

RP-HPLC Estimation of Azelnidipine and Chlorthalidone in Bulk and Combined Dosage Forms

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ABSTRACT

Objectives: A simple, sensitive, robust, precise and efficient RP-HPLC approach for the simultaneous determination of Azelnidipine and Chlorthalidone in combination. **Materials and Methods:** As per ICH Q2 (R1) guidelines, the final chromatographic conditions were optimized with a mobile phase ratio of (30:70% v/v) in Water containing 0.1% formic acid: Methanol containing 0.1% formic acid at a flow rate of 1 mL/min, column temperature of 35°C, injection volume of 20 µL, Interstil C₁₈ analytical column. **Results:** Azelnidipine and Chlorthalidone reported retention times of 2.32 min and 4.09 min, respectively. Validation of method was found to be linear in the range of 0.4-1.2 µg/mL for Azelnidipine and 0.63-1.88 µg/mL for Chlorthalidone. The % Recovery was found to be 99.96-100.18% and 99.91-100.71 for Azelnidipine and Chlorthalidone respectively. The Precision results for both drugs were within the limits while expressed Intraday and Interday. For Azelnidipine, the LOD and LOQ were reported to be 0.164 µg/mL and 0.498 µg/mL, respectively and for Chlorthalidone, 0.148 µg/mL and 0.448 µg/mL. As per ICH Q1A (R2) guidelines, the combination was subjected to acid, base, oxidation, thermal and photolysis stress conditions. **Conclusion:** The results suggest that the developed method could be used for the routine analysis of the drug sample in bulk and combined dosage forms for the determination of shelf life and storage conditions of the formulations.

Keywords: RP-HPLC, Azelnidipine, Chlorthalidone, Forced Degradation Studies, ICH Guidelines.

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Received: 23-05-2025;

Revised: 14-07-2025;

Accepted: 01-09-2025.

INTRODUCTION

Analytical chemistry is the field dedicated to determining the composition and quantity of matter. It encompasses both qualitative and quantitative aspects. Qualitative analysis involves identifying elements, functional groups, or compounds in a sample, while quantitative analysis measures the precise amount of a specific element, species, or compound within the sample. This is vital for ensuring acceptable levels of impurities in samples. Analytical chemistry has diverse applications across industries like pharmaceuticals, food and beverages, forensic science and the oil sector, playing a crucial role in identifying and quantifying substances. Moreover, it aids in detecting adulterants and determining product shelf life.^{1,2}

To carry out analytical procedures on chemical samples, it's necessary to establish specific experimental conditions, known as analytical method development. This involves selecting a precise assay approach to determine a formulation's composition,

ensuring consistent results and setting criteria tailored to the product.³⁻¹¹

Method validation is the process of substantiating that an analytical technique can consistently produce results meeting predefined criteria. It demonstrates the method's suitability for analyzing future samples in a laboratory context. Typically, method validation adheres to ICH Guidelines and involves evaluating parameters like specificity, accuracy, precision, limit of detection, limit of quantitation, robustness and others.³⁻¹¹

Forced degradation studies are conducted to explore how a drug or substance degrades under diverse conditions. These studies assist in establishing storage requirements and shelf life. The ICH Guidelines specify the harsh conditions under which substances are tested, including acidic hydrolysis, basic hydrolysis, oxidative degradation, thermal degradation and photolytic degradation.³⁻¹¹

MATERIALS AND METHODS

Drug Identification

Chlorthalidone and Azelnidipine were chosen as the Active Pharmaceutical Ingredients (APIs) for the experimental study to verify their genuineness, standardization, quality and purity. The drug identification was confirmed on the basis of melting point and IP spectra.¹²⁻¹⁶



DOI: 10.5530/ijper.20265371

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Selection of Chromatographic Condition

The selection of the most suitable LC-MS/MS method depends on the nature of the sample, encompassing factors like the presence of ionic or neutral molecules, molecular weight, pKa and solubility. Following a literature review and considering aspects like simplicity and feasibility, LC-MS/MS for the separation process was initially opted. To refine the chromatographic conditions, the impact of parameters such as mobile phase composition, pH, flow rate and solvent ratio, investigated aiming to enhance the chromatographic settings.¹⁷⁻²⁰

Procedure for Solution Preparation

Preparation of Diluent

A solution was prepared by combining water and methanol in a ratio of 70% water to 30% methanol.

Preparation of Standard Stock Solution of Azelnidipine

Accurately weighed amounts of 8 mg of Azelnidipine API were separately introduced into distinct 100 mL volumetric flasks. Subsequently, they were dissolved in a diluent using ultrasonication and further diluted to the mark, yielding a stock solution with an Azelnidipine concentration of 80 µg/mL.

Preparation of Standard Stock Solution of Chlorthalidone

Accurate measurements of 12.5 mg of Chlorthalidone API were separately introduced into distinct 100 mL volumetric flasks. Subsequently, these were dissolved in the diluent using ultrasonication and further diluted to attain the prescribed concentration, yielding a Chlorthalidone stock solution with a concentration of 125 µg/mL.

Preparation of Working Standard Solution of Azelnidipine

1 mL was withdrawn from the Azelnidipine standard stock solution and suitably diluted with a diluent to reach a concentration of 0.800 µg of Azelnidipine per milliliter.

Preparation of Working Standard Solution of Chlorthalidone

1 mL was withdrawn from the aforementioned standard stock solution of Chlorthalidone and suitably diluted with a diluent to attain a concentration of 1.250 µg/mL for Chlorthalidone.

Combined Preparation of Working Standard Solution of Azelnidipine and Chlorthalidone

1 mL was withdrawn from the stock solutions of both Azelnidipine and Chlorthalidone and introduced into a 100 mL volumetric

flask. Subsequently, diluent was added to reach the required volume, resulting in Azelnidipine at a concentration of 0.800 µg/mL and Chlorthalidone at a concentration of 1.250 µg/mL.

Preparation of Mobile Phase

Mobile phase was prepared by blending 30% methanol with 70% water that includes 0.1% formic acid.

Preparation of Sample Working Solution of Chlorthalidone and Azelnidipine

Dilutions of the sample were prepared in a similar manner as that of the standard so as to achieve a concentration of 1.250 µg/mL for Chlorthalidone and 1.000 µg/mL for Azelnidipine.

Chromatographic Separation

Standard solutions of Chlorthalidone and Azelnidipine were injected into the column using a 20 µL micro-syringe and the system in the mentioned chromatographic conditions, provided the peak information, including area under the peak and retention time.

Validation of LC-MS/MS Method

Specificity

The blank solution, working standard solution and working sample solution of Chlorthalidone and Azelnidipine was introduced in the LC-MS/MS system. Importantly, the chromatogram of the blank remained unaffected by the chromatogram of either the standard or sample.

Linearity and Range

To assess the linearity of each drug, standard solutions with concentrations ranging from 0.630 to 1.880 µg/mL for Chlorthalidone and 0.400 to 1.200 µg/mL for Azelnidipine were analyzed. 0.500, 0.750, 1.000, 1.250 and 1.500 mL portions were pipetted from the stock solutions of Azelnidipine and Chlorthalidone. These were transferred to 100 mL volumetric flasks and appropriately diluted with diluent to obtain concentrations of 0.400, 0.600, 0.800, 1.000 and 1.200 µg/mL for Azelnidipine and 0.630, 0.940, 1.250, 1.560 and 1.880 µg/mL for Chlorthalidone. Correlation coefficients, intercepts and area under the curve were noted.

Precision

Repeatability

To assess repeatability, injected Azelnidipine (0.800 µg/mL) and Chlorthalidone (1.250 µg/mL) were injected into a standard solution 6 times. The peak areas were measured and percent R.S.D. was calculated.

Intraday Precision

On the same day, three analyses of a standard solution containing Azelnidipine (0.625, 1.250, 1.875 µg/mL) and Chlorthalidone (0.400, 0.800, 1.200 µg/mL) were performed.

Interday Precision

Azelnidipine (0.625, 1.250, 1.875 µg/mL) and Chlorthalidone (0.400, 0.800, 1.200 µg/mL) were introduced into a standard solution, which was evaluated three times on different days and the percent R.S.D. was calculated.

Accuracy

Three separate flasks, labeled A, B and C, each contained a 0.500 µg/mL drug solution. Each flask was spiked with 80%, 100% and 120% of the standard solution and diluted them to 100 mL. The peak area for each solution was calculated and the quantities of Azelnidipine and Chlorthalidone were determined at each level, calculating percentage recoveries.

Limit of Detection and Limit of Quantitation

LOD and LOQ were determined from the three calibration curves used to assess the method's linearity.

Robustness

To evaluate robustness, systematic changes were made to parameters and their effects were observed on system suitability for standard preparation. Specifically, the flow rate of the mobile phase was varied (± 0.2 mL/min) to 0.8 mL/min and 1.2 mL/min. Additionally, the ratio of the mobile phase (± 2) was adjusted between Buffer and Methanol, testing both (32:68) and (28:72) ratios.

Analysis of Market Formulation

Tablet powder containing 8.0 mg of Azelnidipine and 12.5 mg of Chlorthalidone was assessed. This powder was added to a 100 mL volumetric flask, shaken for 15 min. and then diluted to the desired volume. After filtering the solution through a 0.45 Millipore filter, the first few drops of the filtrate were discarded.

The solution was further diluted with diluent from 1 mL to 100 mL and 20 µL of the solution were injected into the LC-MS/MS apparatus. Peak areas were measured.

RESULTS AND DISCUSSION

Method Development

After a thorough exploration of various combinations of mobile phases, it was concluded that a mixture of water containing 0.1% formic acid and methanol containing 0.1% formic acid was the optimal choice. This selection was made due to the favorable peak shape and reduced retention time it offered. The chromatographic analysis was performed using an Intertsil ODS, C18 column with dimensions 100 mm \times 2.1 mm and a particle size of 1.6 µm. The mobile phase was delivered at a flow rate of 1.0 mL/min, with an injection volume of 20 µL. The column oven temperature was maintained at 35°C, while the column oven compartment was at ambient temperature. The analysis was conducted in isocratic mode, with a total run time of 9 minutes. The Retention Time (R.T.) for Chlorthalidone was observed at 2.32 min, whereas Azelnidipine eluted at 4.19 min.

Method Validation

Specificity

The developed method exhibits specificity as there is no evidence of interference between the chromatograms of the Chlorthalidone and Azelnidipine blank and the chromatograms of Chlorthalidone and Azelnidipine standards or samples. The chromatograms are depicted in Figures 1-3.

Linearity and Range

To assess the linearity of both Azelnidipine and Chlorthalidone, standard solutions with drug concentrations ranging from 0.63 to 1.88 µg/mL and 0.63 to 1.2 µg/mL, respectively, were analyzed. This analysis resulted in calibration curve correlation coefficients of 0.991 for Azelnidipine and 0.997 for Chlorthalidone. Figures 4 and 5 present the calibration curves for both drugs. The results of linearity and range are mentioned in Table 1.

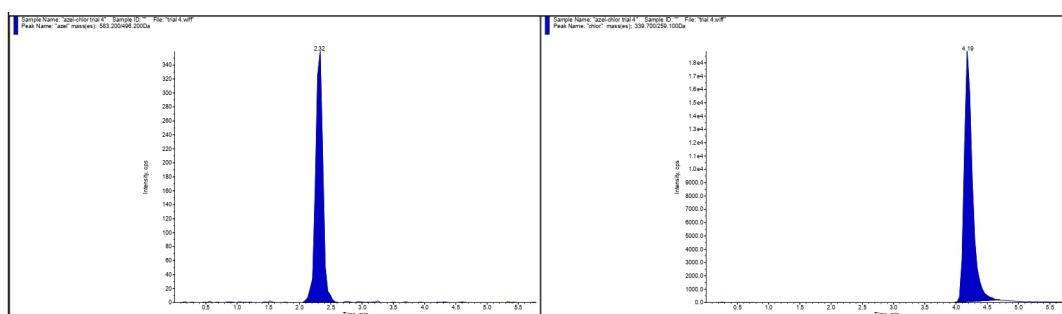


Figure 1: Chromatogram of Chlorthalidone and Azelnidipine in Water: Methanol Water: Methanol: 0.1% formic acid (30:70v/v).

Table 1: Linearity data of Chlorthalidone and Azelnidipine.

Chlorthalidone		
S. No.	Concentration ($\mu\text{g/mL}$)	Area
1	0.400	1740.138
2	0.600	2262.511
3	0.800	3227.408
4	1.000	3966.507
5	1.200	5224.008
Azelnidipine		
S. No.	Concentration ($\mu\text{g/mL}$)	Area
1	0.630	8577.623
2	0.940	11774.920
3	1.250	17261.181
4	1.560	20774.972
5	1.880	25892.903

Table 2: Repeatability Data for Azelnidipine and Chlorthalidone.

Azelnidipine				
S. No.	Conc. ($\mu\text{g/mL}$)	Area	Mean \pm S.D (n=6)	% R.S.D
1.	0.8	3244.389	3231.015 \pm 34.350	1.063
		3290.778		
		3219.440		
		3190.598		
		3211.556		
		3229.331		
Chlorthalidone				
S. No.	Conc. ($\mu\text{g/mL}$)	Area	Mean \pm S.D (n=6)	% R.S.D
1.	1.25	17821.774	17722.263 \pm 237.964	1.343
		17710.330		
		17609.255		
		17323.664		
		18010.225		
		17858.332		

Mean \pm S.D., n=6.

Precision

Repeatability

To assess the consistency of peak area measurements for both Azelnidipine and Chlorthalidone, six measurements of the same solution were taken. The results showed percent Relative Standard Deviations (RSD) of 1.063 for Azelnidipine and 1.343 for Chlorthalidone. Detailed information on the repeatability of Azelnidipine and Chlorthalidone can be found in Table 2.

Intraday precision

Intraday precision data for Chlorthalidone and Azelnidipine were recorded. Azelnidipine exhibited a % R.S.D. for intraday precision ranging from 0.968 to 1.656, while Chlorthalidone had a % R.S.D. ranging from 0.975 to 1.371. Table 3 provides further details on intraday precision readings for these drugs.

Interday precision

Table 3 represents intraday and interday precision of Chlorthalidone and Azelnidipine. Azelnidipine's R.S.D. for interday precision ranged from 0.877 to 1.467, while Chlorthalidone's R.S.D. ranged from 0.555 to 1.346.

Table 3: Intraday and Interday precision data for Estimation of Azelnidipine and Chlorthalidone.

Intraday precision			
S. No.	Conc. (µg/mL)	Area Mean±S.D. (n=3)	% R.S.D
Azelnidipine			
1	0.4	1729.740±28.648	1.656
2	0.8	3260.257±44.919	1.378
3	1.2	5270.121±368.943	0.968
Chlorthalidone			
1	0.63	8590.754±83.727	0.975
2	1.25	17567.425±345.439	1.371
3	1.88	25707.866±395.083	1.344
Interday precision			
S. No.	Conc. (µg/mL)	Area Mean±S.D. (n=3)	% R.S.D
Azelnidipine			
1	0.4	1720.932±18.566	1.079
2	0.8	3263.631±47.886	1.467
3	1.2	5264.022±46.179	0.877
Chlorthalidone			
1	0.63	8547.703±47.432	0.555
2	1.25	17834.678±240.075	1.346
3	1.88	25413.517±306.030	1.204

Mean±S.D., n=3.

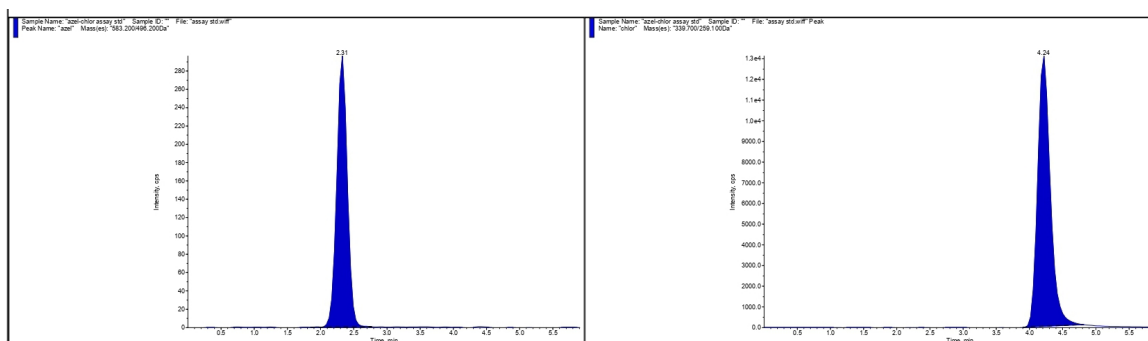


Figure 2: Chromatogram of Chlorthalidone and Azelnidipine Standard.

Accuracy

To confirm the method's accuracy, a recovery analysis was performed using a marketed formulation at a level three standard addition. Table 4 present the results, indicating that Azelnidipine and Chlorthalidone achieved recovery rates of 99.96-100.18% and 99.91-100.71%, respectively.

LOD and LOQ

The LOD and LOQ were determined by repeating the calibration curve five times and calculating the Standard Deviation (SD) of the intercepts. The Limit of Detection (LOD) and Limit of

Quantification (LOQ) for Azelnidipine and Chlorthalidone were calculated using the formulas:

$$\text{LOD} = 3.3 \times (\text{SD} / \text{Slope}) \quad \text{LOQ} = 10 \times (\text{SD} / \text{Slope})$$

For Azelnidipine:

$$\text{LOD} = 3.3 \times (215.792 / 4335.868) = 0.164 \mu\text{g/mL}$$

$$\text{LOQ} = 10 \times (215.792 / 4335.868) = 0.498 \mu\text{g/mL}$$

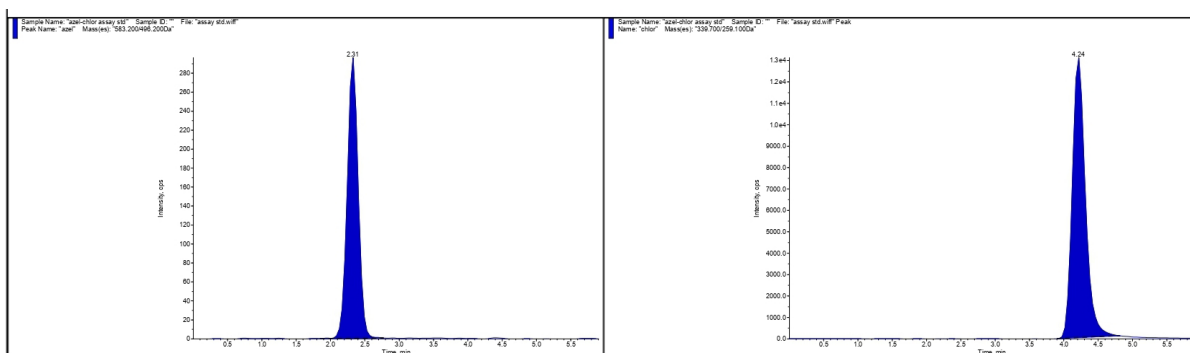
For Chlorthalidone:

$$\text{LOD} = 3.3 \times (625.566 / 13961.796) = 0.148 \mu\text{g/mL}$$

Table 4: Recovery Data for Azelnidipine and Chlorthalidone (n=3).

S. No.	Conc. Level (%)	Sample amount (µg/mL)	Amount Added (µg/mL)	Amount recovered (µg/mL)	% Recovery	% Mean Recovery±S.D
Azelnidipine						
1	80%	0.400	0.320	0.324	101.281	1.263
2		0.400	0.320	0.316	98.862	
3		0.400	0.320	0.322	100.726	
4	100%	0.400	0.400	0.400	100.121	1.098
5		0.400	0.400	0.402	100.430	
6		0.400	0.400	0.394	98.400	
7	120%	0.400	0.480	0.471	98.158	1.243
8		0.400	0.480	0.483	100.629	
9		0.400	0.480	0.477	99.379	
Chlorthalidone						
1	80%	0.625	0.500	0.501	100.141	1.079
2		0.625	0.500	0.505	100.901	
3		0.625	0.500	0.494	98.773	
4	100%	0.625	0.625	0.635	101.602	1.234
5		0.625	0.625	0.627	100.380	
6		0.625	0.625	0.620	99.125	
7	120%	0.625	0.750	0.747	99.574	0.779
8		0.625	0.750	0.759	101.136	
9		0.625	0.750	0.753	100.431	

Mean±S.D., n=3.

**Figure 3:** Chromatogram of Chlorthalidone and Azelnidipine Sample.

$$LOQ = 10 \times (625.566 / 13961.796) = 0.448 \mu\text{g/mL}$$

These values indicate the sensitivity of the method for detecting and quantifying Azelnidipine and Chlorthalidone in the given analytical conditions.

Robustness

The impact of various adjustments on system suitability for standard preparation was assessed. The results fell within an acceptable range, with the Relative Standard Deviation (RSD) not

exceeding 2%. Table 5 contains information regarding the RSD for robustness testing.

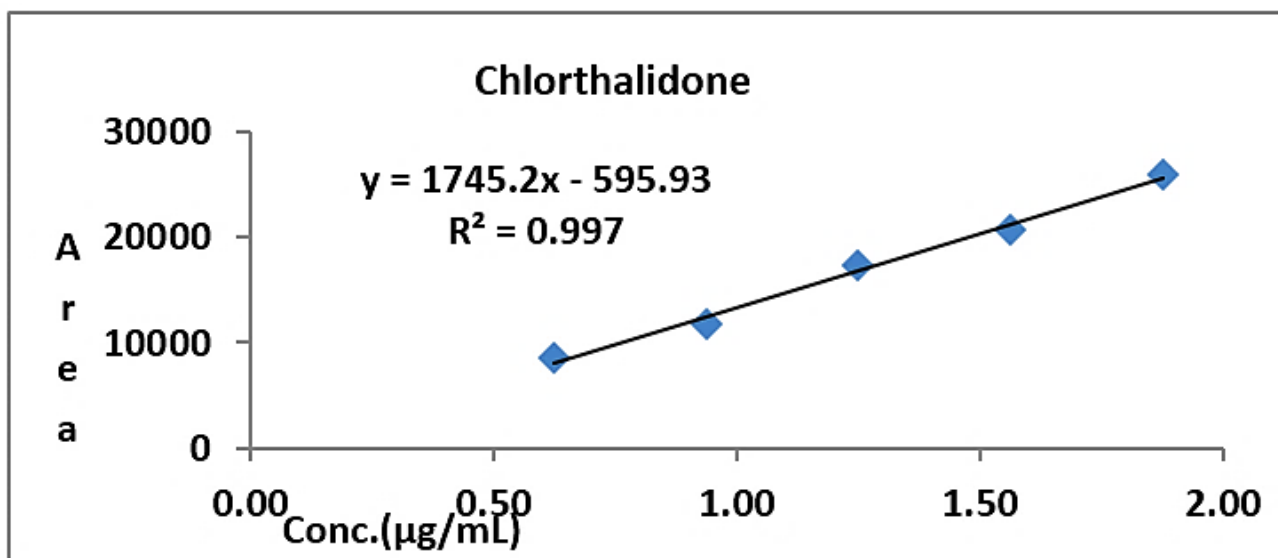
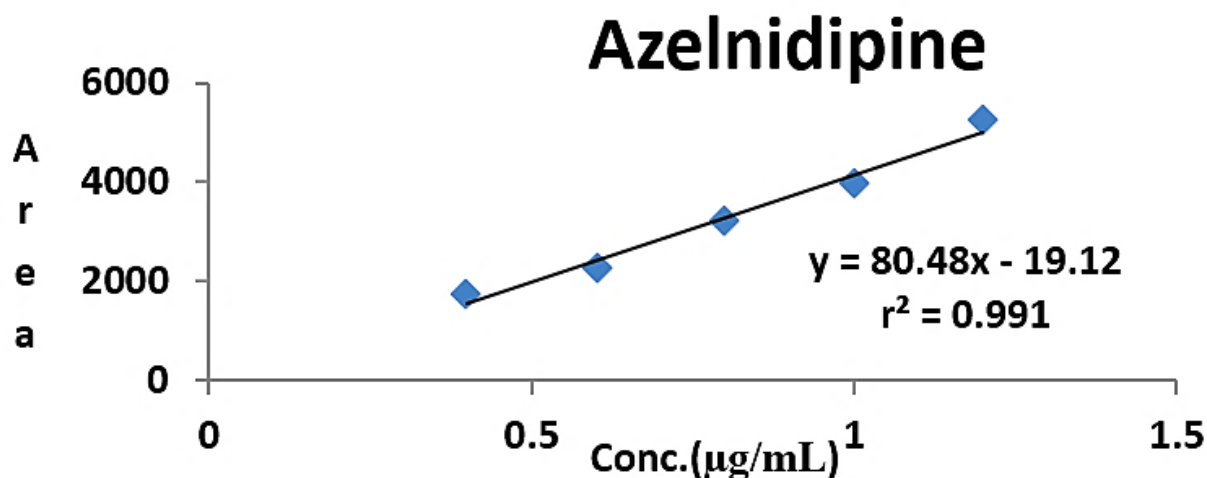
Analysis of marketed formulation by developed method

The applicability of the proposed method was assessed by analyzing the Uniaz-CH tablet formulation, which is readily available in the commercial market. The assay results closely matched the designated values for each drug within the combined dosage form. These outcomes underscore the accuracy, precision,

Table 5: Robustness data for Azelnidipine and Chlorthalidone.

S. No.	Area at Flow rate (- 2.0 mL/min)	Area at Flow rate (+ 2.0 mL/min)	Area at Mobile phase(-2)	Area at Mobile phase(+2)
Azelnidipine				
1	3981.810	3185.114	3877.785	3520.357
2	3890.220	3122.335	3842.691	3582.339
3	3932.443	3201.499	3902.113	3501.667
% R.S.D	1.165	1.318	0.771	1.195
Chlorthalidone				
1	19380.332	14769.220	17851.864	15971.137
2	19581.224	14501.332	17401.263	15590.331
3	19032.879	14280.446	18016.776	15721.286
% R.S.D	1.435	1.686	1.794	1.228

Mean±S.D., n=3.

**Figure 4:** Calibration Curve of Azelnidipine (0.4-1.2 µg/mL).**Figure 5:** Calibration Curve of Chlorthalidone (0.63-1.88 µg/mL).

simplicity and efficiency of the developed method. It can be effectively employed in industrial settings for routine quality control of typical dosage forms. The assay of the Uniaz-CH tablet formulation, containing 8 mg of Azelnidipine and 12.5 mg of Chlorthalidone, was performed to determine the percentage of the labeled claim. The results showed that Azelnidipine had an assay value of $99.270 \pm 1.704\%$, while Chlorthalidone was found to be $99.720 \pm 0.806\%$, indicating that the formulation complies with pharmacopeial standards and ensures the presence of the active ingredients within the acceptable range.

CONCLUSION

A RP-HPLC straightforward, precise and rapid simultaneous estimation method was developed and validated for the routine analysis of Azelnidipine and Chlorthalidone. It is recommended to employ established technique for the routine analysis Azelnidipine and Chlorthalidone mixtures, particularly in manufacturing settings. Azelnidipine and Chlorthalidone quantification was achieved through RP-HPLC method, which was both developed and validated. All method validation parameters adhere to the acceptance criteria outlined in the ICH Q2 (R1) guidelines. Consequently, it is affirmed that this method is this method is not only accurate and precise but also linear and selective. It is well-suited for the routine simultaneous analysis of Azelnidipine and Chlorthalidone. Moreover, the primary peak displayed no indications of degradation and the results fell within acceptable limits. Hence, the developed and validated RP-HPLC method can be reliably employed to determine the concentrations of Azelnidipine and Chlorthalidone in a mixture.

ACKNOWLEDGEMENT

We would like thank School of Pharmacy, DAVV and Modern Institute of Pharmaceutical Sciences, Indore for providing us the platform to work on the project and providing us the facilities required for the work.

ABBREVIATIONS

RP-HPLC: Reverse Phase High Performance Liquid Chromatography; **mL:** Millilitre; **µL:** Microlitre; **RSD:** Relative Standard Deviation.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Cite this article: Singh A, Narsinghani T. RP-HPLC Estimation of Azelnidipine and Chlorthalidone in Bulk and Combined Dosage Forms. *Indian J of Pharmaceutical Education and Research.* 2026;60(1)s:304-s311.