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Research Article

Validation of a Green Analytical Method for the Quantitative Analysis of Antimicrobial Aztreonam in Lyophilized Powder for Injection by Fourier-Transform Infrared Spectroscopy (FT-IR)#

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#This paper is dedicated to my father, Paulo Porto Nunes, who made me love the pharmaceutical sciences since I was a child, on the occasion of his 80th birthday in February 2017. H.R.N.S.

Abstract

Aztreonam is a monocyclic synthetic antimicrobial with bactericidal activity against Gram-negative bacteria, the first agent from the monobactam family to be therapeutically approved. A validated analytical method for aztreonam in powder for injectable quantification was developed and validated using Fourier-transform infrared (FT-IR) transmission spectroscopy. This technique does not use organic solvents, which is one great advantage over the most common analytical methods. This fact contributes to minimize the generation of organic solvent waste by the industry and there by reduces the impact of its activities on the environment. The method involved absorbance measurements of the band corresponding to one of the carbonyl groups in the molecule, centered in the region between 1760 - 1750 cm^{-1}. The method was validated according to international guidelines, showing to be linear (r = 0.9997), accurate, precise and robust, over a concentration range from 1.0 to 3.5 mg. The validated method is able to quantifying the aztreonam in powder for injection preparation and can be used as an environmentally friendly alternative for the routine analysis in quality control.

Keywords: *Aztreonam; Green Method; Infrared; Quality Control; Spectroscopy; Validation*

Introduction

Aztreonam (Figure 1), a synthetic antimicrobial used in the treatment of infections against Gram-negative alert inhibiting the synthesis of the cell appears to protein binding, causing cell lysis [1]. It is, however, ineffective against Gram-positive pathogens and anaerobes [2,3], as it can not bind to proteins of these bacteria [4]. It was discovered in 1978 from a strain of *Chromobacterium violaceum* in New Jersey (USA) [5]. Aztreonam molecular formulae is $C_{13}H_{17}N_5O_8S$, molecular weight of 435.43 g/mol and pH from 4.5-7.5 [6]. This antimicrobial agent is no absorbed in the gastrointestinal tract and therefore can only be used by parenteral route [7]. Due to its low immunogenicity has not crossed the cases of allergy with penicillins or cephalosporins. Aztreonam can produce, rarely, a small increase aminotransferase and alkaline phosphatase, and prolong clotting times. Skin rash, with or without eosinophilia, has been reported in approximately 1% of patients [8-10]. It has a low incidence of nephrotoxicity [11-13].

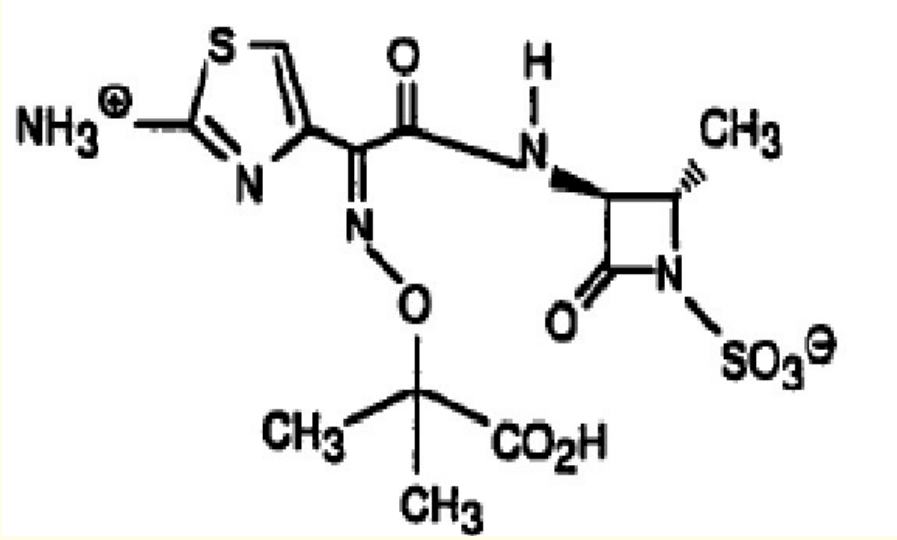

Figure 1: *Structure of aztreonam (CAS: 78110-38-0) [3].*

There are several analytical methods described in the literature for the quantitative determination of aztreonam in pharmaceutical products, such as High Performance Liquid Chromatography [14-18], spectrophotometry and fluorimetry using reaction with ceric acid [19], colorimetric and atomic absorption [20], antimicrobial susceptibility studies [21] and ultraviolet spectrophotometry [22,23]. However, aztreonam has no methodology for testing infrared spectroscopy. Most of them involve the use of organic solvents, which contributes to the generation of this kind of waste by the pharmaceutical industry. Special attention to the environmental preservation gained strength and today has a great impact on society. Thus, the trend is that the industries look for ways to reduce the impacts of their activities on the environment. In this way, they can adopt the posture of reduction, prevention or elimination of process waste. In this regard, there are some steps may be taken, including the replacement of analytical methodologies that employ organic solvents for others that do not use, the substitution of a synthetic process by another cleaner or "green" and the exchange of raw materials and supplies by other less toxic [24,25]. In this context, infrared spectroscopy stands out. This is a method that does not use organic solvents and, although it is formally accepted for the identification of individual compounds, the literature shows some publications that use this method for quantitative analysis [26-31]. The aim of this study is to develop, validate and apply an analytical method of infrared spectroscopy (IR) green, quick and simple for quantitative determination of aztreonam in powder for injectable form.

Experimental

Chemicals

Aztreonam reference substance (RS) (assigned purity 99.2 %), lot number 0908120, was kindly donated by União Química Pharmaceutical Industry (Brazil). Batches of Uni-Aztrenam™, lyophilized powder, containing 1g aztreonam were obtained from commercial sources within its shelf-life.

Potassium bromide (KBr) used for the pellets preparation was analytical grade. Before use, the KBr was dried at 105oC until constant weight.

Instrumentation and analysis conditions

Equipment

The FT-IR spectrophotometer used was Shimadzu (Kyoto, Japan) model IR Prestige-21, the samples were dried in an oven (New Ethics, São Paulo, Brazil). Furthermore, analytical scale used was Kern 410 (Kern, Germany).

Spectroscopy

IR spectra of RS and sample mixture were recorded in 150 mg pellets. The region comprised in the spectral analysis was from 4000-500 cm^{-1}, at 2 cm^{-1} interval. After obtaining the IR spectrum and with the assistance of the IR Solution software, quantitative analysis was carried out in the spectral region between 1760-1750 cm^{-1}, related to a carbonyl band of the aztreonam molecule, and this band had its height analyzed in terms of absorption. Spectroscopy measurements were recorded by using KBr pellet as a blank.

Obtaining of analytical curve

Equivalent amounts of 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mg of aztreonam (previously diluted in KBr 1:10, w/w) were taken and diluted with sufficient amount of KBr to obtain 150 mg pellets. The powders were mixed and ground until obtaining a homogeneous mixture. Thus, this mixture was compressed in a mechanical die press for 15 minutes to obtain translucent pellets, through which the beam of the spectrometer can pass.

Determination of aztreonam in the pharmaceutical dosage form

Preparation of reference substance aztreonam pellets

Amounts of powder equivalent to 2.5 mg of aztreonam (25.0 mg of the 1:10 dilution in KBr) were taken and homogenized with 125 mg of KBr, making the total pellet weight of 150 mg. The determinations were performed in triplicate.

Preparation of aztreonam sample pellets

The content of twenty vials of aztreonam in powder for injection solution were mixed. From this mixture, amounts of 2.5 mg of aztreonam (25.0 mg of the 1:10 dilution in KBr) were taken and well homogenized with 125 mg of KBr, comprising the total pellet weight of 150 mg. The determinations were performed in triplicate.

Calculation of aztreonam content in the sample

The aztreonam concentration in the sample was calculated by Equation 1 and its percentage content was calculated by Equation 2:

$$Cs = As \times \frac{Crs}{Ars} \qquad (1)$$

$$Cs\% = Cs \times \frac{100}{Ct} \qquad (2)$$

Where:

Cs = sample concentration

%Cs = percentage sample concentration

Crs = concentration of reference substance (mg)

As = sample absorbance

Ars = reference substance absorbance

Ct = theoretical concentration of aztreonam in the sample

Method validation

The method was validated by determining the following parameters: linearity, precision, accuracy, robustness, and detection and quantification limits, according to the literature recommendation [32].

Linearity

Linearity, evaluated by linear regression analysis, was obtained with six concentrations (1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mg) of aztreonam RS on three different days (n = 3). Each concentration was assessed in triplicate.

Selectivity

Selectivity was established by analyzing the excipient (L-arginine) present in the aztreonam samples, evaluated by regression analysis in six concentrations ranging from 1.0 to 3.5 mg prepared on three consecutive days (ANOVA). Each concentration was determined in triplicate. The selectivity was also evaluated according to the spectra of solutions of excipient, aztreonam sample and RS at a concentration of 1.0 mg. The excipient solution was prepared under the same conditions as the commercial samples.

Precision

Precision was evaluated with respect to both repeatability and intermediate precision. Repeatability was assessed by the performance of seven determination of the aztreonam RS in a concentration of 2.5 mg/pellet, all in the same day and identical working conditions. Intermediate precision was studied by performing the analysis on three different days (inter-day) and by another analyst in the same laboratory under the same experimental conditions (between-analyst). The relative standard deviation percentage (RSD) values of the determinations were examined [32].

Accuracy

Accuracy was determined via the recovery assay, in which known quantity of aztreonam RS was added to known quantity of the sample [32]. The recovery was performed in three levels, R1, R2 and R3, and the pellets were prepared according to the Table 1, in triplicate.

	Aztreonam sample[1] (mg)	Aztreonam RS[1] (mg)	KBr[2] (mg)	Final concentration (mg/pellet)
Sample	15.0	-	135.0	1.5
R1	15.0	5.0	130.0	2.0
R2	15.0	10.0	125.0	2.5
R3	15.0	15.0	120.0	3.0
Standard	-	15.0	135.0	1.5

Table 1: *Preparation of pellets for the recovery of the assay method of FT-IR spectroscopy in aztreonam.*

[1]Diluted 1:10 (w/w) in KBr.

[2]Sufficient amount for the preparation of pellets with a total weight of 150 mg

Aztreonam RS: Aztreonam Reference Standard

The recovery percentage was calculated by the equation determined by the Association of Official Analytical Chemists [33].

The robustness of the method was evaluated by analysing data after checking the mark of KBr, temperature and time of compression. The concentration of 2.5 mg of aztreonam was used in these experiments.

Limit of Detection (LOD) and limit of quantification (LOQ)

The detection (LOD) and quantitation (LOQ) limits were calculated based on the standard deviation intercept and the curve slope, as described in the literature [32]. Three different curves were performed for the obtainment of the necessary data for the calculation. The values were calculated by equations 3 and 4.

$$LOD = 3.3\left(\frac{SD}{a}\right) \qquad (3)$$

$$LOQ = 10\left(\frac{SD}{a}\right) \qquad (4)$$

where SD is the intersection standard deviation and a is the average slope, obtained from the analytical curves of the linearity study.

Results and Discussion

Method development

The methods described in the literature [14-23] for the determination of aztreonam are time consuming, complex, use toxic solvents, requiring the use of large amounts of organic solvents and high cost. Recently, researchers have shown an increased interest in the validation of green analytical methods. So far, several studies investigating new and innovative methods for drugs have been reported [26-31,34-43]. Correa and Salgado described an overall strategy for the prediction and development of infrared quantitative analytical method in active pharmaceutical ingredients (APIs) and drug products [40]. Green analytical methods are part of the pharmaceutical analysis strategy being undertaken to quantify API and dosage forms.

In this research, a green method for quantification of aztreonam powder for injection was fully validated where we can highlight no use of toxic solvent, simplicity and environmentally friendly IR spectroscopy method. In the spectra obtained was analyzed the carbonyl band between 1760-1750 cm^{-1}. The values of these bands/peaks of absorbance were supplied. There was no interference of the excipient in the spectrum of the carbonyl region; this band is specific and useful in the determination for aztreonam in powder for injection. Our studies were conducted under more severe and exaggerated conditions than those usually used for qualitative tests.

The infrared spectrum of aztreonam is shown in Figure 2.

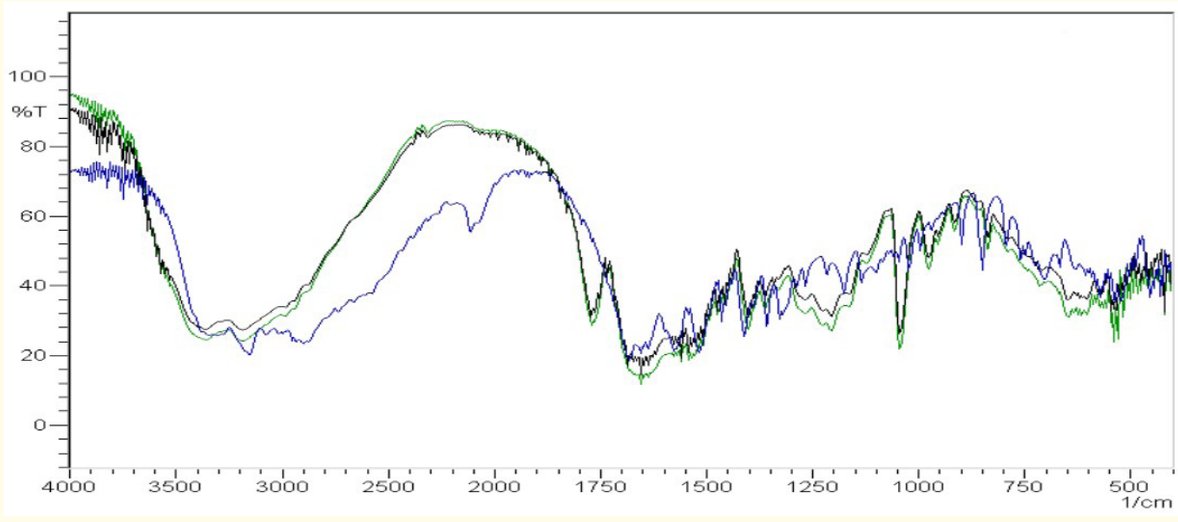

Figure 2: *Spectrum aztreonam reference substance (green), sample (black) and excipient (blue).*

Method validation

Linearity

The analytical curves, resulting on three consecutive days (n = 3) by plotting the mean absorbance values of spectra at 1760 to 1750 cm^{-1} against concentration yielded correlation coefficient greater than 0.9997 (Figure 3), a value close to 1.0, which shows the excellent linearity of the method. Furthermore, data were validated by analysis of variance (Table 2) which showed highly significant regression (Fcalculated > Fcritical, P = 5%).

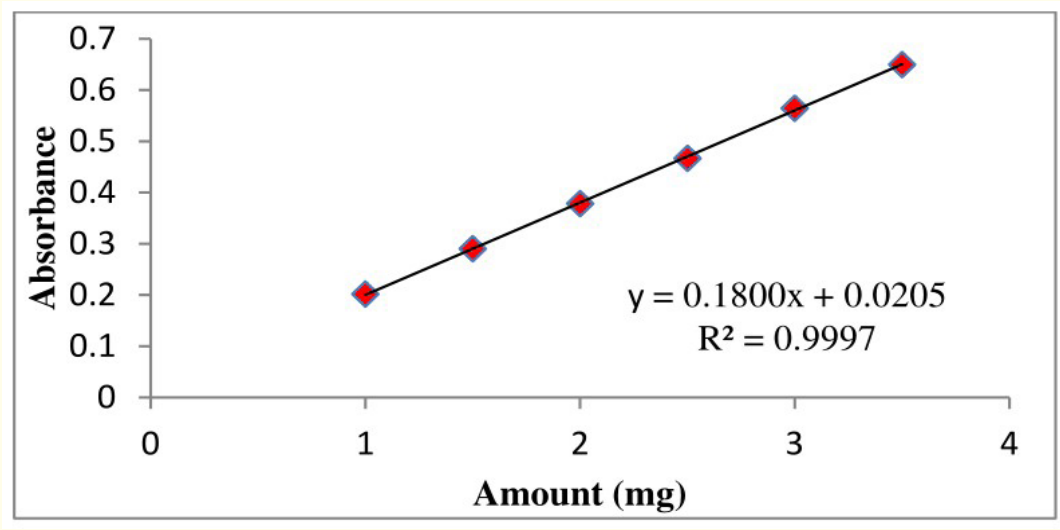

Figure 3: *Graphical representation of the aztreonam analytical curve by FT-IR spectroscopy.*

Source of variation	Degree of freedom	Sum of square	Variability	F calculated	F critical
Between concentration	5	0.42518	0.08504	100.33*	3.11
Linear regression	1	0.42515	0.42515	501.62*	4.75
Deviation of linearity	4	0.00003	0.0000075	0.01	3.26
Residue	12	0.0101706	0.0008475	-	-
Total	17	0.43535	-	-	-

Table 2: *Analysis of variance of absorbance values determined in the obtaining of the calibration curve of aztreonam reference substance using the FT-IR spectroscopy method.*

**Significant p < 0.05%*

Selectivity

Selectivity was established by analyzing the excipient (L-arginine) of aztreonam in the samples prepared on three consecutive days (n = 3). The correlation coefficient was 0.9993 (Figure 4), a value near 1.0, which shows excellent specificity of the method. Furthermore, data were validated by variance analysis (Table 3) which showed highly significant regression (Fcalculated > Fcritical, P = 5%). The selectivity was also evaluated according to the spectra of solutions of excipient, aztreonam sample and reference substance (Figure 2). The spectrum showed that the excipient does not interfere with the infrared spectroscopy.

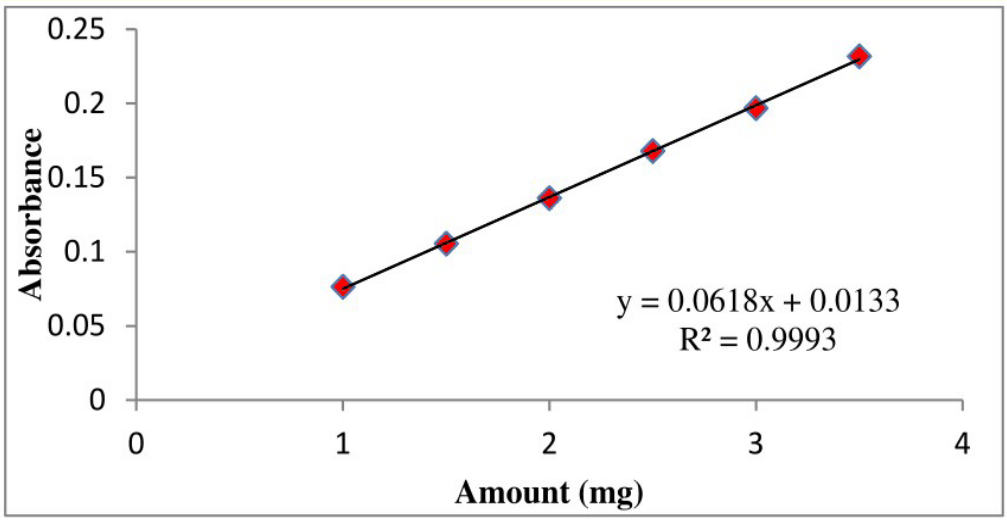

Figure 4: *Graphical representation of the aztreonam analytical curve by FT-IR spectroscopy.*

Source of variation	Degree of freedom	Sum of square	Variability	F calculated	F critical
Between concentration	5	0.05023	0.01005	10.45*	3.11
Linear regression	1	0.05020	0.05020	52.21*	4.75
Deviation of linearity	4	0.00003	0.0000075	0.01	3.26
Residue	12	0.0115382	0.000961	-	-
Total	17	0.06177	-	-	-

Table 3: *Analysis of variance of absorbance values determined in the obtaining of the calibration curve of aztreonam using the FT-IR spectroscopy method.*

**Significant p < 0.05%*

Precision

Precision was evaluated with respect to both repeatability and intermediate precision. Repeatability was determined by calculating the relative standard deviation (RSD) for seven repetitions of the test concentration (2.5 mg). The RSD value obtained was 1.74%. The intermediate precision was evaluated by calculating the recovery of the drug, performed on three different days (inter-day precision). The RSD value obtained was 0.74%, while precision between-analysts, the RSD was 0.75%. Values less than 5% confirms the method is precise. The interday precision was evaluated by analysis of variance while the between-analyst precision was evaluated by Student's t test.

Accuracy

The accuracy of the method was confirmed by determining the average recoveries from the samples by applying the standard addition method. As shown in Table 4, the mean percentage recoveries were 100.60%, with standard deviation of 0.41%. The study results demonstrate that accuracy slight variations in the concentration of aztreonam can be readily quantified by the method as well as no interference of excipients therefore the analytical method developed is sufficiently accurate.

	Added (mg)	Found[1] (mg)	Recovery[1] (%)	Average recovery (%)	R.S.D.[2] (%)
R1	2.0	2.02	101.0	101.6	0.41
R2	2.5	2.47	98.8		
R3	3.0	3.15	105.0		

Table 4: *Determination accuracy of the analytical method for the analysis of aztreonam by FT-IR spectroscopy.*

[1]Average of three determinations

[2]R.S.D.: relative standard deviation

Robustness

The results obtained in robustness test are shown in Table 5. Statistical analysis was performed by small modifications, individually, in the following method parameters: temperature, brand of potassium bromide and time compression. The R.S.D. values show smaller than 2%, which demonstrates the robustness of the method for the aztreonam. Small changes that occurred during the analyses did not affect the absorption intensity of the samples.

Parameters	Condition	RSD[1] (%)
Temperature	18°C 26°C	0.48
KBr brand	Synth Shimadzu	0.83
Time of compression	10 min 15 min 20 min	1.04

Table 5: *Parameters of the robustness evaluation of the analytical method for the analysis of aztreonam by FT-IR spectroscopy.*

[1]RSD: relative standard deviation

Limit of Detection (LOD) and limit of quantification (LOQ)

LOD and LOQ values were found to be 0.28 mg and 0.09 mg, respectively. The values obtained indicate the reliability of the method to detect and quantify the aztreonam in powder for injectable preparation.

Assay of the pharmaceutical product

The validated method was applied to the determination of aztreonam in powder for injection. The results showed that the percentage content of the drug is according to the Brazilian Pharmacopoeia. Spectroscopy is a technique based on the fact that molecules absorb energy, are undergoing a transition to a higher energy state, and only the transitions of vibrational energy occurs in the mid-infrared. The vibrations induced by infrared radiation include stresses and strains inter-atomic bonds and changing angles of links [44]. Considering that the molecule absorbs only selected frequencies in the infrared radiation, in will only absorb those that correspond to its natural vibrational frequency, which causes an increase in the amplitude of vibration motion of its chemical bonds. However, the vibration frequency can be associated with a particular bond type [45]. Besides the fact that it does not use organic solvents, the infrared spectroscopy technique has other advantages. This is a rapid technique which requires minimum or no pretreatment of the sample, provides accuracy

comparable to other methods and also helps in detectionof counterfeiting and impurities. Through this technique, the sample can be scanned, on average, up to 64 times in any physical state and taking less than a minute with high resolution and precision. Furthermore, it is a suitable technique for drugs with solubility problems, since they can be prepared in the pellet form [34-42]. This is an ecological method that does not use organic solvents and, although it is formally accepted for the identification of individual compounds, the literature shows some publications that validated this technique for quantitative analysis [26-31,34-43].

FTIR spectroscopy is an interferometric technique based upon the coherence of the superposition from the sample and reference beams of an interferometer. The raw data gathered by the detector of the instrument are Fourier transformed to extract the frequency dependent information. FTIR spectroscopy is a broad band technique that enables the study several materials in the spectral range which coincides with the wavelength range of light which is highly reflected. The mid infra-red spectral region, together with the far infra-red region (25 - 1000 μm), is known as the molecular fingerprint region. At these wavelengths, rotational and vibration resonant couplings occur between chemical bonds within constituent molecules and the incident radiation. There is great variation in molecular structure between different drugs and also between samples of the class. To date, the assessment of infrared quantitative analysis is limited in the literature even it is been a recognized ecological method. The specific interaction between groups can cause the characteristics of the transmitted radiation. These changes can be detected by a suitable detector, allowing the properties of the target sample to be inferred [43].

The applicability of the method to test pharmaceutical preparations was examined. The results are highly reproducible for determination of the aztreonam pharmaceutical formulation.

Conclusion

In this study, we validate a FTIR spectroscopic method for the quantification of aztreonam in pharmaceutical formulation.

The analytical method of infrared spectroscopy was successfully developed for quantitative determination of aztreonam in powder for injection. All validation parameters were satisfactory, indicating linearity, selectivity, precision, accuracy, robustness and detection and quantification limits appropriate. The method can therefore easily be applied without laboratory quality control since it ensures its safety and efficacy. Furthermore, it has some advantages over other methods described in literature, the main one being that it does not use organic solvents, which contributes to non-generation of such waste, contributing to minimize the environmental impact of the pharmaceutical.

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Declaration of Interest

The authors report no declarations of interest.

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