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**Development and validation of a microbiological assay by turbidimetry to determine the  
potency of cefazolin sodium in lyophilized powder**

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**ABSTRACT**

In many countries, high rates of mortality and morbidity from infectious diseases represent high social and economic costs. Cefazolin sodium is a semi-synthetic  $\beta$ -lactam antimicrobial for parenteral use, belonging to the first-generation cephalosporin's group. Its use in clinical practice stands out for its effectiveness as therapeutic agent and in surgical prophylaxis, having great importance in the fight against many diseases. This paper reports the development and validation of an efficient, accurate, reproducible, and of low cost microbiological assay by turbidimetric method to quantify cefazolin in lyophilized powder. These requirements are essential for the analysis of this cephalosporin in pharmaceutical industry. The assay is based on the inhibitory effect of cefazolin sodium upon the strain of *Staphylococcus aureus* ATCC 26923 used as test microorganism. The method was validated according to ICH guidelines and the results were treated by analysis of variance (ANOVA), showing to be linear ( $r^2= 0.9999$  for reference substance and  $r^2= 0.9995$  for sample), in the selected range from 6 to 11.76  $\mu\text{g mL}^{-1}$ , precise (RSD values  $< 2.0\%$ ), robust and accurate (99.92 %). The developed method showed excellent validation results, and the statistical analysis corroborated for its assessment. Furthermore, a Student's t-test showed no statistically significant difference between the proposed turbidimetric method and an UV spectrophotometry method previously validated. Thus, the validated method is able to quantify cefazolin sodium in powder for injectable solution, while being an economical and rapid alternative for its routine analysis in quality control.

**Keywords:** Cefazolin sodium; Microbiological method; Quality control; Turbidimetric assay; Validation.

## INTRODUCTION

Cephalosporins are among the safest and most effective broad-spectrum bactericidal antimicrobial agents available to the clinician. For this reason, they have become the most widely prescribed of all antibiotics. Cefazolin sodium (CFZ) is a semi-synthetic  $\beta$ -lactam antimicrobial for parenteral use, belonging to the first-generation cephalosporin's group. Its use in clinical practice stands out for its effectiveness as therapeutic agent and in surgical prophylaxis, having great importance in combat against many diseases.<sup>1,2</sup>

A single dose of cefazolin immediately before surgery constitutes the most used prophylaxis on several surgical procedures<sup>1</sup> and reduces the incidence of infections which may represent serious complications in patients undergoing surgery.<sup>3</sup> However, for the drug to be used by people safely and expected to have therapeutic efficacy, the use of methods validated by quality control for evaluating the drug potency against the microorganism is essential and it ensures that the dosage is correct. Failure to comply with these criteria can interfere with biopharmaceutical characteristics, which may lead to serious consequences.

Although there are many physicochemical methods related to the cefazolin sodium available in literature.<sup>4-11</sup> Even though the physicochemical methods are precise and can be employed to quantification of antibiotic, they alone cannot infer analyzes of power in relation to the activity of the drug compared to micro-organisms. Therefore, bioassays continue to play an essential role in manufacturing and quality control of antibiotic medicines, the association of microbiological assays such as agar diffusion method or by turbidimetry corroborates to better evaluate the drug.<sup>12-19</sup> Few papers for microbiological analysis cefazolin sodium were found in literature. The method by agar diffusion was used by Yeh and Chi in 2001<sup>20</sup> to define the activity of cephalothin and cefazolin against *Escherichia coli* and *Klebsiella pneumoniae*. The current

edition of the Brazilian Pharmacopoeia, 2010<sup>21</sup> brings the microbiological assay by agar diffusion with strains of *Staphylococcus aureus* (ATCC 6538p) for analysis of the cefazolin sodium chapter of microbiological assays of antibiotics. Nevertheless, the turbidimetric assay presents some advantages when compared to traditional agar diffusion assays. Besides being a simple and fast method, it does not present drug diffusibility difficulty, since the test is conducted in broth as medium culture.

In this sense, considering that cefazolin sodium is a drug commonly used throughout the world, it justifies the fact that this paper proposes the accomplishment of a microbiological assay to assess the potency of the drug against the microorganism, which is essential for quality assurance in the production of safe and appropriate medication for the use of the population. Therefore considering that the turbidimetric assay has the advantage of reduced analysis time when compared to the agar diffusion method, the aim of this work was to propose a rapid turbidimetric method for the analysis of cefazolin sodium's potency in the dosage form of powder for injectable solution.

## EXPERIMENTAL

### Chemicals

Cefazolin sodium reference substance – RS (purity 98.2 %) and cefazolin sodium lyophilized injectable form containing 1000 mg of the active component, were kindly donated by Laboratory ABL Antibióticos do Brasil Ltda (Cosmópolis-SP, Brazil). The vials do not present excipients. All solutions used in this assay were prepared from ultrapure water obtained from a Milli-Q Plus (Millipore, USA). All other chemicals were of analytical grade.

## Apparatus

In the turbidimetric method, the culture media were sterilized before use in a vertical autoclave AV model (Phoenix Luferco, SP, Brazil). The culture medium used was *Brain Heart Infusion* - BHI broth of brand Acumedia. Incubation of micro-organisms was performed using a Shaker incubator MA420 model (Marconi, SP, Brazil) and an oven ECB Digital 1.2 (Odontobrás, SP, Brazil). For the readings, it was used a spectrophotometer DU 530 (Beckman Coulter, CA, USA). The software Microsoft Excel (2007) was used to construct the calibration curves. Other apparatus also used were: 20-200  $\mu\text{L}$  micropipettes (Digipet, PR, Brazil); H10 analytical scale (Mettler Toledo, Switzerland); B160 semi-analytical scale (Micronal, SP, Brazil).

## Preparation of reference substance and sample solutions

Cefazolin sodium stock solution was prepared by dissolving 20 mg of cefazolin sodium accurately weighed in water purified in 100 mL volumetric flask. Further diluting different volumes for volumetric flask with same solvent three final concentrations were obtained: 6.00; 8.40 and 11.76  $\mu\text{g mL}^{-1}$ .

## Turbidimetric Assay

### *Preparation and standardization of inoculums*

*Staphylococcus aureus* ATCC 26923 used in micro bioassay showed to be more suitable due to their susceptibility to cefazolin sodium. The strain of *Staphylococcus aureus* ATCC 26923 were cultivated and maintained on tryptic soy agar medium in the freezer and pealed to BHI broth / 24h before the assay that were kept at  $35 \pm 2$  °C. The microorganism standardization was carried out according to the procedure described in the Brazilian Pharmacopoeia, 2010. The bacteria, previously incubated in BHI broth, were diluted with BHI broth to achieve a suspension

turbidity of  $25 \pm 2$  % (transmittance) using a spectrophotometer (Beckman, DU 530) with the wavelength at 580 nm using a 10 mm absorption cell, against BHI broth as blank. Aliquots of 0.4 mL of this standardized suspension were added to each 10 mL of BHI broth and the concentration of product used was 6.0; 8.4; 11.76  $\mu\text{g mL}^{-1}$ .

### **The bioassay**

In this experimental work of  $3 \times 3$  design, three dose levels for each standard and sample were used following the procedures described in the Brazilian Pharmacopoeia. It was performed in triplicate. The six test-tubes containing 10 mL of broth BHI were autoclaved, then it was added to each tube 0.4 mL of the standardized *S. aureus* ATCC 26923 suspension. In three standard reference tubes, 200  $\mu\text{L}$  of standard working solutions were added (in the concentrations of 6.00; 8.40 and 11.76  $\mu\text{g mL}^{-1}$ , respectively), and in the sample series, the same was carried out with the working sample solutions. After that, the test-tubes were incubated in a shaker, in water bath, at a temperature of  $35.0 \text{ }^\circ\text{C} \pm 2.0 \text{ }^\circ\text{C}$  for 4 hours. After the incubation period, the multiplication of microorganisms was interrupted by the addition of 0.5 mL of 12 % formaldehyde solution in each tube. Then, the spectrophotometer was reset by the test-tube containing a negative control (10 mL of broth without the microorganism, containing 0.5 mL of the formaldehyde solution) and the absorbance values were performed on each tube at a wavelength of 530 nm in a spectrophotometer. The parameters for carrying out the microbiological assay are shown in Table 1.

#### *Obtaining of analytical curve*

The curve was constructed by the plotting of the logarithm of the concentration versus the average of the absorbance values, with the average absorbance value of each concentration of the

cefazolin sodium - RS. The assay was performed in triplicate on three different days. The mean absorbances observed were plotted in graphical form using the software Microsoft Excel - 2007.

#### *Calculation of Activity*

To calculate the potency of cefazolin sodium, Hewitt equation was used.<sup>22</sup>

#### **UV spectrophotometric method**

The UV spectrophotometric method chosen as a comparative method in the determination of cefazolin sodium in powder for injectable solution was previously developed and validated by our study group. The procedure was performed in UV-Vis spectrophotometer Shimadzu UV-mini 1240 model, using quartz cuvettes 1 cm optical path, in the wavelength  $\lambda=270$  nm and spectrophotometer under controlled temperature 25° C. The UV method presented could detect and quantify the drug obtaining satisfactory results regarding specificity, precision, accuracy and robustness in the linear range of 8 to 28  $\mu\text{g mL}^{-1}$  and showing correlation coefficient of 0.9999.

#### **Method validation**

The method was validated in accordance with the recommendations described in the literature.<sup>23-24</sup>

*Linearity:* The linearity was determined by constructing three independent analytical curves, each with three reference substance concentrations of cefazolin sodium, in the range of 6.0; 8.4; 11.76  $\mu\text{g mL}^{-1}$ . The results were subjected to regression analysis by a least-squares method to calculate the calibration equation in which the determination coefficient, the linearity and parallelism of the method was detected by ANOVA.

*Precision:* Precision was assessed at different levels: Repeatability (intraday), by testing seven evaluations of the same concentration sample of cefazolin sodium of 8.40  $\mu\text{g mL}^{-1}$ , all on



the same day and under the same experimental conditions and intermediate precision (interday) by conducting the analysis on three different standard solution concentrations (high, intermediate and low), in a three different days under the same experimental conditions, and also by other analysts performing the analysis in the same laboratory (between analysts). Precision was evaluated by the calculating the R.S.D.

*Robustness:* The robustness of the method was determined by analyzing the same sample under a variety of conditions. The factors considered consisted of wavelength (525, 530, 535 nm), volume of culture medium (9.8, 10 and 10.2 mL) and brand culture medium (Acumedia and Imedia).

*Accuracy:* To determine the accuracy of the proposed method, the test was performed over 3 concentration levels, 80, 100 and 120 %, covering the specified range. Accurately aliquots of 0.092; 0.190 and 0.288 mL of the reference solution ( $200 \mu\text{g mL}^{-1}$ ) were transferred into 10 mL volumetric flasks together with aliquots of sample solutions ( $0.300 \mu\text{g mL}^{-1}$ ) and diluted with water purified to give final concentrations of 7.84; 9.80 and  $11.76 \mu\text{g mL}^{-1}$ , respectively.

## RESULTS AND DISCUSSION

Analytical methods used for the quantitative determination of substances are the key determinants in generating reproducible and reliable data. In this context, the choice of a suitable analytical methodology is fundamental for quality control of an active substance. Moreover, the availability of equipments and reagents should be considered for the development of accessible and useful methodologies. Considering that the potency of an antibiotic may be demonstrated under suitable conditions by comparing the inhibition of growth of susceptible microorganisms induced by known concentrations of the antibiotic to be tested and the reference standard, a new

microbiological assay was proposed as a suitable method for determination of CFZ sodium in powder for injection pharmaceutical dosage form.

The turbidimetric assay was chosen because it is faster method compared to agar diffusion method. The incubation time is only 4 hours (agar diffusion is 21 hours). Furthermore, the turbidimetric assay is a simple method is based on the inhibition of microbial growth measured by turbidity of the suspension of microorganisms sensitive to the antimicrobial agent, both contained in a broth. The response of the microorganism is a direct function of the concentration of the active substance. While the agar diffusion method has, among its limitation, the difficulty of diffusivity of some drugs in solid medium and also the reactivity between the culture medium and the drug in some situations. In testing by the agar diffusion it is essential to have a homogenous microbial growth, formation of inhibition zones of regular and well-defined inhibition with diameter suitable for validation. Such interfering factors are decreased in the turbidimetric method, since the test is performed in broth as culture medium.

Preliminary tests were carried out in order to obtain a reliable method. Many microorganisms were tested in different drug concentrations in order to find the linear range for the method. The parameters varied were the microorganism, culture media, temperature and time of incubation, dilution solution, the inoculum concentrations and the drug concentrations. After preliminary testing, it was found that *Staphylococcus epidermidis* ATCC 12228 IAL 2150 did not show good growth, indicating positive controls low and near the lower concentration of antimicrobial tested.

The microorganism *Kocuria rhizophila* ATCC 9341 IAL 636 showed no uniform and reproducible results. There was wide variation in absorbance found for the same test on different days and there were clumps of microbial growth in the various inoculated broths, which interfered with the analysis. With *Bacillus subtilis* ATCC 9372 IAL 1027 concentrations up to 40

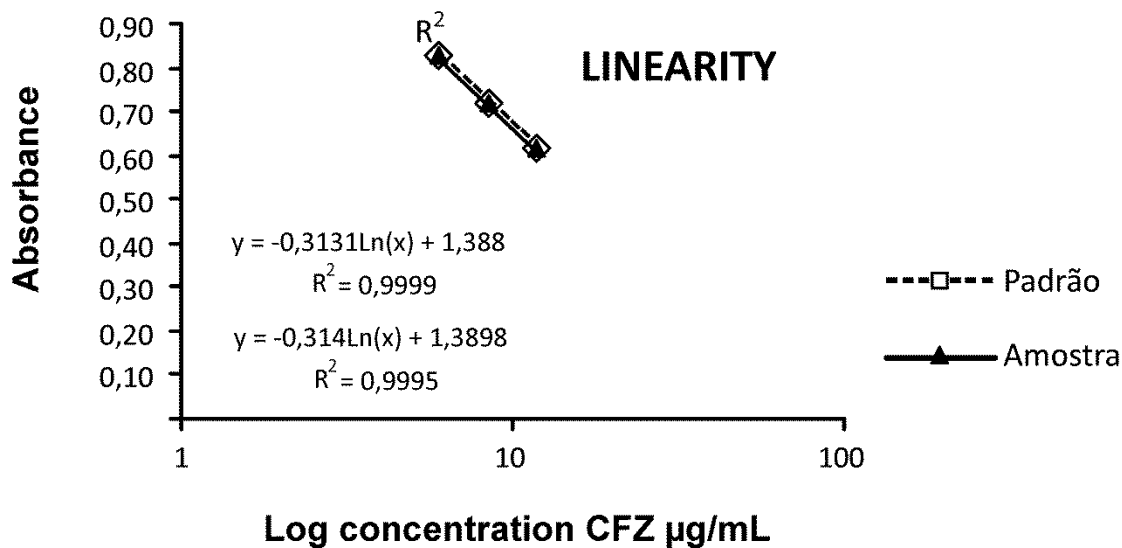
$\mu\text{g ml}^{-1}$  of drug were used, but no antimicrobial activity was observed using only 1 % of standardized inoculum. *Escherichia coli* ATCC 10535 IAL 2393 was also tested, although it is known that the antimicrobial study has modest activity against Gram-negative microorganisms, which was confirmed by microbiological tests.

However, the microorganism *Staphylococcus aureus* showed excellent results in different concentrations tested the method proved to be reproducible. Therefore the choice of the microorganism *Staphylococcus aureus* was based on its sensitivity to the CFZ and its ease of growth, despite a narrow working range (6.00, 8.40 and 11.76  $\mu\text{g ml}^{-1}$ ), the microorganism was sensitive to the study drug and provided accurate and reproducible results, the method was linear in the working concentrations, and allowed the determination of the drug getting power around of 100 % with appropriate parallel to the line of the pattern.

## Method validation

### Linearity

The analytical curve for cefazolin sodium was constructed by plotting the mean absorbance values of three analytical curves in relation to the logarithm of the concentrations, showing linearity in the range between 6.0 and 11.76  $\mu\text{g mL}^{-1}$ . The value of the determination coefficient  $r^2 = 0.9999$  for cefazolin sodium RS and  $r^2 = 0.9995$  for sample, which is considered highly significant for the method. The representative linear equation for cefazolin sodium was  $y = -0.313\text{Ln}(x) + 1.388$  for RS and  $y = -0.314\text{Ln}(x) + 1.3898$  for sample as shown in Figure 1.



**Fig. 1.** Analytical curve obtained by the turbidimetric assay for cefazolin sodium.

The method showed linearity in defined concentrations which allowed measurements of samples with potency around 100 % and appropriate parallelism.

The results for linearity of method were statistically calculated with analysis of variance (ANOVA). According to ANOVA there were no deviations from parallelism and linearity with the obtained results ( $p < 0.05$ ).

### *Precision*

In the precision of the method by repeatability (intraday), seven solutions of cefazolin reference substance with same theoretical concentration ( $8.40 \mu\text{g mL}^{-1}$ ) were submitted to successive analyses and data obtained on the same day under the same experimental conditions, and laboratory analyst were demonstrated by the relative standard deviation of 1.57 %. Whereas in the intermediate precision three samples, on three consecutive days (interday) were analyzed, yielding an average content next to 100 %, and 0.36 % of R.S.D. and also was analyzed by precision between analysts, the samples were evaluated by two analysts and the results were

compared obtaining an power average of 100.45 % and R.S.D. was 0.28 %. Precision showed values R.S.D lower than recommended in the literature.<sup>24-25</sup> Thus the lower values confirm that the proposed method has capacity to generate, for the same sample, reproducible results with low response variation between independent assays.

### *Robustness*

The robustness of the method was evaluated by slight changes in assay such as wavelength (525; 530 and 535 nm), culture medium brand (Acumédia and Imédia) and culture medium volume (9.5; 10.0 and 10.5 mL). The effects resulting from the parameters changed were compared with the parameters established in the assay. The results showed no significant difference, demonstrating the robustness of the method in Table 2.

### *Accuracy*

The accuracy of the method was shown by determining the average recoveries from the samples through the method of standard addition. The results obtained from the bioassay showed good recovery between the true value and the value found. The mean accuracy was 99.92 % and R.S.D. 0.53 % which confirms the ability of the method to determine with accuracy the cefazolin sodium concentration within the range of 80–120 %. Therefore the results indicate that the selected parameters are able to identify and quantify the concentration of cefazolin sodium in pharmaceutical injectable forms indicating that the method is appropriate.

### **UV spectrophotometry**

The comparison between analytical methods is used to verify if two methods are interchangeable. There are yet no article that describes the comparison of the UV method and turbidimetric. The results obtained with the microbiological assay by turbidimetry were

comparable with those obtained by UV spectrophotometry.<sup>10</sup> The percentage contents of cefazolin sodium calculated by both methods are shown in Table 3.

Analysis of variance indicated no significant differences between these methods ( $p < 0.05$ ). Thus, the methods provided statistically the same results and are interchangeable. Furthermore, the amount of CFZ calculated by both methods was within the range between 95 to 102%, recommended by United States pharmacopeia.<sup>25</sup>

Although the statistical analysis has shown that the microbiological and UV methods presented statistically similar results in relation to the determination of CFZ in pharmaceutical form, it is necessary to highlight that the quantification of antibiotic components by chemical methods such as UV spectrophotometry, although precise, cannot provide a true indication of biological activity. Therefore, bioassays continue to play an essential role in manufacturing and quality control of antibiotic medicines. The turbidimetric method provides important information about the biological activity of the pharmaceutical product. Furthermore, the turbidimetric assay is a technique that does not use organic solvents for their analysis, therefore causes no concern for chemical waste and this method requires a lower run time than the required by the agar diffusion assay, which is suggested by the Brazilian pharmacopeias for the analysis of cefazolin sodium.<sup>21</sup> Therefore, the proposed bioassay is a useful methodology for the quality control of cefazolin sodium in pharmaceutical products.

## CONCLUSION

The microbiological assay proved to be able to quantify the cefazolin sodium in its pharmaceutical form. The method showed is sensitive and rapid. The method was validated according to ICH guidelines and demonstrated excellent results, such as linearity, precision,

accuracy and robustness. The turbidimetric assay is more versatile, and fast to apply than agar diffusion assay. Therefore, it is an acceptable alternative method for the routine quality control of cefazolin sodium in lyophilized powder for injection solution.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that there research was conducted in the absence of any commercial or financial relationships that could be construed as a potencial conflict of interest.

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**Table 1.** Standard parameters for evaluation of cefazolin sodium in lyophilized powder by turbidimetric method.

PARAMETERS	DESCRIPTION
Microorganism	<i>Staphylococcus aureus</i> – ATCC 26923
Culture medium	Brain Heart Infusion broth - BHI
Incubation time	4 h
Incubation temperature	35 °C
Inoculum concentration	4%
Diluent solution	Water
Concentration of solutions	6.0; 8.4; 11.76 µg mL <sup>-1</sup>

**Table 2.** Determination of the robustness of the analytical method for the analysis of cefazolin sodium in lyophilized powder by turbidimetric assay.

Variable	Parameters	Potency CFZ (%)	Average <sup>a</sup> (%)	R.S.D. <sup>b</sup> (%)
Wavelength / nm	525	100.57	100.66	0.60
	530	100.10		
	535	101.30		
Volume culture medium / mL	9.5	100.13	99.48	0.59
	10.0	99.32		
	10.5	98.99		
Culture medium brand	Acumedia	100.37	100.47	0.15
	Imedia	100.58		

<sup>a</sup> average of three determinations; <sup>b</sup> R.S.D.: relative standard deviation.

**Table 3.** Values obtained in the determination of CFZ in powder for injectable solution by turbidimetric assay and UV spectrophotometry

	Comparison of methods	
	Turbidimetric assay	UV spectrophotometry
Content (%)	100.25	99.91
	100.30	98.95
	99.66	98.45
Average (%)	100.07	99.10

**Graphical Abstract:**

In this paper a new microbiological method for quantifying and evaluating the potency of cefazolin sodium is presented in order to produce safe medicines to the population.

