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METHOD DEVELOPMENT AND VALIDATION FOR ASSAY AND RELATED SUBTANCES OF AZTREONAM FOR INJECTION 1g AND 2g LYOPHILIZED VIALS (STABLITY INDICATING) BY HPLC TECHNIQUE

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ABSTRACT

Aztreonam for injection contains Aztreonam and Arginine. This is a freeze dried product (Lyophilized). The Aztreonam is a synthetic bactericidal antibiotic used primarily to treat infections caused by gram negative bacteria. It is a synthetic drug and it is resistant to some beta-lactams. But it is inactivated by extended –spectrum beta lactamases and used to treat a wide variety of bacterial infections. The method for determination of Aztreonam and it's related substances in pharmaceutical products is not described in current pharmacopoeias, the aim of this work was to develop and validate a precise, accurate and robust method. The separation was achieved on a reversed Phase C-18 column (5 µm, 25 cm x 0.46 cm, Brand: YMC Pack ODS, AQ) using a mixture of Potassium dihydrogen Phosphate, Tetra hydrofuran and Methanol in the ratio of 50:20:30(%V/V/V) respectively and degassed, at a flow rate of 0.8 ml/min, the flow rate of mobile phase can be adjusted to obtain the retention time of Aztreonam peak between 22 and 23 minutes with help of UV detection wavelength at 254 nm. The method was validated by ICH guidelines and validation parameters showed that it could be used as stability indicating method for determination of Aztreonam and it's related substances in Aztreonam for injection.

Key Words:- Aztreonam and its related substances, HPLC, Validation, Method development.

INTRODUCTION

Analytical method has to ensure good separation of active compounds and their degradation products. These compound, active and placebo, have different properties, and they have to be separated, this method has to be developed as stability-indicating method. The aim of this study was to develop HPLC analytical method (Sorbera et al., 2002; Padhi et al., 2009; Manikandan K et al., 2013; Shaik Saida et al., 2014; Abdul Rasheed et al., 2010; Ye et al., 2016) to determine Aztreonam and it's related substances in Aztreonam for injection and to validate the same analytical method as per ICH guidelines (). The following related substances are listed in Aztreonam for injection.

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METHODOLOGY

Note: (i) The solvents and reagents are used in Gradient and ACS grade.
(ii) Perform analysis under protection of light.
(iii) Use amber colored glassware (Practical HPLC Method Development).

Preparation of dilute Orthophosphoric acid solution:

Dilute 6.9 mL of Orthophosphoric acid to 100 mL with HPLC grade water and mix.

Preparation of Mobile phase A

(1) Weigh and dissolve about 6.8 g of Potassium dihydrogen phosphate anhydrous in 1000 mL of HPLC grade water, adjust the pH to 2.90 ± 0.05 with dilute ortho Phosphoric acid solution and mix well.

(2) Filter through 0.45 µm membrane filter and degas for about 10 minutes.

Preparation of Mobile phase B

(1) Weigh and dissolve about 1.36 g of Potassium dihydrogen phosphate anhydrous in 1000 mL of HPLC grade water, adjust the pH to 2.90 ± 0.05 with dilute ortho phosphoric acid solution and mix well.

(2) Filter through 0.45 µm membrane filter.

(3) Mix above buffer, Tetrahydrofuran and Methanol in the ratio of 50:20:30 (%v/v/v) respectively and degas.

Preparation of Diluent

Mix mobile phase A and Methanol in the ratio of 80:20 (%v/v) respectively and degas.

Chromatographic parameters

(1) Liquid chromatograph equipped with a 254 nm UV detector.

(2) Column : YMC Pack ODS AQ, 250 x 4.6 mm, 5 µm that contains Octadecyl silane chemically bonded to Porous silica particles.
(Mfg by: YMC Co., Ltd; Part # AQ12S05-2546WT)

(3) Column temperature : Ambient.
(4) Sample cooler temperature : 5°C.
(5) Flow rate : *0.8 mL/min.
(6) Injection volume : 15 µL.
(7) Run time : About 65 minutes.
(8) Elution : Gradient.

*Adjust the mobile phase flow rate to obtain the retention time of Aztreonam peak Between 22 to 23 minutes.

Preparation of Resolution solution

Weigh and transfer about 53 mg of Aztreonam standard into a 50 mL volumetric flask, add about 30 mL of diluent, sonicate to dissolve the material completely and heat for about 10 minutes in water bath at about 60°C. Allow to cool the solution at room temperature, dilute to volume with diluent and mix well. Filter through 0.45 µm membrane filter.

Note: Resolution solution can be used up to 7 days when kept in refrigerator

Preparation of Standard solution

Weigh accurately and transfer about 53 mg of Aztreonam standard into a 50 mL volumetric flask, add about 35 mL of diluent sonicate to dissolve the material completely, dilute to volume with diluent and mix.

Preparation of Control solution (About 0.5 ppm)

(1) Pipette 5.0 mL of the above standard solution into a 50 mL volumetric flask, dilute to volume with diluent and mix.

(2) Pipette 5.0 mL of the above solution into a 100 mL volumetric flask, dilute to volume with diluent and mix.

(3) Pipette 5.0 mL of the above solution into a 50 mL volumetric flask, dilute to volume with diluent and mix.

Test preparation

For 1g vials

1) Remove flip off seal and aluminum cap of vial.

2) Weigh the vial and reconstitute with 3 mL of water for injection. Quantitatively transfer the contents of the vial into a 100 mL volumetric flask, rinse the vial with diluent and add to flask. Dissolve and dilute to volume with diluent and mix well. Dry the empty vial and take the weight

3) Pipette 5.0 mL of above solution into a 50 mL volumetric flask, dilute to volume with diluent and mix.

For 2g vials

1) Remove flip off seal and aluminum cap of vial.

2) Weigh the vial and reconstitute with 6 mL of water for injection. Quantitatively transfer the contents of the vial into a 200 mL volumetric flask, rinse the vial with diluent and add to flask. Dissolve and dilute to volume with diluent and mix well. Dissolve and dilute to volume with diluent and mix well. Dry the empty vial and take the weight

3) Pipette 5.0 mL of above solution into a 50 mL volumetric flask, dilute to volume with diluent and mix.

System suitability

(1) Inject 15 µL portion of Blank (diluent) and Resolution solution into chromatographic system, record the chromatograms and measure the response for the major peaks.

(2) The USP Resolution between Aztreonam and degradant impurity (Aztreonam E-Isomer) peaks from Resolution solution should be not less than 10.

(3) Inject 15 µL portion of control solution in single into the chromatographic system, record chromatograms and measure the response for major peak.

(4) Signal to noise ratio of Aztreonam peak from control solution should be not less than 10:1

(5) Inject 15 µL portion of standard solution into the chromatographic system for six times, record the

chromatograms and measure the response for aztreonam peak.
(6) The USP tailing factor for Aztreonam peak from standard solution should be not more than 2.0.
(7) The USP plate count for Aztreonam peak from standard solution should be not less than 10000.
(8) The % RSD of peak areas of six standard solution injections should be not more than 2.0 (United States Pharmacopoeia)

Procedure

Inject 15 µL portion of test preparation into chromatographic system, record the chromatograms and measure the response for the peaks.

Note
(1) Discard the peaks due to a). diluent and inhibit integration after 51 minutes.
(2) Discard the peak due to L-Arginine at about RRT b). 0.12.

SUMMARY OF METHOD VALIDATION

1) System suitability and System Precision

Standard solution, Resolution solution and Control solution were prepared as per test method and injected into the LC System. The system suitability parameters were evaluated for Resolution between degradant peak (Aztreonam E-Isomer) and Aztreonam peak, USP Plate count, USP tailing for Aztreonam peak and the signal to noise ratio for aztreonam peak from control solution were found to be within the limits.

The Standard preparation, prepared by using Aztreonam working standard as per test method was injected ten times into the LC System. The Relative standard deviation for Aztreonam peak area from ten replicate injections was found to be 0.1%.

Specificity

Interference from Placebo peaks

A study was conducted to demonstrate the interference of placebo at the retention time of Aztreonam peak and Aztreonam impurity peaks. Placebo was prepared as per the test method in triplicate and injected into LC system, the chromatograms showed no interference of placebo peaks at the retention time of Aztreonam and impurity peaks.

Interference from Known impurities

A study was conducted to demonstrate the non interference of known impurities (Openring desulfated Aztreonam, Openring Aztreonam, desulfated Aztreonam, Aztreonam E-Isomer, Impurity-I, Impurity-J, Aztreonam Ethyl Ester, In-House Impurity-1 and In-House Impurity-2) with Aztreonam peak.

Spiked the known impurities into test preparation at specification limit, individual impurities were injected into the HPLC system by following test method conditions. In the spiked chromatogram, no interference from those impurity peaks at the retention time of Aztreonam peak was found.

Interference from degradation products

A study was conducted to demonstrate the effective separation of degradant. Separate portions of sample were exposed to following stress conditions to induce degradation.
Acid Stress: Drug product and Placebo were stressed with 0.1M HCl at 90°C for 10 minutes.
Base Stress: Drug product and Placebo were stressed with 0.1M NaOH Immediately injected.
Oxidation Stress: Drug product and Placebo were stressed with 1% Hydrogen peroxide at 40°C for 7 hours in water bath .
Heat Stress: Drug product and Placebo were stressed with heat at 105°C for 8 hours in hot air oven.
Water Stress: Drug product and Placebo were Stressed with water and kept on bench top for 24 hours.
Humidity stress: Drug product and Placebo were stressed at 25°C and with 95% RH for 24 hours.
Photolytic stress: Drug product and Placebo were stressed with visible light for 1.2 million lux hours and UV light for 200 watts /m^2.

Stressed samples were injected into the HPLC system equipped with Photo diode array detector as per test method conditions. All degradants peaks were resolved from Aztreonam peak in the chromatograms of all stressed samples. The chromatograms of the stressed samples were evaluated for peak purity of Aztreonam using Waters Empower software. For all forced degradation samples the peak purity for Aztreonam peak was passed as purity angle less than Purity Threshold and with the absence of purity Flag in purity results table. Thus, this method is considered to be "Stability Indicating".

Limit of Detection and Limit of Quantitation

A study to establish the Limit of detection and limit of Quantitation of known impurities and Aztreonam was conducted. Limit of detection and limit of Quantitation were established based on slope method. A series of different concentration solutions containing Aztreonam and known impurities in the range of LOQ were prepared and injected into LC system. The predicted LOQ concentration and LOD concentration are calculated.

Aztreonam and known impurity solutions were injected at predicted LOQ and LOD concentrations six times into the HPLC system. Calculated the % RSD for peak areas and found to be less than 10%, 33% for LOQ and LOD respectively. Hence the predicted concentrations are considered as LOQ and LOD concentrations.

Precision at LOQ concentration was established for Aztreonam and known impurities. Six sample preparations were prepared by spiking known impurities and Aztreonam into placebo mixture. Calculated the RSD for % of known impurities and Aztreonam from the six injections.

Linearity Of Detector Response

Linearity of detector response for Aztreonam was established. A series of solutions of Aztreonam in the concentration range of Limit of Quantitation to 150% of the target concentration of Aztreonam (1000ppm) and injected into the LC system.

Plot a graph with concentration in µg/mL on X-axis and average area on Y-axis. Evaluated the correlation coefficient, Y-intercept and limit of Y-Intercept response corresponding to the target concentration from the linearity graph.

Linearity of detector response for known impurities and Aztreonam was established by plotting a graph with concentration in µg/mL on X-axis and area on Y-axis. Evaluated the correlation coefficient and Y-intercept from the linearity graph. A series of solutions of Aztreonam and known impurities in the concentration range of Limit of Quantitation to 250% for Open ring desulfatsd aztreonam, aztreonam E-Isomer and desulfated aztreonam, LOQ to 450% for openring aztreonam and In-House Impurity-2, LOQ to 300% Impurity-I and Impurity-J, LOQ to 750% In-House Impurity-1, LOQ to 150% Aztreonam Ethyl Ester and Aztreonam of the specification limit were prepared and injected into the HPLC system.

Linearity of detector response to cover the proposed qualification levels of impurities (about 6.5%) was established by preparing a series of solutions of Aztreonam standard (Aztreonam is used as surrogate standard)at different spike levels covering proposed qualification levels of impurities and injected into HPLC system. The graph was plotted with concentration in µg/mL on x-axis and area response on y-axis. The correlation coefficient (r) and Y-intercept were summarized and the results were given in the tables below. The detector response was found to be linear for Aztreonam and known impurities.

Precision(Assay)

Repeatability

The precision of test method was evaluated by assaying each six samples of Aztreonam for injection 1g vials, 2g vials as per the test method. Calculate the % Assay, Relative Standard Deviation and 95% confidence interval of the assay results and the results were found to be within the acceptance criteria.

Intermediate Precision(Assay)

Intermediate Precision of the test method was evaluated by different analyst on different system and different columns on different days. Calculate the % Assay Relative Standard Deviation, overall % RSD of the precision and intermediate precision assay results and the results were found to be within the acceptance criteria. The results are found to be there is no significant difference between the two means w.r.t F-test or t-test.

Repeatability(Related substances)

The precision of test method was evaluated by analyzing six samples of Aztreonam injection by spiking known impurities at release specification limit. The % impurity and Relative standard deviation for % impurity were calculated and the results were found to be within the acceptance criteria.

Intermediate Precision(Related substances)

Intermediate Precision of the test method was evaluated by different analyst on different system and different columns on different days. The % impurity and Relative standard deviation for % impurity were calculated and the results were found to be within the acceptance criteria.

Accuracy (Assay)

Accuracy study of Aztreonam preparing samples by mixing Aztreonam in placebo at different levels ranging from 25% to 150% of the manufacturing composition (i.e. 25%, 50%, 80%, 100%, 120% and 150%). Analyzed the samples in triplicate for 50%, 80%, 100% and 120% levels and six times for 25% and 150% levels as per the test method. Calculate the % recovery for each individual level and calculate the % RSD for results of lower and higher levels i.e.: 25% and 150%, and the results are found to be within the limits.

The amount of Aztreonam added and recovered in µg/mL was calculated. The % Recovery and precision at lower and highest level were also calculated.

Linearity of Test Method

Plotted linearity graph for Aztreonam with average ‘µg/mL added’ versus average ‘µg / mL recovered

at each accuracy levels of 25% to 150%. The correlation coefficient, Y-intercept were calculated from the linearity graph and found to be within limits.

Accuracy (Chromatographic purity)

Accuracy study of Aztreonam and known impurities was conducted in spiked samples of Aztreonam test solution. Samples were prepared in triplicate for LOQ to 150% levels and for higher level 6 preparations by spiking known impurities in test solution Limit of Quantitation to 250% for Open ring desulfatsd aztreonam, aztreonam E-Isomer and desulfated aztreonam, LOQ to 500% for open ring aztreonam, LOQ to 450% for In-House Impurity-2, LOQ to 300 % Impurity-I and Impurity-J, LOQ to 750% In-House Impurity-1, LOQ to 150 % Aztreonam Ethyl Ester and Aztreonam of the specification limit were prepared and injected into the HPLC system.

Samples were prepared by spiking Aztreonam (surrogate standard for unknown impurity) on placebo at different spike levels covering the proposed qualification levels of impurities at about 6.5%. injected all the samples into LC system ,calculated the % impurity and substracted the % impurity obtained in the unspiked sample.

The % of impurities in all the three samples and higher level six samples was calculated The amount of impurity added and amount recovered in µg/mL were calculated. The % Recovery and % RSD for % impurity at highest level were also calculated.

Linearity of Test Method (Chromatographic purity)

Plotted linearity graph for known impurities with average 'µg/mL added' versus average 'µg / mL recovered at spike levels of Limit of Quantitation to 250% for Open ring desulfatsd aztreonam, aztreonam E-Isomer and desulfated aztreonam, LOQ to 500% for open ring aztreonam, LOQ to 450% for In-House Impurity-2, LOQ to 300 % Impurity-I and Impurity-J, LOQ to 750% In-House Impurity-1, LOQ to 150 % Aztreonam Ethyl Ester and Aztreonam. The correlation coefficient, Y-intercept for known impurities were calculated from the linearity graph and found to be within limits.

Range of Test Method

Range of the test method was established for Aztreonam from the linearity, precision and accuracy data, the range of the test method for Aztreonam is Limit of Quantitation to 150%,Limit of Quantitation to 250% for Open ring desulfatsd aztreonam, aztreonam E-Isomer and desulfated aztreonam, LOQ to 500% for open ring aztreonam, LOQ to 450% for In-House Impurity-2, LOQ to 300 % Impurity-I and Impurity-J, LOQ to 750% In-House Impurity-1, LOQ to 200 % for Azreonam Ethyl ester.

Ruggedness

Bench top stability of mobile phase

A study to establish stability of mobile phase on bench top was conducted over a period of about 2 days. Mobile phase prepared as per the test procedure and kept on bench top in well-closed container. Standard solution and test solutions spiked with known impurities at release specification limit, injected into HPLC system with the mobile phase kept on bench top at initial, 1 day and 2 day. Evaluated the System suitability parameters and RRT of known impurities, the results were found to be within the acceptance criteria. From the above study it was established that the mobile phase is stable for a period of 2 days on bench top.

Refrigerator stability of Resolution solution

A study to establish stability of resolution solution, prepared as per test procedure was injected at Initial, 1 day, 2 days and 7 Days along with fresh resolution solution prepared at each time. Evaluate the system suitability and report for Resolution solution.

Bench top Stability of Standard solution

A study to establish stability of standard solution on bench top was conducted over a period of 7days. The standard solution was prepared as per test procedure was injected at Initial, 1 day, 2 days, 5 days and 7 days against freshly prepared standard on each time. The difference in % Assay for the Aztreonam standard from initial to 7 days was calculated and concluded that the standard solution was stable on bench top for 1 day.

Refrigerator Stability of Standard solution

A study to establish stability of standard solution on Refrigerator was conducted over a period of 7days. The standard solution was prepared as per test procedure was injected at Initial, 2 days, 5 days and 7 days against freshly prepared standard on each time. The difference in % Assay for the Aztreonam standard from initial to 7 days was calculated and concluded that the standard solution was stable on Refrigerator for 7 days.

Refrigerator Stability of Test solution

The stability of test preparation (Unspiked sample) was established in autosampler compartment maintained at 5°C on hourly basis at Initial, 2 Hours, 4 Hours, 8 Hours ,12 Hours, 16 Hours, 20 Hours, 24 Hours, 28 Hours, 32 Hours,36 Hours and 40 Hours, Calculated % of each known impurity, total impurities, % Assay and concluded that the stability of sample solution was stable on Refrigerator (5°C) for 28hours.

Table 1. Aztreonam related substances in Aztreonam injection

S.No	Impurity Name
1	Openring desulfated Aztreonam
2	Impurity-I
3	Impurity-J
4	Openring Aztreonam
5	Desulfated Aztreonam
6	Aztreonam E-Isomer
7	In-House Impurity-1
8	Aztreonam Ethyl Ester
9	In-House Impurity-2
10	Impurity at RRT 0.57

Table 2. Gradient programme

Time (min)	% Mobile phase-A	% Mobile phase-B
0	96	4
5	84	16
15	80	20
25	80	20
40	30	70
50	30	70
51	96	4
65	96	4

Table 3. The RRT's and RRF'S of known impurities with respect to Aztreonam are as follows

S.No.	Component Name	RRT(About)	RRF	LOQ (%)
1	Open ring desulfated Aztreonam	0.52	1.03	0.0281
2	Impurity I	0.56	0.60	0.0125
3	Impurity J	0.59	0.59	0.0333
4	Open ring Aztreonam	0.64	0.59	0.0434
5	Desulfated Aztreonam	1.33	1.01	0.0332
6	Aztreonam E-isomer	1.41	1.02	0.0421
7	In-house impurity 1	1.56	0.59	0.0446
8	Aztreonam Ethyl ester	1.68	0.81	0.0111
9	In-house impurity 2	1.72	0.72	0.0182
10	Impurity at RRT 0.57	0.57	1.00	-----

Table 4. Peak Purity Results of Forced Degradation Studies

Stress Condition	Observation			
	Condition			
	% Deg.	PA	PT	PF
Acid	Stressed with 0.1 M HCI at 90° C for 10 minutes on mantle			
	8	0.093	0.840	No
Base	Stressed with 0.1 M NaOH and injected immediately.			
	5	0.073	0.896	No
Peroxide	Stressed with 1% H2O2 at 40° C for 7 hours in water bath.			
	8	0.085	0.787	No
Water	Stressed the sample in water bath to 90° C for 1 hour.			
	14	0.102	0.727	No
Heat	Heated the sample at 105° C for 8 hours in Hot air oven.			

Stress Condition	Observation			
	Condition			
	% Deg.	PA	PT	PF
	8	0.073	0.952	No
Humidity	Exposed under 95% RH at 25° C for 72 hours.			
	3	0.045	0.637	No
Photolytic	Exposed to UV light for 200 watts/m2 and Visible light for 1.2 million lux hours respectively.			
	3	0.072	1.051	No

Figure 1. Typical Chromatogram of Spiked sample

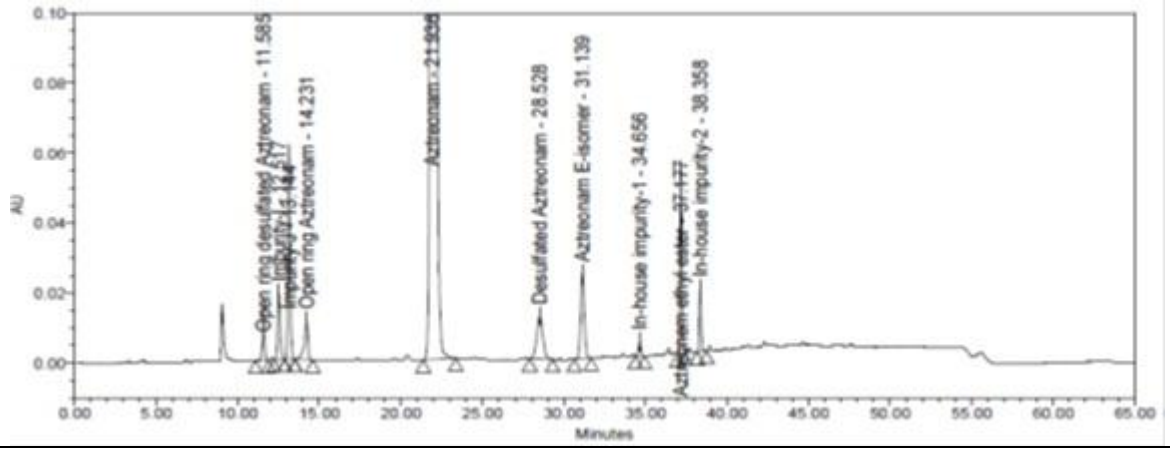
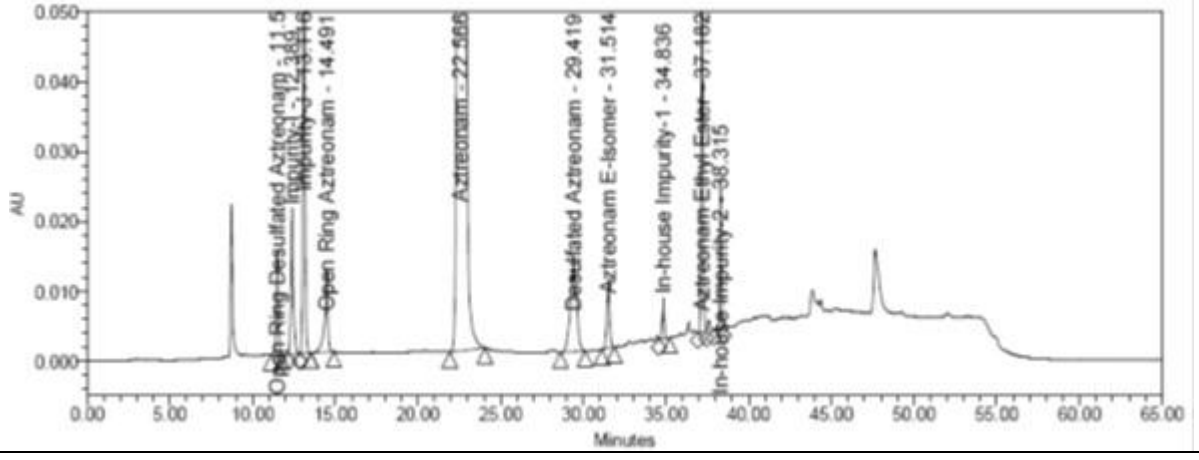


Figure 2. Typical chromatogram of Repeatability spiked sample



CONCLUSION

The proposed method is simple, sensitive, quantitative and economic. Hence this HPLC method has been development and validated for determination of Aztreonam and its related substances of Aztreonam for injection 1g and 2g lyophilized vials with gradient elution. Validation parameters have proved that developed analytical method can be used as stability indicating method to determine the Aztreonam and its related substances in Aztreonam for injection 1g and 2g lyophilized vials.

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