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DEVELOPMENT AND VALIDATION OF REVERSED-PHASE HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF AZILSARTAN MEDOXOMIL AND AMLODIPINE BESYLATE IN TABLET DOSAGE FORM

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ABSTRACT
An isocratic RP-HPLC method was developed and validated for simultaneous estimation of Azilsartan medoxomil and Amlodipine besylate in tablet dosage form. The chromatographic separation was carried out on C18 Inertsil ODS column (250 X 4.6 mm, 5µm particle size) with mixture of 0.1% TFA and ACN in ratio of 75:25, v/v as a mobile phase; at a flow rate of 1.0 mL/min. UV detection was performed at 245 nm. The retention time was found to be 4.626 min and 2.222 min for azilsartan medoxomil and amlodipine besylate, respectively. Calibration plots were linear over the concentration range of 4-20 µg/mL and 1-5 µg/mL for azilsartan medoxomil and amlodipine besylate, respectively. The method was validated for linearity, accuracy, precision, repeatability, LOD and LOQ. The proposed method was successfully used for quantitative estimation of azilsartan medoxomil and amlodipine besylate. Percentage recovery within the limit of 98-102 % and low relative standard deviation confirm the suitability of the proposed method for routine estimation of azilsartan medoxomil and amlodipine besylate in tablet dosage form.

KEYWORDS: RP-HPLC, Azilsartan medoxomil, Amlodipine besylate.

1. INTRODUCTION
Azilsartan Medoxomil (AZM) and Amlodipine Besylate (AMLB) were introduced in the market as combined tablet dosage form. Azilsartan medoxomil (AZM) is chemically 5-methyl-2-oxo-1, 3-dioxol-4-yl) methyl-2-ethoxy-3-[[4-[2-(5-oxo-2H-1,2,4-oxadiazol-3yl) phenyl] phenyl] methyl] benzimidazole-4-carboxylate (Fig. 1).

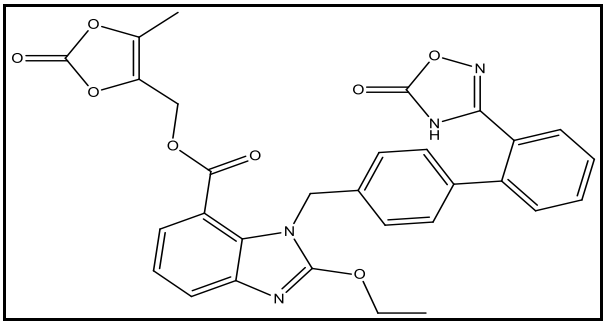

Figure 1: Structure of Azilsartan Medoxomil.

It exists as white crystalline powder, insoluble in distilled water but freely soluble in methanol, dimethyl-formamide, dimethyl-sulfoxide, it is soluble in acetic acid, slightly soluble in acetone, and acetonitrile, very slightly soluble in tetrahydrofuran. It is a selective AT, subtype angiotensin II receptor antagonist applicable for the treatment of hypertension in adult patients, either alone or in combination with other antihypertensive agents.[1-3]

AMLB is chemically 3-Ethyl-5- methyl (±)- 2- [(2-aminoethoxy)methyl]-4-(2- chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate, monobenzenesulphonate (Fig. 2).

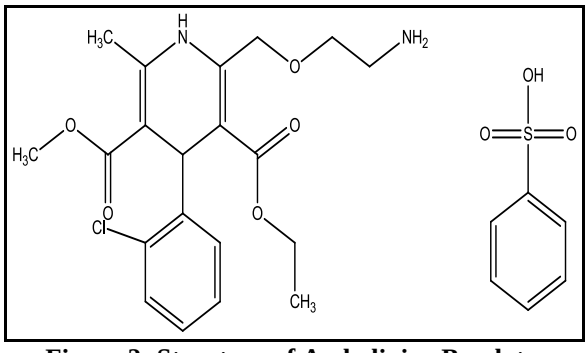

Figure 2: Structure of Amlodipine Besylate.

It is a white crystalline powder. It is a long-acting 1, 4-dihydropyridine calcium channel blocker. It acts primarily on vascular smooth muscle cells by stabilizing voltage-gated L-type calcium channels in their inactive conformation. By inhibiting the influx of calcium in smooth muscle cells, amlodipine prevents calcium-dependent myocyte contraction and vasoconstriction. Amlodipine is used to treat hypertension and chronic stable angina.[4-7]

2. AIMS AND OBJECTIVES

Various methods have been given for determination of AZM[8, 9] and its combination with other drugs[10-12]. Methods have also been developed for determination of AMLB alone[13,15] and in combination with other drugs.[16-20] Few methods have been developed for the determination of AZM and AMLB in combination but it has more scope to develop the method to achieve better resolution and peak shape. The following method has been developed for the estimation of AZM and AMLB in combination.

3. MATERIALS AND METHODS

Chromatographic separation was performed with Shimadzu high performance liquid chromatographic system having C18 Inertsil ODS column (250 mm X 4.6 mm i.d, 5 µm particle size) with photodiode array detector. Chromatographic data were recorded by LC Solution software.

3.1 Preparation of stock and working standard solutions

Accurately weighed 20 mg pure drug of AZM and 10 mg of AMLB and transferred separately in 50 mL and 100 mL clean, dry volumetric flask, sufficient quantity of mobile phase was added and each solution was sonicated to dissolve. The volume was made up to the mark with mobile phase to prepare standard stock solution containing 400 µg/mL AZM and 100 µg/mL AMLB. Further 5 mL each of AZM and AMLB was transferred into separate 50 mL volumetric flask and volume was made up to the mark with mobile phase. Resulting working standard solutions contain 40 µg/mL of AZM and 10 µg/mL of AMLB respectively. Further 3mL from working standard solution was transferred into separate 10 mL volumetric flask and volume was made up to the mark with mobile phase. To prepare mixed working standard solution 3 mL each from AZM and AMLB working standard solution was transferred into a 10 mL volumetric flask and volume was made up to the mark with mobile phase. The solution was used to set the chromatographic conditions.

3.2 Sample preparation

For the estimation of AZM and AMLB in the tablet formulation, 20 tablets were accurately weighed and the average weight per tablet was calculated. The tablets were crushed and finely powdered in glass mortar. Powder equivalent to 40 mg AZM was accurately weighed and transferred into a 100 mL volumetric flask containing sufficient quantity of mobile phase and sonicated to dissolve. The volume was made up to the mark with mobile phase and mixed well to prepare test stock solution containing 400 µg/mL AZM and 100 µg/mL AMLB. The solution was filtered using 0.2 µm membrane filter and degassed by sonication. 5 mL of this solution was transferred into 50 mL volumetric flask and volume was made up to the mark with mobile phase to prepare working test solution containing 40 µg/mL of AZM and 10 µg/mL of AMLB. Further 3 mL from working test solution was transferred into 10 mL volumetric flask and volume was made up to the mark with mobile phase. The resulting solution was used as the sample solution for chromatographic analysis.

After setting the chromatographic conditions and stabilizing the instrument to obtain a steady baseline, the sample solution was loaded in the 20µL sample loop of the injection port. The solution was injected three times and the peak areas were recorded.

3.3 Chromatographic conditions

The chromatographic separation was performed using a C18 Inertsil ODS column (250 × 4.6 mm, 5µm particle size). Several trials were performed with combination of different mobile phase to check the peak shape, retention time, resolution, and other chromatographic parameters. Finally the mobile phase selected consists of a mixture of 0.1% TFA and ACN in ratio of 75:25, v/v. The mobile phase was set at a flow rate of 1 mL/min and the analytes were monitored at 245 nm. The column was maintained at ambient temperature and injected volume was 20 µl. The total run time was 10 min.

3.4 Preparation of calibration curve

Aliquots of standard working solution of AZM and AMLB (1.0, 2.0, 3.0, 4.0 and 5.0 mL) were taken in a series of 10 mL volumetric flasks. The volume was made up to the mark with mobile phase to give the concentration range 4-20 µg/mL for AZM and 1.0-5.0 µg/mL for AMLB. Each solution was injected and a chromatogram was recorded. The calibration curves were plotted using the peak areas against the respective concentrations of the drug.

4. RESULTS AND DISCUSSION

4.1 Method development and optimization

The present study was aimed at developing a precise, sensitive, rapid and accurate HPLC method for the analysis of AZM and AMLB. The chromatographic separation was performed using a C18 Inertsil ODS column (250 × 4.6 mm, 5µm particle size). Several trials were performed with combination of different mobile phase to check the peak shape, retention time, resolution, and other chromatographic parameters. Finally the mobile phase selected consists of a mixture of 0.1% TFA and ACN in ratio of 75:25, v/v. The mobile phase was set at a flow rate of 1 mL/min and the analyte was monitored at 245 nm. The column was maintained at

ambient temperature and injected volume was 20 µl. The total run time was 10 min. The retention time for AZM and AMLB were found to be 4.626 min for AZM and 2.222 min for AMLB. Optimized mobile phase proportion was providing good resolution between AZM and AMLB. (Fig. 3) represents the chromatogram of standard. Results from method development and optimization studies are given in (Table I).

Table I: Optimized chromatographic conditions for estimation of AZM and AMLB.

Column	**C18 Inertsil ODS column (250 mm X 4.6 mm i.d, 5 µm particle size)**
Mobile Phase	0.1% TFA and ACN in a ratio of 75:25, v/v
Flow rate	1.0 mL/min
Column Temperature	Ambient
Detection	245
Injection vol.	20µl
Runtime	10 min
Retention time	4.626 min (AZM) 2.222 min (AMLB)

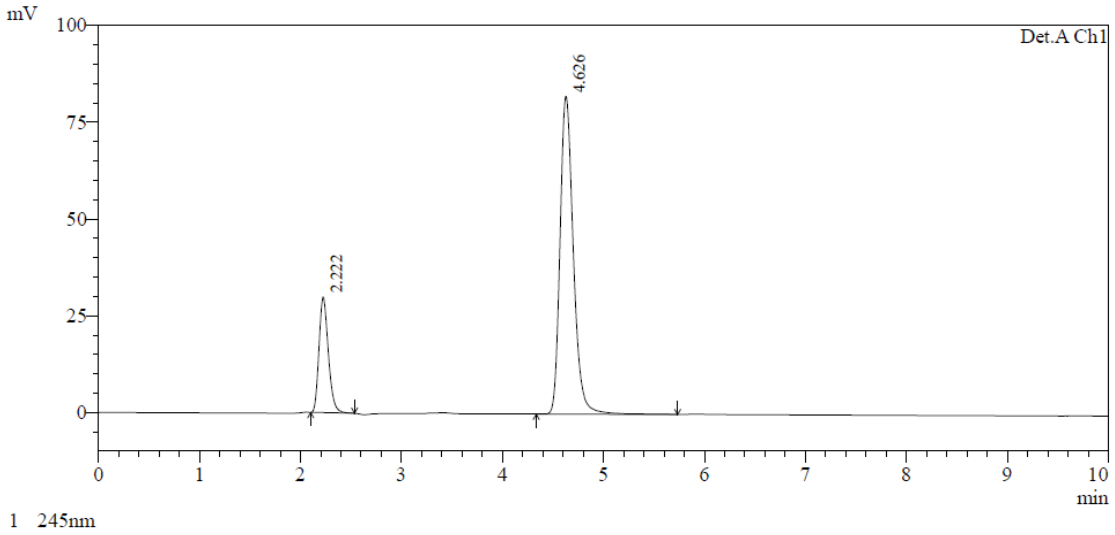

Figure 3: Chromatogram of standard solution of azilsartan medoxomil and amlodipine besylate.

4.2 System suitability

System suitability was evaluated by injecting blank preparation (single injection) and same standard preparation (six replicates) containing AZM and AMLB. The RSD (%) for retention time, peak area, tailing factor and theoretical plate count for both AZM and AMLB were found to be within the limit of 2%, which indicates suitability of the system and is reported in (Table II)**.** The number of theoretical plates and the tailing factor were found within the acceptance criteria of >2000 and ≤2.0, respectively, which indicates good column efficiency and optimum mobile phase composition.

Table II: Results from System-Suitability Study of AZM and AMLB by HPLC.

Parameters	**AZM (12 µg/mL)**		**AMLB (3 µg/mL)**	
	Mean (n = 6)	**%RSD**	**Mean (n = 6)**	**% RSD**
Retention time (t_R)	4.628	0.08	2.224	0.18
Peak area (A)	234433.5	0.17	113597.2	0.16
Tailing factor (T)	1.304	0.03	1.299	0.18
No. of theoretical plates (N)	5866.77	0.11	2373.7	0.48

4.3 Linearity

The linearity response was determined in the concentration range of 4-20 µg/mL for AZM and 1.0-5.0 µg/mL for AMLB. The calibration curve was found to be linear over the range of 4-20 µg/mL for AZM and 1.0-5.0 µg/mL for AMLB (Table III & IV). The method was evaluated by calculating correlation coefficient and intercept from calibration curve. $R^2 \geq 0.999$ and intercept close to zero confirmed the linearity of the method. The standard chromatogram of AZM and AMLB is given in (Fig. 4 & 5).

Table III: Linearity data of AZM by HPLC method.

S.No.	Conc. (µg/ml)	*Peak Area
1.	4.0	80899
2.	8.0	156646
3.	12.0	234085
4.	16.0	319749
5.	20.0	397532

*mean of three replicates

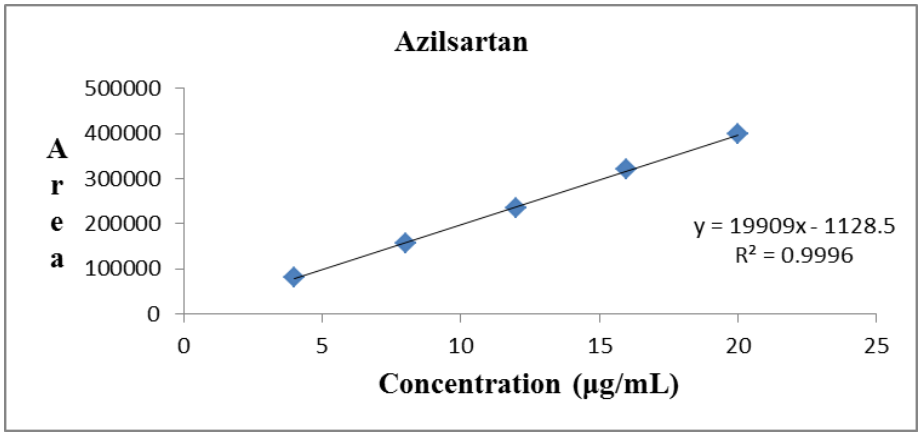

Figure 4: Calibration curve for Azilsartan medoxomil.

Table IV: Linearity data of AMLB by HPLC method.

S.No.	Conc. (µg/ml)	*Peak Area
1.	1.0	38883
2.	2.0	76986
3.	3.0	113457
4.	4.0	154652
5.	5.0	192881

*mean of three replicates

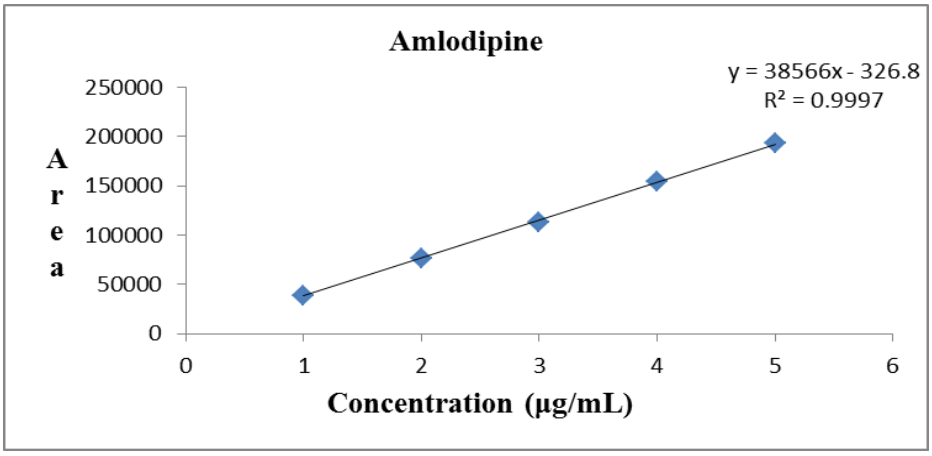

Figure 5: Calibration curve for Amlodipine Besylate.

4.4 Limit of detection and Limit of quantification

LOD and the LOQ of the drugs were calculated using the following equations asper International conference on harmonization (ICH) guidelines. LOD = (3.3 σ) /S, LOQ = (10 σ) /S, Where σ is the standard deviation of the response and S is the slope of calibration curve. The LOD values obtained were 0.5µg/mL and 0.108µg/mL for AZM and AMLB respectively, while the respective LOQ values were 1.515µg/mL and 0.327µg/mL.

4.5 Accuracy

The accuracy of the method was tested by triplicate samples at 3 different concentrations equivalent to 80%, 100% and 120% of the active ingredient, by adding a known amount of AZM and AMLB standard to a fixed amount of the pre-analyzed sample of AZM and AMLB. The recovered amount of AZM and AMLB, % recovery and %RSD of each concentration is calculated to determine the accuracy. The mean (n=3) of % recovery for AZM and AMLB were found to be in the range of 100.24 -100.93 % and 100.39 -100.98% respectively,

which were found to be within the acceptance criteria of ± 2% , results are given in (Table V).

Table V: Accuracy Study for AZM and AMLB by HPLC.

Accuracy Level (%)	Amount Added (µg/ml)		Amount Recovered (µg/ml)		%Recovery		Mean	
	AZM	AMLB	AZM	AMLB	AZM	AMLB	AZM	AMLB
80	6.4	1.6	6.41	1.59	100.08	99.62	100.93	100.39
	6.4	1.6	6.47	1.61	101.17	100.61		
	6.4	1.6	6.50	1.62	101.55	101.94		
100	8	2	8.06	2.02	100.72	101.12	100.71	100.98
	8	2	8.05	2.01	100.63	100.54		
	8	2	8.06	2.03	100.79	101.27		
120	9.6	2.4	9.64	2.43	100.39	101.07	100.24	100.47
	9.6	2.4	9.61	2.41	100.09	101.41		
	9.6	2.4	9.62	2.40	100.25	99.93		

4.6 Precision

Precision of the method was demonstrated by two categories. Repeatability (System precision) study was done by replicate analysis of the standard solutions of AZM and AMLB five times. The % RSD values for AZM and AMLB were found to be 0.17 and 0.16 respectively; the results are given in (Table VI). In addition, to demonstrate the precision of method (Method precision), Intra-day precision was determined by replicate analysis of the solutions (n=3) on the same day under similar conditions. Inter-day precision was determined by repeating analysis of the solution on three different days. Intra-day and inter-day precision was also within acceptance limit of % RSD ≤ 2 for AZM and AMLB, indicating that the developed method has good precision; data is given in (Table VII).

Table VI: Repeatabilty study for AZM and AMLB by HPLC.

S.No.	AZM		AMLB	
	Conc. (µg/ml)	Peak Area	Conc. (µg/ml)	Peak Area
1.	12.0	234085	3.0	113457
2.		234105		113388
3.		234156		113576
4.		234387		113587
5.		234879		113678
6.		234989		113897
Avg		234433.5		113597.2
SD		403.88		179.09
% RSD		0.17		0.16

Table VII: Precision study for AZM and AMLB by HPLC.

Conc. (µg /mL)	Intra-day (n=3)		Inter-day (n=3)	
	Mean ± SD	% RSD	Mean ± SD	%RSD
AZM				
4	81520 ± 741.37	0.90	81851 ± 952.89	1.16
12	234115 ± 36.61	0.02	234751 ± 320.56	0.14
20	397930 ± 406.74	0.12	399561 ± 2426.69	0.61
AMLB				
1	38923 ± 37.81	0.09	39412 ± 494.77	1.26
3	113473 ± 95.10	0.08	113720 ± 1482.33	0.14
5	193524 ± 679.55	0.35	199782 ± 329.00	0.74

4.7 Robustness

Robustness study was done by changing two parameters i.e. flow rate and composition of mobile phase. Small change in these parameters showed results within acceptance criteria of each parameter, indicated that it did not significantly affect the determination of AZM and AMLB. Results for robustness are given in **(**Table VIII).

Table VIII: Robustness study for AZM and AMLB by HPLC.

S. no.	Parameter	Optimized values	Robust conditions	Retention time (t_R), min	Plate count (N)	Tailing factor (T)
AZM						
1	Flow rate	1.0 mL/min	1.1 mL/min 0.9 mL/min	4.209 5.034	5558 6157	1.30 1.29
2	Mobile phase composition (0.1% TFA: ACN)	750:250	725:275 775:225	4.996 4.315	6155 5563	1.29 1.30
AMLB						
1	Flow rate	1.0 mL/min	1.1 mL/min 0.9 mL/min	2.016 2.426	2187 2558	1.31 1.29
2	Mobile phase composition (0.1% TFA: ACNl)	750:250	725:275 775:225	2.256 2.193	2411 2313	1.38 1.28

Acceptance criteria: Tailing Factor (T) < 2.0, Plate count (N) > 2000, significant change in retention time (t_R).

4.8 Assay of Tablet formulation

The developed method in the study was applied for the determination of AZM and AMLB in tablet formulation. From the peak areas the amount of each drug present in tablet was estimated. The drug content was calculated as an average of three determination and assay results were shown in (Table IX). The assay results were very close to the label claim of commercial tablets. The representative sample chromatogram of formulation is shown in (Fig. 6).

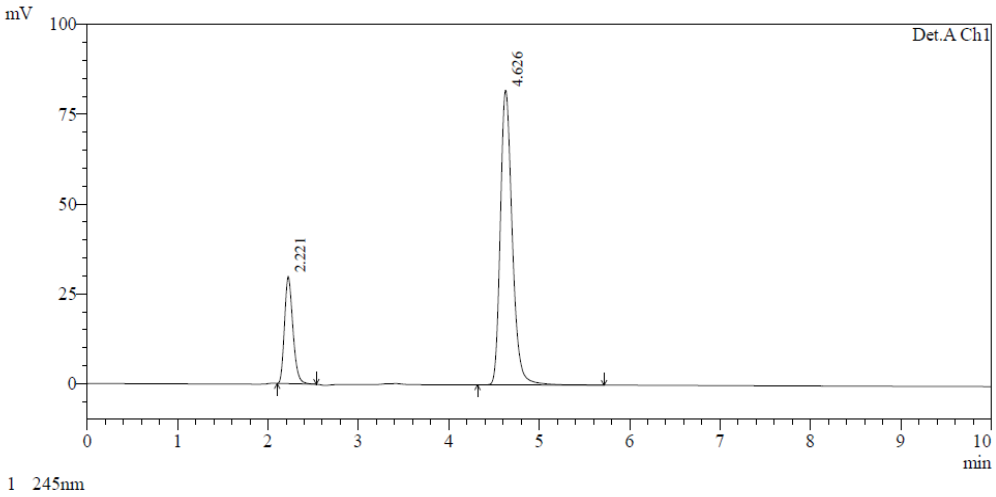

Figure 6: Chromatogram of Azilsartan medoxomil and Amlodipine in Tablet formulation.

Table IX: Results of analysis of Tablet formulation of AZM and AMLB by HPLC.

Formulation	Label Claim (mg)		Amount found (mg)		% Label claim	
	AZM	AMLB	AZM	AMLB	AZM	AMLB
Zarcas	20	5	20.03	5.01	100.13	100.11
	20	5	20.07	5.01	100.34	100.19
	20	5	20.08	5.02	100.39	100.38
Mean			20.06	5.01	100.28	100.23
SD			0.03	0.00	0.13	0.14
%RSD			0.14	0.14	0.13	0.14

5. CONCLUSIONS

A simple and reproducible RP-HPLC method has been developed for simultaneous estimation of azilsartan medoxomil and amlodipine besylate in tablet dosage form. The validation of method was performed for accuracy, precision, linearity and robustness. The method was proved to be superior to most of the reported methods since run time is very short, which can be utilized for rapid estimation of many samples in routine analysis. The mobile phases was simple to prepare and economical. The sample recoveries in the formulation were in good agreement with the irrespective label claim.

Hence the proposed method was found to be rapid, accurate, precise, robust and economical.

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