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Original Article

QUALITY CONTROL ASSAY OF TWO ANTIHYPERTENSIVE REPRESENTATIVES USING RP-HPLC BASED METHODOLOGY: STRESS ASSESSMENT ON ANTIHYPERTENSIVE REPRESENTATIVES

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ABSTRACT

Objective: A quick simultaneous separation and quality control assay of two antihypertensive representatives, Azilsartan (AZIL) and Cilnidipine (CLIN) in bulk and tablet formulation was developed and validated using a Reverse phase (RP) HPLC method within a run time of 10 min.

Methods: All chromatographic separations of AZIL and CILN were operated on a "Supelco C18 column (250 × 4.6 mm, 5 µ)", using a mobile phase of Na_2SO_4 (0.1 M, pH 4.0): methanol at 60:40 (v: v) ratio and the samples were analyzed at 239 nm. Stability assessments of AZIL and CILN were carried out as per the ICH Q1A (R2) regulation. The methodology for determining AZIL and CILN in bulk and formulations tablets was verified by adhering to International Conference on Harmonization (ICH) recommendations.

Results: Retention times of AZIL and CILN samples were 4.023 and 5.732 min, respectively, indicating a quick elution time. Over the tested range of 20–60 µg/ml for AZIL and 5–15 µg/ml for CILN determination, calibration curves have displayed linearity and satisfactory results. LOD of AZIL and CILN are 0.083 µg/ml and 0.056 µg/ml, respectively. The approach suggested herein has satisfactory precision (RSD: 0.1013% for AZIL and 0.4944% for CILN) and accuracy (recovery: 99.20 to 100.34 % for AZIL and 100.17 to 101.59 % for CILN). Furthermore, the approach has also been shown to be effective in detecting degradants of AZIL and CILN and resolving them with high resolution.

Conclusion: This approach is shown to be acceptable for the accurate quality control assay of two antihypertensive representatives, AZIL and CLIN in both bulk and tablet formulation.

Keywords: Antihypertensive, Azilsartan, Cilnidipine, Quality control, HPLC, Degradants

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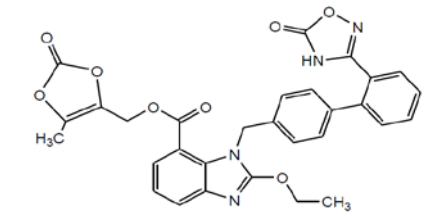
INTRODUCTION

High blood pressure (HBP) affects the arterial linings, leaving them more vulnerable to plaque accumulation that constricts the arteries, eventually leading to heart and brain stroke [1, 2]. In India, HBP has a significant impact on cardiovascular healthcare systems. In India, HBP is wholly accountable to 57% of all stroke fatalities and 24% of all coronary heart disease-related mortality [3]. Hypertension (HTN) is one of the top causes of mortality around the globe, according to the WHO.

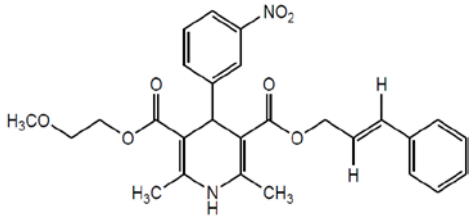
The combination of Azilsartan (AZIL) and Cilnidipine (CLIN) is recommended to alleviate HBP, heart stroke and heart attack [4, 5]. AZIL (fig. 1A) belongs to the angiotensin II receptor antagonist class of drugs. AZIL functions by preventing natural compounds from tightening blood arteries, permitting blood to circulate more freely as well as the heart to circulate more effectively [6]. AZIL also does hardly require dose modifications for people having hepatic or renal impairment, which is also another of its advantages. AZIL lowers blood pressure by inhibiting angiotensin II's action at AT1 receptor, a hormone that constricts blood vessels and decreases urine outflow through the kidneys [7].

Calcium channel blocker CILN (fig. 1B) is a calcium antagonist that also works to block L-type and N-type calcium channels. Despite similar calcium antagonists, CILN has the ability to act upon both N-type and L-type calcium channels [8, 9]. CILN is employed in treating hypertension and associated comorbidities by lowering blood pressure. CILN increases the blood flow of both arterioles and venules with lowering the pressure in the capillary bed by inhibiting N-type and L-type calcium channels. CILN is a vasoselective antiarrhythmic drug with a mild direct dromotropic effect as well as high vasodepressor and arrhythmia-inhibitor.

In the pharmaceutical industry, quality assurance function is critical. Drugs should be sold as secure and therapeutically active compositions providing consistent and anticipated results [10]. New and improved therapeutic agents are indeed developed at a rapid pace. Simultaneously, sophisticated analytical procedures are being developed, enabling their evaluation in a regulatory-compliant manner.



Azilsartan [A]



Cilnidipine [B]

Fig. 1: Chemical structures of [A] AZIL drug and [B] CILN

Shrinivasan *et al.* [11] Masthanamma and Jahnavi [12], Sreenivasulu [13] proposed RP-HPLC [11, 12] and LC-MS [13] for quantification of AZIL drug alone in in bulk and formulations tablets. Sohni *et al.* [14], Naazneen and Sridevi [15], Aher *et al.* [16] proposed RP-HPLC to

quantify AZIL drug along with chlorthalidone molecule in bulk and formulations tablets. Methods for quantifying CLIN along with Olmesartan or Chlorthalidone were proposed by Minase *et al.* [17], Sunitha *et al.* [18], Pawar *et al.* [19], Satyavati *et al.* [20], Patel *et al.* [21], Rao *et al.* [22] and Sawaikar and Kapupara [23]. Jain and Patel reported spectrophotometry techniques to quantify the combination of AZIL and CLIN [24, 25] but the spectrophotometry technique reported by Jain and Patel lacks stability, indicating feature and selectivity.

To support future quality control analysis of AZIL and CLIN in tablet formulation, we aimed at developing and validating stability, indicating RP-HPLC method with a shorter run time. The developed method is fully validated for bulk and tablet formulation in line with ICH method validation guideline.

MATERIALS AND METHODS

Chemicals and reagents

"Merck, India" supplied HCl, NaOH, methanol, Na_2SO_4, and hydrogen peroxide. Milli-Q type water is used throughout this study. AZIL and CILN were procured from local vendor. Myotan CN tablet formulation (40 mg AZIL and 10 mg CLIN) manufactured by "J B Chemicals and Pharmaceuticals Ltd, India" were purchased locally and used.

HPLC system and software

HPLC system of model 2995 equipped with a PDA detector of model 2998 from Waters (India) and empower edition 2.0 software was employed for the entire analytical work.

HPLC conditions

All chromatographic separations of AZIL and CILN were operated on a "Supelco C18 reverse phase column (250 × 4.6mm, 5 µ)" using a mobile phase of Na_2SO_4 (0.1 M, pH 4.0):methanol at 60:40 (v: v) ratio. Across the analysis, the mobile phase column flow rate was held at 1 ml/min and the run time was 8 min under room temperature. The eluents (AZIL and CILN) were detected and analyzed using their peak areas at 239 nm.

Stock solutions of AZIL and CILN

The stock solutions of AZIL and CILN were prepared in mobile phase with 400 µg/ml of AZIL and 100 µg/ml of CILN concentration, respectively. The working solutions of AZIL and CILN were also made in mobile phase by serial dilution of stock solutions of AZIL and CILN. The linearity of AZIL and CILN standards were acquired through dilution of stock solutions of AZIL and CILN appropriately in mobile phase. Five calibration solutions of AZIL and CILN were made within the concentrations range of 20–60 µg/ml for AZIL and 5–15 µg/ml for CILN for linearity check.

Calibration curves of AZIL and CILN

The prepared five dissimilar concentration solutions were injected (10 µl) into HPLC column. At the wavelength (239 nm) chosen, the peak areas for AZIL and CILN in those solutions were recorded from the respective chromatograms. The linear regression factors were derived for AZIL and CILN's peak area values against AZIL and CILN's corresponding concentrations.

Analysis of AZIL and CILN contents in table formulations

Myotan CN tablet formulation (strength: 40 mg AZIL and 10 mg CLIN) was grounded into a fine powder and weighed. AZIL 40 mg and CILN 10 mg weight equivalent powders were deported to 100 ml std. volumetric flask. 50 ml diluent (mobile phase) was added followed by 30 min sonication, filtered through the membrane and filled up to 100 ml indication by diluent. Concentration of AZIL and CILN in Myotan CN stock solution was 400 µg/ml AZIL and 100 µg/ml CILN. One ml Myotan CN stock solution (400 µg/ml AZIL and 100 µg/ml CILN) was mixed with 9 ml diluent. Concentration of AZIL and CILN in the prepared solution was 40 µg/ml AZIL and 10 µg/ml CILN for analysis.

10 µl of Myotan CN sample prepared above was infused into HPLC system for analysis. Conditions given in the section "HPLC conditions" were applied. Chromatograms and peak response of AZIL and CILN were noted. Content of AZIL and CILN in Myotan CN sample was determined by using the peak response data from the chromatograms.

Stability assessments of AZIL and CILN

Stability assessments of AZIL and CILN were carried out with Myotan CN stock solution (400 µg/ml AZIL and 100 µg/ml CILN) as per ICH Q1A (R2) regulation [26].

Peroxide treatment

The sample solution of AZIL and CILN tablets (10 ml) was treated with 10 ml of H_2O_2 (30% in water) at room temperature over 30 min with sonication. Then the resultant solution was diluted to 100 ml and assessed according to the suggested technique.

Acid treatment

The sample solution of AZIL and CILN tablets (10 ml) was treated with 10 ml of HCL (0.1 N) at room temperature for over 30 min with sonication. Then the resultant solution was diluted to 100 ml and assessed according to the suggested technique.

Base treatment

The sample solution of AZIL and CILN tablets (10 ml) was treated with 10 ml of NaOH aqueous solution (0.1 N) at room temperature over 30 min with sonication. Then the resultant solution was diluted to 100 ml and assessed according to the suggested technique.

Thermal treatment

Finely grounded powder of AZIL and CILN tablets were placed on a petri plate and exposed to 60 °C for 30 min. The materials were subsequently cooled, dissolved in the mobile phase and assessed according to the suggested procedure.

Photo treatment

AZIL and CILN tablets were grounded into a fine powder, put on a petri plate and exposed to sunlight over six hrs. The materials were subsequently cooled, dissolved in the mobile phase and assessed according to the suggested procedure.

RESULTS AND DISCUSSION

HPLC method development

Mobile phases of varied solvent combinations and ratios with different columns were explored to acquire appropriate resolution and retention of AZIL and CILN as well as their generated degradants under the varied induced degradation conditions. Based on the chromatographic data obtained, it is established that the "C18 Supelco column (250 × 4.6mm, 5 µ)" and a mobile phase of Na_2SO_4 (0.1 M, pH 4.0):methanol at 60:40 (v: v) ratio with column flow rate at 1 ml/min gave adequate retention of AZIL and CILN and resolved them better from their generated degradants under varied induced degradation conditions (fig. 2). The wavelength of maximal absorption for AZIL and CILN was determined as 239 nm.

Method validation

The current method for quality control of AZIL and CILN in formulation tablets was evaluated to fulfill the requirements of ICH guidelines [27].

System suitability

10 µl of AZIL (40 µg/ml) and CILN (10 µg/ml) solution was injected to HPLC system. RSD (%) was calculated for AZIL and CILN peak response. The plate count, resolution, and tailing symmetry were also measured (table 1).

Selectivity

A test to determine selectivity was undertaken with blank mobile phase, AZIL and CILN solution (40 µg/ml AZIL and 10 µg/ml CILN) and Myotan CN sample (40 µg/ml AZIL and 10 µg/ml CILN). These three samples were made and injected (10 µl) to HPLC system. Fig. 3 shows the related chromatograms.

Linearity

Five calibration AZIL (range: 20–60 µg/ml) and CILN (range: 5–15 µg/ml) solutions were made. Each concentration solution was injected to the chromatographic system and peak areas of AZIL and

CILN peaks were measured after plotting peak areas against concentration. The linear regression equation and regression coefficient were calculated. Regression coefficient was 1.000 in the concentration range of 20–60 µg/ml of AZIL and 0.9998 in the concentration range of 5–15 µg/ml of CILN. Good linear relationship is observed within the studied concentration range.

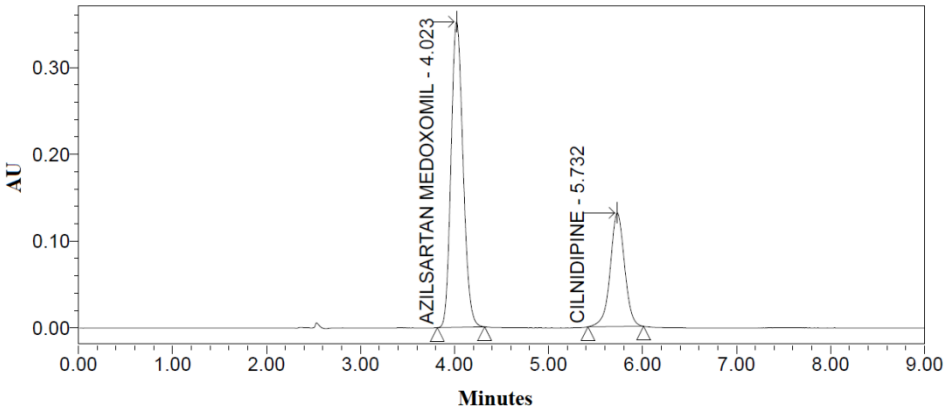


Fig. 2: RP-HPLC chromatogram of Myotan CN tablet formulation containing AZIL and CILN. Elution conditions: "C18 Supelco column (250 × 4.6 mm, 5 µ)" and a mobile phase of Na_2SO_4 (0.1 M, pH 4.0): methanol at 60:40 (v: v) ratio with column flow rate at 1 ml/min under room temperature. The injection volume was 10 µl

Table 1: Analysis of system suitability for AZIL and CILN

Statistics ↓	Retention time (min)	Area counts	Plate counts	Tailing factor	Resolution
AZIL					
Mean* value	4.0444	3168064	4591	1.156	-
SD value	0.0121	22852.8265	88.9579	0.0089	-
RSD value	0.2991	0.7213	1.9377	0.7737	-
CILN					
Mean* value	5.7832	1415455.4	6903.8	0.99	6.598
SD value	0.0267	10189.8303	86.1609	0.0071	0.0164
RSD value	0.4618	0.7199	1.2480	0.7142	0.2490

*Mean of five replicate analyses

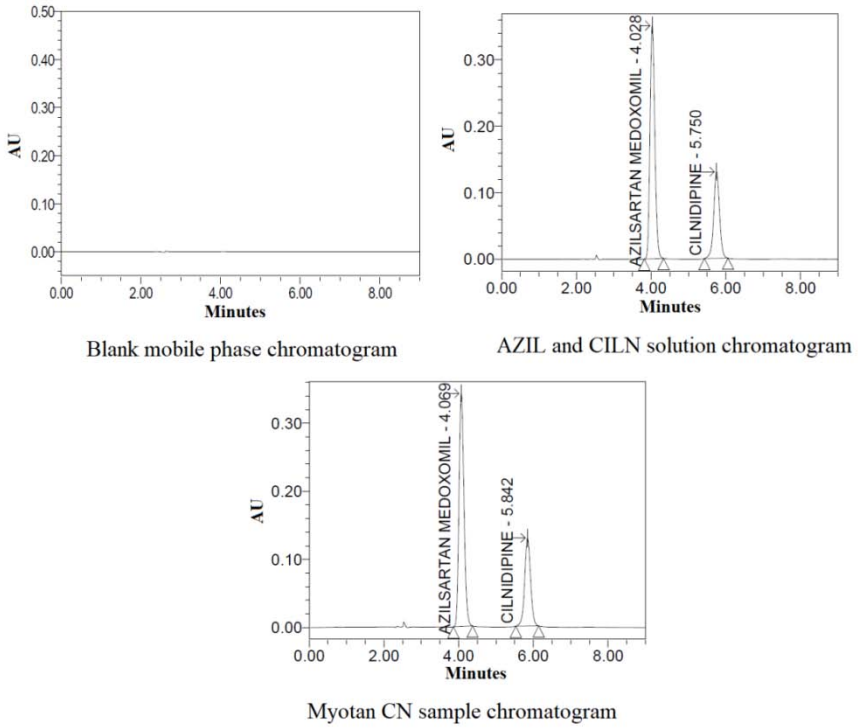


Fig. 3: RP-HPLC chromatograms of AZIL and CILN for selectivity study with mobile phase as blank. Experimental parameters were the same as those described in the legend of fig. 2

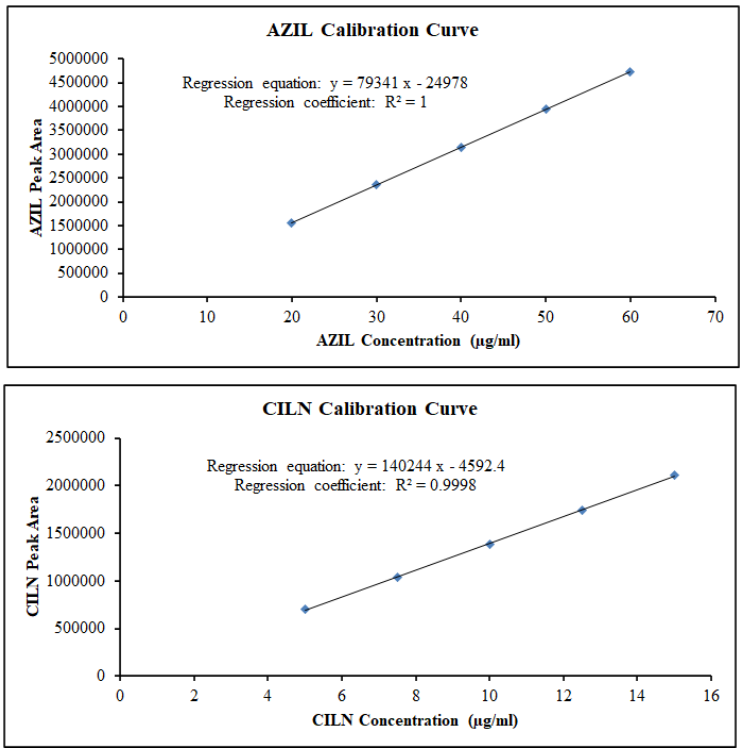


Fig. 4: Linearity curves of (A) AZIL and (B) CILN

The signal-to-noise (S/N) proportion was determined directly. The concentrations of AZIL and CILN that offer a signal-to-noise ratio of 3:1 are accepted as their limit of detection (LOD) value, while the concentrations that provide a signal-to-noise ratio of 10:1 are taken as their limit of quantitation (LOQ) value [12]. LOD values of AZIL and CILN are 0.082±0.0014 µg/ml and 0.055±0.0009 µg/ml, whereas LOQ values of AZIL and CILN are 0.272±0.0046 µg/ml and 0.183±0.0028 µg/ml, respectively.

Accuracy

The accuracy was evaluated by a recovery study of AZIL and CILN from Myotan CN tablets. Exact quantities of AZIL and CILN were combined with Myotan CN sample containing AZIL and CILN at three different concentrations. These three concentrations correspond to 50, 100 and 150% of the objective concentration, i.e., about 19.8, 39.60, and 59.40 µg/ml for AZIL and about 4.90, 9.80, and 14.7 µg/ml for CILN. Each concentration level of AZIL and CILN was made and tested in three replicates. For replicate specimens of AZIL and CILN, the recovery percentage of AZIL and CILN added was calculated (table 2).

Precision

Six standard solutions of each drug (AZIL 40 µg/ml and CILN 10 µg/ml) were made and injected into HPLC column. The RSD (%) for peak response of AZIL and CILN was calculated. The mean area counts of AZIL (40 µg/ml) and CILN (10 µg/ml) are 3147664±3188 (n = 6) and 1390139±6873 (n = 6) respectively, whereas the RSD (%) for AZIL is 0.1013 and that of CILN is 0.4944. It is within the recommended range of precision (0.3-3.0%) as suggested by ICH guidelines.

AZIL and CILN degradation profile

The information about the degradation and drug remaining after applying five different stress conditions are analyzed (table 3 and fig. 5). Five degradants of AZIL and CILN are found in acid stress condition. In both thermal and photo stress condition, four degradants of AZIL and CILN are identified. Three degradants of AZIL and CILN are observed in alkali treatment and peroxide stress condition.

Table 2: AZIL and CILN analysis-accuracy

Drugs	AZIL				CILN			
Added Level	µg/ml added	% Mean*	SD* value	RSD value	µg/ml added	% Mean*	SD* value	RSD value (%)
50%	19.80	99.20	0.3729	0.3759	4.9	101.59	0.1804	0.1775
100%	39.60	100.01	0.1861	0.1861	9.8	100.01	0.5914	0.5913
150%	59.40	100.34	0.0416	0.0415	14.70	101.14	0.1769	0.1749

*Three replicate analyses

Table 3: AZIL and CILN analysis–specificity/stability indicating

Stress conditions	AZIL area	*AZIL remained (%)	AZIL degraded (%)	CILN area	*CILN remained (%)	CILN degraded (%)
Acid treatment	2823927	88.57±0.293	11.22	1281967	90.35±0.479	9.61
Alkali treatment	2909066	91.64±0.256	8.54	1306064	92.49±0.564	7.91
Peroxide treatment	3021044	95.18±0.215	5.02	1326979	93.43±0.417	6.44
Thermal treatment	2884539	90.57±0.245	9.31	1266856	89.59±0.434	10.68
Photo treatment	2963587	93.09±0.533	6.83	1297827	91.17±0.559	8.49

*mean±SD; No. of experiments = 5

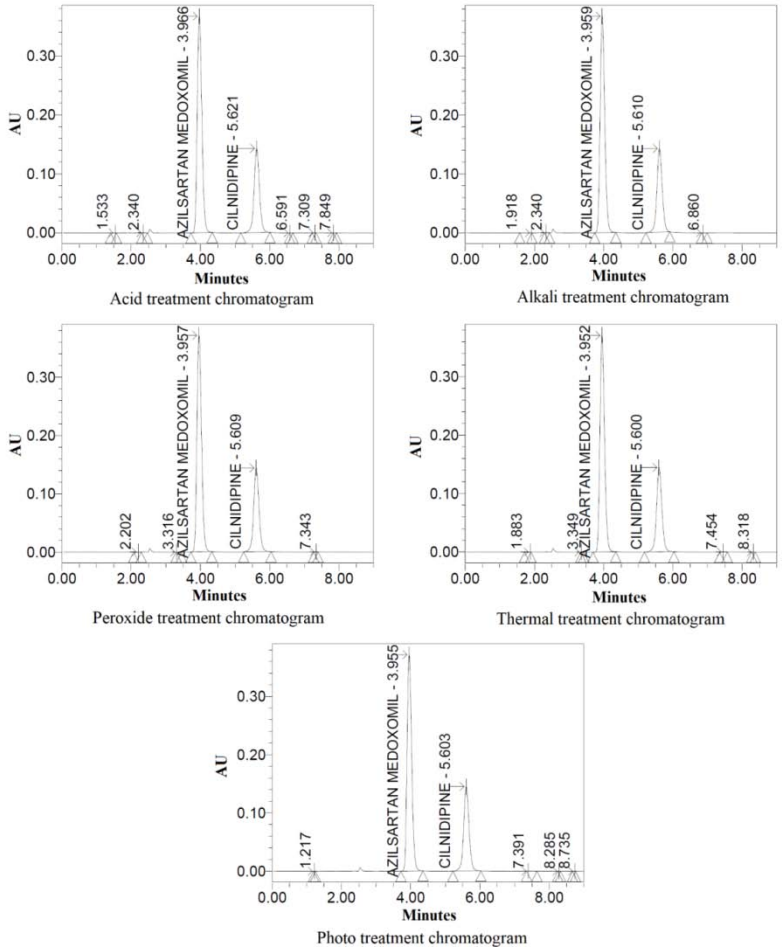


Fig. 5: RP-HPLC chromatograms of Myotan CN samples after degradation under different stress conditions: (a) acid treatment (b) alkali treatment (c) hydrogen peroxide treatment (d) thermal treatment (e) photo treatment

Robustness

AZIL (40 µg/ml) and CILN (10 µg/ml) solution is injected into HPLC column and analysed after the slight variation to the following parameters. The composition of Mobile phase was varied with respect to methanol content (±5%); flow rate was varied at the rate of±0.1 ml/min; column temperature was varied within±2 ℃; mobile phase pH was varied within±0.2 units and wavelength was varied within±2 nm.

The peak response, plate count, resolution, and tailing symmetry are measured and summarized in table 4.

Table 4: AZIL and CILN analysis–robustness

Drug	AZIL			CILN			
Statistics ↓	**Area**	**Plate count**	**Tailing**	**Area**	**Plate count**	**Tailing**	**Resolution**
Mobile phase variation: ±5% methanol ratio							
Mean* value	3120842.7	4735.7	1.2	1385931.3	6981.3	1.0	6.5
RSD value	1.2	1.5	1.5	1.9	1.5	1.2	1.3
SD* value	36062.1	73.1	0.0	26225.6	103.1	0.0	0.1
Flow rate variation: ±0.1 ml/min							
Mean* value	3120385.1	4679.7	1.2	1384609.3	7107.3	1.0	6.5
RSD value	1.8	1.6	1.5	1.7	0.6	1.2	1.7
SD* value	55287.7	72.8	0.0	24216.0	41.3	0.0	0.1
Temperature variation: ±2 °C							
Mean* value	3118078.0	4725	1.2	1380189.3	6986	1.0	6.6
RSD value	1.6	0.7	1.8	1.8	1.5	1.0	1.4
SD* value	50042.4	35.2	0.0	25100.1	103.5	0.0	0.1
pH variation: ±0.2 units							
Mean* value	3162552.8	4697.3	1.2	1410500.0	6950.3	1.0	6.6
RSD value	1.4	1.2	0.3	1.7	1.7	0.4	0.2
SD* value	42763.6	57.5	0.0	24398.1	119.8	0.0	0.0
Wavelength variation: ±2 nm							
Mean* value	3090243.3	4740.3	1.2	1385500.0	7030.1	1.0	6.6
RSD value	1.1	1.0	0.5	1.6	1.3	1.2	0.3
SD* value	33874.2	45.5	0.0	21734.1	89.2	0.0	0.0

*Mean of three replicate analyses

Swati and Anna (2022) reported the RP-HPLC method for quality control analysis of AZIL and CLIN blend in marketed formulations [28]. Riddhi and Satish quantified AZIL and CLIN blend in mixtures developed at lab using spectrophotometry [25]. It was discovered that the Swati and Anna method [28] had an excessive retention time, which increased the time for analysis and the cost of study. Riddhi and Satish [25] did not extend their approach to formulations, nor did they conduct degradation experiments on AZIL and CLIN.

System suitability evaluations are indeed an essential component of chromatographic procedures, and they are used to ensure that the system's accuracy and reliability are appropriate for the required analysis. Plate count, resolution, and tailing symmetry are evaluated and compared to the technique requirements [29]. Plate count, resolution, and tailing symmetry values measured during system suitability criteria testing are found in the limits of ICH acceptance criteria, which proves the suitability of the developed method [27].

The specificity/selectivity of the chromatographic methodology was examined to confirm that there's no intervention from inactive ingredients of formulations, mobile phase solvents or/and stress degradants [30-32]. Interference from excipients, mobile phase at RT of AZIL and CILN has not been noticed, which proves selectivity for AZIL and CILN analysis in Myotan CN sample. AZIL degradation is more in acidic stress condition than that of peroxide stress condition. Similarly, AZIL degradation is more in thermal stress condition compared with peroxide condition. The degradation analysis also proves the stability indicating power of the method [26].

The method's linearity must be verified to establish a proportionate correlation between response and analyte concentration across the method operating range. The correlation coefficient is frequently used to assess the adequacy of linearity information [33]. Linear regression assessment is being used to determine the graph's linearity. The outcome (low slope and intercept values and regression correlation greater than 0.999) proves linearity. The linearity findings demonstrate good linearity of the method [26].

LOD is regarded as the smallest quantity of analyte detectable above baseline noise, which is generally triple the value of noise intensity. LOQ is regarded as the smallest quantity of analyte that can be reproducibly quantitated beyond baseline noise, which is generally ten times the value of noise intensity [34]. Values of LOD and LOQ prove good sensitivity for analyzing AZIL and CILN.

The proximity of test findings achieved by an analytical methodology to the real value is defined as its accuracy. The recovery of known quantities of analyte which is spiked in blank matrices or formulations is used to check accuracy [35]. Recovery of AZIL and CILN (%) is near to 100 percent, proving accuracy of the present developed method.

Precision is the measurement of an analytical strategy's repeatability within normal conditions, and it is generally portrayed as a percent relative standard variation for statistically substantial sample size [36]. During the precision evaluation, RSD was less than 2.0%, demonstrating good precision of the method.

The robustness of an experimental methodology is indeed an indicator of its potential to stay unaffected by minute but purposeful alterations in procedure parameters, and it offers an indicator of its dependability under normal conditions [36]. During the robustness study, it is observed that system suitability execution values are inside the required limits.

CONCLUSION

For simultaneous quality control assay of AZIL and CILN in tablet formulation, a rapid selective and robust stability indicating HPLC technique is developed in this study. The method is assessed in accordance with ICH requirements and found that it is suitable for the intended use. The method is capable of providing accurate and precise quantitative results under slight variations in chromatographic circumstances.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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