017b).

Following Brazilian regulation, when the absorbance values present homoscedasticity and normal distribution, the slope of the regression and intercept must be calculated by the ordinary least squares method. For a calibration curve to be considered valid, the slope of the regression line must be significantly different from zero, and the intercept value must be statistically equal to zero. The statistical analysis of the calibration curve slope of the curve in acidic medium, as shown in **Table 3**, demonstrated that this parameter was statistically different from zero (p-value < 0.05), confirming the presence of a linear relationship between concentration and absorbance. In contrast, the intercept was statistically equal to zero (p-value > 0.05), indicating no significant systematic deviation at the origin (Brazil, 2017b). Similar results were found for the curve in PBS 7.4, as the slope was statistically different from zero, and the intercept was statistically equivalent to zero.

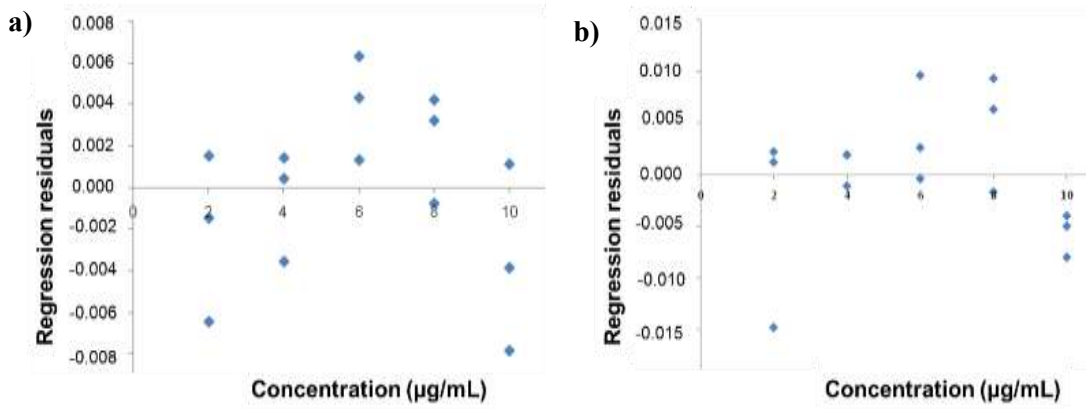

Figure 5. Dispersion of regression residuals in acetic acid 2% (v/v) **(a)** and PBS 7.4 **(b)**.
Source: Elaborated by the authors.

Table 3. Evaluation of regression quality using the ANOVA method.

Medium	Parameter	Slope	Intercept	R^2	Standard error of regression
Acetic acid 2%	value	0.123	0.000	0.999	0.004
	p-value	1.03×10^{-26}	0.888		
PBS 7.4	value	0.104	0.006	0.999	0.007
	p-value	3.28×10^{-23}	0.192		

Source: Elaborated by the authors.

In addition, the regression quality should be demonstrated to verify its statistical significance. The linear model fit was evaluated by analyzing the lack of fit and regression significance (Brazil, 2017b). Analysis of variance (ANOVA) was used to assess this parameter, and the calculated F significance (F statistic) value should be less than 0.05. This result indicates that the regression slope has statistical validity for a 95% significance level (Bernardo *et al.*, 2024). As shown in **Table 4**, the F significance value was much lower than 0.05 for both solvents, confirming that the regression has statistical significance.

Table 4. Regression quality using the ANOVA method.

Solvent		Degrees of freedom	Sum of Squares	Mean of squares	F	F statistic
Acetic acid 2%	Regression	1	1.80	1.80	102294.56	1.03×10^{-26}
	Residue	13	0.00	1.76×10^{-5}		
	Total	14	1.80			
PBS 7.4	Regression	1	1.29	1.29	29563.56	3.28×10^{-23}
	Residue	13	0.00	4.36×10^{-5}		
	Total	14	1.29			

Source: Elaborated by the authors.

3.4. Matrix effect

The matrix refers to the sample component that differs from the substance being analyzed and generally interferes with the analysis process, affecting the accuracy of the results (Jiang *et al.*, 2023). To analyze the matrix effect, Resolution RDC No. 166, dated July 24, 2017, establishes that a comparison must be made between the angular coefficients of the calibration curves constructed with the chemical reference standards (SQR) in solvent and with the sample fortified with the analyte SQR. The parallelism of the lines indicates the absence of interference from the matrix constituents, and its demonstration must be carried out through appropriate statistical evaluation (Brazil, 2024).

It is possible to observe in **Fig. 6** that the lines constructed from the solvent and the fortified matrix with the SQR overlapped, indicating that they are parallel; that is, the matrix effect can be ignored graphically. Furthermore, the statistical analysis of the slope values of the obtained lines, performed using the Student's t-test hypothesis test, showed a *p*-value > 0.05. This result indicates that the slopes of both curves are statistically equal. In other words, there is no statistical difference between the slopes of the lines (Brazil, 2011).

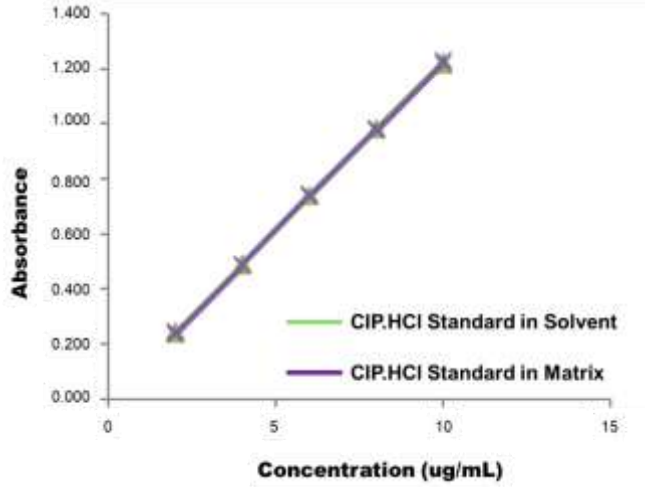

Figure 6. Evaluation of the effect of the matrix in acetic acid 2% (v/v).
Source: Elaborated by the authors.

3.5. Precision

Precision is a parameter that evaluates the dispersion among results obtained from independent assays, repeated on the same sample, similar samples, or standards under defined conditions. This parameter should be expressed through repeatability, intermediate precision or reproducibility. Repeatability and intermediate precision can be evaluated by preparing a set of samples consisting of blank matrices fortified with the substances to be analyzed, at a minimum of three concentration levels and in sextuplicate for each level. However, to evaluate intermediate precision, the preparation and reading of the samples must be performed on different days (Brazil, 2011). The concentrations must cover the linear range and include low, medium, and high concentrations from the analytical curve (Brazil, 2017a).

Thus, the method's precision was evaluated regarding repeatability (intra-day precision) and intermediate precision (inter-day precision). To evaluate these parameters, blank matrices fortified with the standard at concentration levels 2, 6 and 10 μg mL^{-1} were read, with each level evaluated in sextuplicate. The results are presented in **Table 5**.

Table 5. Precision test results on acetic acid 2% (v/v).

Level (μg mL^{-1})	Repeatability		Intermediate precision	
	Concentration (μg mL^{-1}) n = 6	Relative standard deviation (%)	Concentration (μg mL^{-1}) n = 6	Relative standard deviation (%)
2	2.03	1.84	1.99	2.07
6	6.01	1.42	6.05	0.99
10	10.02	0.97	9.99	0.38

Source: Elaborated by the authors.

Similarly, intra-day and intermediate precision in PBS 7.4 was evaluated using sextuplicate at the three concentration levels. As shown in **Table 6**, the relative standard deviation for all levels remained below 2%. It has been demonstrated that a relative standard deviation (RSD) < 2% for precision indicates that the method is reliable, meaning it has acceptable precision and is compatible with the literature (Elgendy *et al.*, 2023; Scherer *et al.*, 2014).

Table 6. Precision test results on PBS 7.4.

Level (μg mL^{-1})	Repeatability		Intermediate precision	
	Concentration (μg mL^{-1}) n = 6	Relative standard deviation (%)	Concentration (μg mL^{-1}) n = 6	Relative standard deviation (%)
2	1.96	1.82	1.93	1.19
6	6.01	0.87	5.98	0.85
10	9.94	0.41	9.92	0.36

Source: Elaborated by the authors.

As observed, the obtained RSD values for all levels were close to or below 2% for both repeatability and intermediate precision. Therefore, based on these RSD values, the method was considered to demonstrate precision (Barbosa *et al.*, 2017; Uncu *et al.*, 2019). The MAPA establishes limits of 20% for repeatability and 30% for intermediate precision for concentration ranges between 1 mg $kg^{-1} \leq C < 10$ mg kg^{-1} (Brazil, 2011). However, the Association of Official Analytical Collaboration (AOAC) sets more stringent limits of 7–11% for intra-day precision and 11–16% for inter-day precision (AOAC, 2016).

3.6. Accuracy

Accuracy is the degree of agreement between the individual results of the method under study and an accepted true value. The procedure can be applied to a matrix containing all the components of the pharmaceutical formulation, except for the target substance. However, a known quantity of the analyte must be added (ICH, 2023). The added analyte should be the SQR, and at least three concentration levels should be evaluated, being them low, medium, and high levels of the analytical range used in the method (Brazil, 2017a).

The accuracy evaluation through the recovery method in this study demonstrated that methodologies for ciprofloxacin determination in ophthalmic solutions yielded recovery ranges between 99 and 101%, with RSD of 2.44%, which was considered acceptable for the method (Cazedey and Salgado, 2012). Similarly, methods for determining CIP.HCl in ear drops recovered between 99 and 101% (Uncu *et al.*, 2019).

In this study, accuracy was evaluated by reading sextuplicate of fortified blank matrix samples (FP) at low (2 μg mL^{-1}), intermediate (6 μg mL^{-1}), and high (10 μg mL^{-1}) levels of the calibration curve, and recoveries are calculated taking as reference the theoretical concentration of each level. As shown in **Table 7**, the mean recovery in the acid medium for the lowest level was 102%, for the intermediate level 100%, and for the highest level 100.20%. The recovered percentages for basic medium ranged from 98 to 100%.

Table 7. Accuracy test results in acetic acid 2% (v/v) and PBS 7.4.

Level (μg mL^{-1})	Acetic acid 2% (v/v)		PBS 7.4	
	Average recovery n = 6	Relative standard deviation (%)	Average recovery n = 6	Relative standard deviation (%)
2	102.0	2.0	98.0	2.0
6	100.0	1.0	100.0	1.0
10	100.0	1.0	99.4	0.4

Source: Elaborated by the authors.

MAPA and AOAC establish acceptable recovery ranges for analyte concentration levels between 1 and 10 μg mL^{-1}, set between 80 and 110% (Brazil, 2011; AOAC, 2016). The recovery values were considered acceptable, as they fell within the specified range and were compatible with the literature (Uncu *et al.*, 2019).

https://doi.org/10.26850/1678-4618.eq.v51.2026.e1643

3.7. Detection limits (LD) and quantification limits (LQ)

The detection limit is the parameter that evaluates the lowest concentration at which the analyte in the sample can be detected, but not necessarily quantified with an exact value, and the quantification limit is the lowest concentration at which the analyte can be quantified with precision and accuracy (ICH, 2023). These limits are typically associated with a 95% probability of obtaining a correct result in the determination (Holst-Jensen, 2007). The detection or quantification limit values can be defined using visual methods, the signal-to-noise ratio, blank determination, or calibration curve parameters (Brazil, 2017a).

This study determined the detection and quantification limits based on the response's standard deviation and the analytical curve's slope. The value of σ considered for calculating these parameters was the standard error of regression shown in **Table 3**. The value used for S was the slope of the calibration curve (Brazil, 2017a; Dolan, 2021; ICH, 2023). The results of detection and quantification limits are presented in **Table 8**. The values found are close to those reported in the literature for ciprofloxacin in an acidic medium (Scherer *et al.*, 2014). No reference values for these parameters in PBS 7.4 were found in the literature.

Table 8. Results of limit of detection and quantification in 2% (v/v) acetic acid and PBS 7.4.

Parameter	Acetic acid 2% (v/v) Value (μg mL^{-1})	PBS 7.4 Value (μg mL^{-1})
LD	0.11	0.21
LQ	0.34	0.64

Source: Elaborated by the authors.

These results demonstrate that the method possesses adequate analytical sensitivity for the selected working range, as the lower limit of the constructed calibration curve is approximately six times higher than the quantification limit for the acidic medium and three times higher for the basic medium. However, in analytical practice, the analytical curve's lower limit is considered the analytical method's detection limit, as signal values and concentrations outside the working range should be avoided (Skoog *et al.*, 2006).

3.8. Robustness

Robustness is the parameter that evaluates the ability of the analytical procedure to meet expected performance criteria under normal usage, and the test is conducted through deliberate variations in analytical method parameters (ICH, 2023). The method used to determine the robustness of ciprofloxacin hydrochloride in the literature has involved minor variations in the reading wavelength of the solutions. Variations of ±1 and ±3 nm have been reported. Methods are considered robust if the relative standard deviation of absorbance readings remains below 2% for each wavelength (Prasad and Ratna, 2018; Uncu *et al.*, 2019).

This study evaluated the method's robustness by constructing an analytical curve with a ±3 nm variation in the reading wavelength for blank matrices fortified with the standard. In the acid medium, the parallelism of the curves obtained for the wavelengths 274, 277 and 281 nm suggests the method's robustness. For robustness evaluation in a basic medium, wavelength variations of ±3 nm were applied, and the parallelism of the curves obtained at 268 nm and 274 nm was evaluated, taking the curve at 271 nm as a reference. Accordingly, **Fig. 7** presents the results obtained, demonstrating visual parallelism in the acidic (**Fig. 7a**) and the basic medium (**Fig. 7b**).

From the obtained curves, the slopes were calculated, and to confirm parallelism, the slopes for each wavelength were subjected to a Student's t-test. The reference wavelengths were 277 nm for the acidic medium and 271 nm for the basic medium. A *p*-value greater than 0.05 indicates no statistically significant difference between the slopes. As shown in **Table 9**, the *p*-values for the slopes were all greater than 0.05. Thus, it was assumed that the curves exhibited parallelism.

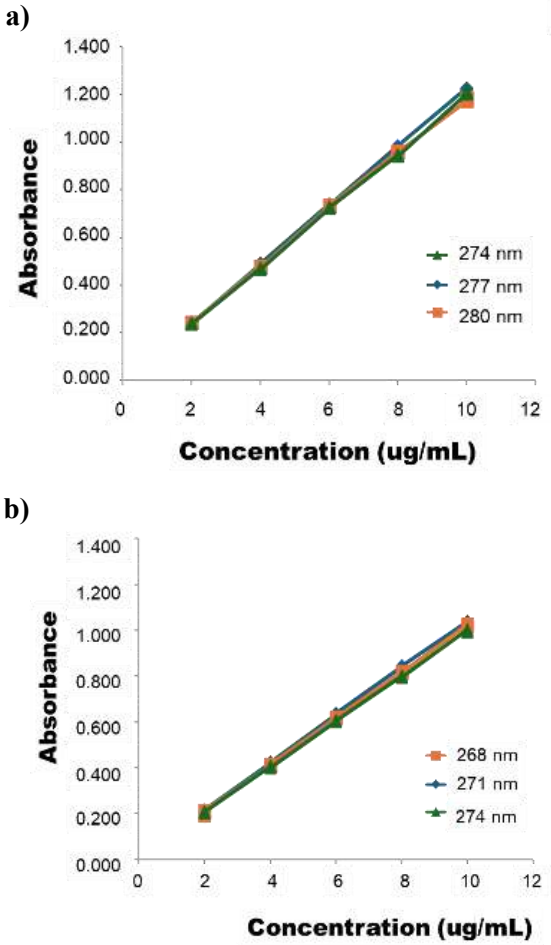

Figure 7. Robustness evaluation in acetic acid 2% (v/v) **(a)** and PBS 7.4 **(b)**.
Source: Elaborated by the authors.

Table 9. Slope of the curves obtained with the variation of ± 3 nm in 2% (v/v) acetic acid and PBS 7.4.

Medium	Wavelength (nm)	Slope
Acetic acid 2% (v/v)	274	0.120
	277	0.123
	280	0.112
	p-value	> 0.05
PBS 7.4	268	0.102
	271	0.104
	274	0.099
	p-value	> 0.05

Source: Elaborated by the authors.

3.9. Evaluation of ciprofloxacin hydrochloride content in chitosan films

The drug content in chitosan films was measured at 277 nm, and the results are shown in **Table 10**. The CIP.HCl content found in the films was approximately 100%, with an RSD of 0.5% for acidic medium.

For physiological medium (PBS 7.4), the drug content in chitosan films was measured at the wavelength of 271 nm, and the results are also shown in **Table 10**. The CIP.HCl content range was 93 to 104% with an RSD of 5.5%. Both results are consistent with the content range of 90.0 to 110.0% required by the Brazilian Pharmacopoeia for solid pharmaceutical forms (Brazil, 2024), and the relative standard deviation (RSD) was below the 7% limit accepted by AOAC.

Table 10. Results of ciprofloxacin hydrochloride content in acidic solvent and PBS 7.4 solvent.

Sample	Theoretical content (mg/film)	Real content in acetic acid (%)	Real content in PBS 7.4 (%)
1	18.24	100.50 ± 0.01	98.9
2	18.24	100.10 ± 0.03	92.9
3	18.23	101.10 ± 0.02	103.7
Average	18.24	100.6	98.5
RSD	0.006	0.5	5.5

Note: RSD = relative standard deviation; mg/film = milligram of ciprofloxacin hydrochloride per film.
Source: Elaborated by the authors.

4. Conclusions

Osteomyelitis is a severe and challenging infectious disease that demands effective therapeutic strategies. Conventional systemic treatments have demonstrated limited efficacy, often contributing to disease progression and increasing the risk of irreversible tissue damage. In this context, developing localized drug delivery systems, such as chitosan-based polymeric matrices incorporating ciprofloxacin as the active pharmaceutical ingredient, emerges as a promising alternative. To ensure the safety, efficacy, and quality control of these therapeutic devices, the establishment and validation of robust analytical methods for drug quantification within the matrix are of paramount importance.

The spectrophotometric methods for quantifying ciprofloxacin hydrochloride in acidic and slightly basic conditions were developed and validated based on maximum absorption wavelength, selectivity, working range, linearity, matrix effect, precision, accuracy, and robustness. The absorption maximum was determined at 277 nm for acidic and 271 nm for basic conditions, confirming the expected spectral shifts and ensuring reliable quantification. Selectivity was established by demonstrating the method's ability to differentiate the analyte from matrix components without significant interference. The method exhibited high linearity, with correlation coefficients close to 0.999, homoscedasticity, and normal distribution of residuals, meeting regulatory validation criteria. Precision was confirmed by relative standard deviation values close to 2%, significantly lower than AOAC limits (7–11% for intra-day and 11–16% for inter-day precision), ensuring method reproducibility under normal operational conditions. Accuracy was validated through recovery values ranging from 100.0 to 101.7% in acidic and 98.0 to 100.1% in basic pH, within the acceptable 80–110% range defined by regulatory guidelines.

Detection and quantification limits were determined based on the response's standard deviation and the analytical curve's slope, confirming the method's sensitivity in detecting and quantifying low analyte concentrations in the nanogram range. These values remained well below the lower limit of the calibration curve, reinforcing method reliability. Robustness was evaluated by varying the reading wavelength by ±3 nm, demonstrating that these changes did not compromise results. Analytical curves remained parallel to the reference, and analyte recovery remained acceptable with relative standard deviations below 2%. These findings confirm the developed method as reliable, precise, accurate, and robust for the spectrophotometric quantification of ciprofloxacin hydrochloride in different conditions. These results were confirmed by the drug dosage values found for the films to be within the range acceptable by the Brazilian pharmacopoeia.

Authors' contribution

Conceptualization: Samuel Beiral Alves Pessoa; André Augusto Gomes Faraco; **Data curation**: Samuel Beiral Alves Pessoa; **Formal Analysis**: Samuel Beiral Alves Pessoa; **Funding acquisition**: André Augusto Gomes Faraco; **Investigation**: Nicole Maia Pedrosa; Lídia Vieira Gomes; Gabriela Regina de Sousa Rezende; **Methodology**: Samuel Beiral Alves Pessoa; Nicole Maia Pedrosa; Lídia Vieira Gomes; Gabriela Regina de Sousa Rezende; **Project administration**: André Augusto Gomes Faraco; **Resources**: André Augusto Gomes Faraco; **Software**: Not applicable; **Supervision**: André Augusto Gomes Faraco; **Validation**: Samuel Beiral Alves Pessoa; **Visualization**: Samuel Beiral Alves Pessoa; **Writing – original draft**: Samuel Beiral Alves Pessoa; **Writing – review & editing**: André Augusto Gomes Faraco

Conflict of interest

The authors declare that there is no conflict of interest.

Data availability statement

All data sets were generated or analyzed in the current study.

Artificial Intelligence usage statement

The authors declare that they did not use Artificial Intelligence tools at any stage of the preparation, correction, or evaluation of this work.

Funding

This project was developed with resources provided by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). Processo No: 305551/2021-3 and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) Processo No: APQ-01791-21

Acknowledgments

Not applicable.

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