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"ADVANCEMENTS IN AZILSARTAN MEDOXOMIL ANALYSIS: RP-HPLC METHOD DEVELOPMENT AND VALIDATION BY USING ICH GUIDELINE"

Dipali N. Bhure[1]*, Sachin N. Naik[2], Kalleshvar P. Jatte[3], Kalpana Gaikwad[4], Shital S. Shelke[5], Ruchika Rajani[6], Smruti Navthare[7]

[1]Department of Pharmaceutical analysis, Mauli College of Pharmacy, Udgir, 413517
[2]Department of Pharmaceutics, Dinesh Bembade College of Pharmacy and research, Mahalangra, Latur, 413513
[3]Department of Pharmaceutical chemistry, Dinesh Bembade College of Pharmacy and Research, Mahalangra, Latur, 413513
[4]Department of Pharmaceutical Quality Assurance, Shramjivi College of Pharmacy, Omerga, 413606
[5]Department of Pharmaceutical Quality Assurance, Shivai Charitable Trust College of Pharmacy, Omerga, 413606
[6]Department of Pharmaceutics, Sri Aurobindo Institute of Pharmacy, Indore, Madhya Pradesh, 453555
[7]Department of Pharmaceutics, Sri Aurobindo Institute of Pharmacy, Indore, Madhya Pradesh, 453555

Corresponding Author: Dipali N. Bhure[1]*,
Department of Pharmaceutical analysis, Mauli College of Pharmacy, Udgir, 413517
Email Id:dipaleebhure3@gmail.com

Abstract:
Hypertension remains a significant global health concern, necessitating the development of effective pharmaceutical interventions. Azilsartan medoxomil, members of the angiotensin receptor blocker have emerged as promising therapeutic agents for the management of hypertension. This research proposal outlines a comprehensive study aimed at developing and validating a novel RP-HPLC method for the quantification of Azilsartan medoxomil in pharmaceutical formulations. The proposed method involves the utilization of a Shimadzu LC 2010 AT system equipped with a C18 Chromasil column (4.6 x 250mm, 5µm). A systematic optimization of chromatographic conditions, including mobile phase composition (Water (0.1% Ortho-phosphoric acid): Acetonitrile 25:75), flow rate (1 ml/min), and detection wavelength (250nm), will be conducted to achieve optimal separation, resolution, and sensitivity. Several mobile phase compositions will be evaluated, with a focus on achieving efficient chromatographic resolution within a reasonable analysis time. The retention time of Azilsartan medoxomil was found to be 5.31 min. The validation of the developed RP-HPLC method will be performed in accordance with International Conference on Harmonisation (ICH) guidelines, encompassing parameters such as specificity, linearity, accuracy, precision, robustness, and system suitability. Calibration curves will be constructed over a suitable concentration range for compounds to assess linearity, with consideration given to the method's sensitivity and dynamic range.

INTRODUCTION

Chromatography Techniques

In chromatographic methods, separation is based on variation in the distribution of different compounds between two dissimilar phases a stationary phase and a mobile phase. Further differentiation can be made between chromatographic procedures in which the individual components are monitored online (HPLC) and procedures in which the components are measured in situ on the chromatographic stationary phase (TLC) [1].

High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is an advanced form of liquid chromatography used in separating the complex mixture of molecules encountered in chemical and biological systems, to understand better the role of individual molecules. In liquid chromatography, a mixture of molecules dissolved in a solution (mobile phase) is separated into its constituent parts by passing through a column of tightly packed solid particles (stationary phase). Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase [2]. A block diagram of the HPLC System is as given below. In HPLC, a pump provides the higher pressure required to move the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. The velocity of the solution depends on the nature of the sample and the compositions of the stationary (column) phase. The time at which a specific sample elutes (comes out of the end of the column) is called the retention time.

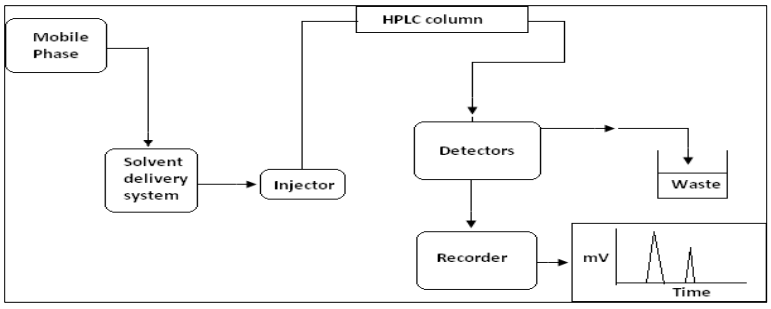


Figure 1: Block diagram of HPLC System

Normal phase HPLC:

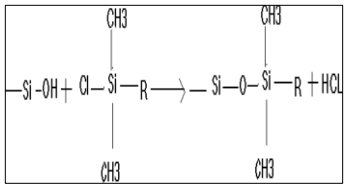
Normal phase chromatography utilizes a polar stationary phase and a non-polar mobile phase. Generally, more polar compounds elute later than non-polar compounds

Reversed-phase HPLC:

About 75% of current HPLC analysis is performed using the reverse phase [3]. In reversed phase chromatography the Stationary phase is mainly silica chemically bonded through a siloxane (Si- o-Si-C) linkage to a low polar function group. These phases are prepared by treating the surface silanol groups of silica with an organochorosilane reagent [4].

The polarity of the column can be changed by varying the alkyl chain length in R.

Figure 2: Structure of organochorosilane



Where R= C6H13 (Hexyl), C8H17 (Octyl) or C18H17 (Octadecyl).

HPLC Detectors

Based on the method or principle used in detection the detectors available are RI detectors, UV detectors, Fluorescent detectors, electrochemical detectors, and Photo diode-array detectors (PDA).For the present study, UV and PDA detectors are mainly used. A refractive index detector is known as a Universal detector, but it is not a very sensitive detector [5,6].

MATERIALS AND METHODS

Materials

Azilsartan Medoxomil was obtained as a gift sample from Tokyo Chemical Industry (TCI), HPLC Grade Water, O-Phosphoric acid, Acetonitrile, Methanol were obtained from Dipa Chemical Industry

Method Development by Uv visible spectrophotometry

Preliminary Studies and Spectral Studies of Azilsartan Medoxomil

FT-IR Studies and UV Spectrometry studies of Azilsartan medoxomil

The FT-IR spectra for Azilsartan medoxomil drug were recorded by using FT–IR (Brukers Alpha) to confirm the identity of the drugs. Solubility of both the drugs was determined by dissolving the drugs in various solvents varying in their polarity.

Identification by IR Spectroscopy

Each 20mg of Azilsartan medoxomil API and KBr was mixed properly then carefully triturated in a mortar pestle. Make thin plate, place in IR chamber and IR Spectrum was scanned.

Method Development by Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

The objective of this experiment was to developed simple, accurate, sensitive, reproducible validated HPLC method for estimation of Azilsartan medoxomilin tablet dosage form by RP-HPLC.

Preparation of Standard Stock Solution:

Accurately weighed equal amounts 40 mg of Azilsartan Medoxomil working standards were transferred into three 100 mL volumetric flasks. 70 mL of diluents is added to each flask, sonicated and made up the volume to100 mL with diluent to 400 µg/ml concentration of Azilsartan medoxomil.

Preparation of Sample Solution:

Take tablet and triturate it by using mortar and pestle, take a powder equivalent to the dose of the drugs of sample (40 mg equivalent to Azilsartan Medoxomil is weighed and add into a 100 mL volumetric flask. 100 mL of diluent is added, sonicated to dissolve and made up to volume with diluent. Further diluted 10 to 100 mL with the diluent. Filtered through 0.45µ Nylon syringe filter. Injected 20µL of Standard preparation five times and Sample preparation into the Chromatographic column. The chromatograms are recorded and the peak responses for Azilsartan Medoxomil was measured. The System suitability parameters are met. From the peak responses, the content of Azilsartan Medoxomil in the sample is calculated.

Optimization of Chromatographic Conditions and Method Development

Method Validation by Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Validation study was intended to show that the method is suitable for assay and stability studies of Azilsartan medoxomilin tablet dosage form. The method validation was carried out as per ICH guidelines for specificity, forced degradation, precision, linearity, accuracy and stability in analytical solution (ICH 1996, Q2 (R1) ICH, 2005).

System Suitability Study

Calibration Standard were prepared and 20 µl of standard preparations in five replicates previously were injected. The chromatograms and the peak responses were measured for Azilsartan medoxomil. System suitability of the method was evaluated in terms of Retention time (RT), peak area, tailing factor, resolution and theoretical plate.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Blank solution, individual standard solution and mixed standard solution of Azilsartan Medoxomil (20 µg/ml) was injected into the HPLC system. The peak purity data of Azilsartan Medoxomil was compared there should not be any interference at the retention time of the main peaks.

Precision

a) System Precision

Six replicates of the mixed standard solution containing the Azilsartan Medoxomil (20 µg/ml) was injected into HPLC system. Prepared solutions were analyzed as per the proposed method. The mean, SD and % RSD were calculated.

b) Method Precision

Six samples containing the known amounts of Azilsartan medoxomil (40 µg/ml) was analyzed as per test method and the % assay and % RSD for both the drugs was calculated.

c) Intraday and Inter-day Precision

The intraday precision of the assay method for Azilsartan Medoxomil was evaluated at three concentration levels prepared from the sample stock solution (Azilsartan Medoxomil 20, 40, 60 µg/ml) by performing analysis at an interval of 2hrs for 12 hrs. The inter-day precision study was also performed on three different days i.e. day 1, day 2 and day 3 at three different concentration levels as used for intraday study.

Accuracy (Recovery Study)

Accuracy study of pre-optimized method was calculated using recovery studies by performing the standard addition method. Three levels of percent i.e. 80, 100 and 120 % amount was added externally to the solutions with predefined amount of (Azilsartan medoxomil 20, 40, 60 µg/ml and the % recovery was calculated.

$$\% \text{ Recovery} = A/B+C \times 100$$

A=Total drug estimated (mg)

B = Wt. (mg) of drug contributed by tablet powder

C= Amount of pure drug added (mg)

Linearity and Range

Linearity for the Azilsartan Medoxomil was determined by preparing the standard solutions at five concentrations in six replicates levels in the range of 20-120 µg/ml for Azilsartan Medoxomil from the stock solutions. 20 µl of each solution was injected into the HPLC system and the peak area of the chromatogram obtained was noted. The mean area with its standard deviation and % relative standard deviation of peak areas were calculated. Mean AUC was plotted against concentration to obtain the calibration curve. Regression equations, correlation coefficients were computed from calibration curves

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ for Azilsartan Medoxomil was calculated from slope and standard deviation of the response for Azilsartan medoxomil. The LOD and LOQ were determined using equations.

$$LOD=3.3\times SD/S$$

$$LOQ=10\times SD/S$$

Where; σ=Standard deviation of response

S=Slope of calibration curve

Robustness

Pre-analyzed sample solution containing mixture of 20µg/ml of Azilsartan medoxomil, was prepared and analyzed as per proposed method by changing the flow rate to 1.2 ml/min and 0.8 ml/min. The system suitability parameters and peak areas (or % assay) was evaluated in each condition and the results were compared with method precision results.

Ruggedness

Ruggedness of the method was determined by carrying out the analysis of Azilsartan medoxomil 20, 40, 60 µg/ml at three different (25°C, 37°C and 60°C) temperatures and area were noted and % RSD was calculated**.**

Results and Discussion

Preliminary Studies and Spectral Studies of Azilsartan medoxomil

FT-IR Studies and UV Spectrometry studies of Azilsartan medoxomil

The preliminary identification was carried out by recording the FTIR spectrum for Azilsartan medoxomil. The observed group frequencies are tabulated in table 1.

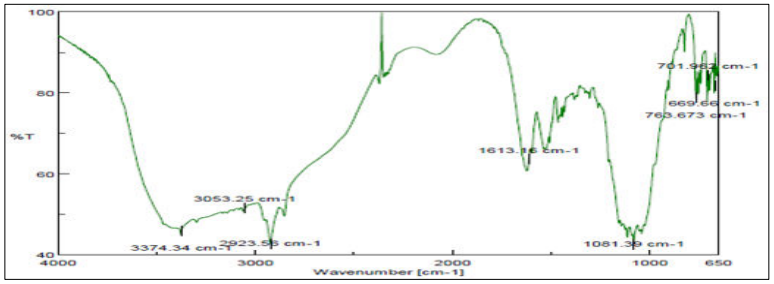


Figure 3: FT-IR Spectra of Azilsartan medoxomil

Table 1: Observed Group of Azilsartan medoxomil Frequencies by FT-IR

Name of Drug	Observed IR Frequencies	Functional group Present
Azilsartan medoxomil	3374.48	N-H stretching, aliphatic primary amine
	3053.25	C-H stretching
	1823.77	C=O stretching
	1613.16	C=O stretching
	1467.21	N-H Bending
	1251.86	C-O stretching, primary alcohol

Method Development by Reverse Phase High Performance Liquid Chromatography (RP-HPLC)
Optimization of Chromatographic Conditions and Method Development

In order to achieve the optimized chromatographic conditions to separate and quantify Azilsartan medoxomilone or two parameters were modified at each trial and chromatograms were recorded with all specified chromatographic conditions. Various trials [figure 4 & 5] were carried out to finalize the optimized chromatographic conditions. Few of them are mentioned in the Table 2. Poor resolution, bad peak shapes, disturbances in base line were the few reasons of the rejections of the trials.

Table 2: Various Trials and Optimization of Chromatographic Conditions

Trial No	HPLC System	Chromatographic Conditions	Observation	Remarks
1	HPLC (Shimazdu LC 2010 with Uv detector)	**Mobile Phase-**Water:Methanol (50:50) **Column -**Inertsil C18 (4.6 x 250mm, 5µm) **Flow rate-** 1 ml/min **Injection Volume-** 20µl **Pump mode-**Isocratic **Column temperature-**Ambient **Wavelength-**250nm	Peaks were not clearly separated. Base line is not clear.	**Rejected**
2	HPLC (Shimazdu LC 2010 with Uv detector)	**Mobile Phase-**Buffer:Methanol (50:50) **Column -**Inertsil C18 (4.6 x 250mm, 5µm) **Flow rate-** 1 ml/min **Injection Volume-** 20µl **Pump mode-**Isocratic **Column temperature-**Ambient **Wavelength-**250nm	Peaks were not clear. Baseline is not clear.	**Rejected**

3	HPLC (Shimazdu LC 2010 with Uv detector)	**Mobile Phase-**Water:Acetonitrile (30:70) **Column -**Inertsil C18 (4.6 x 250mm, 5µm) **Flow rate-** 1 ml/min **Injection Volume-** 20µl **Pump mode-**Isocratic **Column temperature-**Ambient **Wavelength-**250 nm	Peak shape was not good	**Rejected**
4	HPLC (Shimazdu LC 2010 with Uv detector)	**MobilePhase-**Water (0.1% Ortho-phosphoric acid): Acetonitrile 25:75 **Column -**Inertsil C18 (4.6 x 250mm, 5µm) **Flow rate-** 1 ml/min **Injection Volume-** 20µl **Pump mode-**Isocratic **Column temperature-**Ambient **Wavelength-**250nm	Peaks shape were good,with good resolution and intensity	**Accepted**

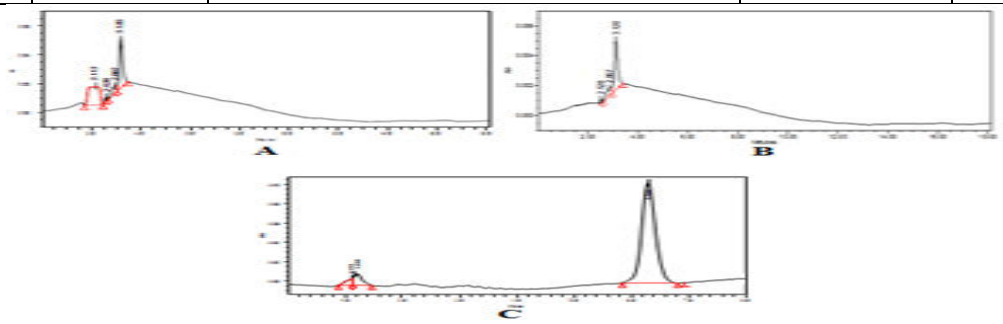


Figure 4: Trials for Method development of Azilsartan medoxomil

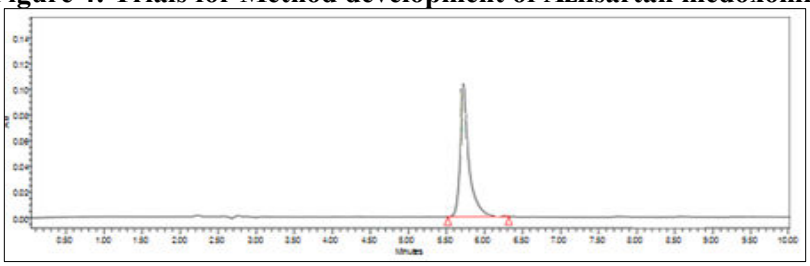


Figure 5: Optimized trial for Method development of Azilsartan medoxomil

System Suitability

The HPLC method has been developed for the determination of the percentage assay of Azilsartan medoxomilin its tablet dosage form. The chromatograms of standard drugs alone and in their mixture are shown in figure 6, 7 and 8.The Retention time for Azilsartan Medoxomil was found to be 5.30 min and other parameters like, resolution, tailing factor, and theoretical plates were found to be within acceptable limit.

Table 3: System Suitability Parameters for Azilsartan medoxomil

Sr. No.	Name	Retention Time (min)	Area	Tailing Factor	Theoretical Plate Count
1	Azilsartan medoxomil	5.31	47258	1.354	5627

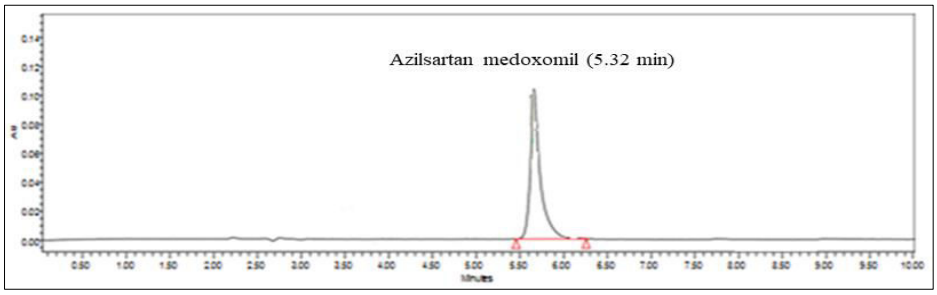


Figure 6: Standard Chromatogram of Azilsartan medoxomil

Specificity

The absence of additional peaks in the chromatogram indicates non- interference of excipients. There was no interference from the blank at the retention time of analyte peaks. The peak purity data of sample solution was compared with standard solution. The peak purity plots are shown in figure 7 & 8 which reveals the homogenous peaks.

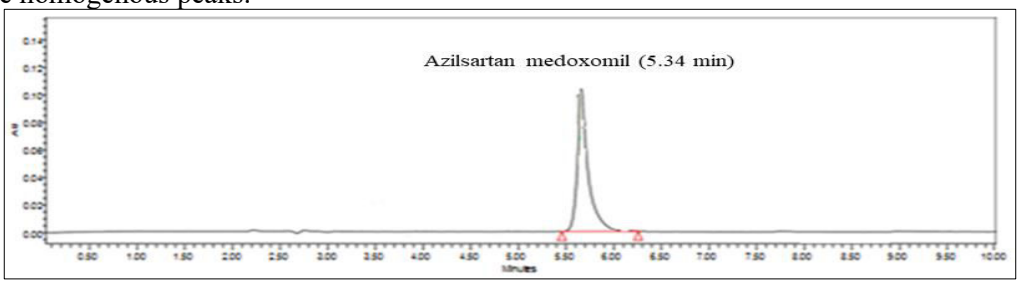


Figure 7: Purity Chromatogram of Standard Azilsartan medoxomil

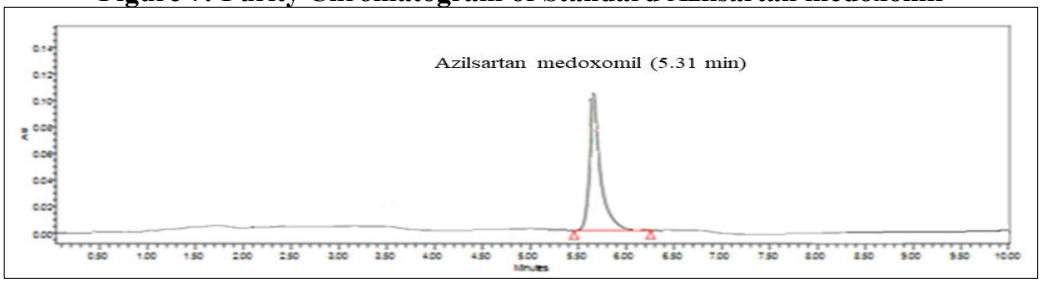


Figure 8: Purity Chromatogram of Sample of Azilsartan medoxomil

Precision

a) System Precision

The system precision was performed by measuring the peak response for standard drugs solutions in six replicates. Peak responses, mean, standard deviation and % relative standard deviation (%RSD) for Azilsartan Medoxomil was found to be 0.425 %.The results are shown in table 4 and were found well within the acceptable criteria.

Table 4: System Precision Data of Azilsartan medoxomil

Sr. No.	Peak areas of Azilsartan medoxomil
1.	47265
2.	47368
3.	47684
4.	47536
5.	47135
6.	47267
Mean	**47375.3**
SD(±)	**201.401**
RSD(%)	**0.425**
Acceptance criteria	**% RSD should not be more than 2**

b) Method Precision

The % assay for Azilsartan medoxomilin six samples was calculated. The results of % assay and % RSD are shown in table 5. The chromatograms for the method precision are shown in figure are 9-14.

Table 5: Method Precision Data of Azilsartan medoxomil

Sample No.	% Assay of Azilsartan Medoxomil (w/w)
1.	99.46
2.	100.24
3.	100.00
4.	99.84
5.	101.56
6.	100.26
Mean	**100.22**
SD(±)	**0.716**
RSD(%)	**0.714**
Acceptancec riteria	**% RSD should not be more than 2**

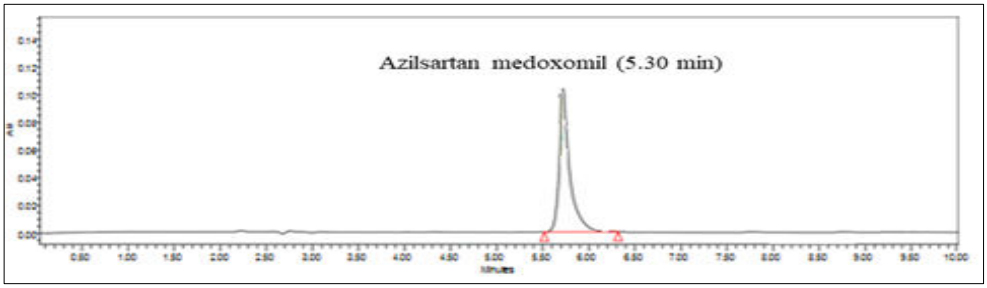


Figure 9: Chromatogram of Method precision 1

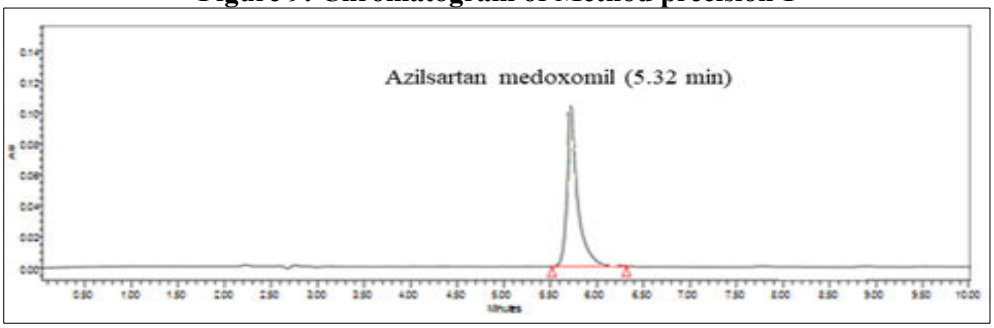


Figure 10: Chromatogram of Method precision 2

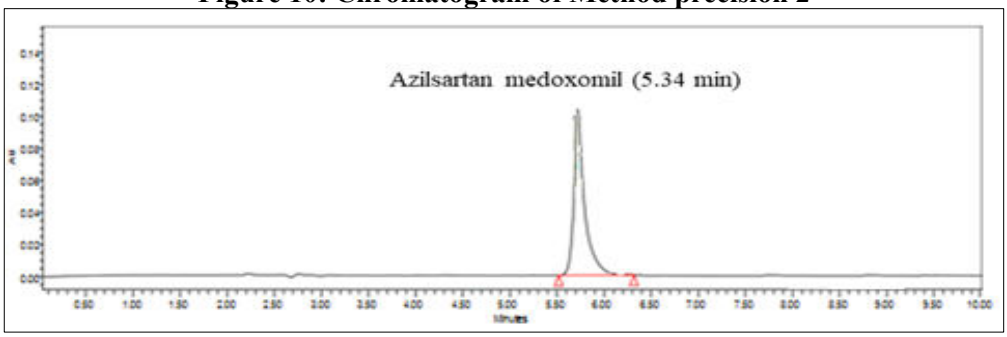


Figure 11: Chromatogram of Method precision 3

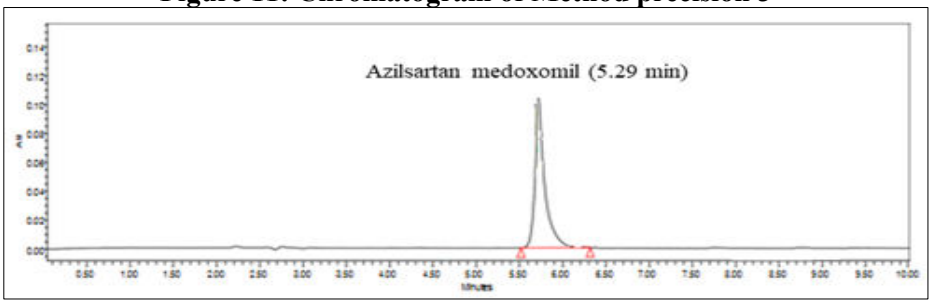


Figure 12: Chromatogram of Method precision 4

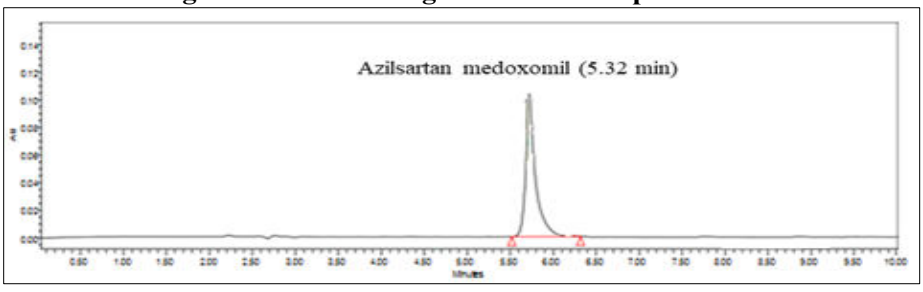


Figure 13: Chromatogram of Method precision 5

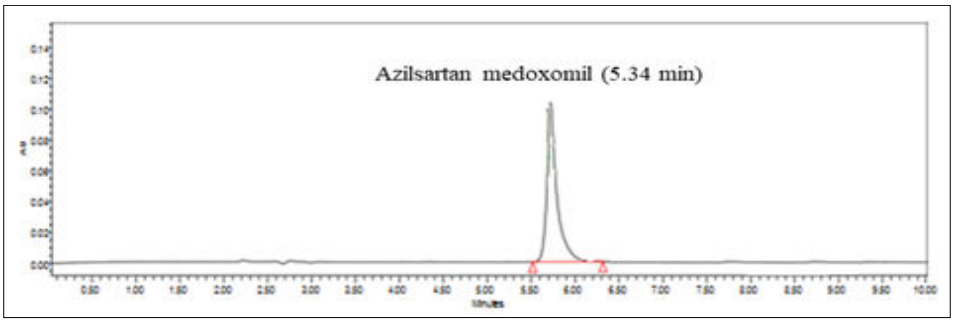


Figure 14: Chromatogram of Method precision 6

Intraday and Inter-day Precision

In inter-day precision % RSD for Azilsartan Medoxomil (20, 60, 100 µg/ml) was found to be 0.685, 0.352, 0.042 %. The results obtained are mentioned in the table 6, 7**.**

Accuracy (Recovery Study)

The mean % recovery was found to be 99.94 % for Azilsartan medoxomil. The results of the recovery study are shown in the table 8.

Table 6: Intraday Precision data of Azilsartan medoxomil

Sr. no.	Conc. (µg/ml)	Area	Mean peak area	SD(±)	%RSD
1	20	22734	22549	194.103	0.860
		22567			
		22347			
2	60	70548	70469	205.614	0.292
		70624			
		70236			
3	100	117426	117460	116.216	0.098
		117589			
		117364			

Table 7: Inter-day Precision data of Azilsartan medoxomil

Sr. no.	Day	Conc. (µg/ml)	Peak Area	Mean Peak Area	SD (±)	%RSD
1	Day 1	20	22460	22326	152.978	0.685
	Day 2		22159			
	Day 3		22357			
2	Day 1	60	70635	70353	247.946	0.352
	Day 2		70259			
	Day 3		70167			
3	Day 1	100	117264	117285	49.722	0.042
	Day 2		117341			
	Day 3		117248			

Table 8: Recovery study for Azilsartan medoxomil

Azilsartan medoxomil							
Level	**Set**	**Amount added(µg/ml)**	**Amount found(µg/ml)**	**%Recovery**	**Mean**	**SD**	**%RSD**
80%	1	20	19.918	99.59	99.74	0.435	0.437
	2	20	20.045	100.23			
	3	20	19.879	99.39			
100%	1	40	40.043	100.11	100.11	0.223	0.223
	2	40	39.353	99.88			
	3	40	40.131	100.32			
120%	1	60	59.941	99.90	99.98	0.188	0.189
	2	60	60.116	100.19			
	3	60	59.903	99.84			

Linearity and Range

Linearity for Azilsartan medoxomilwas found to be in the range of 20 - 120µg /ml with correlation coefficient value (r2) 0.9992 for Azilsartan medoxomil. The results were tabulated in table 9 and graphically represented in figure 15 and 16. The overlay chromatogram of the all concentration is shown in figure 18.

Table 9: Linearity and Range for Azilsartan medoxomil

Concentrations (µg/ml)	**Average Peak Area**
20	22896
40	47167
60	70616
80	94258
100	117724
120	137684

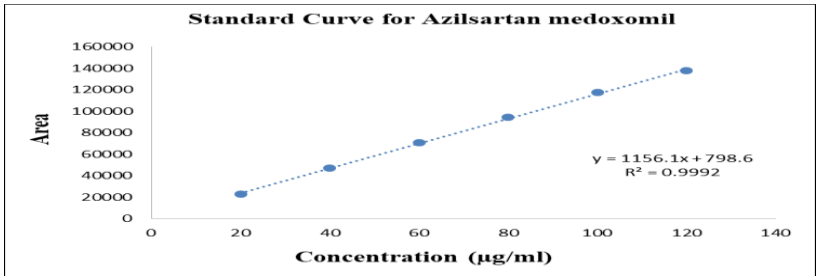


Figure 15: Standard Curve for Azilsartan medoxomil

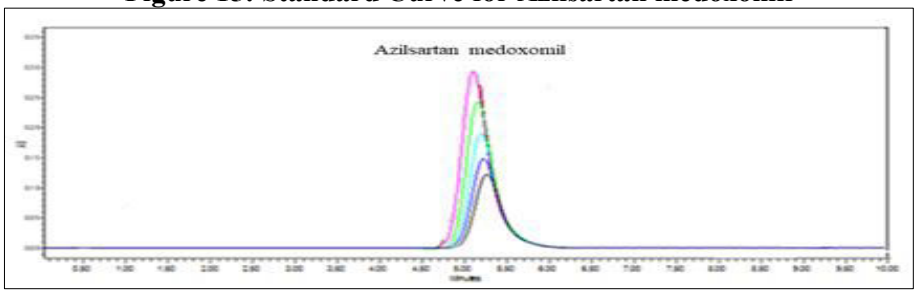


Figure 16: Overlay chromatogram of all concentrations of Azilsartan medoxomil

Stability in Analytical Solution

No significant difference was found in the % Assay of both drugs before and after storing for 24 hrs in refrigerator and room temperature. This confirms the stability of the drugs in solutions. The percentage assay is tabulated in table 10.

Table 10: Solution Stability Data of Azilsartan medoxomil

Time level	Refrigerator (25°C)	Room Condition(37°C)
Initial	100.62 (±0.19)	100.69 (±0.22)
After24hrs	100.12 (±0.36)	99.84 (±0.41)

*Average of Six determination

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

For Azilsartan medoxomilthe LOD and LOQ were found to be 0.114µg/ml and 0.346µg/ml respectively. These values indicate that the method is suitable for the determination of the lower concentration and confirms that proposed method is sensitive for the determination.

Robustness

The system suitability parameters and peak areas were evaluated in each condition and the results were compared with method precision results. %RSD at each condition was found less than 2. This indicates the robustness of the method. The results are tabulated in table 11.

Table 11: Robustness data of Azilsartan medoxomil

Flow Rate	**Parameters**			
0.8 ml/min				
	RT	**Area**	**TheoreticalPlates**	**Tailing factor**
Average	5.409	22755	2700.33	0.745
S. D	0.083	97.736	47.648	0.010
%RSD	**1.541**	**0.429**	**1.764**	**1.446**
1.2 ml/min				
Average	5.263	22230	2547	0.960
S. D	0.022	127.986	26.457	0.014
%RSD	**0.427**	**0.575**	**1.038**	**1.773**

Robustness

The robustness parameter was determined by analyzing the different concentration at different temperature. The results were showed in table 12.

Table 12: Data of Robustness for Azilsartan medoxomil

Area of Standard	Mean	SD	%RSD
47215	47203.3	70.230	**0.148**
47128			
47267			
47256	47298.7	48.232	**0.101**
47289			
47351			
47105	47086.3	91.440	**0.194**
46987			
47167			

Conclusion

The results clearly indicate that the RP-HPLC technique can be successfully applied for the estimation of Azilsartan medoxomil drug in their formulation. The method was validated in accordance with ICH guidelines. The result of recovery study was satisfactory and show that there is no interference of excipients in tablet dosage form. The mobile phase is simple to prepare and economical and as the process is precise and accurate, drug is also stable for long hours. In addition, the main features of the developed method are short run time and retention time around 5.31±0.25 min. In the current research, the method shows good reproducibility, moreover the RP-HPLC method is accurate, precise, specific, reproducible, sensitive and cost effective for the analysis of Azilsartan medoxomil. Hence this method can be easily and conveniently adopted for routine quality control analysis of Azilsartan medoxomil.

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