Stability Indicating RP-HPLC Method for Azilsartan Related Substances in Solid Dosage Forms

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Abstract:-

Objective: **The main objective of the research work was to develop a simple, accurate, stability indicating RP-HPLC method for the quantification of Azilsartan and its related substances.**

Method: **The method was developed by Hitachi Lachrome HPLC with the Develosil ODS HG-5 RP C18, (5µm,15 cm x4.6mm) , it has a mobile phase of buffer, methanol and acetonitrile(ACN) in the ratio of 60:30:10v/v/v was used. The flow rate was set at 1.0 ml/ min. ml/min with a detection wavelength of 243nm using VWD detector. The method was validated for analytical parameters such as specificity, accuracy, precision, robustness and ruggedness as per ICH guidelines.**

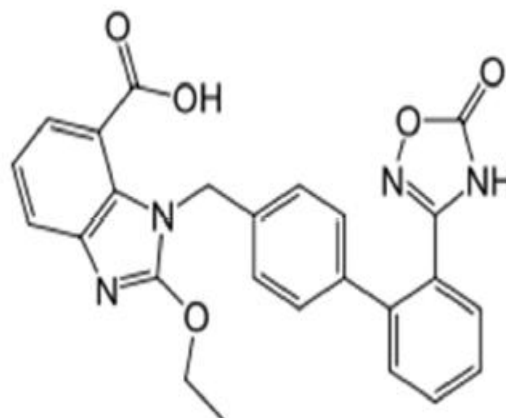
Results: **Under the specificity experiment, samples were stressed under various stress conditions and analyzed along with unstressed samples. AZM was found to be very stable under all degradation conditions. The developed method can be used for routine analysis because the linearity found in AZM, Impurity A, Impurity B, Impurity C and Impurity D was nearing 1 that is 0.999, 0.998, 0.997, 0.999 and 0.998 respectively which shows the good regression for linearity. The results from solution stability experiments confirmed that standard and sample solutions were stable up to 24 h for both assay and related substances analysis. Maximum recovery is obtained by this developed method and the mean percentage recovery for each component was nearing 100%.**

Conclusion: **Statistical validation of the data shows that the proposed method can be successfully applied for the routine analysis of the AZM and related substances. The satisfying % recoveries and low % RSD values were confirmed the suitability of the developed method for the usual analysis of AZM and related substances in pharmaceuticals.**

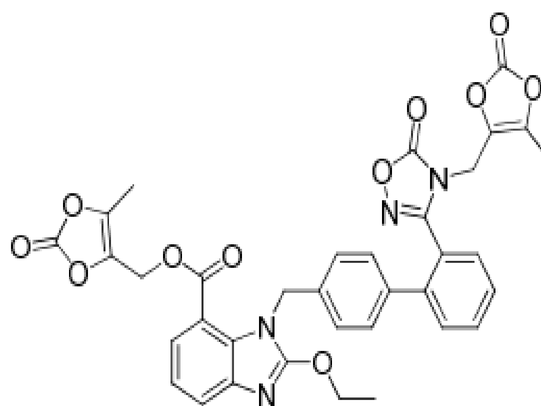
Keywords: **Azilsartan, HPLC, Stability indicating, method development, validation**

I. INTRODUCTION

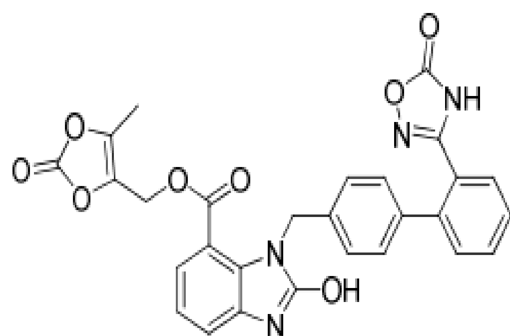
Azilsartan medoxomil (AZM) is chemically known as 5-methyl-2-oxo-1,3-dioxol-4-yl)methyl2-ethoxy-1-([2'-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]methyl)-1Hbenzimidazole-7-carboxylate. The chemical formula ofAzilsartan medoxomil is $C_{25}H_{20}N_4O_5$with molecular weight of 456.46 g/Mol. Azilsartan medoxomil is white powder which is practically insoluble in water and freely soluble in methanol The development and validation of an analytical method is to ensure a specific, accurate and precise method for a particular analyte. The principal objective for that is to enhance the conditions and parameters, which should be observed in the evolution and establishment. Literature review reveals that a few analytical methods [3-11] were developed for the determination of AZM in combinations of other drugs in bulk and capsules. So far there is no method for the determination of AZM and its impurities using HPLC. Hence the author developed a new simple, accurate and stability indicating HPLC method for the determination of AZM drug along with its impurities. The method developed was validated as per ICH guidelines. The structure of AZM and its impurities are shown in the fig.1



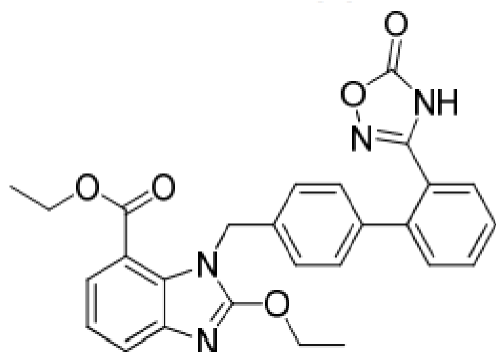
Azilsartan medoxomil



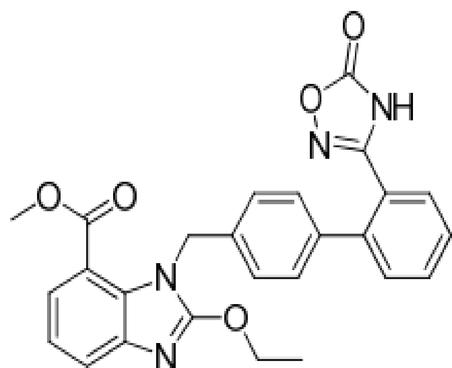
Impurity A



Impurity B



Impurity C



Impurity D

Figure 1: Structure of AZM and its impurities

II. EXPERIMENTAL

Materials and reagents

The reference sample of AZM and its impurities A,B,C&D were received as a gift sample from Veeprho labs pvt.Ltd, Talegaon DabhadeDist, Pune.Milli-Q-water was used throughout this research. All other analytical reagents such as Potassium phosphate, Acetonitrile, Methanol, Phosphoric acid, Hydrochloric acid, Sodium hydroxide and Hydrogen peroxide (30%) were obtained from S.D Fine Chemicals, Mumbai, India.

Instrumentation

This work has been performed on Hitachi Lachrome (HPLC) instrument. It has binary gradient pump (Smash HTA Pump), L6530 diode array detector (DAD), AS2000 auto sampler and L2300 column compartment. Chromatogram was analysed using PEAK chromatographic chemistration version B.02.01.

Preparation of solutions

Standard solution of AZM

10mg of AZM was weighed accurately and transferred into 100 ml volumetric flask. About 10 ml of HPLC grade methanol was added and sonicated to dissolve. The volume was made up to the mark with same solvent. The final solution contained about 40 µg/ml of Azilisartan.

Impurity stock solution:

20mg, 2.0mg, 2.2mg and 2.2mg of Impurity-A, Impurity-B, Impurity-C and Impurity-D were accurately weighed individually and transferred into 100ml, 10ml, 10ml and 10ml volumetric flask respectively. Then the volume was made up to the mark using diluent individually. From each impurity solutions 0.75ml was pipetted out and transferred into 50ml volumetric flask individually. Then the volume was made upto the mark using diluent.

Buffer solution

2.7gm of mono basic potassium phosphate was dissolved in 1000mL of HPLC grade water. pH was adjusted to 3.0 with 10% phosphoric acid.

Mobile phase

Mixture of Buffer, methanol and acetonitrile(ACN) in the ratio of 60:30:10v/v/v was used. Mobile phase was filtered through 0.45µM membrane filter.

Diluent

Diluent buffer was prepared by adding 2ml of TEA and 2ml of phosphoric acid in 1000ml of HPLC water. Mixture of Diluent buffer and acetonitrile in the ratio of 45:55v/v was used as a diluent.

System suitability solution

Accurately weighed amount of about 25mg of AZM working standard or reference standard was transferred into a 50ml volumetric flask. 10ml of diluent was added and sonicated to dissolve. 1ml of above impurity stock solution was added and then diluted to volume with diluent.

Procedure

20µL of diluted standard and sample of AZM and individual impurity solutions were injected individually with five replicate injection. Chromatogram was recorded individually and peak responses were measured and reported in the figure 2

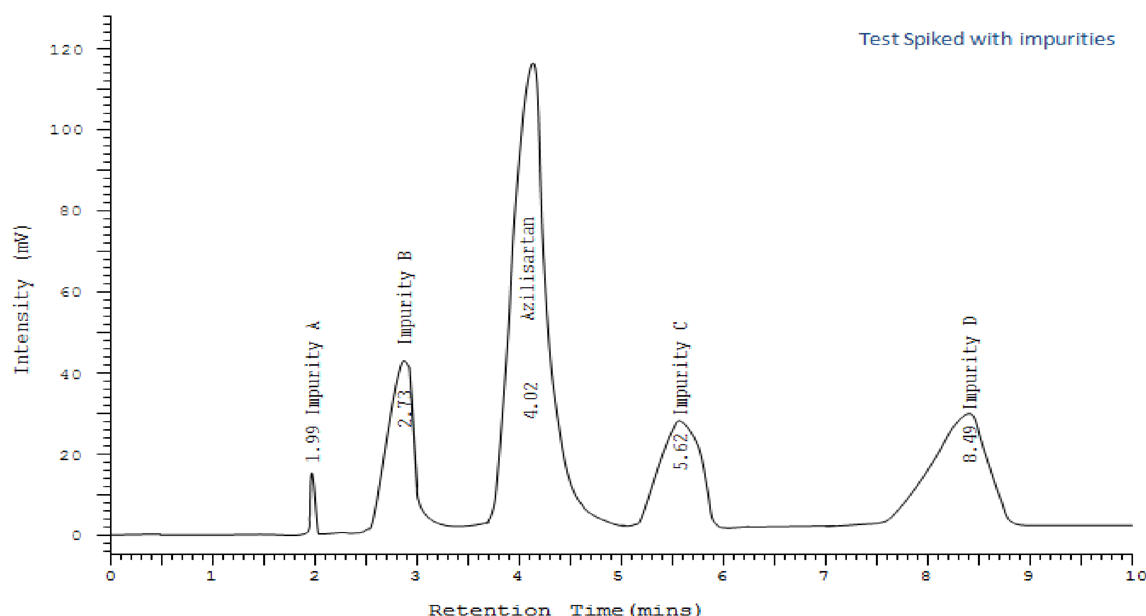


Figure 2: Chromatogram of Azilsartan and its impurities

III. METHOD VALIDATION

The proposed method was validated for the analysis of AZM using following parameters. System-suitability studies are an intact part of method development and are practiced to ensure satisfactory performance of the chromatographic system. For five replicate injections of the drugs Number of theoretical plates (N) and tailing factor (T) were assessed. Linearity was established by plotting a graph between concentration versus peak area and the correlation coefficient was determined. To obtain proportionality, the slope and intercept of the regression line and correlation coefficient were calculated statistically from the calibration curve of the AZM. To find out variations in the test methods precision was studied for AZM of spiked test preparation with AZM blend solution to get 0.5% of each impurity with respect to test concentration and analyzed as per test method when analysis carried out by Analyst to Analyst, System to System and Column to Column Variation (ruggedness). The mentioned solution was injected six times and the area was measured for all six injections in HPLC. The % relative standard deviation (%RSD) and % content results were used for assessment of precision and ruggedness. The accuracy of the method was demonstrated by analyzing AZM of spiked test preparation with LOQ, 100% and 200% of target concentration. After injection, recovery values for individual drugs were estimated. Specificity is the ability of a method to differentiate the analyte(s) of interest from other components in the sample. Placebo was prepared as per the marketed product formulas of drugs. Placebo interference from excipients was studied. Robustness of the method was determined by varying flow rate, and filtration. Bench top stability (25^0C & 60 % RH) and Refrigerator (8^0C & 55%RH) stability were determined on the 1st and 2nd day. Forced degradation study was conducted to demonstrate the effective separation of degradants from AZM. AZM was exposed to the following stress conditions such as refluxed with 3N HCl solution for about 24 hours at 60ºC (Acid). Refluxed with 3N NaOH solution for about 24 hours at 60ºC (Base).Treated with 10% Hydrogen peroxide (H_2O_2) for 24 hours at 6oºC (Peroxide).Dry heat at 105° C for about 24 hrs in an oven.

IV. RESULTS & DISCUSSION

An Isocratic reverse – phase HPLC procedure was suggested as a suitable method for the analysis of AZM related substances. System suitability parameters like theoretical plate, % relative standard deviation and tailing factor for AZM, Impurity A, Impurity B, Impurity C and Impurity D were reported.

System suitability studies

The system suitability was evaluated by injecting a known Volume of sample containing a known amount of AZM into the chromatograph and calculated the resolution between AZM and its impurities, the number of theoretical plates, relative standard deviation (RSD) for six injections. The resolution was found to be 2.87; the number of theoretical plates ,\was calculated 30794. The relative standard deviation was calculated as 0.32 % and the asymmetry ofAZM peak was found to be 0.96 which showed that the selected column is suitable for the analysis.

Standard solutions were prepared as per test procedure and injected into the HPLC system as per test method. Evaluated system suitability parameters are Summarized in the table 1

Table 1: System suitability results

System suitability parameters		**Observed value**	**Acceptance criteria**
Theoretical Plates	AZM	4693	Should be NLT 2000
	Impurity-A	3642	
	Impurity B	3216	
	Impurity C	3935	
	Impurity D	4120	
%RSD	AZM	0.13	Should be NMT 5.0
	Impurity-A	0.12	
	Impurity B	0.14	
	Impurity C	0.72	
	Impurity D	0.66	
Tailing factor	AZM	1.0	Should be NMT 2.0
	Impurity-A	1.0	
	Impurity B	1.0	
	Impurity C	1.0	
	Impurity D	1.0	

System and method Precision

The system precision of test method was evaluated by analyzing six test preparations by spiking test preparation with AZM and its related substances blend solution to get 0.2% of each impurity with respect to test concentration and analyzed as per test method.

Results of system precision were reported in the table 2. Percentage relative standard deviation of system precision reports was with in 2. From the results, the method has a good system precision.

Table 2: System precision results

Injection N°	Response				
	AZM	Impurity-A	Impurity-B	Impurity-C	Impurity-D
01	795969	151967	402869	183572	258600
02	709036	147209	402586	185214	261077
03	800545	163802	318251	190228	260998
04	854769	149188	302569	185261	260994
05	764305	149731	302896	196512	255872
06	879951	143027	318519	185254	256435
Mean	800762.5	150820.6667	341281.7	187673.5	258996
Standard deviation	61568.5726	7035.160913	48108.01	4882.420127	2400.228
% Relative standard deviation	1.76	1.93	0.54	0.13	0.92

Method Precision:

Method precision results were given in percentage content.

The individual results of Aziliartan and its impurities were reported in the table 3.

Table 3: Method precision data for Azilisartan and its impurities:

Injection	**AZM**	**Impurity-A**	**Impurity-B**	**Impurity-C**	**Impurity-D**	**PercentageofImpurityAin spiked sample**	**Percentage of impurityB in spiked sample**	**Percentage of impurity-C present in spiked sample**	**Percentage of impurity-D present in spiked sample**
1	99.94	97.62	98.29	97.39	97.12	0.19	0.18	0.19	0.18
2	100.76	99.26	103.42	99.42	99.93	0.20	0.19	0.21	0.21
3	98.32	99.31	97.5	100.36	97.07	0.18	0.19	0.21	0.19

4	102.61	100.39	98.71	96.61	98.73	0.23	0.21	0.22	0.21
5	101.37	96.41	98.92	101.37	100.71	0.21	0.20	0.18	0.19
6	97.61	98.79	98.61	102.99	99.62	0.18	0.19	0.19	0.18
Mean	98.72	102.39	100.07	99.87	98.53	0.20	0.21	0.19	0.20
SD	99.904	99.16	99.36	99.71	98.81	0.2	0.2	0.2	0.2
RSD	1.79	1.92	1.94	2.19	1.38	0.017	0.0107	0.0124	0.013

Accuracy (%Recovery)

A study of accuracy of AZM impurities from spiked samples of test preparation was conducted. Samples were prepared in triplicate at each level by spiking test preparation with LOQ, 50%, 80%, 100%, 150% and 200% of target concentration (i.e., 0.5% of each impurity) of AZM impurities.The mean % recovery of AZM impurities at mentioned concentration level were reported in the table 4. The AZM and its known impurities recovery is should be within the acceptance limit between 85.0% to 115.0%.

Table 4: Recovery data for AZM impurities.

S.No	Sample Name	Mean % Recovery			
		Impurity-A	Impurity-B	Impurity-C	Impurity-D
1	Unspiked	-	-	-	-
2	100% spiked sample-1	98.6	99.7	98.7	98.6
3	100% spiked sample-2	99.3	98.64	101.4	97.5
4	200% spiked sample-1	97.4	99.8	99.7	99.8
5	200% spiked sample-2	102.8	98.3	103.1	97.4

Linearity

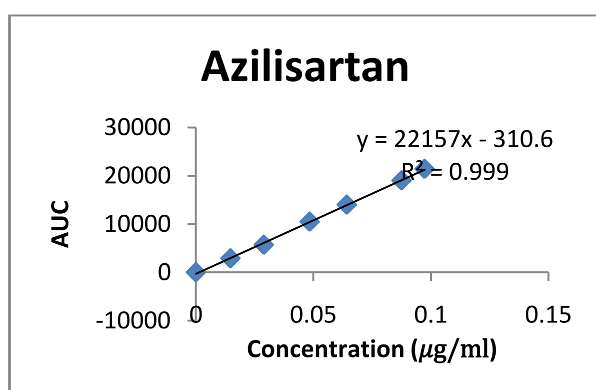
Linearity was established by plotting a graph between concentration versus peak area and the correlation coefficient was determined. A series of solutions of AZM related substances with concentrations ranging from LOQ% to 120% of specification limit prepared and injected into the HPLC system. Different concentration of AZM and impurities were analysed. Correlation coefficient of drugs and its impurities were above 0.99. The Linearity results were summarized in the table-5 and table-6. The linearity graphs were shown in figure-3.

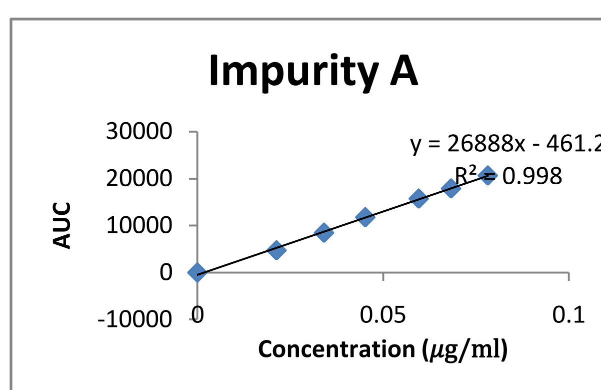
Table-5 .Linearity data for AZM, Impurity A and B.

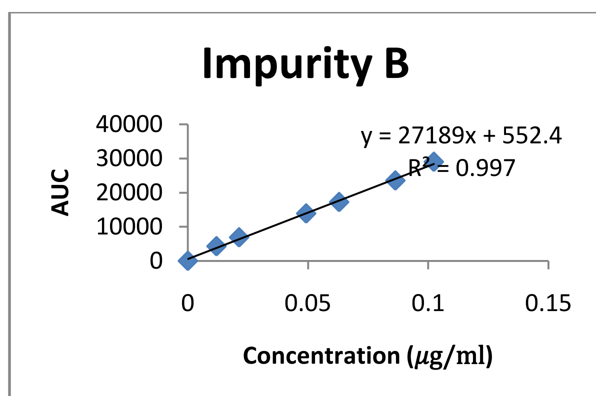
AZM		Impurity A		Impurity B	
Mean Conc. (µg/mL)	Mean± SD	Mean Conc. (µg/mL)	Mean± SD	Mean Conc. (µg/mL)	Mean± SD
0	0	0	0	0	0
0.0147	2876±12	0.0213	4716± 36	0.0119	4274± 56
0.0289	5683± 24	0.0341	8472± 42	0.0213	6876± 39
0.0484	10458± 56	0.0452	11769±69	0.0492	13854± 43
0.0642	13983± 78	0.0596	15734± 72	0.0629	17196± 78
0.0875	18993± 84	0.0683	17895± 84	0.0863	23491± 85
Slope	22157± 95		26888 ± 70		27189± 64
Intercept	- 310.6		- 461.2		+ 552.4
Correlation coefficient	0.999		0.998		0.997

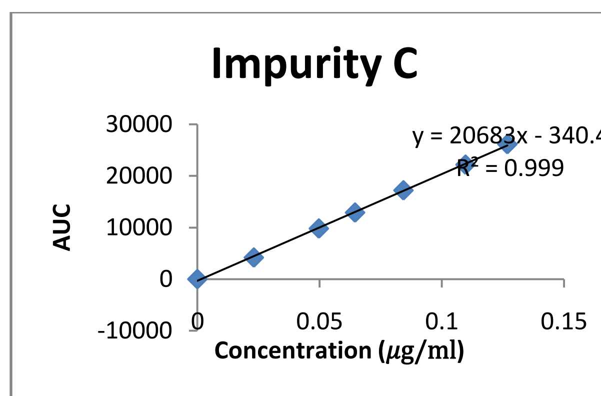
Table-6 .Linearity data for Impurity C and Impurity D.

Impurity C		Impurity D	
Mean Concentration (µg/mL)	**Mean± Standard deviation**	**Mean Concentration (µg/mL)**	**Mean± Standard deviation**
0	0	0	0
0.0231	4179± 24	0.0113	3349
0.0497	9801± 41	0.0321	7642
0.0645	12891± 27	0.0546	12985
0.0843	17196± 47	0.0764	17184
0.1097	22167± 53	0.0952	21094
Slope	20683		21965
Intercept	- 340.4		+ 518.2
Correlation coefficient	0.999		0.998









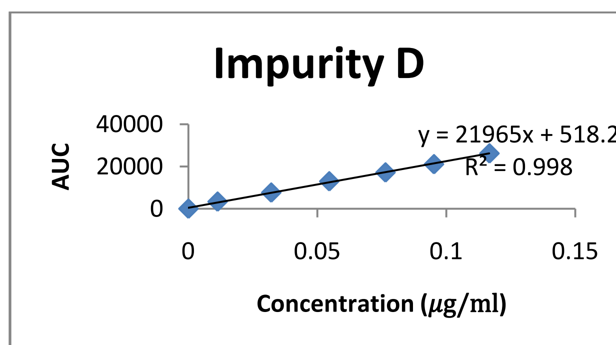


Figure: Linearity curves for Azilisartan

Limit of quantitation

Limit of quantitation was established by identifying the concentration which gives signal to noise ratio about 10.Results of LOQ were reported in the table-7. The LOQ value for the impurities was below reporting threshold (0.05%). The test concentration was optimized as 500ppm.

Table-7 : LOQ results

Impurity name	% LOQ
AZM	1.2
Impurity-A	0.0137
Impurity-B	0.0121
Impurity-C	0.0101
Impurity-D	0.0098

V. FORCED DEGRADATION STUDIES

The study was performed by subjecting the drug substance to acidic, alkaline, oxidizing, thermal and photolytic conditions. Purity factor AZM by forced degradation studies was mentioned in table-8 and Purity factor of AZM was found within the threshold level in all forced degradation studies. Main peak was separated from known impurity and unknown impurities in forced degradation. Mass balance values were within the acceptance limit. (NLT 95.0). The peak purity of AZM was passed in all degradation samples.

Table -8. Forced degradation studies

S.No.	AZM	% of Degradation	% of Assay	Mass balance
1	Unstressed sample	0.0541	98.97	98.16
2	Acid stressed	0.0769	99.92	99.64
3	Base stressed	0.0986	98.93	101.54
4	Thermal Stressed	0.0317	100.36	102.39
5	H_2O_2 stressed	0.0783	100.42	99.78
6	Humidity stressed	0.1328	98.76	98.63
7	UV stressed	0.0327	98.69	99.81
8	Under sunlight	0.0673	100.05	101.42
9	By Hydrolysis	0.0767	97.04	97.23

VI. CONCLUSION

Validation was performed on the developed analytical method for its acceptable performance to ensure suitability of indent purpose. The validation parameters like accuracy, precision, specificity, detection limit, quantitation limit, linearity, range, ruggedness and robustness were executed and established method conditions to meet the requirements to execute the analysis of AZM and its impurities. Under the specificity experiment samples were stressed various stress conditions and analyzed along with unstressed samples. AZM was found to be very stable under all degradation conditions . The developed method can be used for routine analysis because the linearity found in AZM, Impurity A, Impurity B, Impurity C and Impurity D was nearing 1 that is 0.999, 0.998, 0.997, 0.999 and 0.998 respectively which shows the good regression for linearity. The results from solution stability experiments confirmed that standard and sample solutions were stable up to 24 h for both assay and related substances analysis. Maximum recovery is obtained by this developed method and the mean percentage recovery for each component was nearing 100%. Data of repeat experiment were showed <2% RSD (relative standard deviation) for assay and <2% RSD for impurities. In all the deliberate varied chromatographic conditions like flow rate (±0.2 mL/min), column temperature (±5°C), composition of organic solvent (±10% of method organic solvent) and pH of mobile-phase buffer (±0.2), all analyte and impurities were adequately resolved and elution orders remained unchanged. The resolution between all pair compounds was >2.0. These results are conforming good precision of the method. Therefore this method can be used for the routine analysis and one most important reason is that the developed method does not involve the use of expensive reagents. Also, our proposed method requires less time for the determination of AZM and its known impurities simultaneously when compared to other methods. The developed method is uncomplicated, accurate, sensitive and precise for the determination of related substances in the AZM. The satisfying % recoveries and low % RSD Values were confirmed the suitability of the developed method for the usual analysis of AZM and related substances in pharmaceuticals.

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