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Research Article

Development of Stability Indicating RP-HPLC Method for Simultaneous Estimation of Amlodipine and Olmesartan in Pure and Pharmaceutical Dosage Form

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ABSTRACT
A simple reverse phase HPLC method was developed for the simultaneous estimation of Amlodipine and Olmesartan in bulk and tablet form. Chromatography was performed by isocratic reverse phase separation on a stainless steel column 4.6 x 150mm, symmetry column packed with octa decyl silane bonded to porous silica (C18) with particle size 5 micron with mobile phase containing TEA Buffer of pH 3.0 and Acetonitrile in proportion of 25:75 respectively. The flow rate was 1.0 ml/ min and effluent was monitored at 258 nm. The retention times were 2.39 min and 3.33 min respectively. The standard curve was linear over a working range of 05–35 µg/ml for both Amlodipine and Olmesartan and gave an average correlation coefficient of 0.999, and 0.999 for Amlodipine and Olmesartan respectively. The limit of quantitation (LOQ) of this method was 2µg/ml for Amlodipine and Olmesartan. The absolute recovery was 100% for Amlodipine and 100.3 for Olmesartan. Degradation products produced as a result of stress studies did not interfere with the detection of Amlodipine and Olmesartan and the assay can thus be considered stability-indicating.

Keywords: Amlodipine, Olmesartan, RP-HPLC, TEA Buffer: Acetonitrile, Validation.

INTRODUCTION

Amlodipine 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. A second proposed mechanism for the drug's vasodilatory effects involves pH-dependent inhibition of calcium influx via inhibition of smooth muscle carbonic anhydrase. Some studies have shown that amlodipine also exerts inhibitory effects on voltage-gated N-type calcium channels. N-type calcium channels located in the central nervous system may be involved in nociceptive signaling and pain sensation. Amlodipine is used to treat hypertension and chronic stable angina[1]. Amlodipine decreases arterial smooth muscle contractility and subsequent vasoconstriction by inhibiting the influx of calcium ions through L-type calcium channels. Calcium ions entering the cell through these channels bind to calmodulin. Calcium-bound calmodulin then binds to and activates myosin light chain kinase (MLCK). Activated MLCK catalyzes the phosphorylation of the regulatory light chain subunit of myosin, a key step in muscle contraction. Amlodipine is slowly and almost completely absorbed from the gastrointestinal tract. Peak plasma concentrations are reached 6-12 hour following oral administration. Its estimated bioavailability is 64-90%. Absorption is not affected by food. It is metabolized extensively (90%) to inactive metabolites via the cytochrome P450 3A4 isozyme[2].

Olmesartan is an antihypertensive agent, which belongs to the class of medications called angiotensin II receptor blockers. It is indicated for the treatment of high blood pressure and is marketed under the name Olmetec®. The FDA label includes a black-box warning of injury and death to the fetus, so women of child-bearing age need to be warned and take the necessary precautions. Olmesartan is also contraindicated in diabetes mellitus patients taking aliskiren. Olmesartan is an ARB that selectively inhibits the binding of angiotensin II to AT1, which is found in many tissues such as vascular smooth muscle and the adrenal glands. This effectively inhibits the AT1-mediated vasoconstrictive and aldosterone-secreting effects of angiotensin II and results in a decrease in vascular resistance and blood pressure. Olmesartan is selective for AT1 and has a 12,500 times greater affinity for AT1 than the AT2 receptor. Also unlike the well-known ARB losartan, olmesartan does not have an active metabolite or possess uricosuric effects

MATERIALS AND METHODS:

Drugs

Pure pharmaceutical sample of AML and OLM was obtained from Yucca Pharma. Commercial tablet of amlodipine besylate (5mg), olmesartan

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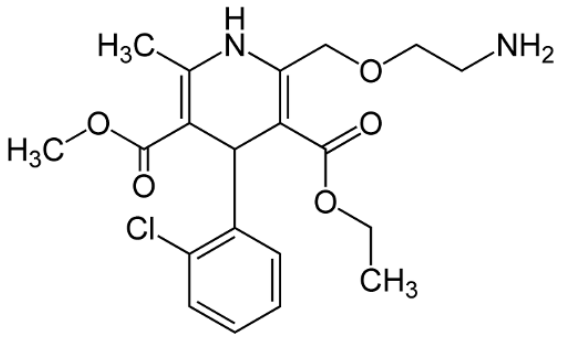

Figure 1: Amlodipine

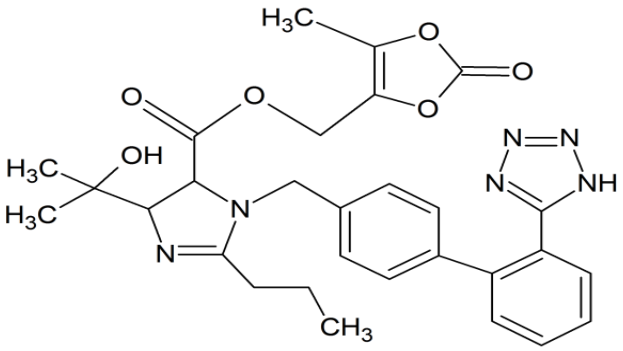

Figure 2: Olmesartan

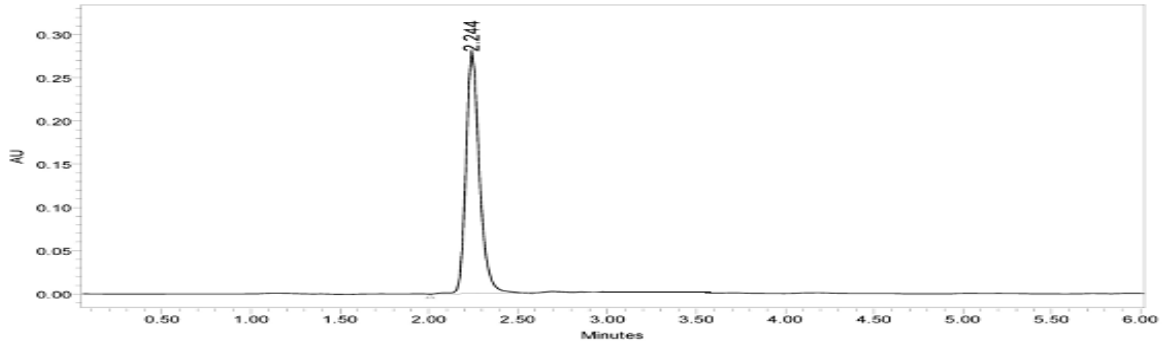

Figure 3: chromatogram of Amlodipine (Rt-2.395min).

S.No.	Peak Name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Amlodipine	2.316	1232142	194123	3.6	1.2	4651

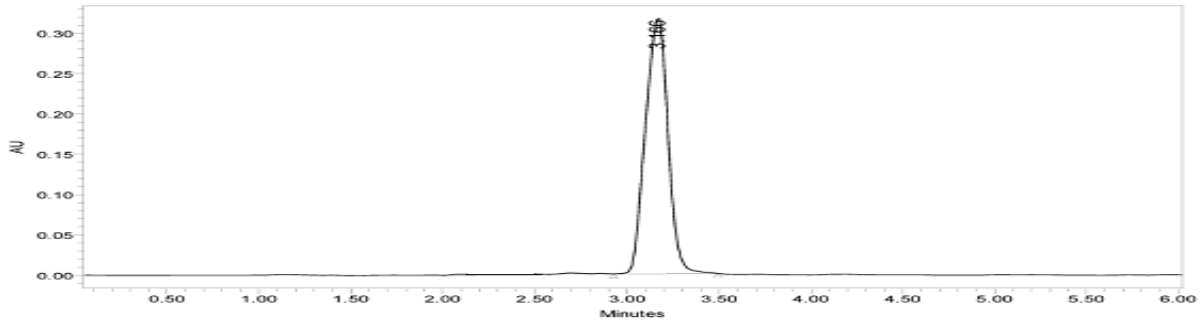

Figure 4: chromatogram of Olmesartan (Rt-3.339min).

S.No.	Peak Name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Olmesartan	3.304	1491465	176582	5.6	1.5	3982

medoxamil(20mg) Olmark A (Intas Pharmaceuticals Ltd) 20mg/5mg were procured from the local drug market.

Chemicals

Sodium dihydrogen phosphate (AR Grade), 85% Orthophosphoric acid (AR Grade), Acetonitrile (HPLC Grade), Hydrochloric Acid (AR Grade), Triethyl-Amine (AR Grade), Sodium Hydroxide (AR Grade) were purchased from Sd fine-Chem limited[3].

Instrument

Liquid chromatographic system from Waters alliance 2695 with Waters UV detector equipped with Empower software was used.

Preparation of mobile phase

Mobile phase was prepared by dissolving Buffer of pH 3 in Acetonitrile in the ratio of 25:75. The Mobile phase was filtered through 0.45 µm membrane filter and degassed under ultrasonic bath prior to use. The mobile phase was pumped through the column at a flow rate of 1.0 ml/min[5].

Preparation of TEA buffer (PH-3)

1.5 ml of Triethyl amine dissolved in 250 ml of HPLC Water. Adjusted pH 3.00 with ortho phosphoric acid[4].

Diluent preparation

The Mobile phase was used as the diluent

Stock solutions and standards

A stock solution of drugs were prepared by transferring accurately weighed 25 mg of AML and OLM in two seperate 25 ml volumetric flask and dissolved in 15 ml of mobile phase. The solutions were sonicated and the volumes were made up to mark with mobile phase to get concentration of 1000µg/ml of AML and OLM.

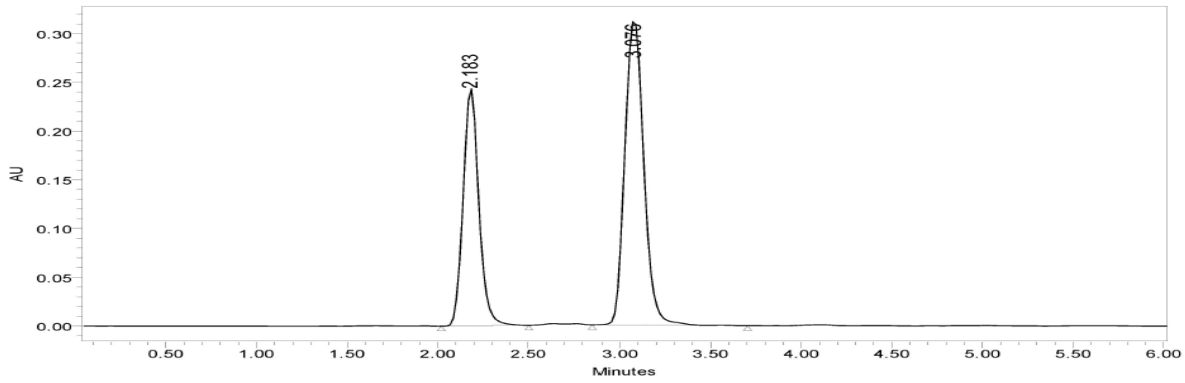

Figure 5: chromatogram of Amlodipine (Rt-2.395min) and Olmesartan (Rt-3.339min).

S.No.	Peak Name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Amlodipine	2.395	1242388	197332		1.1	4741
2	Olmesartan	3.339	1494848	177825	5.2	1.2	3793

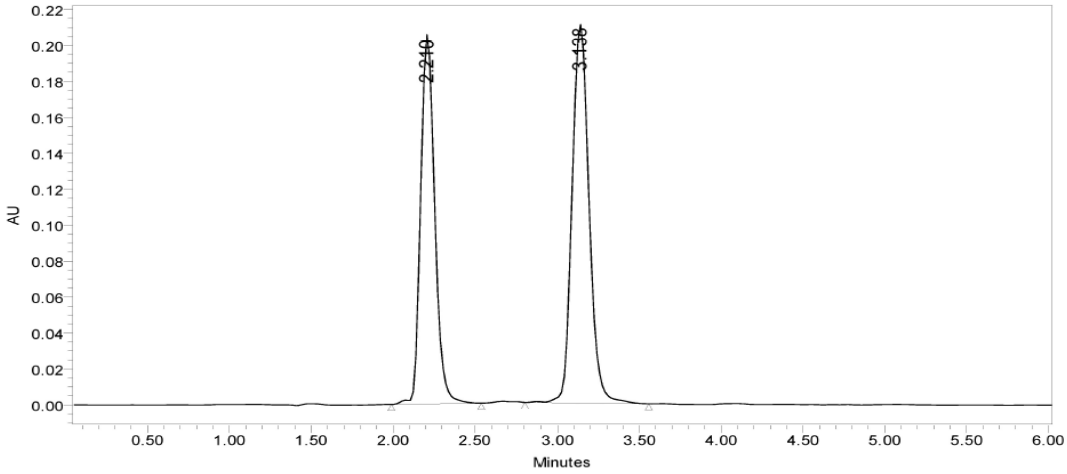

Figure 6: Chromatogram showing degradation for Amlodipine and Olmesartan in 0.1 N HCl

S.No.	Peak Name	R_t	Area	Height	USP Tailing	USP plate count
1	Amlodipine	2.210	1113179	198754	1.2	4854
2	Olmesartan	3.138	1339383	176582	1.3	3872

Table 1: System suitability parameters.

Instrument used	Waters HPLC with auto sampler and UV detector
Temperature	Ambient
Column	Symmetry C18 (4.6mm x 150mm, 5µm, Make: Waters)
Buffer	1.5ml of Triethyl amine dissolve in 250ml of HPLCwater. Adjust pH-3.00 with orthophosphoric acid.
pH	3
Mobile phase	TEA Buffer (pH-3.00), Acetonitrile in proportion of 25:75
Flow rate	1 ml per min
Wavelength	258 nm
Injection volume	20 µl
Run time	6 min

Preparation of Sub Stock Solution

1ml was pippeted from Amlodipine stock solution and 4 ml from Olmesartan stock solution and transferred in 100 ml volumetric flask separately. The volume was made up to the mark with mobile phase it gives final concentration of 10 µg/ml and 40 µg/ml solution of AML and OLM respectively.

Preparation of sample solution

Accurately weighed ten tablets were taken and crushed in mortar and pestle. 100 mg equivalent weight of powdered

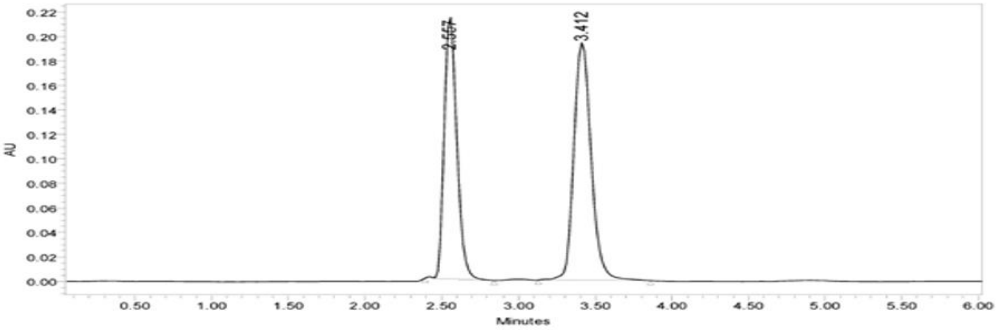

Figure 7: Chromatogram showing degradation related impurity in 0.1 N NaOH.

S.No.	Peak Name	R_t	Area	Height	USP Tailing	USP plate count
1	Amlodipine	2.557	1153184	198574	1.0	4658
2	Olmesartan	3.412	1387517	187452	1.1	3694

Table 2: Results of forced degradation studies of Amlodipine and Olmesartan API.

Stress condition	Time	Assay of degraded products	Assay of active substance	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	10.4	89.6	100
Basic Hydrolysis (0.I M NaOH)	24Hrs.	7.18	92.82	100
Thermal Degradation (50 ^{0}C)	24Hrs.	4.92	95.08	100
UV (254nm)	24Hrs.	2.44	97.56	100
3 % Hydrogen peroxide	24Hrs.	9.78	90.22	100

Table 3: Linearity results: (for Amlodipine).

Concentration of AML in ppm	Peak area
0	0
5	224748
10	475848
15	692648
20	944621
25	1180741
30	1390935
35	1598929

Table 4: Linearity results: (for Olmersartan).

Concentration of OLM in ppm	Peak area of Olmesartan
0	0
5	1234613
10	2472924
15	3570426
20	4853049
25	6053925
30	6990601
35	7817235

drug containing OLM and AML (marketed formulation- dose of OLM is 20 mg, dose of AML is 5mg in combination tablet) were transferred into a 100 mL volumetric flask and made the volume up to the mark with the solvent. (Stock solution). Further 2 ml pipetted out from stock solution into a 50 ml volumetric flask and diluted up to the mark with diluent[6].

Stability Study

Tablet powder equivalent to the weight of one tablet was transferred to 250 ml round bottomed flask and treated under acidic, alkaline, oxidizing, thermal and photolytic stress conditions. When degradation was complete, the solution were left to equilibrate to room temperature and diluted with diluents to furnish solutions of concentration equivalent to 40 µg/ml OLM and 10µg/ml AML. The specific conditions are described below. In acidic degradation drug was heated under reflux with 1M hydrochloric acid for 30 min at 80° and the drug was treated with 0.1N NaOH at room temperature for 2 h in alkaline degradation. Then resulting solution was neutralized. The drug was treated with 2% (v/v) H2O2 at room temperature for 2 hour in oxidative degradation. Thermal degradation was performed by exposing the solid drug to dry heat in a convection oven at 70° for 72 h and photolytic degradation was performed by exposing the drug to sunlight for 72 h.

Apparatus and Chromatographic conditions

Quantitative HPLC was performed on Waters HPLC system with UV detector. empower software is used along with a stainless steel column 4.6 x 150mm, packed with Octa decyl silane bonded to porous silica (C18) with particle size 5 micron. To develop a suitable and robust HPLC method for the determination of OLM and AML, different mobile phases containing TEA buffer and Acetonitrile were used in different compositions like (30:70, 40:60, 50:50, 70:30, 80:20) at different flow rates (0.5,0.75,1.0, 1.2, 1.5, ml/min). The mobile phase TEA buffer and Acetonitrile with a flow rate of 1.0 ml/ min gave peaks of good resolution and were eluted at retention times

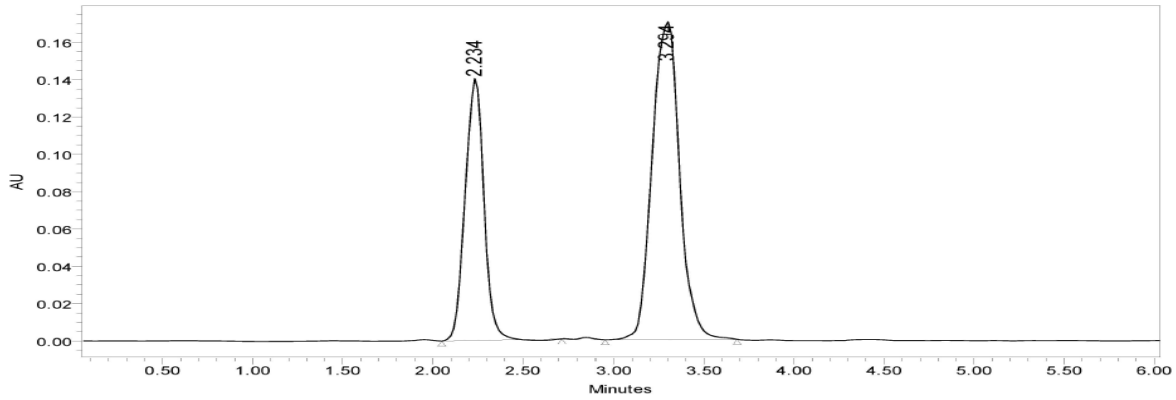

Figure 8: Chromatogram showing thermal degradation studies.

S.No.	Peak Name	R_t	Area	Height	USP Tailing	USP plate count
1	Amlodipine	2.234	1181262	198698	1.2	4821
2	Olmesartan	3.294	1432363	169587	1.4	3365

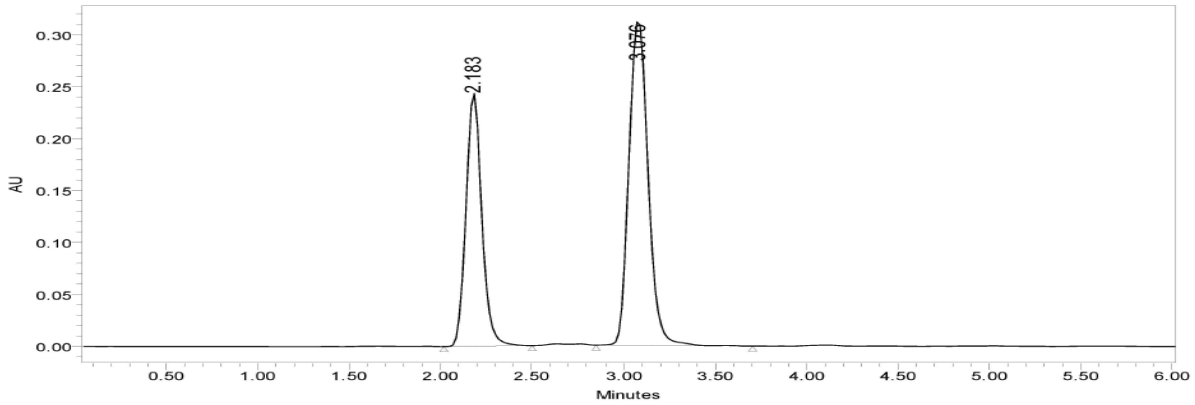

Figure 9: Chromatogram is showing photolytic degradation.

S.No.	Peak Name	R_t	Area	Height	USP Tailing	USP plate count
1	Amlodipine	2.183	1212073	186954	1.0	4857
2	Olmesartan	3.076	1458373	169587	1.2	3635

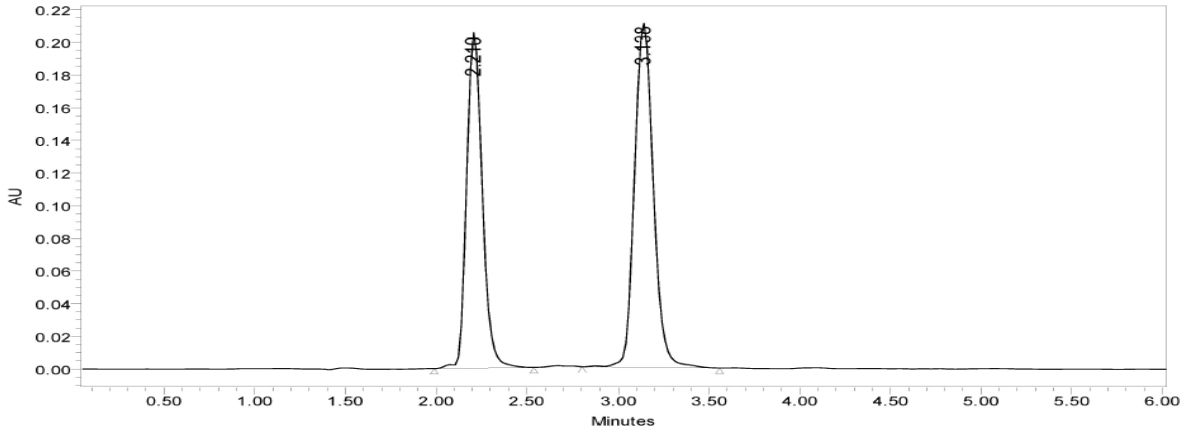

Figure 10: Chromatogram shows oxidative degradation.

S.No.	Peak Name	R_t	Area	Height	USP Tailing	USP plate count
1	Amlodipine	2.210	1120882	198596	1.2	4635
2	Olmesartan	3.138	1348651	177854	1.5	3458

around 2.39 min, 3.33 min with symmetric peak shape. The detection is performed at the wavelength 258 nm[7].

Running the standard solution of Amlodipine

1 ml of stock solution (1000ppm) was pipetted out into a 100 ml volumetric flask. The volume was made up to the mark with mobile phase. The solution was filtered through the 0.45 □m membrane filter and degassed under

Table 5: Results of method precession for Amlodipine.

S. No.	Peak name	Rt	Area (µV*sec)	USP Plate Count	USP Tailing
1	Amlodipine	2.234	1010585	1.0	3802
2	Amlodipine	2.261	1011075	1.1	3546
3	Amlodipine	2.183	1011924	1.4	4633
4	Amlodipine	2.244	1014299	1.1	4812
5	Amlodipine	2.458	1022159	1.0	3802
	Mean		1014008		
	Std. Dev		4774.567		
	% RSD		0.470861		

Table 6: Results of method precession for Olmesartan.

S.No.	Peak Name	Rt	Area (µV)	USP Tailing	USP Plate Count
1	Olmesartan	3.294	1513391	1.2	4759
2	Olmesartan	3.191	1513391	1.1	3695
3	Olmesartan	3.076	1526673	1.1	4741
4	Olmesartan	3.166	1560819	1.2	3793
5	Olmesartan	3.319	1560819	1.1	4741
	Mean		1535019		
	Std. Dev.		24168.56		
	% RSD		1.57448		

Table 7: Accuracy studies for Amlodipine.

% Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% recovery	Mean Recovery
80%	605652.5	4	4.0	100.0%	
100%	1246314	5	4.94	98.0%	99.9%
120%	1869868	6	6.1	101.6%	

Table 8: Accuracy results for Olmesartan.

% Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
80%	774787.7	16	15.9	99.37%	99.8%
100%	1537580	20	19.9	99.5%	
120%	2285575	24	24.1	100.4%	

ultrasonic bath prior to use. The solution was injected into the HPLC system. The chromatogram obtained is shown in figure 3.

Running the standard solution of Olmesartan

4 ml of stock solution was pipetted into a 100 ml volumetric flask. The volume was made up to the mark with mobile phase. The solution was filtered through the 0.45 µm membrane filter and degassed under ultrasonic bath prior to use. The solution was injected into the HPLC system. The chromatogram obtained is shown in figure 4.

Running the standard solution of Amlodipine and Olmesartan

1 ml of AML stock solution and 4 ml OLM stock solution was pipetted into a 100 ml volumetric flask. The volume was made up to the mark with mobile phase. The solution was filtered through the 0.45 µm membrane filter and degassed under ultrasonic bath prior to use. The solution was injected into the HPLC system. The chromatogram obtained is shown in figure 5.

RESULTS AND DISCUSSION

Method development and optimization

The main target of the chromatographic method is to get the separation of closely eluting drugs Amlodipine and Olmesartan, The drugs were co-eluted by using different stationary phases like C18, C8 with varying lengths and different mobile phases containing buffers like phosphate, sulphate and acetate with different pH (2-7) and using organic modifiers like acetonitrile, methanol and ethanol in the mobile phase. pH of the buffer has played a significant role in achieving the separation between drugsl. The chromatographic separation was achieved on a stainless steel column (4.6 x 250mm) column packed with Octa decyl silane bonded to porous silica (C18) with particle size 5 micron, by using solutions TEA Buffer and Acetonitrile in the ratio of (25:75), pH adjusted to 3 using ortho phosphoric acid. The flow rate of the mobile phase was maintained at 1.0 ml/min. At 25^0 C of column temperature, the peak shape of AML AND OLM was found symmetrical with mobile phase 60:40 ratio. In the optimized conditions AML AND OLM were well separated with a good resolution and the typical retention times of AML AND OLM were about 2.3 min and 3.3 min, respectively. The system suitability results are given in table no.1 and the developed LC method was validated.

Table 9: Results of specificity studies for Amlodipine and Olmesartan.

Speficity data for Amlodipine						
% Concentration (at specification Level)	Area	Drug Added (mg)	Excipient Added (mg) (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	444310	5	2.5	`4.98	99.6%	
100%	885413	5	5	4.97	99.7%	99.6%
150%	1319238	5	7.5	4.96	99.7%	
Specificity data for Olmesartan						
%Concentration (at specification Level)	Area	Drug Added (mg)	Excipient Added (mg) (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	50577	20	10	19.97	99.4%	
100%	104365	20	20	19.96	99.6%	99.5%
150%	156541	20	35	19.95	99.6%	

Table 10: Results of robustness for Amlodipine.

Change in parameter	% RSD
Flow (1.1 ml/min)	1.03
Flow (0.9 ml/min)	0.68
Temperature (27^0C)	0.42
Temperature (23^0C)	0.57
Wavelength of Detection (250 nm)	0.23
Wavelength of detection (266 nm)	0.12

Stability Studies

Acid Hydrolysis

An accurately weighed 25 mg of pure drugs AML and 100 mg of OLM were transferred to a clean & dry 25 ml volumetric flask separately. To which 0.1 N Hydrochloric acid was added & made up to the mark & kept for 24 hrs. From both drug solutions 0.5 ml was taken and transferred in to a 50 ml volumetric flask & made up to the mark with mobile phase, then injected into the HPLC system against a blank of HCl (after all optimized conditions).

Basic Hydrolysis

An accurately weighed 25 mg of pure drugs AML and 100 mg of OLM were transferred to a clean & dry 25 ml volumetric flask separately. To which 0.5N Sodium hydroxide was added & make up to the mark & kept for 24 hrs, From both drug solution 0.5 ml was taken in to a 50 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of NaOH (after all optimized conditions).

Dry Heat Degradation

An accurately weighed 25 mg of pure drugs AML and 100 mg of OLM were transferred in to a 25 ml volumetric flask, volume was made up to the mark with mobile phase & maintained at 50 ^{0}C for 24 hrs. From both drug solutions 0.5 ml was taken in to a 50 ml volumetric flask & make up to the mark with mobile phase. Further it is injected into the HPLC system against a blank of mobile phase.

Photolytic Degradation

Approximately 25 mg of pure drugs AML and 100 mg of OLM were taken in a clean & dry Petri dish. It was kept in a UV cabinet at 254 nm wavelength for 24 hours without interruption. Accurately weighed 1 mg of AML and 4 mg of OLM the UV exposed drug was transferred to a clean & dry 100 ml. volumetric flask. First the UV exposed drug was dissolved in methanol & make up to the mark. Then injected into the HPLC system against a blank of mobile phase (after all optimized conditions).

Oxidation With (3%) H_2O_2

Accurately weighed 1 mg of AML and 4 mg of OLM of pure drugs were taken in a clean & dry 100 ml. volumetric flask. 30 ml. of 3% H_2O_2 and a little methanol was added to it to make it soluble & then kept as such in dark for 24 hours. Final volume was made up to 100 ml. using water to prepare 10 ppm and 40 ppm of AML and OLM solution respectively. The above sample was injected into the HPLC system.

Results of forced degradation studies

The results of the stress studies indicated the specificity of the method that has been developed. Amlodipine and Olmesartan were stable in photolytic, thermal and basic stress conditions. The result of forced degradation studies are given in the following table 2.

Results of method validation

Linearity

Linear calibration plot for assay method was obtained over the calibration ranges tested, i.e. 1- 3 µg/ml for Amlodipine and 2µg/ml to 30µg/ml for Olmesartan and the correlation coefficient obtained was greater than 0.999. The results show that an excellent correlation existed between the peak area and concentration of the analyte which is given in table 3 and 4.

Intermediate precision/ruggedness

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day by using different make column of same dimensions.

Recovery and accuracy

The percentage recovery of AML and OLM in bulk drugs samples was ranged from 99.4 - 99.6% which indicates that the method was accurate which is given in table no.7.

Accuracy results

The accuracy of the method was determined by preparing solutions of different concentrations of AML and OLM that is 80%, 100% and 120% in which the amount of marketed formulation (AML and OLM 5 mg and 20 mg respectively) was kept constant and the amount of pure drug was varied that is 4 mg, 5mg and 6mg for AML and

Table 11: Results of robustness for Olmesartan.

Change in parameter	% RSD
Flow (1.1 ml/min)	0.03
Flow (0.9 ml/min)	0.08
Temperature (27^0C)	0.19
Temperature (23^0C)	0.73
Wavelength of Detection (250 nm)	0.82
Wavelength of detection (266 nm)	0.46

16mg, 20mg and 24 mg for OLM i.e. 80%, 100% and 120%respectively. The solutions were prepared in triplicates and the accuracy. similarly was indicated by % recovery in table 7 and 8.

Specificity

5mg/ml of AML was spiked with 50% (2.5mg), 100% (5mg), and 150% (7.5mg) of excipient mix (Magnesium Stearate), Further 01 ml is pippeted out from the all three samples and diluted to 100 ml in three separate volumetric flask, and analysed for % recovery of AML.Similarly 20 mg/ml OLM sample were prepared and analysed.

LOD and LOQ

Detection limit and Quantitation limit of described method were observed as 0.653 mg/ml and 1.959 mg/ml for AML, 0.646 mg/ml and 1.638 mg/ml for OLM,

Robustness

Robustness is a measure of capacity of a method to remain unaffected by small, but deliberate variations in the method conditions, and is indications of the reliability of the method. A method is robust, if it is unaffected by small changes in operating conditions. To determine the robustness of this method, the experimental conditions were deliberately altered at three different levels and retention time and chromatographic response were evaluated. One factor at a time was changed to study the effect. Variation of wavelength (235 and 239 nm) and mobile phase flow rate by 0.1 ml/min (0.9 and 1.1ml/min) had no significant effect on the retention time and chromatographic response of the 50 µg/ml solution, indicating that the method was robust.

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature ($\pm 2^0$C), Wavelength of detection (±2nm) & Acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-19, % RSD < 2%) the developed RP-HPLC method for the analysis of Amlodipine.

CONCLUSION

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of Amlodipine and Olmesartan was done by RP-HPLC. The proposed method was found to be simple, precise, accurate and rapid for determination of AML and OLM in pure and dosage form. The mobile phase is simple to prepare and economical. The sample recoveries in all formulations were in good agreement within the limit. Hence, this method can be easily and conveniently adopted for routine analysis of AML and OLM in pure form and dosage form.

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