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Development and validation of a RP-HPLC method for the determination of aztreonam and its related impurities in bulk substances

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ABSTRACT

A facile reverse phase high-performance liquid chromatographic method, for the determination of related substances of Aztreonam drug substance has been developed and validated. The degraded products and the isolated impurities were analysed by RP-HPLC utilizing a Octadecylsilane Column (Waters Symmetry C-18, 250×4.6 mm, 5µ), followed by ultraviolet detection at 210 nm followed by 254 nm and a mixture of acetonitrile and phosphate buffer 0.02M with pH 3.2, used as a mobile phase in a gradient elution. This method was validated in terms of Selectivity, Linearity, Precision, Accuracy, Robustness, Limit of detection (LOD), Limit of quantitation (LOQ). This method has been successfully applied for drug substance of Aztreonam.

KEYWORDS

Aztreonam;
β-Lactam;
Validation;
HPLC;
Impurities.

1. INTRODUCTION

Aztreonam,3-[2-(2-azaniumyl-1,3-thiazol-4-yl)-2-(1-hydroxy-2-methyl-1-oxo-propan-2-yl) oxyimino-acetyl]amino-2-methyl-4-oxo-azetidine-1-sulfonate. Aztreonam is similar in action to penicillin. It inhibits mucopeptide synthesis in the bacterial cell wall. It has a very high affinity for penicillin-binding protein 3 (PBP-3) and mild affinity for PBP-1a. Aztreonam binds the penicillin-binding proteins of gram-positive and anaerobic bacteria very poorly and is largely ineffective against them[1,2]. It is known to be effective against a wide range of bacteria including *Citrobacter*, *Enterobacter*, *E.coli*, *Haemophilus*, *Klebsiella*, *Proteus*, and *Serratia* species[3]. The determination of Aztreonam using HPLC was extensively studied[4-12]. Our strategy was to develop a valid high-performance liquid chromatography using UV detection method to analyse the process related impurities as well as the degraded products of Aztreonam. This paper explicates the method development and validation to determine the related substances of Aztreonam drug substance and formulated products.

2. EXPERIMENTAL

2.1. Samples and reagents

The well-examined sample of Aztreonam bulk material (Batch No-VSS/S-378/S) was obtained from Orchid Chemicals and Pharmaceuticals Ltd., Chennai, India. The process related impurities and other degraded impurities (Figure 1) were isolated and characterized

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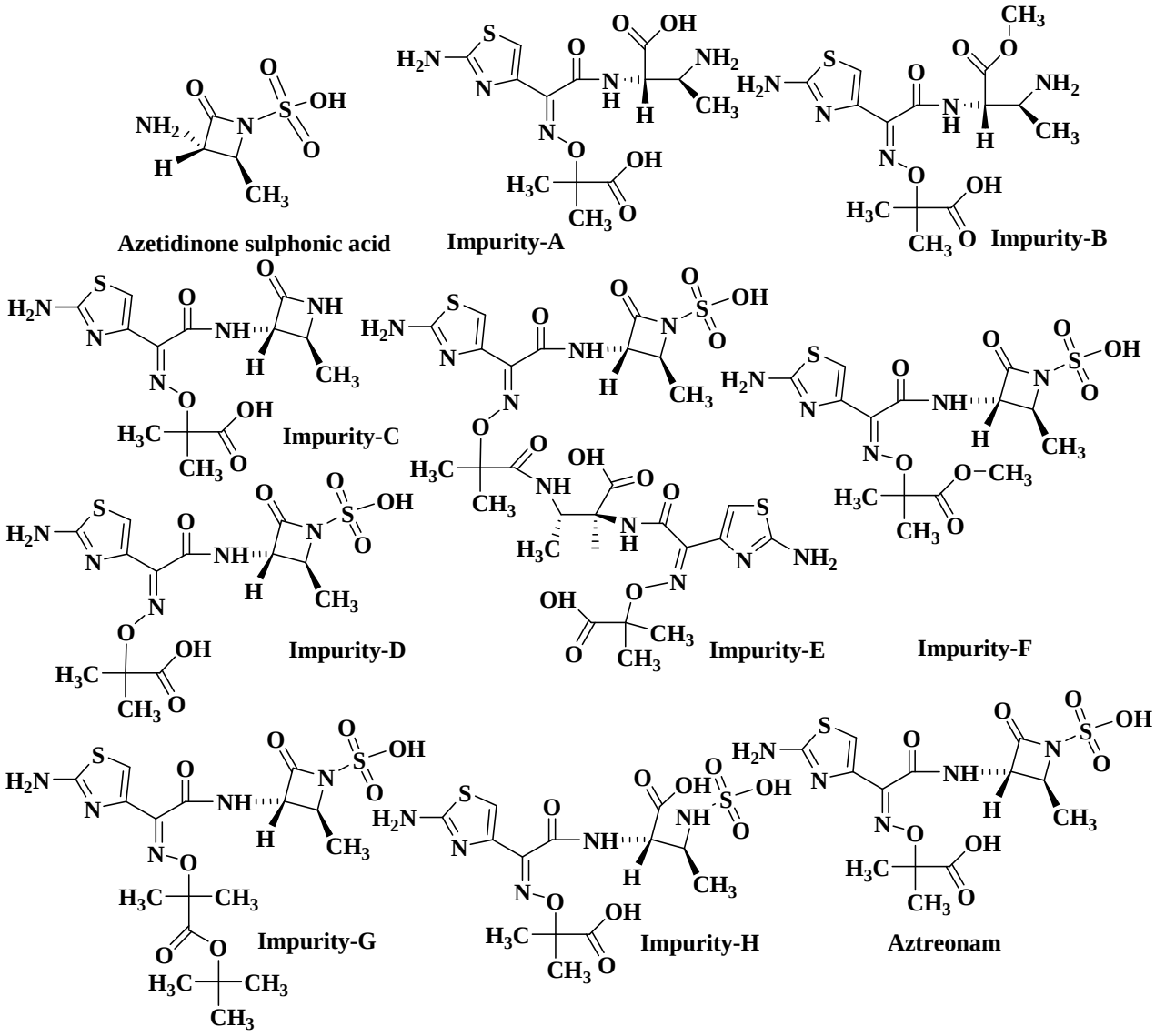

Figure 1: Structures of Aztronam and its impurities

by analytical division of Orchid research laboratories Ltd.

Sodium dihydrogen orthophosphate dihydrate and Orthophosphoric acid (85% v/v), all AR grade were obtained from Qualigens, India.Acetonitrile HPLC grade was obtained from Merck, India. High pure Milli-Q water was used with the help of Millipore Milli-Q plus purification system (MILLIPORE SA, 67120 MOL SHEM, France).

2.2. Apparatus

A Waters Model Alliance 2695 separation model equipped with a Waters 996 photo diode array UV detector and 2690 separation model equipped with a Waters 2487 UV-VIS detector were used. Waters Empower Chromatography software was used for calculation of results. Samples were weighed in Mettler Toledo Model AT 261 and Sartorius microbalance MC5.

2.3. HPLC conditions

An in-house liquid chromatography method was developed for the analysis of Aztreonam, its impurities and the intermediates, where a C-18 Column (Waters Symmetry C-18 250 × 4.6 mm, 5µ) with a mobile phase consisting of a mixture of 0.02M NaH2PO4.2H2O (pH 3.2; 0.02 M) and acetonitrile in the gradient elution was used with UV detection at 210nm for 5 minutes and

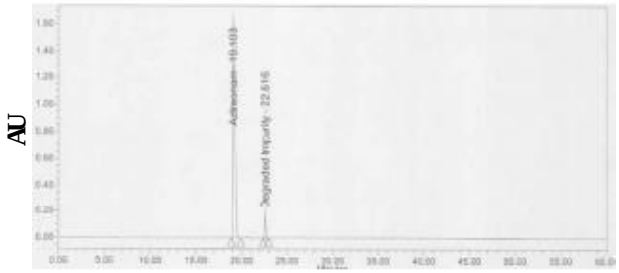

Figure 2: Chromatogram of system suitability

Aztreonam related substances

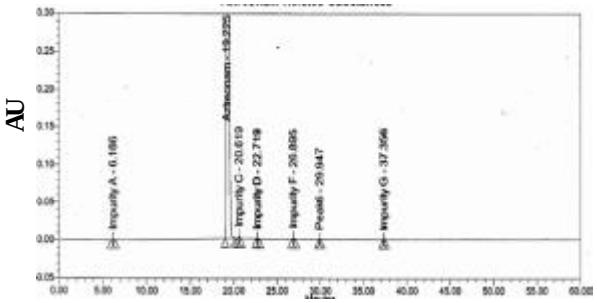

Figure 3: Chromatogram of Aztreonam sample

then at 254nm till the end of the run at a constant flow rate of 1.0 ml/min for the resolution of all impurities. The gradient condition started with 95% of phosphate buffer and5 % of acetonitrile up to 10 minutes and then the acetonitrile was raised to 15 % at 20 minutes, slowly raised to 30% at 30 minutes and linearly raised to 70 % at 45 minutes and finally the 70 % of acetonitrile was maintained up to 55 minutes isocratically. In every injection the delay time of 10 minutes was maintained at the initial gradient condition. The overall analysis was performed at ambient temperature and the injection volume 20µL.

2.4. Preparation of resolution solution and evaluation of system suitability

The accurately weighed quantity (50 mg) of Aztreonam working standard was transferred to 50 ml volumetric flask and dissolved in phosphate buffer solution (pH 3.2), diluted to the final concentration of 1.0 mg ml^{-1}. The solution was heated at 90^0C for about 30 minutes in an oven and then cooled. The cooled solution was taken for analysis, 20µL was injected to check the system is suitable for analysis; the resolution between the peaks corresponding to degraded impurity and Aztreonam was not less than 3.0. The tailing factor for Aztreonam peak was not more than 2.0. (Figure 2).

2.5. Preparation of standard, Sample solutions and quantitative determination for related substances

Around 25 mg of Aztreonam working standard was weighed and dissolved in phosphate buffer solution in 25 ml volumetric flask, diluted to the final concentration of 0.01 mg ml^{-1}, and 20µL was injected.

The Aztreonam sample to be examined was weighed about 25 mg, diluted with the same phosphate buffer solution to the final concentration of 1.0 mg ml^{-1}, and 20µL was injected. The following formula was used to calculate the content of related substance.

% of each related substance = $A_T \times DS \times P/A_S \times DT$

Where A_T and A_S represent the individual impurity peak area of sample and the peak area of standard respectively, DT and DS represent the dilution factor of sample solution and standard solution respectively and the P represents the purity (%w/w) of Aztreonam working standard.

2.6. Stability of Aztreonam under stressed conditions

Stability of the solid state of both Aztreonam powdered and compacted forms were demonstrated by storing for 5 weeks at 90^0C in a Petri dish. Aztreonam drug substance was separately treated with 5N hydrochloric acid, 0.2N sodium hydroxide and 30 % w/w solution of hydrogen peroxide. The sample was irradiated at UV light for 159 Hrs at 254nm; also the sample was subjected to humidity degradation by keeping at 25^0C and 97% relative humidity for about 185 Hrs.

3. RESULTS AND DISCUSSION

3.1. Optimization of HPLC conditions

In order to obtain a precise and rugged method, several trials with low pH, higher pH and the different buffers like acetate and citrate were tried in the mobile phase. Finally the complete resolution among all the related substances of Aztreonam and the degraded products was achieved by using phosphate buffer and acetonitrile mixture with ODS column in a selective gradient condition. Instead of acetonitrile, methanol was tried in the gradient elution; some of the related substances impurity-G and impurity-F were highly non-polar

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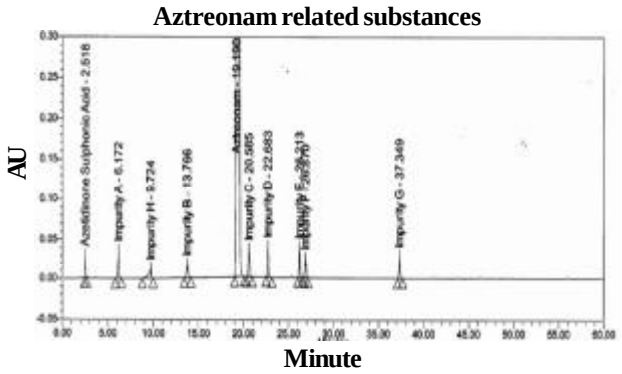

Figure 4: Chromatogram of Aztreonam sample spiked with its impurities in 1% level

TABLE 1: The relative retention time (RRT) of known peaks with respect to Aztreonam

S.no	Name	RRT
1	Azetidinone sulfonic acid*	0.131
2	Impurity A	0.322
3	Impurity B*	0.717
4	Impurity C	1.073
5	Impurity D	1.182
6	Impurity E*	1.366
7	Impurity F	1.400
8	ImpurityG	1.946
9	Impurity H*	0.507

***Which are not present in the final substance**

TABLE 2 : Degradation of Aztreonam

Mode of degradation	Conditions	% of degradation
Acid	5N HCl (1.0Hrs/RT)	6
Alkali	0.2N NaOH (1.0Hr/RT)	6
Oxidative	H_2O_2 30%w/w (Initial /RT)	3
Thermal	120°C (23Hrs)	4
Photolytic	UV irradiation (159Hrs)	7
Humidity	25°C/97%RH (185Hrs)	0

TABLE 3 : Linearity

Name	Conc. (µg ml-1) Lowest-Highest	Slope	Intercept	Correlation Coefficient
Azetidinone sulfonic acid	0.123-12.031	17277	-72	0.99999
Impurity A	0.124-12.112	27302	294	0.99998
Impurity B	0.122-11.972	25589	-310	0.99998
Impurity C	0.126-11.856	29985	21	0.99999
Impurity D	0.124-12.054	26414	26	0.99999
Impurity E	0.123-12.121	21588	-100	0.99999
Impurity F	0.126-12.067	21693	-384	0.99994
Impurity G	0.125-12.010	21858	481	0.99999
Impurity H	0.125-12.010	25282	-1279	0.99997

when compared to Aztreonam were not eluted out. Since the azetidinone sulfonic acid was detected at 210nm, the wavelength program was performed up to 5 minutes and then turned at 254 nm till the end of the run time to detect the Aztreonam and other related substances. (Figures 3 and 4).

3.2. Validation of determination of related substance

After optimization of analytical conditions, the evaluation of parameters such as specificity, linearity, LOD, LOQ, precision, accuracy, ruggedness and robustness were completed for the validation of the method.

Specificity

In order to show this method is highly specific, each related substances and the intermediates of Aztreonam were injected individually in the concentration about 0.5 mg ml^{-1}. Further to confirm the specificity, the known related substances and the intermediates of Aztreonam were spiked with the sample in 1% level to the Aztreonam concentration 1.0 mg ml^{-1}. It was observed that the related substances are well separated from each other and also from the Aztreonam peak (Figure 2). The relative retention time (RRT) of all well resolved peaks were tabulated in TABLE 1.

This method is not only specific in the normal analysis, but also in the analysis of Aztreonam samples, which endure in stressed conditions. The percentages of degradation values of Aztreonam were given in TABLE 2.

Linearity

The solutions of Aztreonam and its known related impurities were prepared at low concentrations from 0.12µg ml^{-1} and at higher concentrations 12µg ml^{-1}, and the relationship between peak area (Y) and concentration (X) was observed. An excellent linearity [for Aztreonam Y = 28297 X + 1518 (r = 0.99999)] was obtained within the above concentration range for all related substances. Microsoft Excel software used to plot the peak areas versus micrograms injected. (TABLE 3).

Limit of quantitation and detection

The limit of quantitation (LOQ) of known related substances of Aztreonam were determined by using the residual standard deviation [STEYX, that is the standard error of the predicted Y value for each X in the regression. The standard error is a measure of the amount of error in the prediction of Y for an individual X] and the slope values from the linearity data of respective related substances using the following formula [LOQ =

TABLE 4: Limit of quantitation

Name	LOD (%w/w)	Area of LOD preparation						%RSD*
		1	2	3	4	5	6	
ASA#	0.019	2837	2849	2840	2898	2895	2853	0.94
Impurity A	0.009	2461	2262	2475	2436	2430	2608	4.54
Impurity B	0.008	1591	1593	1736	1772	1543	1635	5.47
Impurity C	0.006	1833	1865	1753	1815	1807	1965	3.91
Impurity D	0.008	2051	2097	2136	2170	2257	2207	3.48
Impurity E	0.007	1653	1555	1579	1590	1559	1551	2.40
Impurity F	0.013	2758	2822	2677	2671	2856	2736	2.72
Impurity G	0.007	1725	1522	1647	1654	1691	1781	5.27
Impurity H	0.011	2150	2235	2157	2015	2274	2063	4.56

#Azetidinone sulfonic acid, *The acceptance criteria are the %RSD should not more than 10% for LOQ

TABLE 5: Limit of detection

Name	LOD (%w/w)	Area of LOD preparation						%RSD*
		1	2	3	4	5	6	
ASA#	0.006	0979	0826	0851	0748	0940	0699	12.85
Impurity A	0.004	1054	0910	0827	0643	0786	0701	18.05
Impurity B	0.004	0871	0714	0749	0796	0962	0676	13.34
Impurity C	0.003	0826	1027	0852	0757	0733	0879	12.42
Impurity D	0.004	1269	0926	1124	0941	1215	1266	13.89
Impurity E	0.003	0776	0848	0726	0661	0640	0659	11.28
Impurity F	0.007	1316	1157	1205	1128	1257	1669	15.36
Impurity G	0.004	1151	1194	1077	0959	0929	1221	11.21
Impurity H	0.006	1169	0777	0779	0642	1047	0705	24.26

#Azetidinone sulfonic acid, *The acceptance criteria are the %RSD should not more than 33% and not less the 10% for LOD

TABLE 6 : Precision

Name	%w/w of Impurities						%RSD*
	1	2	3	4	5	6	
Impurity A	0.057	0.054	0.057	0.057	0.057	0.057	2.11
Impurity C	0.095	0.095	0.094	0.094	0.094	0.096	0.84
Impurity D	0.037	0.039	0.040	0.045	0.046	0.046	9.52
Impurity F	0.104	0.103	0.103	0.103	0.103	0.104	0.49
Impurity G	0.028	0.028	0.028	0.028	0.028	0.028	0.00
Highest unknown	0.023	0.023	0.023	0.023	0.023	0.024	1.74
Total unknown	0.023	0.023	0.023	0.023	0.023	0.024	1.74
Total related substances	0.344	0.342	0.345	0.350	0.351	0.355	1.44

***The acceptance criteria are the %RSD should not more than 10%**

TABLE 7 : Ruggedness

Analysis	(Total related substances in %w/w)	
	Analysit-1	Analyst-2
1	0.344	0.354
2	0.342	0.348
3	0.345	0.357
4	0.350	0.344
5	0.351	0.350
6	0.355	0.348
%RSD	1.420	1.330
Over all % RSD*	1.361	

*** % RSD of over all values of both analysts.**

(STEYX / slope) ×10]. The each related substance solutions were prepared at about the predicted LOQ concentration level and its precision was verified. (TABLE 4).

Similarly the limits of detection (LOD) of known related substances of Aztreonam were determined by using the following formula [LOD = (STEYX / slope) ×3.3]. The each related substance solutions were prepared at about the predicted LOD concentration level and its precision was verified. (TABLE 5).

Precision or reproducibility and ruggedness

The precision of the method was determined by preparing a sample solution of single batch of Aztreonam drug substance (in the concentration of 1.0 mg ml^{-1}) six times and analyzed as per the proposed method. The related substances of Aztreonam were calculated against the Aztreonam standard. (TABLE 6).

Two different analysts conducted the six replicate determination of Aztreonam drug substance in the same concentration on different days using different instruments in two different columns of same brand. The comparative results are summarized in TABLE 7. There is no significant deviation between the results of two different values, it has clearly indicates that this method is

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TABLE 8 : Accuracy

Name	20%			60%			120%		
	AA*	AR*	Rec*	AA	AR	Rec	AA	AR	Re
ASA#	2.003	1.982	98.95	6.008	5.963	99.25	12.016	12.045	100.24
	2.003	1.977	98.70	6.008	6.023	100.25	12.016	12.067	100.42
	2.003	1.939	96.80	6.008	5.935	98.78	12.016	12.103	100.72
Impurity A	2.219	2.146	96.71	6.658	6.498	97.60	13.315	13.387	100.54
	2.219	2.143	96.58	6.658	6.541	98.24	13.315	13.791	103.57
	2.219	2.140	96.44	6.658	6.499	97.61	13.315	13.833	103.89
Impurity B	2.016	1.856	92.06	6.048	5.715	94.49	12.095	11.853	98.00
	2.016	1.861	92.31	6.048	5.719	94.56	12.095	11.832	97.83
	2.016	1.837	91.12	6.048	5.693	94.13	12.095	11.825	97.77
Impurity C	2.012	1.995	99.16	6.036	5.889	97.56	12.071	12.202	101.09
	2.012	2.010	99.90	6.036	5.979	99.06	12.071	11.973	99.19
	2.012	1.991	98.96	6.036	5.946	98.51	12.071	12.130	100.49
Impurity D	2.407	2.322	96.47	7.220	7.411	102.65	14.441	15.191	105.19
	2.407	2.384	99.04	7.220	7.560	104.71	14.441	15.168	105.03
	2.407	2.370	98.46	7.220	7.443	103.09	14.441	15.308	106.00
Impurity E	2.020	2.058	101.88	6.059	6.197	102.28	12.119	12.506	103.19
	2.020	2.047	101.34	6.059	6.247	103.10	12.119	12.506	102.95
	2.020	2.043	101.14	6.059	6.202	102.36	12.119	12.506	103.31
Impurity F	2.000	1.880	94.00	5.999	5.528	92.15	11.998	11.229	93.59
	2.000	1.859	92.95	5.999	5.552	92.55	11.998	11.231	93.61
	2.000	1.850	92.50	5.999	5.566	92.78	11.98	11.213	93.46
Impurity G	2.003	1.946	97.15	6.009	5.814	96.75	12.018	11.882	98.87
	2.003	1.959	97.80	6.009	5.904	98.25	12.018	11.858	98.67
	2.003	1.927	96.21	6.009	5.846	97.29	12.018	11.850	98.52
Impurity H	2.003	1.884	94.06	6.008	5.553	92.43	12.017	11.664	97.06
	2.003	1.879	93.81	6.008	5.579	92.86	12.017	11.655	96.99
	2.003	1.894	94.56	6.008	5.512	91.74	12.017	11.689	97.27

The % RSD of recovery of all substances in three levels are <3 % ; #Azetidinone sulfonic acid, *AA-Amount added in mg, AR-Amount recovered in mg and Rec- recovery in percentage

precise and rugged.

Accuracy

Method accuracy was demonstrated by spiking a known amount of related substances of Aztreonam in the sample preparation (1.0 mg ml^{-1}) in three different levels, like 20%, 60% and 120% in the concentration of 2.0μg ml^{-1}, 6.0μg ml^{-1} and 12.0μg ml^{-1} respectively in triplicate. There is no significant change in the values between the amount added and the amount recovered after the corrections of the known sample, which is already present. The percentage recoveries of all substances were in between 90 to 106. (The acceptance criteria is *80 % to 120 %*) The % RSD of recovery of three levels were < 3.0. (TABLE 8).

Stability of analytical solution

The solution (1.0 mg ml^{-1}) of Aztreonam with the known impurities (spiked in 1% level) was studied at room temperature at different time intervals. The cumulative %RSD of each related substances were calculated and concluded that the Aztreonam and its related substances were stable for about 10 hrs at room temperature (≅ 25°C).

Robustness

The chromatographic conditions were deliberately changed to demonstrate the robustness. The flow rate (± 10 %), detection wavelength (± 5 nm), the composition of acetonitrile (± 2 % absolute) and the column oven temperature (at 35^0C) were changed to check the difference in the resolution between the all related substances of Aztreonam. There is no noteworthy variation in results were clearly indicates that this method is robust.

System suitability

The system suitability testing, which is part of an integral part of chromatographic methods, and used to verify that the resolution and reproducibility of the sys-

tem are adequate for the analysis to be performed.

4. CONCLUTIONS

According to complete validation studies, the Aztreonam peak of both powdered and compacted were free of interference from the related substances and its degradation products, point out that the proposed RP-HPLC method is simple, precise, accurate, rugged and robust in all situation.

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