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Development and validation of an ecological, new and rapid stability-indicating High Performance Liquid Chromatographic method for quantitative determination of aztreonam in lyophilized powder for injection

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Aztreonam is a monocyclic synthetic antimicrobial with bactericidal activity against Gram-negative bacteria, the first agent from the monobactam family to be therapeutically approved. It was developed and validated analytical method using high performance liquid chromatography with UV detection at 292 nm to quantify the aztreonam. Furthermore, assessing stability through stress tests was carried out. The chromatographic separation was carried out by reverse phase on an Agilent C_{18} column (250 x 4.6 mm, 5 µm) with a mobile phase composed of water:ethanol (70:30, v/v) adjusted to pH 2.5 with acetic acid, pumped isocratically at a flow rate of 0.5 $mL.min^{-1}$. The validation parameters linearity, selectivity, precision, accuracy, robustness, limits of detection and quantification were determined. The method proposed provides linear response within the concentration range of 45-95 $\mu g.mL^{-1}$ for aztreonam. Results obtained were found to be satisfactory. The proposed method is linear, accurate, precise, selective, and robust being able to quantify the aztreonam in pharmaceutical preparations. The validated method was suitable for applications in quality control laboratories.

Keywords: aztreonam; analytical methods; HPLC; quality control; validation

Introduction

Aztreonam (Figure 1), chemically named 2-[[[1-(2-amino-4-thiazolyl)-2-[(2-methyl-4-oxo-1sulfo-3-azetidinyl)amino]-2-oxoethylidene]amino]oxy]2-methyl, is an antibiotic belonging to the monocyclic monobactam family (1-3). Nowadays, it is used to treat infections caused by Gram-negative bacteria. It is active against *Escherichia coli*, *Klebsiella* spp., *Proteus* spp., *Serratia marcescens*, *Salmonella* spp, *Enterobacter* spp., *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Neisseria meningitides* (4-6). It is, however, ineffective against Gram-positive and anaerobic pathogens (7,8). It is indicated in cases of urinary tract infections, skin infections and intra-abdominal infections (9). This antimicrobial agent is not absorbed from the gastrointestinal tract and therefore can be used only by parenteral route (10). After intramuscular administration, peak serum concentrations are reached within one hour (11). Regarding the mechanism of action, aztreonam inhibits cell wall biosynthesis in Gram-negative bacteria by binding to proteins, which causes cell lysis (12).

Several methods are described for the quantitative determination of aztreonam in pharmaceutical preparations, as a raw material or biological material. Some published papers bring HPLC analysis for aztreonam (13-16), but they use toxic solvents. The aim of this study is to optimize the system, improve the constitution of the mobile phase, decrease analysis time, use less toxic reagents and mainly the offspring less residue. In this context, the objective of this study is to develop and validate an analytical method using a cheap, simple and helpful mobile phase to quantify aztreonam. Furthermore, assessing stability through stress tests to improve quality control and ensure effective therapy (17,18).

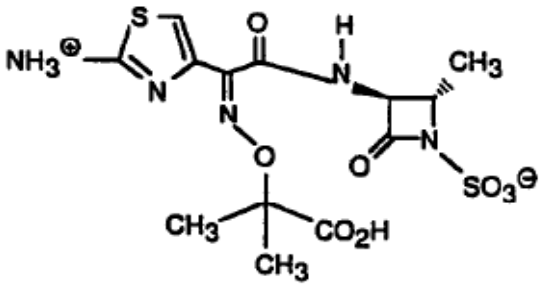

Figure 1 Chemical structure of aztreonam (CAS: 78110-38-0)(12).

Experimental

Chemicals

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

The adjuvant present and tested was L-arginine. Ethanol and acetic acid were obtained from Tedia (Darmstadt, Germany). The water was obtained by a Milli-Q system (Millipore, Milford, USA). The solutions were filtered through a hydrophilic Millipore filtration membrane (13-mm, 0.45-mm pore size).

Equipments and chromatographic conditions

The liquid chromatographic system used consisted of a Waters chromatograph equipped with Waters 1525 pump binary grade, Rheodyne Breeze-7725i injection valve with 20 µL loop, UV-Vis Waters 2487 detector and reserved phase Agilent C18 column (250x4.6 mm i.d., 5 mm particle size) and a Waters C18 column (250x4.6 mm i.d., 5 mm particle size) to achieve robustness. The analyses were performed at room temperature under isocratic conditions with a flow rate of 0.5 $mL.min^{-1}$. The mobile phase, consisted of water:ethanol (70:30) adjusted to pH 2.5 with acetic acid, was vacuum filtered and degassed under ultrasonic liquid (Model XL 2020) for 30 minutes. Aztreonam solution, prepared in mobile phase at a concentration of 200 $\mu g.mL^{-1}$, was filtered on 0.45 µm hydrophilic membrane. The injection volume was 20 µL. UV detection of the analyte was carried out at 292 nm.

Preparation of solutions

The stock and sample (a pool of 20 batches with an average weight of 1.6861 g) solutions were prepared by accurately weighing 20 mg of aztreonam RS, transferring to a 100 mL volumetric flask and dissolved in mobile phase to give a concentration of 200 $\mu g.mL^{-1}$. Appropriate dilutions of the stock solution were transferred to 10 mL volumetric flasks, and volume was completed with mobile phase. The solutions were filtered through membranes of 0.45 µm x 13-mm.

Degradation conditions

Since stability testing is an important part of the process of drug product development, a forced degradation study was carried by exposing the drug to acidic, alkaline, neutral, oxidative and photolytic conditions (19,20). Forced degradation studies were performed to evaluate the stability indicating properties and specificity of the method (17).

Preparation of sample solution

Acidic, alkaline and neutral degradation studies

Acid degradation was carried out in 0.1 *M* HCl (Synth, Brazil) at a concentration of 75 $\mu g.mL^{-1}$ aztreonam for 48 hours. Stress studies in basic conditions were conducted using a drug concentration of 75 $\mu g.mL^{-1}$ in 0.01 M NaOH (Synth, Brazil) for 168 hours. These solutions were exposed to a temperature of 60 ºC. Additionally, the aztreonam in mobile phase at a concentration of 75 $\mu g.mL^{-1}$ was heated at 60 ºC for 168 hours.

Oxidative degradation

Oxidative degradation carried out in H_2O_2 3% (Vetec Química, Brazil) at a concentration of 75 $\mu g.mL^{-1}$ aztreonam. These solutions were exposed to a temperature of 60ºC for 1 hour.

Photostability

Aztreonam was exposed the photolytic degradation using a mirrored chamber with UV C light for 168 hours in a concentration of 75 $\mu g.mL^{-1}$.

System suitability

System Suitability tests are an integral part of a liquid chromatographic method, and they were used to verify that the proposed method was able to produce good resolution with high reproducibility (2). The system suitability was determined by six replicate injections from prepared standard solutions and analyzing the peak area, theoretical plates (N) and tailing factors (T).

Method validation

The validation parameters for linearity, specificity, precision, accuracy, robustness, limit of detection (LOD) and limit of quantification (LOQ) was according to the parameters established in guidelines (17,18,22-28).

Linearity

Linearity test solutions for the assay method were prepared from a stock solution at six concentrations of 45, 55, 65, 75, 85, 95 $\mu g.mL^{-1}$. The analytical curve was evaluated on three different days. Each concentration was determined in triplicate. Linearity was analyzed with least squares linear regression, fitting the data using the least squares method.

Specificity

Specificity was established by analyzing the excipient (L-arginine) present in the samples aztreonam, evaluated by regression analysis in six concentrations ranging from 45 to 95 $\mu g.mL^{-1}$ prepared on three consecutive days. Each concentration was determined in triplicate. The specificity was also assessed following the chromatograms of solutions of excipient, aztreonam sample and RS at a concentration of 200 $\mu g.mL^{-1}$ and the stress test. The excipient solution was prepared under the same conditions as the commercial samples. The specificity of the developed HPLC method for aztreonam was carried out in the presence of its degradation products. Intentional degradation was attempted to stress conditions exposing to evaluate the ability of the proposed method to separate aztreonam from its degradation products.

Precision

To study the precision, it was used the concentration of 45 $\mu g.mL^{-1}$ of aztreonam (test concentration). Precision was evaluated with respect to

repeatability and intermediate precision. Repeatability was assessed by analysis of standard solutions of aztreonam in the same concentration and the same day, performing seven repetitions. 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-analysts). Data from the relative standard deviation (RSD) were evaluated.

Accuracy

The accuracy of the method was determined by recovering the standard solution and sample in triplicate on three levels 80, 100 and 120% concentration method (45 $\mu g.mL^{-1}$), in accordance with the ICH recommendations.[17] In 10 mL volumetric flasks, aliquots of 0.3, 0.95 and 1.4 mL of this solution (concentrations 36, 45 and 54 $\mu g.mL^{-1}$, respectively) were each added to 1.3 mL solutions of sample. The recoveries of aztreonam for testing and relative standard deviation (RSD) were determined.

Robustness

To evaluate robustness of the method, it was used aztreonam concentration of 45 $\mu g.mL^{-1}$ (concentration test). The robustness of the method was verified by the variation in wavelength (292 nm normal, 290 and 294 nm changed), temperature (26°C normal, 18°C changed), column (Waters normal, Agilent changed), flow (0.50 $mL.min^{-1}$ normal, 0.48 and 0.52 $mL.min^{-1}$ changed), volume of injection (20 µL normal, 18 and 22 µL changed), ethanol supplier (Tedia normal, J.T.Baker changed) and preparation of solution in mobile phase (normal) and water (changed).

Limits of detection and quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) of the method were obtained from equations (1) and (2),

$$\mathrm{LOD} = \frac{3.3\,\sigma}{\mathrm{SD}} \qquad \text{Eq. 1}$$

$$\mathrm{LOQ} = \frac{10\,\sigma}{\mathrm{SD}} \qquad \text{Eq. 2}$$

where S.D. is the intersection standard deviation and α is the average slope, obtained from the analytical curves of the linearity study.

Results and Discussion

Method development

The methods described in the literature for the determination of aztreonam are time consuming, complex, use toxic solvents and require the use of large amounts of organic solvents and high cost. In this paper, the selection of the mobile phase was based on peak parameters (height, tail symmetry, and tailings), analysis time, ease of preparation of the mobile phase and sample solvent, and costs less toxic. Furthermore, the mobile phase composition is simple and the column gave longer life and reduced risk of precipitations associated with the use buffers. The peak retention time was 5.9 minutes (Figure 2). This is a good value for a routine procedure in quality control and possible to analyze large numbers of samples on the same day.

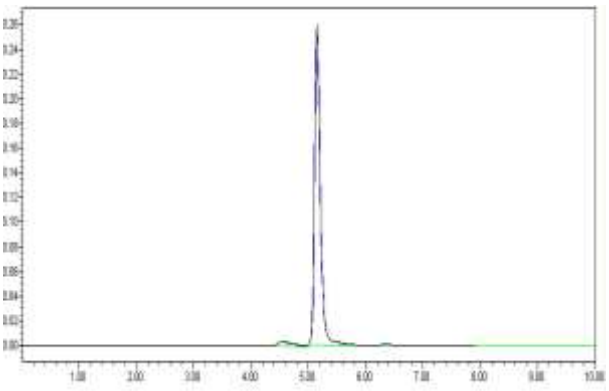

Figure 2 Chromatogram of standard (black), pharmaceutical product (blue) and excipient (green).

Stress degradation experiments

Stability testing is an important part of the process of drug product development. The purpose of stability testing is to provide evidence of how the quality of a drug substance or drug product varies with time under a variety of environmental conditions (19). The forced degradation studies provide information about the conditions in which the drug is unstable so that measures can be taken during manufacturing to avoid potential instabilities. The stability indicating capability of the method was established from the peak purity of aztreonam in degraded samples. The peak purity index and threshold for aztreonam in all cases was more than 0.99 (Table 1). Typical chromatograms obtained following the assay of stressed samples are shown in Figure 3.

Table 1 Summary of forced degradation conditions for aztreonam quantitation by HPLC method.

Stress conditions	Time (hours)	Degradation (%)
Acid hydrolysis (0.1 *M* HCl)	48	14.86
Alkaline hydrolysis (0.01 *M* NaOH)	168	0.82
Photostability	168	1.53
Oxidation (3% H_2O_2)	1*	18.02
Neutral (60ºC)	168	19.04

*hour

Aztreonam has deteriorated rapidly in oxidative degradation, the main peak lost 18.02% of area after 1 hour. In acidic conditions, aztreonam 14.86% degraded after 48 hours and neutral at 60°C, 19.04% after 168 hours.

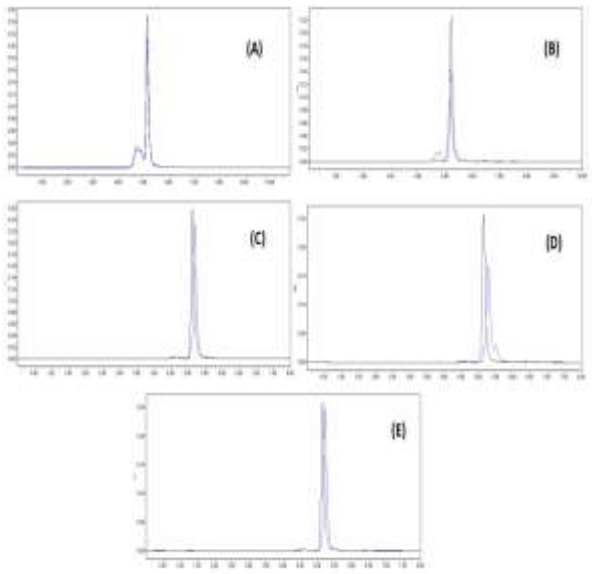

Figure 3 Chromatograms of aztreonam sample after (A) oxidative degradation (H_20_2 3% for 1 hour), (B) acid degradation (HCl 0.1 *M*, for 48 h at 60°C), (C) alkaline degradation (NaOH 0.01 *M*, for 168 hours at 60°C), (D) neutral degradation (mobile phase, for 168 hours at 60°C), (E) photolytic degradation (UV C light for 168 hours at 25°C).

Method Validation

Linearity

The analytical curves, resulting in from three consecutive days were fitted by linear regression analysis, whose straight line equation is: y = 23817.3810x+1342.5926. The coefficient correlation was 0.9999 (Figure 4). Furthermore, data was validated by an analysis of variance (ANOVA), which showed highly significant regression, since the F values calculated were smaller than those *F* critical values (Table 2).

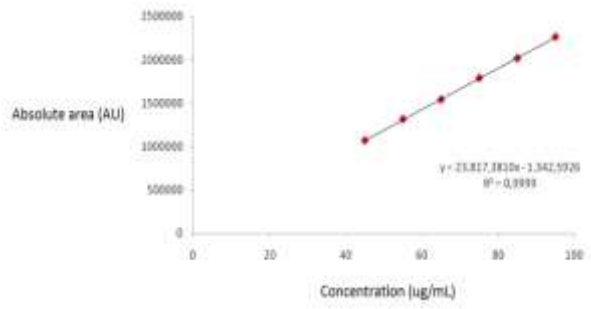

Figure 4 Calibration curve constructed for aztreonam standard solutions in the range of 45 to 95 µg mL^{-1}.

Specificity

Data from the calibration curve resulting from the average of three sample curves generated on three consecutive days, whose straight line equation is: y = 22048.3038x + 22341.0111. The correlation coefficient was 0.9998. The data were validated by analysis of variance, and F calculated values were lower than the F critical values (Table 3).Moreover, specificity was demonstrated by the absence of any interfering peak in the chromatogram obtained, showing that the carrier does not interfere with the peak of aztreonam (Figure 2). The specificity was also confirmed by studies of stress.

Table 2 Analysis of variance (ANOVA) of peak's area determined in the obtaining of the calibration curve of aztreonam using HPLC method.

Source of variation	DF	Sum of square	Variability	*F* calculated	*F* critical
Between concentration	5	297857	595714219	361.04*	3.11
Linear regression	1	297857	297857109	1805.21*	4.75
Deviation of linearity	4	0.00003	0.0000075	0.10	3.26
Residue	12	197998	164998898	-	-
Total	17	299837	-	-	-

Table 3 Analysis of variance (ANOVA) of peak's area determined in the obtaining of the calibration curve of aztreonam using HLPC method.

Source of variation	DF	Sum of square	Variability	*F* calculated	*F* critical
Between concentration	5	27348154	54696309142	817.59*	3.11
Linear regression	1	27348154	27348154571	4087.96*	4.75
Deviation of linearity	4	0.00003	0.0000075	0.00	3.26
Residue	12	80279202	66899335764	-	-
Total	17	274284337	-	-	-

Precision

Precision was evaluated with respect to repeatability and intermediate precision. Repeatability was determined by calculating the relative standard deviation (RSD) for seven repetitions of the test concentration (75 µg.mL^{-1}) on the same day and under the same experimental conditions. The RSD value obtained was 0.55%. 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 0.31%. Finally, the between-analysts precision shows RSD = 1.31%. Values lower than 5% confirm 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, as shown in Tables 4 and 5.

Table 4 Analysis of variance (ANOVA) for interday precision using HPLC method for determination of aztreonam.

Source of variation	DF	Sum of square	Average squares	*F* calculated	*F* critical
Between groups	2	873946361.3	4369731	7.04	9.55
Within groups	3	186149292	6204976	-	-
Total	5	1060095653	-	-	-

Table 5 Student's *t* test for precision between analysts by HPLC method for analysis of aztreonam.

Source of variation	Average	Degree of freedom	Variance	*t* calculated	*t* critical
Analyst 1	1754654.5'	7	10268502	-0.58	2.17
Analyst 2	1757528	7	64371204		

Accuracy

The accuracy of the method was confirmed by determining the average recoveries of samples using the standard addition method. As shown in Table 5, the percentage recovery was 100.57%, with a standard deviation was of 0.21%. The results demonstrate that 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.

Table 6 Determination of the accuracy of the analytical method for the analysis of aztreonam by HPLC.

Sample 200 µg. mL^{-1}	RS concentration ($\mu g.mL^{-1}$)		Recovery (%)	RSD (%)	Mean recovery (%)
	Added	Found			
	60	58.98	98.3	0.50	
	75	74.14	98.8	0.87	98.56
	90	88.80	98.6	1.23	

Robustness

The robustness was found to be reliable, as determined by the RSD (<5%). We observed the constancy of peak area with change in the experimental parameters wavelength, temperature, column, flow, injection volume, ethanol supplier and sample preparation in water and mobile phase (Table 7). Small changes that occurred during the analysis did not affect the peak's area of the samples.

Table 7 Robustness test for aztreonam by HPLC method.

Parameters (Normal – Changed)	RSD (%)
Wavelength (292 nm – 290/294 nm)	0.11
Temperature (26 °C – 18 °C)	0.23
Column (Waters – Agilent)	0.37
Flow rate (0.50 mL/min – 0.48/0.52 mL/min)	0.24
Injection volume (20 µL – 18/22 µL)	0.20
Solvent supplier (Tedia – J.T.Baker)	0.07
Sample preparation (mobile phase – water)	0.83

RSD = relative standard deviation

Limits of detection (LOD) and quantitation (LOQ)

LOD and LOQ values were found to be 0.48 $\mu g.mL^{-1}$ and 1.46 $\mu g.mL^{-1}$, respectively. The method is sensitive to small concentrations.

System suitability

System suitability tests are used to verify that the resolution and reproducibility were adequate for the analysis performed. The parameters measured were tailing factor, capacity factor, theoretical plates, retention time (5.013 min, RSD 0.61%) and repeatability as %RSD (0.47%) of peak area for six injections of a standard solution of aztreonam (100% concentration). The tailing factor showed less than 2 (0.5, RSD 0.0%), the capacity factor was more than 1.5 (2.25, RSD 0.89%) and the theoretical plates were more than 2000 (3475, RSD 0.87%).

Green Chemistry

The concept Green Chemistry has had a growing importance in the last decade and it is stimulated by the academy, industry segment and society. For this reason a new method was developed which uses a green mobile phase composed by water and ethanol to quantify aztreonam in pharmaceutical dosage forms. This mobile phase contributes to the environment, not only to waste parameter but also to operator. Moreover, these both solvents have a low price, and they could quantify aztreonam in a short time. Each analytical method can be characterized by its specific requirements. Therefore, it is very important to evaluate and to optimize the greenness of an analytical method or procedure and to focus on its least green aspect (29-31).

The rapid analysis of aztreonam has been particularly of interest by pharmaceutical industries and quality control laboratories. In this aspect, it is possible to reduce residues, make a faster and cheaper analysis, and it is not time consuming.

Despite the characteristic points of each analytical method and drugs or other substances determined, sample as well operator take an important role in every analysis. Moreover, the methods can also require the use of equipment, reagent and produce analytical residues. Although pharmaceutical analysis is a process comprising several steps influencing each other, Gałuszka and coworkers suggested six critical issues for Green Chemistry principles (28).

Solvents as ethanol and water were not frequently used as mobile phase in chromatographic system some years ago because the use of ethanol increased the working pressure due to it incorporates a higher viscosity to the system. However, nowadays modern instrumentation makes possible the use of ethanol as mobile phase solvent, as these devices support much higher pressures (32). Recently there are many researches published which could demonstrated that ethanol or methanol and water mixtures have excellent properties when used in chromatographic system of reverse phase C_8 and C_{18} columns (32-41). Finally,

many analytical methods for quantitative determination of aztreonam in pharmaceutical products are described in the literature (13-16,42,43). Nevertheless, this research validates a new useful HPLC method, which shows the important characteristics such as environmentally healthy with green solvents and low residues amount.

Conclusions

The chromatographic method was successfully validated for quantitative determination of aztreonam. This method has advantages over other existing methods that are simplicity, speed, precision, accuracy and low cost. The main advantage of this method was the use of a mobile phase of very simple composition, which gave the column longer lifetime, and reducing risk of rainfall associated with the use of buffers in the solvent system. All validation parameters were found to be highly satisfactory, including linearity, selectivity, precision, accuracy, robustness and limit of detection and quantification appropriate. Furthermore, there was no interference from any components of pharmaceutical products or degradation. The method can therefore easily be applied in quality control laboratories providing new perspectives for process control.

Declaration of Interest

The authors report no declarations of interest.

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