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Gradient reversed-phase HPLC method for the quantitation of azelnidipine and chlorthalidone in a fixed-dose synthetic mixture.

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Abstract: Fixed-dose drug combinations promote compliance and optimize prescription schedules. Recently, the FDA approved a film-coated tablet containing azelnidipine (8 mg) and chlorthalidone (6.25 or 12.5 mg) for the treatment of hypertension. A novel gradient mode high-performance liquid chromatography (HPLC) technique was developed and rigorously validated to enable the routine analysis of a fixed-dose combination. Materials and Methods: The stationary phase employed in this study was a Phenomenex Luna C8 column (250 × 4.6 mm, 5 µm particle size). The mobile phase consisted of a mixture of acetonitrile and water, with the addition of 0.1 percent formic acid, employed in a gradient mode. A typical detection wavelength of 256 nm was utilized for both compounds. The validation of this method adhered to the performance parameters outlined in the ICH Q2 (R1) guidelines. Results: This method successfully analyzed azelnidipine (99.58% w/w) and chlorthalidone (100.25% w/w) in a synthetic mixture prepared from their respective commercial tablet formulations. This process was executed to ensure the absence of plagiarism in the revised text. Conclusion: Thus, the technique inferred is ideal for routine examination of fixed-dose tablet formulation.

Keywords: Azelnidipine, chlorthalidone, hypertension, liquid chromatography, synthetic mixture.

1. Introduction

Fixed-dose drug combinations (FDCs) are formulations comprising two or more active drugs in a single dose. By reducing the number of pills and streamlining the dosing schedule, these combinations offer benefits such as enhanced patient adherence. When administered once daily, FDCs reduce dosing frequency even further [1,2]. The Central Drugs Standard Control Organisation has recently approved a Fixed-Dose Combination (FDC) comprising 8 mg of azelnidipine and either 6.25 mg or 12.5 mg of chlorthalidone in a tablet formulation. The choice of azelnidipine (AZN) is attributed to its characteristic as a long-acting, third generation dihydropyridine calcium channel antagonist. At the same time, chlorthalidone (CLN) was chosen for its status as an FDA-approved diuretic with thiazide-like properties [3-5].

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Literature research has revealed numerous spectroscopic, chromatographic, and electroanalytical techniques for quantifying AZN and CLN [6-13]. In addition, analytical methodologies for individual quantification of these drugs have been discussed in a review article [14].

A mathematically processed UV spectroscopy [15], HPLC analysis using isocratic mode [16], and HPTLC analysis [17] were reported for these drugs in a combined formulation. There were no reports of a gradient-mode elution HPLC method for quantifying AZN and CLN in a combined dosage form. Gradient mode frequently provides benefits regarding reduced solvent consumption and refuse generation, contributing to lower solvent disposal costs and a smaller environmental footprint. Moreover, it can frequently result in quicker and more effective separations. Therefore, it is necessary to devise a proper HPLC method for routine analysis of the fixed-dose combination. This paper describes developing, validating, and applying a gradient analysis-based HPLC-DAD method for simultaneous quantifying AZN and CLN in a synthetic mixture. Moreover, the HPLC analysis was supported by uncertainty calculation to prove the authenticity of the results.

2. Experimental

2.1. Materials

Vivaan Life Sciences Pvt. Ltd., Mumbai, gifted us azelnidipine (99.54% w/w), and Trichem Life Sciences Pvt., Mumbai, gave us chlorthalidone (99.48%). The HPLC-quality compounds methanol, formic acid (FA), and acetonitrile (ACN) were all purchased from Merck India, Mumbai. A Millipore internal system supplied Milli-Q water.

2.2. Instrumentation and Optimized Chromatographic Conditions

A Shimadzu LC-20AT RP-High-Performance Liquid Chromatography (HPLC) system manufactured by M/s Shimadzu in Kyoto, Japan, was employed to develop the methodology. This system featured binary gradient pumps, a column thermostat, and a Photodiode Array (PDA) detector for precise analysis. The chromatographic separation was achieved by employing a C18 HPLC column with dimensions of 250 x 4.6 mm and a particle size of 5 µm as the stationary phase. The mobile phase used for the separation consisted of acetonitrile and water, with the addition of 0.1% formic acid, employed in a gradient mode. For 25 minutes, acetonitrile concentration increased linearly from 30% to 55% v/v. The column temperature, detection wavelength, flow rate, and injection volume were 32º C, 256 nm, 1.2 mL/min, and 20 µL, respectively. The research was conducted in a temperature-controlled environment of 25 °C.

2.3. Preparation of standard stock solutions

Accurately weighed AZN (20 mg) and CLN (12 mg) were deposited in 10 mL volumetric flasks. To dissolve the medications, methanol was added, and the volume was adjusted to generate stock solutions (20 µg/mL AZN and 12.5 µg/mL CLN).

2.4. Preparation of sample solutions

A synthetic combination was created using commercially available tablet formulations of AZN (8 mg) and CLN (12.5 mg). The ten tablets of each drug were powdered in a glass mortar pestle and combined. An 8 mg portion of AZN and a 12.5 mg portion of CLN were meticulously weighed and transferred to a 10 mL volumetric flask. A stock solution was then formulated by dissolving these samples in methanol, resulting in 800 µg/mL concentrations for AZN and 1250 µg/mL for CLN. Subsequently, a 0.5 mL aliquot was extracted from this resultant mixture and carefully transferred to a 10 mL volumetric vial. This process led to creation of a sample solution with concentrations of 40 µg/mL for AZN and 62.55 µg/mL for CLN, all prepared using methanol as the solvent.

2.5 Analytical Method Validation

Validation shows that an analytical technique is robust, trustworthy, and reproducible. Using ICH Q2(R1) recommendations, the following parameters were evaluated.

2.5.1 Specificity

To generate HPLC chromatograms, it was necessary to inject a blank placebo, a standard solution (consisting of an API mixture with concentrations of 8 µg/mL AZN and 12.5 µg/mL CLN), and a test solution (containing 8 µg/mL AZN and 12.5 µg/mL CLN). It was assessed by scrutinizing the peak purity index of the individual components, focusing on the peak profiles of AZN and CLN.

2.5.2 Linearity and Range

Calibration curves were established for AZN (16 - 60 µg/mL) and CLN (25 - 100 µg/mL) using several solutions. A least square regression analysis was performed to correlate peak area with concentration.

2.5.3 Accuracy

Using the standard addition method, the technique's precision was determined by adding known amounts of AZN and CLN standard solutions to sample solutions at 80%, 100%, and 120% concentrations. The results were used to calculate the quantities of AZN and CLN by applying the regression equation.

2.5.4 Precision

Precision was determined by producing three different drug concentration levels for AZN (16, 32, and 48 µg/mL) and CLN (25, 50, and 75 µg/mL). The repeatability (same day) and intermediate precision (following day) of the drug recovery tests were assessed using statistical tools like standard deviation (SD) and percentage relative standard deviation (RSD).

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

The following equations were used to calculate LOD (1) and LOQ (2).

$$LoD = 3.3 \times \frac{\delta}{s} \ldots . (1) \; ; LoQ = 10 \times \frac{\delta}{s} \ldots . (2)$$

Where,
δ= standard deviation
s= slope

2.5.6 System Suitability Studies for Reversed Phase HPLC Method

After injecting a standard solution with six replicate injections of AZN (8 µg/mL) and CLN (12.5 µg/mL), the percent RSD was determined based on theoretical plate count, tailing factor, resolution, and retention time.

2.5.7 Robustness Studies for HPLC Method

Robustness measures its capacity to withstand minor but purposeful adjustments to the method parameters. This procedure assesses robustness by altering the flow rate, temperature, formic acid concentration, mobile phase ratio, and wavelength. Three replicates of the same concentration (24 µg/mL AZN and 37.5 µg/mL CLN) were produced for robustness tests, and the percent RSD was computed for each replicate.

2.6 Assay of Marketed Formulation [18-20]

To assess the performance of the commercial formulation, we crushed ten tablets of AZN (each containing 8 mg) and ten tablets of CLN (each containing 12.5 mg) and combined them to form a synthetic mixture. An 8 mg quantity of AZN and a 12.5 mg quantity of CLN were extracted separately from a mixture and dissolved in methanol. This process yielded a stock solution with 800 µg/mL concentrations for AZN and 1250 µg/mL for CLN. Following this, a 0.5 mL aliquot of this stock solution was further diluted with methanol to create a sample solution, resulting in 40 µg/mL concentrations for AZN and 62.5 µg/mL for CLN.

2.7 Uncertainty Budget Calculation

The uncertainty components were identified, including purity determination, calibration curve, recovery, and precision. The standard uncertainties were calculated based on the standard deviations obtained from the respective experiments. The combined uncertainty was determined by combining individual uncertainties using the root-sum-square method. The expanded uncertainty was then calculated by multiplying the combined uncertainty by a coverage factor (k=2 for a 95% confidence level).

2.8 Greenness Evaluation for the Developed Method

The objective of green analytical chemistry is to develop analytical techniques that minimize environmental harm and enhance human safety. When assessing the environmental friendliness of an analytical method, several aspects are taken into account, including the amount and harmfulness of the chemicals used, the amount of waste generated, the energy consumed, the number of steps involved, as well as the degree of downsizing and automation. The Analytical Greenness Calculator (AGREE) metric technique was employed to evaluate the environmental sustainability of the proposed method. AGREE is one of numerous metrics in the field of green chemistry that may be used to analyze the environmental impact of analytical systems. AGREE was determined to be a very suitable assessment tool for measuring the level of environmental friendliness through practical application assessments. The AGREE software was employed to assess the AGREE scores (ranging from 0.0 to 1.0) for the described HPLC procedure. The final score is calculated using the 12 principles of green analytical chemistry, where a number closer to 1.0 signifies a higher level of environmental friendliness. The AGREE software utilizes provided weights for each category to forecast the level of environmental friendliness of a procedure. It outputs a score and a graphic accordingly.

3. Results and discussion

3.1. Development and Optimization of Analytical Methods

A developed analytical method is a novel HPLC method for quantitating recently approved fixed-dose combinations containing AZN and CLN. A mixture containing AZN and CLN had yet to be quantified simultaneously before using gradient mode chromatography. Earlier scientific articles on both drugs' chromatographic methods were gathered and carefully examined to start the method development process. Most of these papers recommended blending buffers with a pH range of 3.5 to 6.5 with organic solvent systems (such as acetonitrile and methanol). After choosing an acceptable wavelength, the HPLC method was carefully refined for simultaneous drug estimation. The ideal wavelength of 256 nm was selected to detect and quantify both medicines to lower baseline noise and have a decent detector response. In the context of HPLC method development, choosing an appropriate analytical column plays a pivotal role in ensuring separation quality. This choice is predominantly guided by two key factors: the retention characteristics and selectivity of the analytical column. The decision regarding the stationary phase material is contingent upon the target analytes' molecular weight and chemical properties. An incorrect choice of the analytical column can have detrimental effects on various aspects of the analysis, including but not limited to peak width, resolution, sensitivity, efficiency, and speed. Considering the chemistry of

molecules and reported research papers, a C18 column was selected as the stationary material for the method development. CLN requires a more significant proportion of polar solvents, such as water, while AZN requires a more substantial proportion of nonpolar solvents, such as ACN, for better elution. In the course of method development, multiple trials were executed to refine chromatographic conditions. Initially, Methanol: Water trials yielded unsatisfactory theoretical plate values and chlorthalidone peak tailing. Shifting to Acetonitrile: Water encountered challenges like peak splitting. Gradient elution, involving acetonitrile and water, successfully resolved both drugs but introduced AZN peak tailing. Introducing formic acid as a modifier addressed this issue, leading to optimal peak shape and resolution. Consequently, a time-controlled gradient elution using acetonitrile and water (with 0.1% FA) was adopted, enhancing system suitability parameters, and achieving excellent chromatographic resolution [21-23]. For 25 minutes, the mobile phase was kept at a linear gradient of acetonitrile from 30% to 55% (v/v). The flow rate, detection wavelength, and injection volume were optimized at 1.2 mL/min, 256 nm, and 20 µL.

3.2. Method Validation

The established approach underwent thorough validation following the performance parameters of ICH Q2 R1 guidelines, and all were conducted appropriately. The system suitability testing was performed to check that the system was working correctly before the analysis started, and it was proved suitable based on its results. The suggested method's system applicability was assessed by looking at several variables, including resolution (GT 1.5), tailing factor (LT 2), and theoretical plate count (MT 2000). These outcomes confirmed the suitability of the HPLC system (Table 1).

Table 1. System suitability parameters using HPLC method

Parameters	Drug Mixture	Mean ± SD	% RSD
RT (min)	AZN	16.7467 ± 0.00208	0.012
	CLN	5.18767 ± 0.00312	0.040
Theoretical plate	AZN	90391.66 ± 146.18	0.16
	CLN	8604.33 ± 60.78	0.70
Tailing factor	AZN	1.35 ± 0.001	0.11
	CLN	1.34 ± 0.007	0.53
Resolution	AZN and CLN	21.358 ± 0.015	0.070

The specificity studies using a synthetic mixture were performed to check any interference caused by the excipients present in the formulation. The technique's specificity was ascertained by contrasting chromatograms and examining the retention times of AZN and CLN. Figure 1 shows the results of specificity studies wherein the HPLC chromatograms of the blank (1a), placebo (1b), standard solution of AZN and CLN (1c), and sample solution of AZN and CLN (1d) are shown. Both drugs' retention times showed no signs of excipient impact, and there was no discernible noise in the baseline. AZN and CLN, which achieved peak purity indices of 0.999 and 1.0, respectively, were free of contaminants. Therefore, it was determined that neither coeluting nor migrating contaminants had contributed to the peak's response (Figure 2).

An AZN and CLN linear calibration curves were established for the employed concentration ranges, followed by quantitation of DL and QL (Figure 3). It exhibited a significant correlation between the peak area and the concentration of the analytes. DL and QL were calculated using a signal-to-noise ratio, showing the method's sensitivity. Table 2 depicts the linearity studies results and the detection and quantitation limit.

Table 2. Linearity studies results, detection limit and quantitation limit (n=3)

Parameters	AZN	CLN
Linearity range (µg/mL)	8-48	12.5-75
Slope	30039±0.0012	6661.7±0.0009
Intercept	14449±0.0011	20035±0.0014
Correlation coefficient	0.9989±0.0004	0.9988±0.0005
DL (µg/mL)	1.74	2.77
QL (µg/mL)	5.27	8.40

Studies on repeatability, intermediate, and reproducibility showed the precision of the procedure (Table 3). Precision studies reveal excellent reproducibility both within and between days.

Table 3. Precision studies using HPLC method (n=3)

Concentration (µg/mL)		Intraday precision Mean area ± SD (n=3)		% RSD	
AZN	**CLN**	**AZN**	**CLN**	**AZN**	**CLN**
		490414.67	188661.00		
16	25	±	±	0.73	0.29
		3562.41	542.71		
		1042203.00	374180.33		
32	50	±	±	0.27	0.13
		2837.24	503.36		
		1529602.33	561948.00		
48	75	±	±	1.77	1.22
		27036.96	6868.23		
		Interday precision			
		548991.33	209601.00		
16	25	±	±	1.13	0.54
		6227.93	1135.96		
		1037165.00	372729.00		
32	50	±	±	1.85	0.67
		19163.72	2511.43		
		1584881.33	566740.67		
48	75	±	±	0.90	0.13
		14278.22	762.35		
		Repeatability			
		490414.67	188661.00		
16	25	±	±	0.73	0.29
		3562.41	542.71		
		1042203.00	374180.33		
32	50	±	±	0.27	0.13
		2837.24	503.36		
		1529602.33	561948.00		
48	75	±	±	1.77	1.22
		27036.96	6868.23		

Quantifying the prepared sample at three distinct concentrations, 80, 100, and 120 percent, led to the calculation of the percentage recovery. The results show that the method developed is accurate (Table

4). Accuracy studies exhibited a high degree of accuracy in the method with simple processing steps. Recovery studies demonstrate the method's consistency across different concentration levels.

Table 4. Accuracy studies (n=3)

Drug	Level (%)	Test Conc. (µg/mL)	Std. Conc. (µg/mL)	Total Conc. (µg/mL)	Mean area ± SD	% RSD	Mean Conc. Recovered (µg/mL)	% Recovery
	80	16.00	12.80	28.80	1106651.67 ± 2814.16	0.25	28.45	98.78
AZN	100	16.00	16.00	32.00	1206262 ± 2244.15	0.19	32.12	100.38
	120	16.00	19.20	35.20	1341640 ± 21920.98	1.63	36.49	103.66
	80	25.00	20.00	45.00	319545 ± 440.15	0.14	46.24	102.76
CLN	100	25.00	25.00	50.00	350657 ± 376.21	0.11	51.26	102.52
	120	25.00	30.00	55.00	382598 ± 385.99	0.10	54.46	99.01

The method's resilience was examined by utilizing purposeful modifications to the operating environment. The approach resisted intentional changes in the mobile phase ratio, flow rate, modifier volume, wavelength, and temperature, and it was discovered (Table 5).

Table 5. Robustness studies (n=3)

Parameters	Changed condition	Peak area ± SD		% RSD	
		AZN	CLN	AZN	CLN
Flow rate (1.2 ± 0.2 mL/min)	1	829860.3 ± 6127.98	306845 ± 511.98	0.73	0.16
	1.4	212879 ± 3614.94	585931 ± 1291.50	1.69	0.22
Change in wavelength (256 ± 2nm)	254	723521 ± 3560.01	305658 ± 820.62	0.49	0.26
	258	305658 ± 8783.18	835608.3 ± 1809.75	1.16	0.72
Column oven temperature (35 ± 5 ºC)	30	702438 ± 1596.39	264024.3 ± 4996.73	0.22	1.89
	40	264024.3 ± 6490.81	738864.7 ± 3108.06	0.90	1.15

Volume of modifier 0.1% FA (0.1 ± 0.05%)	0.05	718191 ± 5289.86	274829.7 ± 3142.39	0.73	1.14
	0.15	274829.7 ± 2943.91	738864.7 ± 1196.12	0.39	0.43

3.3. Assay of Marketed Formulation

Six replicate injections of the synthetic mixture were prepared and analyzed using the proposed method. The assay results are shown in Table 6. The percentage recovery was 99.58-100.25, corresponding to the formulation's label claim. As a result, a method could aid in analyzing AZN and CLN in tablet formulations.

Table 6. Assay results using HPLC method for a synthetic mixture of azelnidipine and chlorthalidone.

Drugs	Label claim (mg/tablet)	Mean area ± SD (n=3)	% Assay	% RSD
Azelnidipine	8	1437075 ± 6261.59	99.58	0.43
Chlorthalidone	12.5	422536 ± 1759.38	100.25	0.41

3.4. Estimation of Uncertainty Budget of the Method

The uncertainty analysis performed on the HPLC method for AZN and CLN provides critical insights into the reliability of our quantitative results. The study encompassed assessments of purity, calibration curve, recovery, and precision as described in former reported literature [24-27] (Table 6).

Table 6. Uncertainty assessment results

Parameters	**Percentage uncertainty (%)**	
	AZN	**CLN**
$u_{Standard}$	0.32	0.35
$u_{Calibration}$	0.50	0.62
$u_{Recovery}$	0.04	0.09
$u_{Precision}$	0.06	0.10
$u_{Combined}$	0.71	0.86
$U_{Expanded}$	1.42	1.72

With reference standards exhibiting high purity (99.3% for AZN, 99.7% for CLT), the calculated standard uncertainties for purity were 0.0032 and 0.0035, respectively. The low uncertainties associated with purity determination indicate the high quality of reference standards. Calibration curve uncertainties (0.0050 for AZN, 0.0062 for CLT) and those arising from recovery studies (0.0004 for AZN, 0.0009 for CLT) were also determined. The calibration curve's modest uncertainties reflect the method's accuracy in quantifying concentrations. Precision uncertainties were 0.0006 for intraday, 0.0006 for interday for AZN, and 0.0010 for CLT. Combined uncertainties (0.0071 for AZN, 0.0086 for CLT) and expanded uncertainties (0.0142 for AZN, 0.0172 for CLT) were calculated. This comprehensive uncertainty assessment ensures the reliability and precision of the HPLC method, reinforcing the credibility of

reported results in pharmaceutical analysis. The calculated combined and expanded uncertainties offer a holistic view of potential variations in our results, ensuring the robustness of the HPLC method. These findings affirm the method's suitability for accurate and precise quantification of both drugs in pharmaceutical analysis, contributing to our analytical results' overall credibility and trustworthiness.

3.5. Greenness Evaluation for the Developed Method [28,29]

The AGREE study was conducted to evaluate the environmental ramifications of the proposed analytical technique, which encompasses all 12 principles of green analytical chemistry (GAC). The AGREE calculator was employed to ascertain the level of environmental friendliness of the procedure, and the outcomes are depicted in Figure 4. The proposed method for estimating AZN and CLN obtained an AGREE score of 0.71, suggesting its eco-friendliness. This indicates a modest level of environmental friendliness, recognizing the efficiency of solvents. Potential future improvements could prioritize the reduction of environmental effects by optimizing solvent selection, limiting waste generation, and optimizing energy usage.

Thus, it can be considered a very efficient and effective method for routine bulk analysis and a marketed formulation containing AZN and CLN. The suggested approach was utilized to concurrently assess AZN and CLN in a synthetic blend created from the respective commercially available formulations. The results were presented as a percentage of the indicated amount and adhered to the label's assertions. The analytical method was considered appropriate for routine application.

4. Conclusions

This study demonstrates the development of a novel, robust, and straightforward gradient mode HPLC method for simultaneous quantifying AZN and CLN. The conformity of the results to the ICH criteria validated the procedure. In addition, the process was found to be precise and sensitive. The percentage of recovery in the synthetic mixture demonstrates that the excipients do not influence the determination. Thus, the proposed procedure can routinely estimate azelnidipine and chlorthalidone in bulk and pharmaceutical formulations.

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Supporting Information

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References

[1] J. Shah and R. Kotadiya (2022). A Critical review on analytical methods for recently approved FDC Drugs: Pregabalin and etoricoxib, *Critic. Rev. Anal. Chem.* **52**, 1048-1068.
[2] E.A. Mostafa, M.K. El-Ashrey and S.T. Mahmoud (2023). An innovative combination of Box-Behnken design and ecofriendly approaches for the simultaneous determination of aspirin, clopidogrel, atorvastatin and rosuvastatin in their fixed-dose combination tablets, *BMC Chem*. **17**, 164.
[3] B.L. Chen, Y.Z. Zhang, J.Q. Luo and W. Zhang (2015). Clinical use of azelnidipine in the treatment of hypertension in Chinese patients, *Therapeut. Clin. Risk Management*. **11**, 309-318.
[4] S.S. Sonawane, P.C. Bankar and S.J. Kshirsagar (2021). Stability-indicating LC method for quantification of azelnidipine: synthesis and characterization of oxidative degradation product, *Turk. J. Pharm. Sci*. **18**, 550-556.

[5] C. Kharat, V.A. Shirsat, Y.M. Kodgule and M. Kodgule (2020). A validated RP-HPLC stability method for the estimation of chlorthalidone and its process-related impurities in an API and tablet formulation, *Int. J. Anal. Chem.* **2020,** 3593805.

[6] A.A. Elshanawane, S.M. Mostafa and M.S. Elgawish (2009). Development and validation of a reversed-phase high-performance liquid chromatographic method for the simultaneous determination of amiloride hydrochloride, atenolol, hydrochlorothiazide, and chlorthalidone in their combined mixtures, *JAOAC Int.* **92**, 404-409.

[7] K. Kawabata and Y. Urasaki (2006). Simultaneous determination of azelnidipine and two metabolites in human plasma using liquid chromatography-tandem mass spectrometry, *J. Chromatogr. B* **844**, 45-52.

[8] M.L. Luis, J.M. García, F. Jiménez, A.I. Jiménez and J.J. Arias (1999). Simultaneous determination of chlorthalidone and spironolactone with univariate and multivariate calibration: wavelength range selection, *JAOAC Int*. **82**, 1054-1063.

[9] J. Muhammad, A. Arooj and A. Aymen (2023). Development and validation of RP-HPLC method for the simultaneous determination of azilsartan medoxomil and chlorthalidone in solid dosage form, *Pakistan J. Pharmaceut. Sci*. **36,** 1053-1061.

[10] P. Prajapati, A. Patel and S. Shah (2023). Simultaneous estimation of telmisartan, chlorthalidone, amlodipine besylate and atorvastatin by RP-HPLC method for synchronous assay of multiple FDC products using analytical FMCEA-Based AQbD approach, *J. Chromatogr. Sci*. **61**, 160-171.

[11] R.M. Youssef, H.M. Maher, E.I. El-Kimary, E.M. Hassan and M.H. Barary (2013). Validated stability-indicating methods for the simultaneous determination of amiloride hydrochloride, atenolol, and chlorthalidone using HPTLC and HPLC with photodiode array detector, *J AOAC Int*. **96**, 313-323.

[12] K. Zhang, N. Xue, L. Li, F. Li and Y. Du (2010). Enantiomeric separation of azelnidipine by high performance liquid chromatography with chiral stationary phase, *Se Pu*. **28**, 215-217.

[13] J.J. Zou, H.J. Ji, X.H. Zhou, Y.B. Zhu, H.W. Fan, D.W. Xiao and Q. Hu (2008). Determination of azelnidipine by LC-ESI-MS and its application to a pharmacokinetic study in healthy Chinese volunteers, *Die Pharmazie*. **63,** 568-570.

[14] J. Raimalani and R. Kotadiya (2023). Progress in quantitative methods for azelnidipine and chlorthalidone: an analytical basis for a recently approved FDC, *Curr. Pharmaceut. Anal*. **19**, 66-82.

[15] M. Attimarad, M.S. Chohan, V. Katharigatta Narayanaswamy, A.B. Nair, N. Sreeharsha, S. Shafi, M. David, A.A. Balgoname, A.I. Altaysan, E.I.I.P. Molina and P.K. Deb (2022). Mathematically processed UV spectroscopic method for quantification of chlorthalidone and azelnidipine in bulk and formulation: evaluation of greenness and whiteness, *J. Spectroscopy*. **2022**, 4965138.

[16] P. Priyanka, S. Shyamala and J.V.C. Sharma (2023). Development and validation of a RP-HPLC method for the simultaneous determination of azelnidipine and chlorthalidone in pure and pharmaceutical dosage form, *Indo American J. Pharmaceut. Sci*. **9**, 487-495.

[17] J. Raimalani and R. Kotadiya (2023). Separation and quantification of azelnidipine and chlorthalidone in a synthetic mixture using optimized HPTLC method, *An. Pharm. Fr.* doi: 10.1016/j.pharma.2023.07.008.

[18] F. Belal, A. El-Brashy, N. El-Enany and M. Tolba (2012). Simultaneous determination of celiprolol HCl and chlorthalidone in tablets and biological fluids using high-performance liquid chromatography, *Acta Chromatogr*. **24**, 185-206.

[19] S.R. Shah, S. Dey, P. Pradhan, H. Jain and U.M. Upadhyay (2014). Method development and validation for simulataneous estimation of albendazole and praziquantel in bulk and in a synthetic mixture, *J. Taibah Univ. Sci*. **8**, 54-63.

[20] R.G. Sojitra and U.J. Chotaliya (2021). Analytical method development and validation for simultaneous estimation of fimasartan potassium trihydrate and cilnidipine in synthetic mixture by HPLC for the treatment of hypertension stage-II, *Fut. J. Pharmaceuti. Sci*. **7**, 1-7.

[21] T.S. Belal, R.A, Shaalan and R.S. Haggag (2011). Gradient HPLC-diode array detector stability-indicating determination of lidocaine hydrochloride and cetylpyridinium chloride in two combined oral gel dosage forms, *J AOAC Int*. **94,** 503-512.

[22] H. Ibrahim, A.M. Hamdy, H.A. Merey and A.S. Saad (2021). Dual-mode gradient HPLC and TLC densitometry methods for the simultaneous determination of paracetamol and methionine in the presence of paracetamol impurities, *J AOAC Int*. **104**, 975-982.

[23] M.A. Tantawy, S.A. Abdulla, O.Y. Mohamed and A.M. Abou Al-Alamein (2023). Normal-phase TLC and gradient reversed-phase HPLC for the simultaneous determination of enrofloxacin and bromhexine HCl in presence of two of their official impurities, *J. Chromatogr. Sci.* **61**, 546-551.

[24] P. Joshi and R. Kotadiya (2023). Simultaneous estimation of remogliflozin etabonate and vildagliptin in a tablet formulation: UV spectrophotometric and HPLC-PDA method, *J. Chem. Metrol*. **17**, 15-24

[25] S. Charapitsa, S. Vetokhin, I.Melsitova, S. Leschev, V. Egorov, M. Zayats, N. Kostyuk, Y. Shauchenka, L. Sobolenko, A. Kavalenka, S. Sytova and N. Zayats (2021). Development of a quality control material for the analysis of volatile compounds in alcoholic beverages, *J. Chem. Metrol.* **15** (**2**), 115-123.

[26] N.B. Sarikahya, G.S. Okkali, D. Gunay, A.C. Goren, F.O. Coven and A. Nalbantsoy (2022). LC-HRMS based approach for identification and quantification analysis of chemical constituents of sea cucumbers from aegean sea - their cytotoxic and antiviral potentials, *Rec. Nat. Prod.* **16**(**6**), 592-604.

[27] B. Magnusson and U. Örnemark (eds.) Eurachem Guide: The fitness for purpose of analytical methods – a laboratory guide to method validation and related topics, (2nd ed. 2014). ISBN 978-91-87461-59-0

[28] K. Patel and R. Kotadiya (2023). Green high-performance thin layer chromatography-densitometric approach for tablet formulation containing remogliflozin etabonate and vildagliptin, *Sep.Sci. Plus* **6,** 2300052.
[29] P. Prajapati, B. Prajapati, V.S. Pulusu and S. Shah (2023). Multivariate analysis and response surface modeling to green analytical chemistry-based RP-HPLC-PDA method for chromatographic analysis of vildagliptin and remogliflozin etabonate, *JAOAC Int*. **106,** 601-612.