INTERNATIONAL JOURNAL FOR INNOVATIVE RESEARCH IN MULTIDISCIPLINARY FIELD ISSN: 2455-0620 Volume - 6, Issue - 9, Sept – 2020
Monthly, Peer-Reviewed, Refereed, Indexed Journal with IC Value: 86.87 Impact Factor: 6.719
Received Date: 08/09/2020 Acceptance Date: 28/09/2020 Publication Date: 30/09/2020

Assay Method Development and Validation for Azilsartan using High Performance Liquid Chromatography

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Abstract: *A simple, accurate, precise, stability indicating HPLC method was carried for the determination of Azilsartan along with its impurities. The method was developed by Hitachi Lachrome HPLC with the Develosil ODS HG-5 RP C18, (5µm,15 cm x4.6mm) with 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 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.*

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

1. 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 of Azilsartan 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. Azilsartan is an angiotension receptor blocker that lowers blood pressure by blocking the angiotensin II a vasopressin hormone. 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[1-2]

2. Materials and Methods

Chemicals 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 Dabhade Dist, 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. The structure of AZM and its impurities are shown in the fig.1

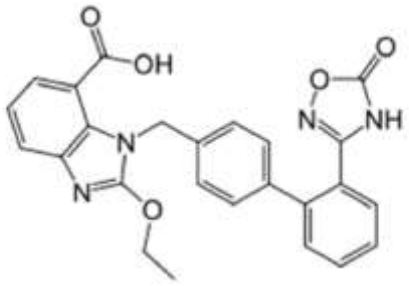
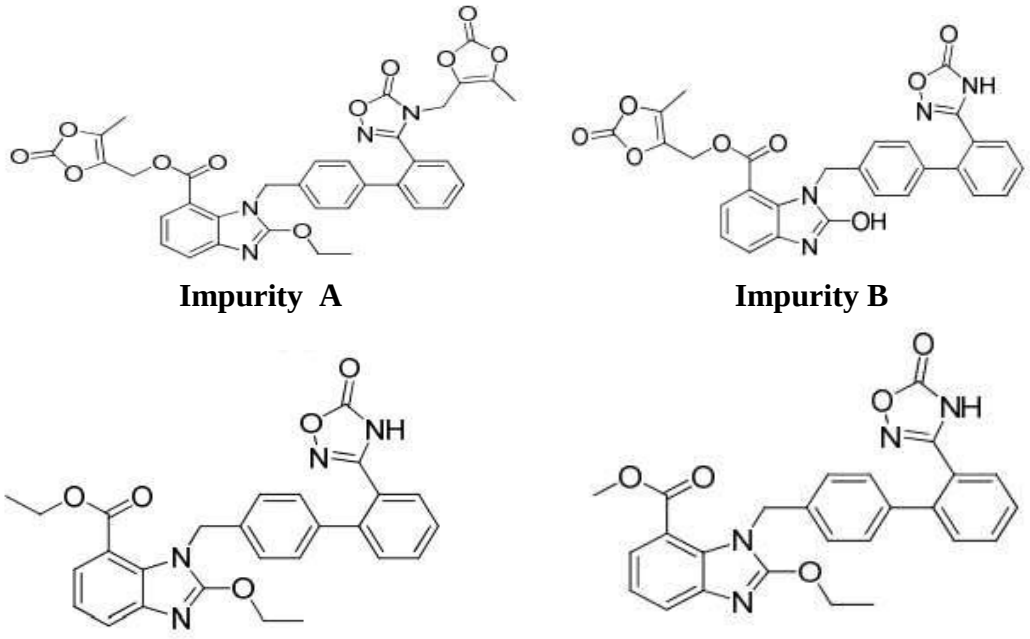


Fig.1AZM



Impurity A **Impurity B**

Impurity C **Impurity D**

Figure 2: Structure of AZM and its impurities

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.

3. Method Development:

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 as mobile phase which was filtered through 0.45µM membrane filter.

Diluent

Diluent buffer was prepared by adding 2ml of triethylamine 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 3.

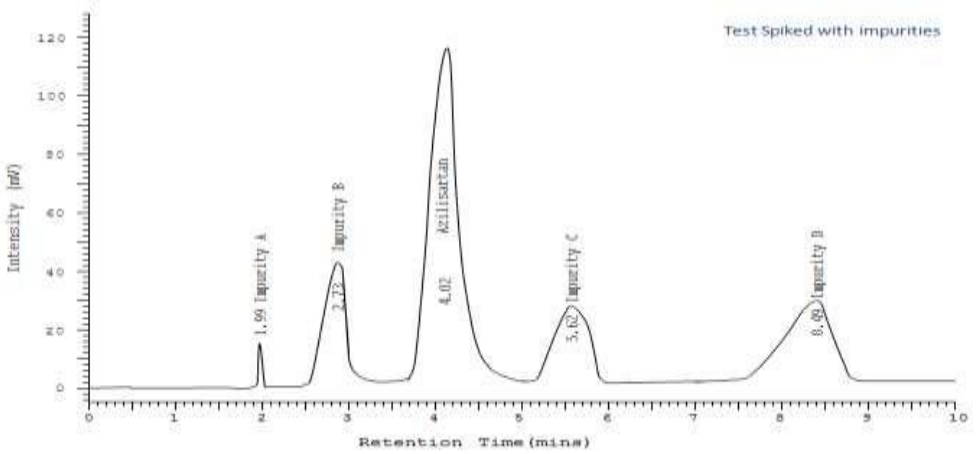


Figure 3: Blend chromatogram of Optimized method

4.RESULTS AND DISCUSSION:

METHOD VALIDATION

The newly developed method was extensively validated for typical validation parameters e.g specificity, Limit of detection, Limit of quantification, precision linearity, accuracy, ruggedness, robustness and solution stability as per the ICH guidelines.(ICH Q2B: Validation of Analytical Procedure.2003)

SYSTEM SUITABILITY STUDIES

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	0.132778	Should be NMT 5.0
	Impurity-A	0.121334	
	Impurity B	0.142576	
	Impurity C	0.719693	
	Impurity D	0.659393	
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	

Limit of quantitation and limit of detection

Limit of quantitation was established by identifying the concentration which gives signal to noise ratio about 10. The LOQ values for the impurities was below reporting threshold (0.05%). The test concentration was optimized as 500ppm. Results of LOQ were reported in the table 2.

Table 2: LOD & LOQ Values

Name	Concentration		Signal to Noise Ratio	
	LOD	LOQ	LOD	LOQ
AZM	0.0002	0.0004	3.1	9.8
Impurity-A	0.0001	0.0007	3.6	10.1
Impurity-B	0.0003	0.0005	3.5	9.3
Impurity-C	0.0002	0.0006	3.2	9.4
Impurity-D	0.0003	0.0002	3.4	9.7

Accuracy (% Recovery)

A study of accuracy of AZM impurities from spiked samples of test preparation was conducted.. The AZM and its known impurities recovery is should be within the acceptance limit between 85.0% to 115.0%. The mean % recovery of AZM impurities at mentioned concentration level were reported in the table 3

Table 3: 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.

System precision

Percentage relative standard deviations of system precision reports were with in 2. From the results, the method has a good system precision

Ruggedness (Intermediate Precision):

Intermediate precision (Ruggedness) was performed by analyzing six test preparations of same precision sample with different analyst, different system and varying column. Intermediate precision was performed by changing analyst to analyst. Different analyst used different system and different column. Results of intermediate precision were expressed as theoretical plate, %RSD and tailing factor which were within the criteria. Results of ruggedness were mentioned in table 4 and 5

Table 4: Intermediate precision for Azilisartan and its impurities

	Azilisartan		Percentage of impurity A present in Azilisartan		Percentage of impurity B present in Azilisartan	
No of Injections	Analyst 1	Analyst 2	Analyst 1	Analyst 2	Analyst 1	Analyst 2
1	10.113	10.006	0.198	0.186	0.186	0.189
2	10.124	10.198	0.203	0.197	0.196	0.191
3	10.119	10.023	0.187	0.184	0.193	0.188
4	10.006	10.212	0.235	0.209	0.211	0.207
5	9.7683	9.8453	0.219	0.219	0.206	0.201
6	9.3247	9.9321	0.189	0.191	0.193	0.198
Mean	9.909	10.0360	0.2051	0.1976	0.1975	0.1956
Standard deviation	0.3170	0.1452	0.018595	0.013794	0.00926	0.00758
% RSD	0.00031	0.00014	0.000906	0.000698	0.00046	0.000387
Mean difference	0.0001		0.0051		0.0001	

Table 5: Intermediate precision for Azilisartan and its impurities

	Percentage of impurity C present in Azilisartan		Percentage of impurity D present in Azilisartan	
No of Injections	Analyst 1	Analyst 2	Analyst 1	Analyst 2
1	0.191	0.197	0.186	0.191
2	0.212	0.219	0.215	0.208
3	0.212	0.209	0.199	0.210
4	0.221	0.218	0.214	0.211
5	0.189	0.198	0.192	0.189
6	0.197	0.201	0.183	0.191
Mean	0.203667	0.207	0.198167	0.2
Standard deviation	0.01311	0.009859	0.01379	0.010658
% RSD	0.000644	0.000476	0.000696	0.000533
Mean difference	0.0002		0.0001	

Stability studies:

Top Stability of 100% spiked sample in Accuracy (Recovery) solutions

Similarity factor at 24 hrs were found to be 1.00, 1.00, 1.02 and 1.01 for impurity A, impurity B, impurity C and impurity D respectively.

Refrigerator Stability of 100% spiked sample in Accuracy (Recovery) solutions

Similarity factor at 24 hrs were found to be 1.02, 1.01, 1.00 and 1.01 for impurity A, impurity B, impurity C and impurity D respectively. Percentage concentration of mentioned impurities resultswere reported in the table 6. The test solution was stable on bench top stability and refrigerator stability up to 24 hours.

Table 6: Stability of 100% spiked sample preparation at ambient temperature about (25ºC) and refrigerator temperature about (8ºC)

Sample Name (% concentration)	Initial		B. top Day-1		Difference		Refrigerator Day-1		Difference	
	test_1	test_2	test_1	test_2	test_1	test_2	test_1	test_2	test_1	test_2
Impurity C	0.2619	0.2714	0.2761	0.2879	0.00	0.01	0.2421	0.2576	0.00	0.01
Impurity D	0.2353	0.2427	0.2815	0.2931	0.00	0.01	0.2513	0.2546	0.00	0.01
Impurity A	0.2719	0.2781	0.3642	0.3520	0.00	0.01	0.3437	0.3569	0.01	0.01
Impurity B	0.2619	0.2517	0.3216	0.3126	0.01	0.01	0.3185	0.3290	0.00	0.01
Total impurity	1.2312	1.2317	1.1976	1.1865	0.00	0.01	1.1997	1.9870	0.01	0.02

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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. The forced degradation studies were reported in table 7

Table 7. 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

5.CONCLUSION: A Simple and sensitive HPLC method for the determination of AZM and its impurities has been successfully developed and validated. The proposed method is simple, accurate, precise and highly sensitive. Hence this method can be used for routine analysis in pharmacy.

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