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Validation and Development of Reverse-Phase High-Performance Liquid Chromatography Methods for Anti-hypertensive Drugs

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Objective: This work aimed to establish a simple and accurate approach employing RP-HPLC technology for estimating the amounts of azilsartan and chlorthalidone in tablet and bulk form.
Methods: The mobile phase is a blend of buffer and acetonitrile in a 30:70 (v/v) ratio. It is passed through a 5 µm particle size Thermo Scientific Syncronis C-8 column, measuring 250 x 4.6 mm (i.d.). The flow rate of the mobile phase is 1.2 milliliters per minute. A constant 25°C was kept in the column oven. A wavelength of 254 nanometers was chosen. Using the diluent, stock, and working solutions, they were made. A ten-minute runtime was established, and the experiment was carried out in the mobile phase.
Results: Chlorthalidone and azolsartan separated adequately at retention times of 2.3 and 4.6, respectively. Plate count, tailing factor, and other system compatibility criteria are used to determine the permissible range set by the International Council for Harmonization (ICH). According to linearity studies, the linear relationship between AzilsartanMedoxomil and Chlorthalidone was linear in the concentration range of 10–50 µg/ml. For chlorthalidone, the regression equation and correlation coefficient were R2 = 0.9983 and y = 8792x + 1660.9, respectively; for AzilsartanMedoxomil, they were y = 27382x - 5157 and R2 = 0.9996. Azilsartan, Medoxomil, and Chlorthalidone were found to have recovery percentages between 99.89% and 99.96%, indicating a high degree of accuracy in the suggested task. After analysis, the concentration of the tablet was found to be 100.15%.
Conclusion: According to the study, every parameter met the requirements outlined in the guidelines of the "International Council for Harmonization" (ICH). Furthermore, the method was simple and economical because the retention times

were lower than those documented in the literature, which led to a shorter run time overall.

1. Introduction

Azilsartan is a substance that is precisely Benzimidazole-7-carboxylic acid, a subclass of benzimidazole-carboxylic acids. 2-Ethoxy-3-[[4-[2-(5-oxo-4H-1,2,4-oxadiazol-Benzimidazole-4-carboxylic acid [1] (Fig 1). One drug that is categorized as an angiotensin 2 receptor blocker is Abelian. The mechanism of action of azalisartan is influenced by its role as an antagonist of the Angiotensin 2 Type 1 receptor [2]. Azilisartan's physiological effect is attained by lowering blood pressure. A medication called azilartan is administered to treat high blood pressure. It belongs to the class of angiotensin II receptor blockers (ARBs) [3]. There are no documented examples of acute liver injury associated with it, even though it is linked to the uncommon occurrence of transient elevations in serum aminotransferase levels. Chlorthalidone is a sulfonamide that belongs to the monochlorobenzene and isoindole families of chemicals [4]. The molecule is known as 2-chloro-5-(1-hydroxy-3-oxo-2H-isoindol-1-yl) benzene sulfonamide (Fig 2). Chlorthalidone is a thiazide-type diuretic that is prescribed to treat high blood pressure and manage fluid retention caused by illnesses such as heart failure or renal failure [5]. Chlorthalidone reduces water absorption, and edema, and improves blood pressure management by inhibiting the Na+/Cl− symporter in the kidneys' distal convoluted tubule cells. The precise mechanism by which chlorthalidone decreases blood pressure is still up for discussion [6]. Conversely, it is believed that the drug's ability to elicit diuresis results in a reduction in extracellular fluid and plasma, which lowers cardiac output and thus lowers blood pressure.

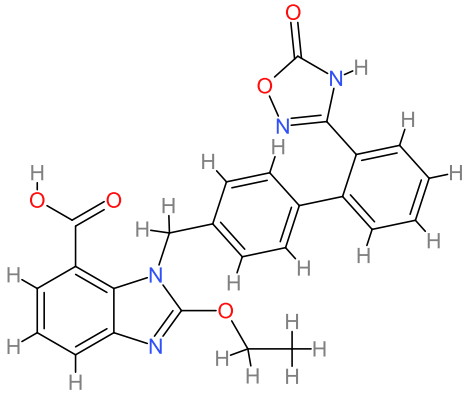

Fig. 1: Chemical Structure of Azilsartan.

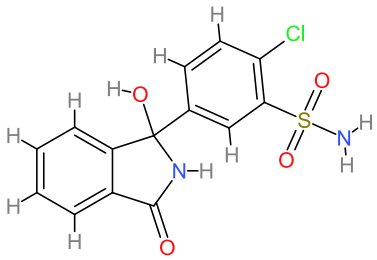

Fig. 2: Chemical Structure of Chlorthalidone.

2. Materials and Methods

Chemicals regents

The FDA in Uttarakhand provided us with complimentary samples of azilartan and chlorthalidone. The commercially available Zilarta-CT 40 formulation was obtained from Microlabs Ltd. in the local market, while Merck Life Sciences Pvt. Ltd. in Mumbai supplied HPLC-grade acetonitrile, methanol, and water. In Mumbai, S D Fine-Chem Ltd. was the supplier of potassium dihydrogen phosphate. Specialty of Merck Pvt. Ltd. The remaining reagents were all analytical grade.

Instrument

The Shimadzu HPLC i-Series LC-2050 HPLC system, which has a UV detector, is the one being used. Thermo Scientific Syncronis C-8, 5 µm particle size, Dim. (mm) 250x4.6 i.d. is the column that is being applied. Instrument components are detailed in (Table 1.)

Table 1: HPLC Instrument Information.

Components of Instruments	Details
System	HPLC (Binary Gradient System)
Model no.	HPLC (i- Series LC -2050)
Company	Shimadzu Corporation
Pump	Shimadzu
Column	Thermo Scientific Syncronis C 8, 5 µm particle size, Dim. (mm) 250x4.6 i.d.
Detector	UV
Software	Lab Solutions

Preparation of a standard solution

A separate 100 ml volumetric container should be used to hold about 50 mg of Azilsartan, Chlorthalidone working standard, which should be weighed exactly. To dissolve the material in the flask, add 30 milliliters of diluent sonicate. Additionally, make a 50 ml solution of 1 ml chlorthalidone and 4 ml azilsartan by properly combining the diluent and passing it through a 0.45µm nylon syringe filter. It is recommended to discard the first 5 milliliters of filtrate. Adjust its concentration to the proper level

Sample solution

To make twenty tablets equal forty milligrams of Azilsartan, weigh and crush the tablets precisely so that the powder is fine. The powdered material should be poured into a 100-

milliliter volumetric flask. Add approximately 60.0 ml of methanol after 15 minutes of sonication. Mark correction: Apply methanol. It is also necessary to dilute 5 ml of this solution with 50 ml of diluent mix very thoroughly before filtering through a 0.45µm nylon syringe filter. The filtrate's first five milliliters should be discarded

Method Development

Preparation of buffer for the Mobile phase

Measure/, then transfer to bring the pH to 3.0, 2.7 grams of potassium dihydrogen phosphate, 1000 milliliters of Milli Q water, and diluted orthophosphoric acid is mixed with a 0.45µm membrane filter.

Mobile phase

(Buffer: Acetonitrile) (30: 70) v/v.

Diluents

Mobile phase

Method Validation

The following parameters—specificity, linearity, sensitivity, precision, accuracy, and stability of analytical solutions—were used to validate the developed RP-HPLC technique. The International Conference on Harmonization (ICH) recommendations for the validation of analytical processes were followed in the execution of the validation. [7]

System suitability and system repeatability

The assessment of system appropriateness and repeatability was conducted by injecting two standards into the chromatographic system. The %RSD for peak area, %RD between standards, retention duration, tailing factor, theoretical plates, and resolution were computed and recorded.

Specificity

To show that there is no interference with the elution of eptifibatide in standard samples or pharmaceutical formulations, the specificity and selectivity of the method were examined by injecting blank samples (deionized water and citric acid buffer as excipient)

Linearity

To examine the linearity of the analytical process, three independent replicates of five working standard solutions at varying concentration ranges (10-50µg/ml) were introduced into the HPLC system. A calibration curve was created by graphing the average peak area on the Y-axis and the concentration of eptifibatide on the X-axis to establish that the experimental response was directly proportional to the analyte concentration. Linear regression analysis was used to compute the regression equation and the co-relation coefficient value

Sensitivity

The limit of quantification (LOQ) and the limit of detection (LOD) were used to determine the analytical technique's sensitivity. Based on a signal-to-noise ratio of 3:1 and 10:1, respectively,

LOD and LOQ were determined. The lower limit of quantification (LOQ) for eptifibatide that could be obtained quantitatively with reasonable precision and accuracy was defined [8].

Precision and accuracy

Three distinct concentration levels of QC samples (0.375, 0.75, and 1.5 mg/mL) were examined three times in a single day (intra-day precision) and three times in a series of days (inter-day precision). By computing the relative standard deviation (RSD) values for intra-day and inter-day analyses with acceptance criteria of no more than 2%, the suggested method's precision was determined. Methodological recovery was used to evaluate the measurement method'saccuracy. The recovery of the procedure was represented as a percentage variation between the theoretical concentration and the determined concentration of the QC sample [9,10].

Robustness

Robustness testing was performed using filter compatibility tests, varied sonication durations, and deliberate modifications of chromatographic parameters, such as temperature and wavelength, to evaluate the system's stability and performance under many situations.

Table 2: System Suitability Parameters.

System suitability parameters	**Azilsartan**	**Chlorthalidone**
Retention time	5.28	2.40
Theoretical plate no	9593	4355
Tailing factor	1.227	1.536
Resolution	---	---

Stability of analytical solutions

To assess sample stability throughout the study, repeated analysis (N = 3) was used to verify sample stability under three distinct settings. Following 24 hours of short-term storage (at 25°C), two weeks of long-term storage (at 2–8°C), and three cycles of freeze-thaw (from -20 to room temperature every 24 hours), aged QC samples were reanalyzed against a freshly made standard solution [11].

Range

The linearity and precision of Azilsartan and Chlorthalidone were validated within a concentration range of 10–50 µg/ml. The method's range was determined from the outcomes of linearity, accuracy, and precision assessments, providing dependable quantification and uniform performance within this concentration range.

3. RESULT AND DISCUSSION:

Development and optimisation of chromatographic methods

The mobile phase employed in the experiment consists of a buffer and acetonitrile in a 30:70 (v/v) ratio. The mixture is processed using a Thermo Scientific Syncronis C-8 column, with a particle size of 5 µm and dimensions of 250 x 4.6 mm (i.d.). The stationary phase is kept at an ambient temperature of approximately 25 °C throughout method development and optimisation. The mobile phase flow rate is established at 1.2 millilitres per minute, while the

column oven temperature is maintained at a constant 25°C. The selected detection wavelength for the experiment is 254 nanometres. Stock and working solutions were formulated with the suitable diluent. The experiment's overall duration was set at 10 minutes, during which the mobile phase facilitated sample analysis. This configuration enabled efficient technique development and optimisation, assuring repeatability and accuracy in the outcomes.Summary of Optimised Chromatographic Conditionsshown (Table3.)

Table-3: Summary of Optimised Chromatographic Conditions.

Instrument	**HPLC Equipped with UV Detector**
'Column'	Thermo Scientific Syncronis C 8, 5 µm particle size, Dim. (mm) 250x4.6 i.d.
'Detection wavelength'	254 nm
'Column oven temperature'	25^0C
'Sampler cooler temperature'	25^0C
'Flow rate'	1.2 ml/min
'Injection volume'	20
'Run time'	10 min

Method validation

System suitability and repeatability

System suitability and repeatability are critical elements of analytical methods to guarantee the reliability of the chromatographic system. System reproducibility was assessed using the parameters specified in (Table 2.) These evaluations are vital for confirming the system's performance, ensuring consistent outcomes, and preserving method accuracy.[12,13]

Specificity

The absence of interference at the peaks of Azilsartan and Chlorthalidone indicates the specificity of the approach (Fig. 3,4).

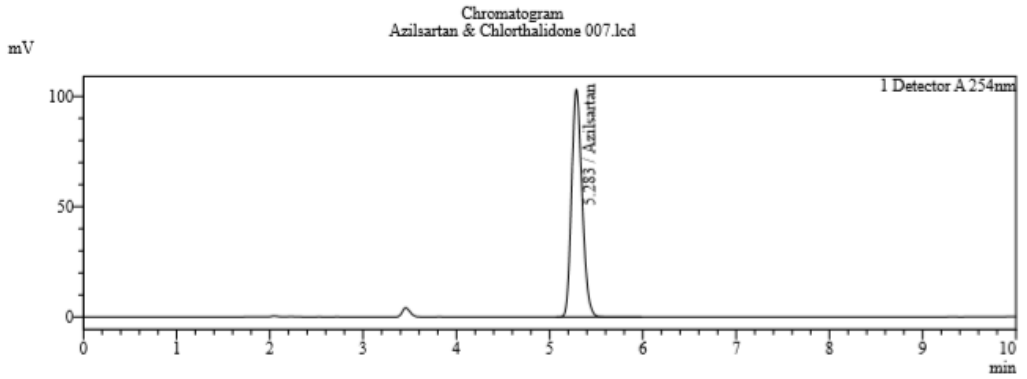

Fig. 3: RT Chromatogram of standard solution for Azilsartan

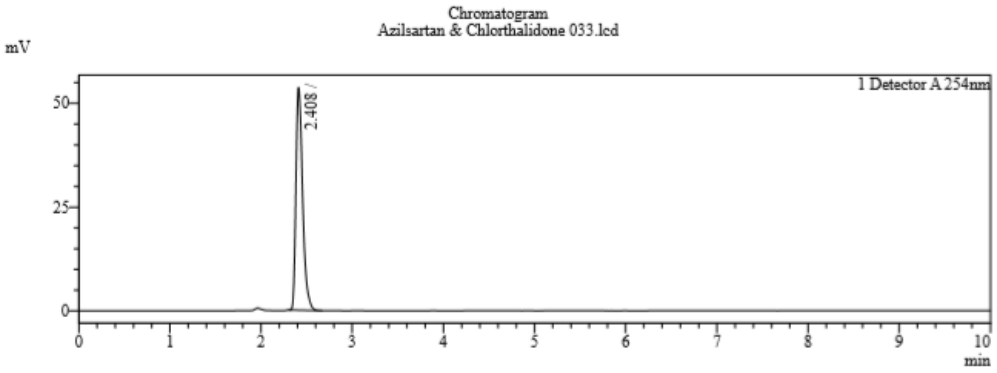

Fig. 4: RT Chromatogram of sample solution for Chlorthalidone.

Linearity

The approach exhibited remarkable linearity within the concentration range of 10-50 µg/mL for both Azilsartan (R^2 = 0.9996) and Chlorthalidone (R^2 = 0.998), confirming its appropriateness for analysis. The linearity curves illustrated in Figure 6 validate the method's accuracy and precision (Table 4).

Table 4: Linearity Studies for Azilsartan& Chlorthalidone.

Drug	Concentration (µg/ml)	Area
Azilsartan	10	2,70,167
	20	5,41,498
	30	8,07,975
	40	10,77,944
	50	13,78,782
Chlorthalidone	10	89,968
	20	1,79,969
	30	2,71,376
	40	3,40,775
	50	4,46,672

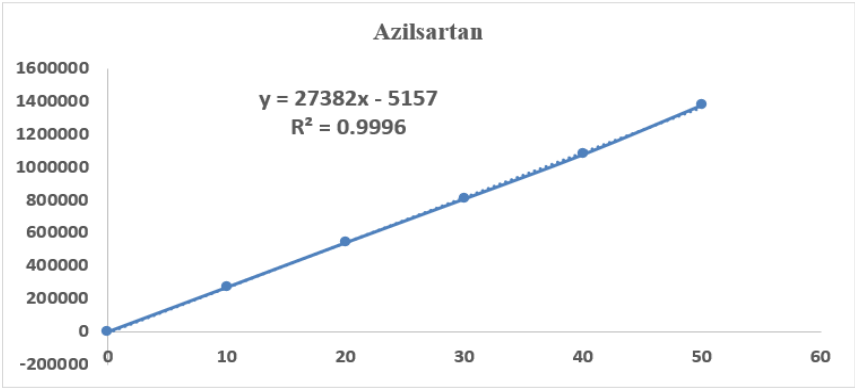

Fig. 5: Calibration Curve of Azilsartan.

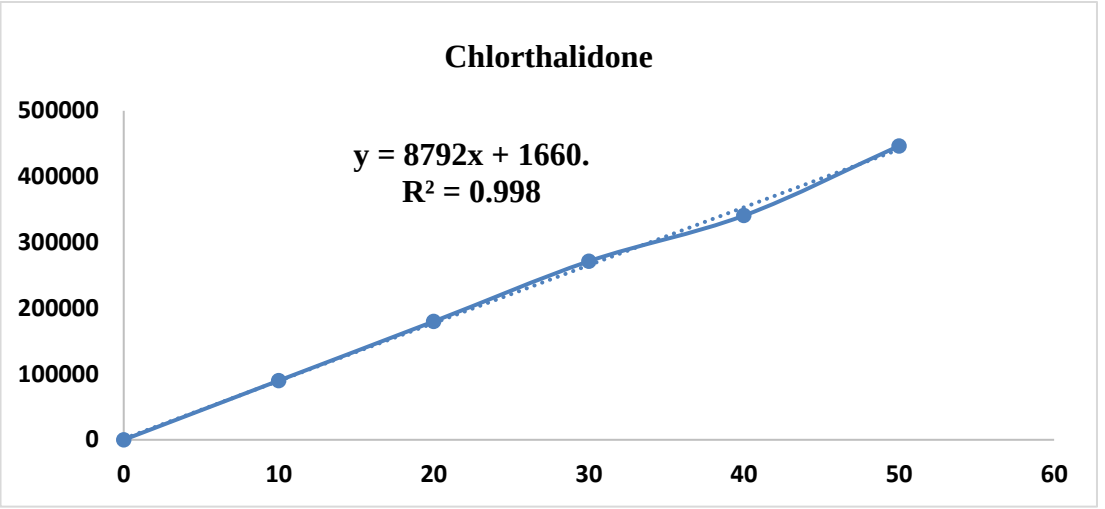

Fig. 6: Calibration Curve of Chlorthalidone.

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

(Table 5) shows the LOD and LOQ for Azilsartan and Chlorthalidone. For Azilsartan, the LOD was 3.10 µg/mL and the LOQ was 10.32 µg/mL. For Chlorthalidone, the LOD was 7.14 µg/mL and the LOQ was 23.81 µg/mL. The LOD and LOQ show the method's sensitivity, with the LOD being the lowest detectable concentration and the LOQ the least measurable concentration with acceptable accuracy and precision These findings demonstrate the method's ability to identify and quantify both substances.[14,15]

Table 5: LOD and LOQ Studies for Azilsartan and Chlorthalidone.

Parameters	**Azilsartan (µg/ml)**	**Chlorthalidone (µg/ml)**
LOD	3.10	7.14
LOQ	10.32	23.81

Accuracy

The average percentage recovery findings for Azilsartan at recovery levels of 50%, 100%, and 150% were 99.56%, 100.08%, and 99.73%, respectively. The typical recoveries for Chlorthalidone were 99.6%, 100.42%, and 100.20%. The results in (Table 6,7.) indicate that the technique exhibits good accuracy and is not influenced by excipient interference, hence affirming its trustworthiness for the studies of Azilsartan and Chlorthalidone.

Table 6: Data for Accuracy study of Azilsartanby HPLC method

Conc (%)	Sample amount (µg/ml)	Sample amount (µg/ml)	Amount recovered (µg/ml)	% recovery	% mean recovery
50 %	20	10	30.1	100.33	99.56
	20	10	29.8	99.33	
	20	10	29.7	99.00	
100%	20	20	40.2	100.50	100.08
	20	20	39.8	99.50	
	20	20	40.1	100.25	
150%	20	30	50.3	100.60	99.73
	20	30	49.8	99.60	
	20	30	49.5	99.00	

Table 7: Data for Accuracy study of Chlorthalidone by HPLC method.

Conc (%)	Sample amount (µg/ml)	Sample amount (µg/ml)	Amount recovered (µg/ml)	% recovery	% mean recovery
50 %	20	10	29.5	98.33	99.67
	20	10	30.5	101.67	
	20	10	29.7	99.00	
100%	20	20	40.6	101.50	100.42
	20	20	39.9	99.75	
	20	20	40.0	100.00	
150%	20	30	50.3	100.60	100.20
	20	30	49.2	98.40	
	20	30	50.8	101.60	

Repeatability and Precision:

The established method demonstrates excellent repeatability and precision, with % RSD values for the assay remaining below 2.0 for both Azilsartan and Chlorthalidone. These findings, outlined in (Table 8,9,10.) illustrate the method's robustness in terms of repeatability and intermediate precision, providing consistent and dependable performance.

Repeatability:

Table 8: Data for Repeatability study of Azilsartan and Chlorthalidone by HPLC method.

Repeatability (Azilsartan) µg/ml	**Area**	**Repeatability** (Chlorthalidone.) µg/ml	**Area**
30	8,07,023	30	2,73,796
30	8,07,123	30	2,73,610
30	8,07,035	30	2,72,796
30	7,98,874	30	2,71,501
30	8,06,731	30	2,70,795
Mean	8,05,357	Mean	2,72,500
SD	3627.23	SD	1313.81
%RSD	0.45	%RSD	0.48

Intraday precision:

Table 9: Data for Precision study of Azilsartan and Chlorthalidone by HPLC method.

Azilsartan(30 µg/ml)		**Chlorthalidone (30 µg/ml)**	
	Area		Area
	8,14,319		2,74,030
	8,06,117		2,72,345
Morning	8,05,674	**Morning**	2,74,200
	7,95,786		2,70,988
	8,10,944		2,71,243
Evening	8,06,107	**Evening**	2,72,305
Mean	806491.2	**Mean**	272518.5
SD	6271.50	**SD**	1353.30
% RSD	0.78	**% RSD**	0.50

Inter day precision:

Table 10: Data for Precision study of Azilsartan and Chlorthalidone by HPLC method.

Azilsartan(30 µg/ml)		**Chlorthalidone (30 µg/ml)**	
Morning	Area	**Morning**	Area
	8,15,391		2,74,171

	8,09,593		2,74,246
	8,08,519		2,74,241
Evening	8,05,531	**Evening**	2,74,005
	8,00,969		2,73,465
	7,89,845		2,72,987
Mean	804974.7	**Mean**	273852.5
SD	8805.15	**SD**	515.70
% RSD	1.09	**% RSD**	0.19

Robustness

The robustness testing was carried out by utilising the change in temperature and wavelength, which resulted in the calculation of the findings in (Table 9,) and the RSD was found to be not more than 2%. [16.17]

Table 11: Data for Robustness study of Azilsartan and Chlorthalidone by HPLC method.

Parameters	Azilsartan(30 µg/ml)	Chlorthalidone (30 µg/ml)
Wavelength	14,91,429	3,06,464
	14,80,323	3,06,654
	14,68,287	3,05,598
Mean	14,80,013	3,06,239
SD	11574.11	562.91
RSD	0.78	0.18
Temperature	14,81,770	2,71,299
	14,62,661	2,68,512
	14,79,451	2,70,987
Mean	14,74,627	2,70,266
SD	10427.81	1527.00
RSD	0.71	0.56

Analytical solution stability

To ensure sample stability throughout the investigation, repeated analysis (N = 3) was performed in three different conditions. After 24 hours of short-term storage (25°C), two weeks of long-term storage (2–8°C), and three cycles of freeze-thaw (-20 to room temperature every 24 hours), aged QC samples were reanalysed against a freshly prepared standard solution.

Assay:

To prepare twenty tablets equivalent to forty milligrammes of Azilsartan, accurately weigh and finely crush the tablets. Transfer the powdered substance into a 100-milliliter volumetric flask. After 15 minutes of sonication, add approximately 60.0 ml of methanol. The assay results for Azilsartan and Chlorthalidone at concentrations of 40 and 10 µg/ml are 99.81% and 100.14%, respectively, as indicated in (Table 13.). Chromatograms of the standard solution and the test solution for Azilsartan and Chlorthalidone are shown in Fig. 7 and 8.

Table 12: Assay data Azilsartan and Chlorthalidone by HPLC method.

Drug name	Composition (µg/ml)	Area of standard	Area of sample	% Assay
Azilsartan	40	1080396	1061441	99.81
Chlorthalidone	10	109611	107657	100.14

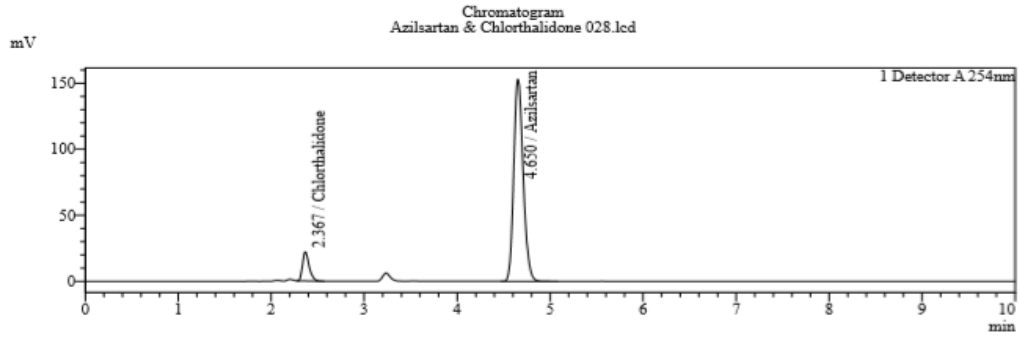

Fig. 7: Chromatogram of standard solution for Azilsartan and Chlorthalidone

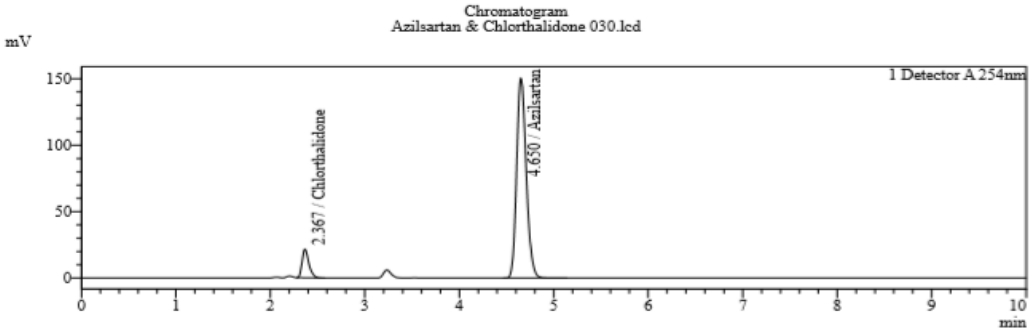

Fig. 8: Chromatogram of sample solution for Azilsartan and Chlorthalidone.

The applied analytical method was Shimadzu HPLC i-Series LC-2050, an HPLC system with a UV detector, is the one beingused. A Thermo Scientific Syncronis C 8 column with a 5 µmparticle size and dimensions of 250x4.6 i.d. is being used. A variety of mobile phase combinations were examined to assess how they affected other system suitability variables such as capacity factor, theoretical plate count, and resolution. The separation was ultimately accomplished using a recently constructed mobile phase. In the mobile phase, acetonitrile and pH 3.0 phosphate buffers are combined. A 1.2 milliliter per minute flow rate wasused. It was discovered at a wavelength of 254 nm.

4. Conclusion

The separation of chlorthalidone and azalisartan run times of 10 minutes showed good resolution. The standards of the ICH are met by metrics for system suitability like plate count and tailing factor. The linearity study revealed a linear relationship between 10–50 µg/ml of AzilsartanMedoxomil and Chlorthalidone In the mobile phase, it travels at 1.2 ml/min. A constant temperature of 25 °C is maintained in the column oven. A wavelength of 254 nanometers was used. The mobile phase was 30:70 v/v acetonitrile in a pH 3.0 phosphate buffer. It was measured at 254 nm, and the flow rate was regulated to 1.2 ml/min. Retention times for the medicines after separation and elution were 2.3 and 4.6, respectively.

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