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Research Article

Mathematically Processed UV Spectroscopic Method for Quantification of Chlorthalidone and Azelnidipine in Bulk and Formulation: Evaluation of Greenness and Whiteness

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A simple, eco-friendly four analytical methods were established by improving the selectivity through the application of mathematical processing of UV absorption spectra for concurrent quantification of chlorthalidone (CTL) and azelnidipine (AZE). The UV absorption spectra were recorded using environment-friendly ethanol (10% v/v) and were mathematically processed using simple software provided with a UV spectrophotometer. The analytes' peak amplitude was determined using zero-crossing point first derivative spectra and ratio first derivative spectra of CTL and AZE, which were measured at 238.5 nm and 239.5 nm for CTL and 272.1 nm and 342.1 nm for AZE, respectively. The peak amplitude difference was determined from the ratio spectra of CTL and AZE by measuring the peak amplitudes at 211.8 and 267.2 nm for CTL and 328.4 and 286.1 nm for AZE. Further, ratio spectra of CTL and AZE were converted into zero-order spectra by subtracting the constant followed by multiplication with divisor spectra, and the peak amplitudes were measured at 226.9 nm and 257.3 nm for CTL and AZE zero-order spectra, respectively. Further, validation results of all the four methods confirmed the accuracy and precision of the methods by displaying good recovery (98.37–100.34%) and percentage relative standard deviation (0.397–1.758%), respectively. Good linearity was observed in the range of 1–15 μg/mL for both analytes with less than a 1 μg/mL limit of quantification. Further, the greenness and whiteness of the methods were evaluated by recently proposed AGREEness, complexGAPI, and white analytical chemistry techniques. The proposed UV spectroscopic methods were environmentally friendly, safe, economic, and effective, hence, could be used for regular quality control study of a formulation containing CTL and AZE.

1. Introduction

Cardiovascular disease (CVD) is a leading cause of death in elderly patients, and it is increasing in developing countries. WHO predicted that more than 75% of CVD events could be prevented [1]. Control of blood pressure is an important part of CVD management. The use of a combination of different antihypertensive agents with a different mechanism of action reduced mortality and morbidity due to CVD [2]. A fixed-dose combination of calcium channel blocker (azelnidipine) and diuretics (chlorthalidone) showed a positive effect in reducing the risk of stroke, myocardial infraction, and cardiovascular mortality [3]. Chlorthalidone (CTL, Figure 1(a)) is an oral diuretic agent and due to its prolonged effect, it is used as an antihypertensive drug [4]. It acts by decreasing the plasma volume, cardiac output, and extracellular fluid volume, by increasing the excretion of sodium, chloride, and water, along with the vasodilation effect by inhibiting carbonic anhydrase [5]. Azelnidipine (AZE, Figure 1(b)) is a third-generation, dihydropyridine, long-acting calcium channel antagonist, antihypertensive effect is produced by inhibiting L-type Ca^{+2} channels followed by vasodilation of vascular smooth muscle. AZE also showed cardio-protective, cerebro-protective, lipid-lowering effects along with improvement in insulin resistance [6, 7]. Hence, the fixed-dose combination of CTL and AZE has been developed for the treatment of hypertension.

The literature reported few analysis methods for quantification of CTL and AZE alone and in combination with other antihypertensive drugs. Several spectrophotometric methods [8–14], spectrofluorometric methods [8, 13], HPLC [14–16], and HPTLC [17] methods were carried out for the estimation of CTL and AZE with other analytes in the formulations. Stability indicating RP-HPLC methods [18–22] were also reported in the literature for quantification of CTL and AZE separately. In addition, LCMS methods [23, 24] were utilized for the determination of CTL and AZE separately and with other drugs in biological samples. However, no analytical method has been developed for simultaneous determination of CTL and AZE in bulk and formulation.

The development of eco-friendly analytical methods is the present trend to make environmental sustainability and reduce or eliminate the use and generation of toxic chemicals during the analysis [25]. The extensively used analytical method for quality control of pharmaceuticals is the liquid chromatographic method, because of its sensitivity and specificity. However, chromatographic methods use an enormous amount of hazardous solvents creating a large quantity of waste having toxic effects and requiring a long analysis time. Hence, the alternative analytical method needs to be used for the routine quality control of pharmaceuticals. UV spectroscopic method is simple and fast, and there are many official assay methods in the pharmacopeia [26]. However, it suffers from the selectivity of multicomponent formulation due to good absorption of UV light by most pharmaceuticals and complete overlapping of spectra. The selectivity issue of the UV spectroscopic method can be solved by developing mathematically processed techniques such as derivative and ratio derivative spectroscopic methods, without the use of any additional hazardous chemicals [27, 28]. Derivatization increases the resolution of the individual spectrum and allows the quantification of an analyte in the presence of other analytes and formulation excipients without prior separation [29–34]. To make more greener analytical method, 12 green analytical principles were adopted during the development of the derivative UV spectroscopic method for simultaneous determination of CTL and AZE in bulk and fixed-dose formulation. Finally, different greenness and whiteness evaluation methods were applied to confirm the eco-friendly nature of the developed analytical methods.

2. Materials and Methods

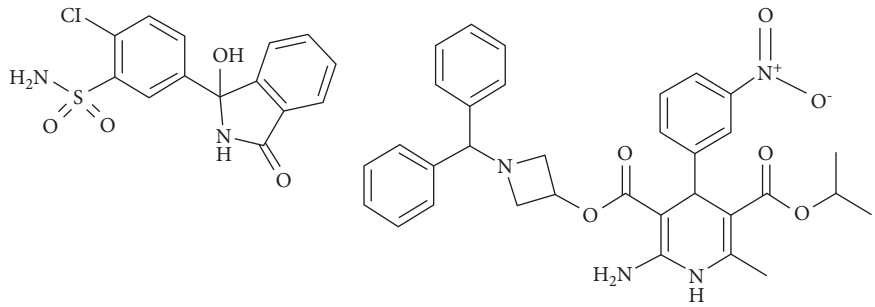
2.1. Chemicals and Reagents. Analytically pure standards of chlorthalidone (purity >99.5%) and azelnidipine (purity >98.9%) were purchased from Biochemix India Limited (Hyderabad, India). Absolute ethyl alcohol (ethanol) used for the preparation of standard and sample solutions was of analytical grade procured from Sigma Aldrich (MA, USA). Ultrapure water was prepared using Milli Q (Millipore, USA) water purifier and was used throughout the experiments. The fixed-dose combination of CTL and AZE is not available in the market; hence, separate tablets consisting of CTL (6.25 mg and 12.5 mg) and AZE (8 mg) were purchased from the local market.

2.2. Apparatus and Software. UV spectrophotometer (1650, Shimadzu, Japan) connected to an autosampler (6 cuvettes) and a personal computer was used for recording the UV absorption spectra. The identical quartz cuvettes with 10 mm path length were used for both standard, sample, and blank solutions. UV spectrophotometer was adjusted to fast scanning speed with a slit width of 0.1 nm. The spectra were mathematically processed using UV probe software provided with the instrument (Ver. 2.2, Shimadzu, Japan).

2.3. Preparation of Standard and Working Standard Solutions. Stock solutions of CTL and AZE (1 mg/mL) were prepared by transferring 50 mg of analytes into two volumetric flasks (50 mL) separately. The working standard solutions, for validation of the developed methods, were prepared by diluting the required amount of stock solutions of both the analytes with water.

2.4. Procedure for Each Method

2.4.1. First Derivative (1DD). Working standard solutions of CTL (1–15 μg/mL) and AZE (1–15 μg/mL) were prepared separately transferring an aliquot of CTL and AZE stock solutions into a 5 mL volumetric flask. The final volume was adjusted with water by maintaining 500 μl of ethanol in each flask. The UV absorption spectra were recorded for all solutions against ethanol-water (10 v/v) as blank. The spectra were converted into first derivative spectra using 10 nm as $\Delta\lambda$ with a scaling factor of 100. The peak amplitudes were



(a) (b)

Figure 1: Chemical structure of (a) chlorthalidone and (b) azelnidipine.

recorded at 238.5 nm and 272.1 nm from CTL and AZE first derivative spectra, respectively. The calibration curve was constructed between peak amplitudes and corresponding concentrations for both analytes.

2.4.2. Ratio Absorption Difference (RAD). Above-recorded UV absorption spectra of CTL (1–15 μg/mL) and AZE (1–15 μg/mL) were divided separately by the spectrum of AZE (2 μg/mL) and CTL (8 μg/mL), respectively, to generate the ratio spectra of CTL and AZE respectively. The peak amplitude difference was calculated from the ratio spectra of CTL by subtracting the peak amplitude at 267.2 nm from the peak amplitude of 211.8 nm, and the calibration curve was constructed by plotting against the corresponding concentration. Similarly, the peak amplitude difference was calculated from the ratio spectra of AZE by subtracting the peak amplitude at 286.1 nm from the peak amplitude of 328.4 nm, and the calibration curve was constructed by plotting against the corresponding concentration.

2.4.3. Ratio First Derivative (R^1DD). The above-generated ration spectra of CTL (1–15 μg/mL) and AZE (1–15 μg/mL) were converted into first derivative spectra using 10 nm as $\Delta\lambda$ with a scaling factor of 100. Then the peak amplitude was recorded at 239.8 nm and 342.1 nm from the first derivative spectra of CTL and AZE, respectively. The linearity curve was plotted against corresponding concentrations of both the analytes.

2.4.4. Ratio Constant Subtraction (RCS). Working standard solutions consisting of a mixture of both analytes in the concentration of 1–15 μg/mL were prepared, and UV absorption was measured between 200 and 400 nm. For measurement of CTL, combined spectra were divided by the spectrum of AZE, to get the ratio spectra, and a constant absorption value sufficient to bring the spectra to the baseline was subtracted. Later the spectra were multiplied with the spectrum of AZE to generate the zero-order spectra of CTL. The calibration curve was constructed by measuring the absorption at 226.9 nm against the corresponding concentration. Similarly, to generate the AZE zero-order spectra, combined spectra were divided by the spectrum of CTL to generate ratio spectra, followed by subtraction of a constant, resulting spectrum was multiplied by the CTL spectrum. Absorption was measured at 257.3 nm and plotted against the corresponding concentration of AZE.

2.5. Procedure for Laboratory Mixed Solutions. The laboratory mixed solutions were prepared by transferring the required amount of CTL and AZE stock solutions in the ratios of 1 : 15, 8 : 8, 15 : 1, 4 : 12, and 12 : 4 μg/mL, slightly below and above the formulation concentration and within the calibration curve range, respectively. The laboratory mixed solutions were scanned in the wavelength range of 200–400 nm, and the concentration of each analyte was determined following the general procedure of all four methods.

2.6. Procedure for Pharmaceutical Dosage Form. The newly approved medicine consisting of CTL and AZE is not manufactured and sold yet in the local market. The fixed-dose combination of this medicine was formulated in two strengths (6.25 mg CTL + 8 mg AZE and 12.5 mg CTL + 8 mg AZE). Hence, tablets consisting of CTL (6.25 mg and 12.5 mg) and AZE (8 mg) were procured from the local market. To mimic the real sample, ten tablets of CTL 6.25 mg and ten tablets of AZE 8 mg were weighed and crushed into powder. Similarly, ten tablets of CTL 12.5 mg and ten tablets of AZE 8 mg were weighed and crushed into powder. Powder equivalent to 6.25 mg of CTL + 8 mg of AZE and 12.5 mg of CTL + 8 mg of AZE was weighed and dissolved in 50 mL of ethanol separately and filtered to get clear solutions of sample solutions consisting of 125 μg/mL + 160 μg/mL and 250 μg/mL + 160 μg/mL of CTL and AZE, respectively. The sample solutions were diluted with water to bring the concentration in the range of the calibration curve and scanned in the wavelength range of 200–400 nm. Finally, the concentration of each analyte was determined following the general procedure of all four methods using corresponding regression equations.

3. Results and Discussion

Molecular absorption spectroscopy is extensively used in the quality control department for the quantification of drugs in the formulation due to its simple, accurate, and rapid

analysis. However, most of the drugs have high UV absorption due to the presence of highly conjugated systems and aromatic and heterocyclic rings. Hence, the development of the UV abstraction spectroscopic method for simultaneous determination of multicomponent formulations becomes difficult due to the complete overlap of spectra of the analytes (Figure 2(a)). However, resolution between the two spectra is possible by mathematical modification of overlapped spectra, such as different order derivatization, and creation of ratio spectra to quantify specifically one analyte in presence of another [27–33]. Hence, in the present work, four mathematically processed simple, eco-friendly UV absorption spectroscopic methods were established for simultaneous determination of CTL and AZE in coformulated medicine.

3.1. First Derivative (^{1}DD). Salinas et al. [28] established derivatization of UV absorption spectra for quantification of multicomponent formulations. Where the measurement of the peak amplitude of the derivative spectra at the zero crossing point of one of the components, where another compound show some absorption, allows us to quantify analytes in presence of another analyte. The first derivative spectrum of CTL showed two zero-crossing points at 238.5 nm and 248.8 nm. However, at 238.5 nm, the peak amplitude for AZE was better and showed good sensitivity. Similarly, AZE showed three zero-crossing points at 272.1 nm, 324.8 nm, and 375.2 nm; however, CTL showed good peak amplitude at 272.1 nm and better recovery. Further, wavelength ($\Delta\lambda$) for derivation has been investigated, different wavelengths starting from 5, 10, 20, 30, and 50 nm were tried, with higher wavelength smooth spectra that were obtained, but important peaks were merged; however, with 10 nm good shape, smooth spectrum was obtained. The intensity of the first derivative spectra was low for both the analytes; hence, scaling factor 10 was used to get good peak amplitude during derivatization. Further, a comparison of the first derivative spectra of a mixture of both the analytes and pure analytes consisting of the same amount of analytes showed the same peak amplitudes (Figure 2(b)). In the present work, AZE (1–15 μg/mL) and CTL (1–15 μg/mL) were converted into first derivative spectra with a scaling factor of 100 and peak amplitudes were measured at 238.5 nm and 272.1 nm, respectively (Figures 2(c) and 2(d)).

3.2. Ratio Absorption Difference (RAD). The ratio absorption difference method [33, 34] involves two steps, the first step is the formation of ratio spectra, and the second step is the determination of peak amplitude difference, which is directly proportional to the concentration of one of the analytes. If the two analytes have overlapping spectra, ratio absorption spectra were generated by dividing the mixture spectra ($A_{TZ} = \mathcal{E}_Z C_Z + \mathcal{E}_T C_T$) with one of the analyte spectra ($A_T = \mathcal{E}_{T'} C_{T'}$) with concentration $C_{T'}$, which represents the ratio spectra and expressed as follows:

$$\frac{A_{TZ}}{A_{T_1}} = \frac{\varepsilon_Z C_Z}{\varepsilon_T C_{T_1}} + \frac{\varepsilon_T C_T}{\varepsilon_{T_1} C_T}, \tag{1}$$

where $\mathcal{E}_Z$ and $\mathcal{E}_T$ are molar absorptivity of AZE and CTL, respectively, and C_Z and C_T are the concentration of AZE and CTL, respectively.

This can be simplified as below

$$\frac{A_{TZ}}{A_{T_1}} = \frac{A_Z}{A_{T_1}} + \frac{C_T}{C_{T_1}} \text{ (constant)}, \tag{2}$$

Equation (2) can be simplified as

$$R_{TZ} = R_z + K, \tag{3}$$

where R_{TZ} is $A_{TZ}/A_{T'}$ ratio absorption spectra optioned by dividing the mixture spectra with one of the component spectra at a certain concentration ($C_{T'}$), R_z is $A_Z/A_{T'}$ ratio spectra of AZE to the CTL, and K is constant.

In Equation (3), the constant along with other instrumental errors and interference from formulation excipients can be eliminated by subtracting peak amplitude at two different wavelengths (λ_1 and λ_2):

$$\Delta R = R_{Z1} - R_{Z2} = \left[\frac{A_Z}{A_{T_1}} + \text{constant}\right]_{\lambda 1} - \left[\frac{A_Z}{A_{T_1}} + \text{constant}\right]_{\lambda 2}, \tag{4}$$

where R_{Z1} and R_{Z2} are peak amplitudes at two different wavelengths λ_1 and λ_2, respectively. ΔR is the difference in the peak amplitudes at λ_1 and λ_2, representing the absorption of only one component (AZE), eliminating the effect of CTL.

The effect of concentration of dividing spectrum was envisaged by selecting 0different concentration spectra of both the analytes in the range of 1–15 μg/mL; however, CTL spectrum with 8 μg/mL and AZE spectrum with 2 μg/mL showed good recovery of AZE and CTL from the laboratory mixed solutions. Further, a comparison of the ratio spectra generated from a mixture of both the analytes and pure analytes showed the same peak amplitude difference, indicating the elimination of the absorption effect of one of the components (Figures 3(a) and 3(b)).

In the present work, ratio spectra of CTL (Figure 3(c)) were generated by dividing CTL spectra (1–15 μg/mL) with a spectrum of AZE (2 μg/mL), and the peak amplitude difference was determined by subtracting the peak amplitude at 267.2 nm from the 211.8 nm, and the calibration curve was constructed by plotting against the corresponding concentration of AZE. Similarly, ratio spectra of AZE (Figure 3(d)) were generated by dividing the spectra of AZE (1–15 μg/mL) by the spectrum of CTL (8 μg/mL), and the peak amplitude difference was determined by subtracting the peak amplitude at 286.1 nm from the 328.4 nm, and the calibration curve was constructed by plotting against the corresponding concentration of AZE.

3.3. Ratio First Derivative (R^1DD). The derivatization of ratio spectra resolves the effect of one of the components of

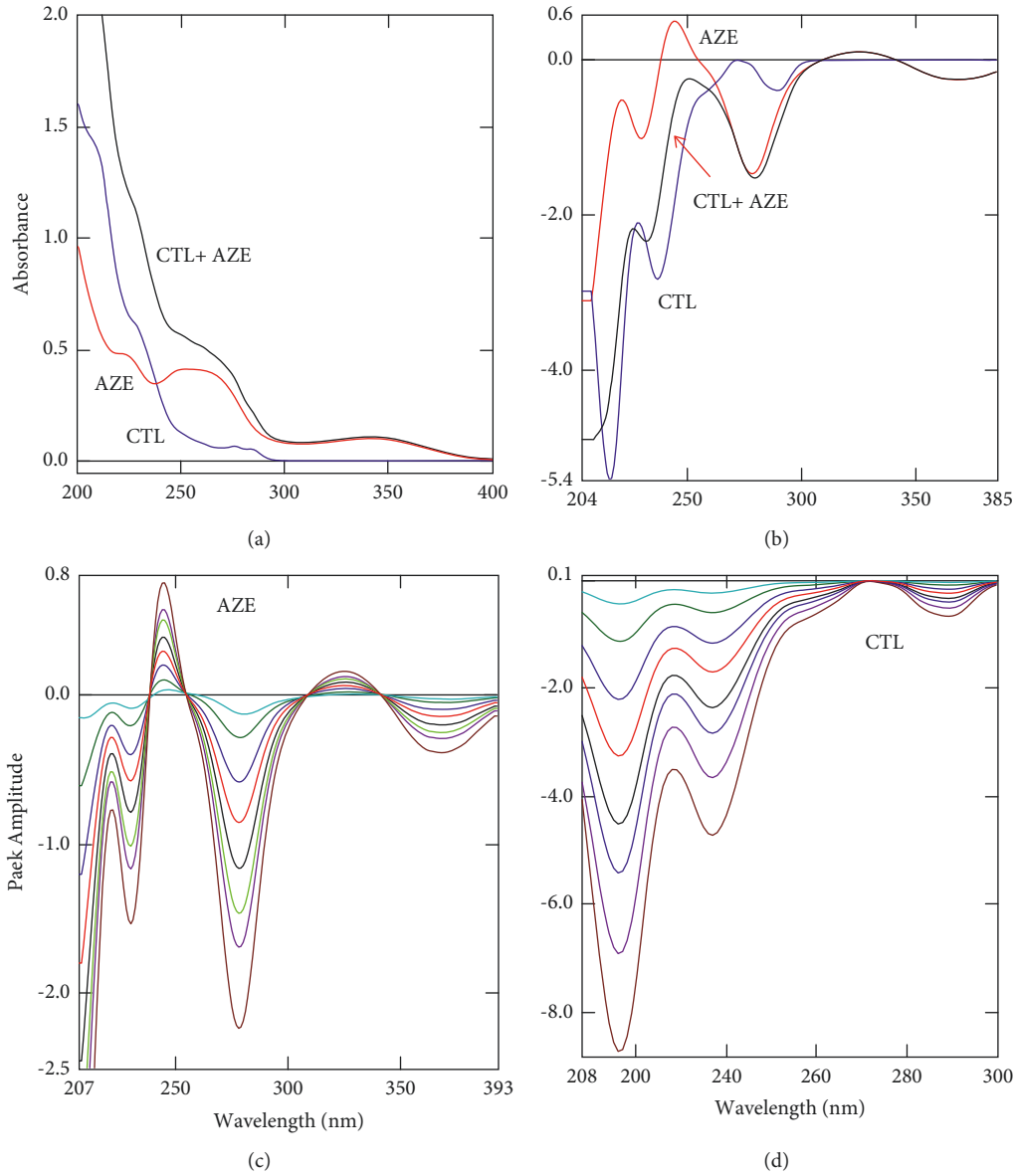


Figure 2: (a) Normal spectra of CTL (blue), AZE (red), and Mixture (black); (b) first derivative spectra of CTL (blue), AZE (red), and mixture (black); (c) first derivative spectra (1–15 μg/mL) of AZE; (d) first derivative spectra (1–15 μg/mL) of CTL.

completely overlapping spectra [28, 33, 34]. It also allows us to use the highest peak amplitudes for quantification of analytes due to the presence of many maxima and minima and is it not necessary to look for zero-crossing points. Further, ratio spectra followed by derivation allow direct quantification of one of the analytes in the presence of another and formulation excipients. In the present work, the ratio spectra of CTL and AZE were converted into first derivative spectra using 10 nm as $\Delta\lambda$. The wavelength was selected based on the peak amplitude and recovery study. The ratio first derivative spectra of CTL showed three minima at −219.2 nm, −239.8 nm, and −290.9 nm. However, the linearity was good at all three wavelengths, but the recovery of CTL was better at 239.8 nm. Similarly, for the quantification, AZE peak amplitudes were measured at two maxima at 297.2 nm and 320.8 nm and two minima at −342.1 nm and −365.2 nm, respectively. The linearity was good at all wavelengths, the percentage recovery was better at 342.1 nm due to the higher signal-noise ratio. Further, the ratio first derivative spectra of a mixture of both analytes and the pure analytes with the same concentration showed the same amplitude at 239.8 nm and 342.1 nm for CTL and AZE, respectively (Figures 4(a) and 4(b)). In the present work, the ratio first derivative of CTL and AZE (Figures 4(c) and 4(d)) showed good linearity in the concentration range of 1–15 μg/mL with a good regression coefficient at 239.8 nm and for CTL and at −342.1 nm for AZE.

3.4. Ratio Constant Subtraction (RCS). The ratio spectra that represent Equation 2 were developed by dividing the spectra of the mixture of both the analytes CTL (T) and AZE (Z) with a carefully selected concentration spectrum of standard

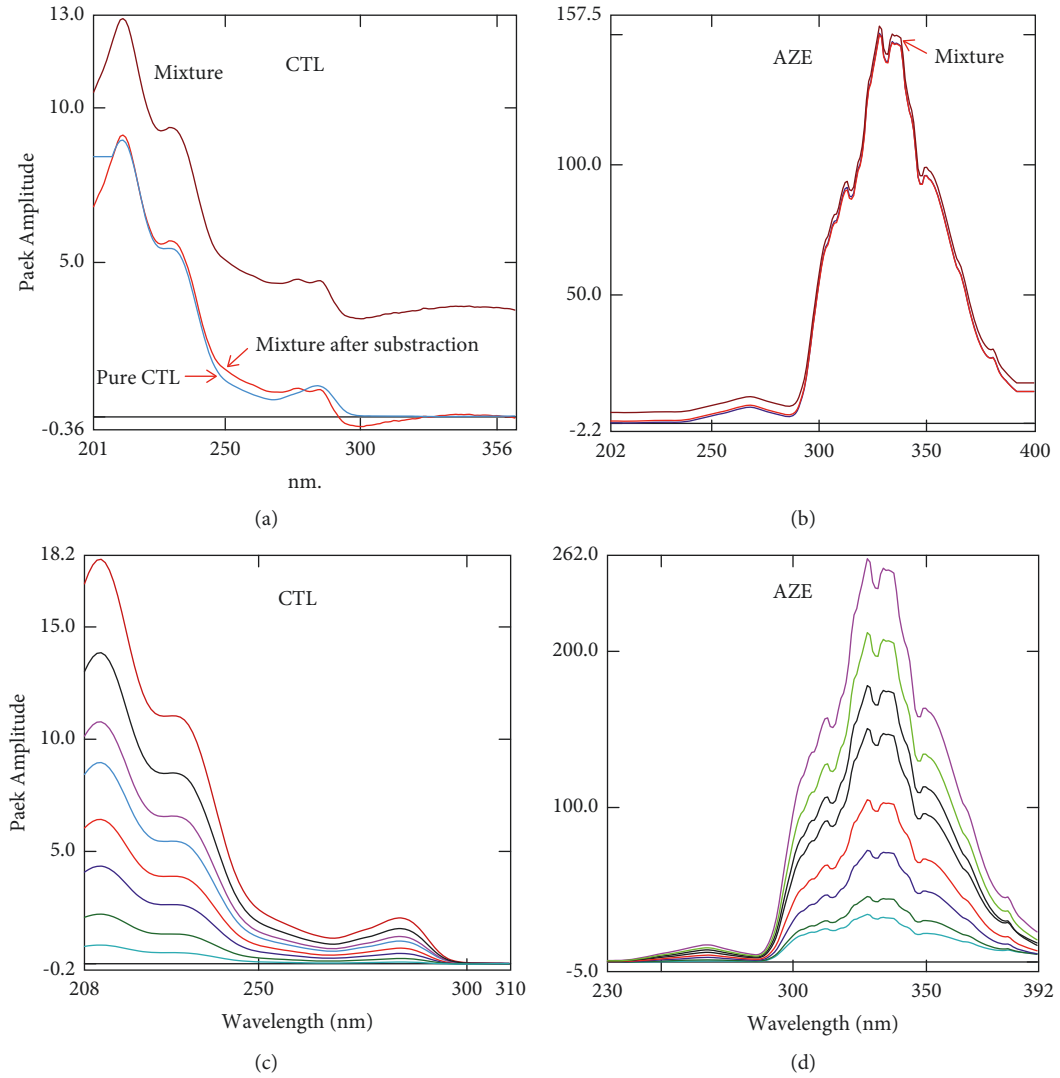


Figure 3: (a) A comparison of ratio spectra generated from pure CTL and mixture of CTL + AZE. (b) A comparison of ratio spectra generated from pure AZE and mixture of CTL + AZE (b). (c) Ratio spectra of CTL 1–15 μg/mL. (d) Ratio spectra of AZE 1–15 μg/mL.

CTL (T') [34]. The constant CT/CT' absorption was subtracted at 325 nm to bring the ratio spectra to the baseline, followed by multiplication with the standard spectrum of CTL ($A_{T'}$ used as a divisor to generate spectra) to generate the zero-order spectra of AZE [33]:

$$\frac{A_{TZ}}{A_{T'}} = \frac{A_Z}{A_{T'}} + \frac{C_T}{C_{T'}} \text{ (constant)},$$

$$\frac{A_{TZ} * A_{T'}}{A_{T'}} = \frac{A_Z * A_{T'}}{A_{T'}}, \tag{5}$$

$$A_{TZ} = A_Z.$$

Finally, zero-order spectra of AZE (1–15 μg/mL) (Figure 5(a)) were used for the construction of the calibration curve by measuring the peak amplitude at 257.3 nm against the corresponding concentration. Similarly, zero-order spectra for CTL (1–15 μg/mL) were generated (Figure 5(b)) by subtracting the constant from the ratio spectra at 211 nm followed by multiplication with the spectrum of AZE, used as a divisor to generate the ratio spectra of CTL. Peak amplitude was measured at 226.8 nm, and the calibration curve was constructed against the corresponding concentration of CTL. Further, the same peak amplitude was observed at wavelengths 257.3 and 226.8 nm of zero-order spectra generated from the mixture ratio spectra and pure zero-order spectra for AZE and CTL, respectively (Figures 5(c) and 5(d)).

3.5. Method Validation. Confirmation of the linearity, sensitivity, accuracy, precision, and selectivity of the proposed methods was carried out as per the ICH guidelines [35] by performing the validation experiments by simultaneous determination of CTL and AZE using the developed UV spectroscopic method.

3.5.1. Linearity. Seven concentrations of both the analytes in the range of 1–15 μg/mL were analyzed three times on

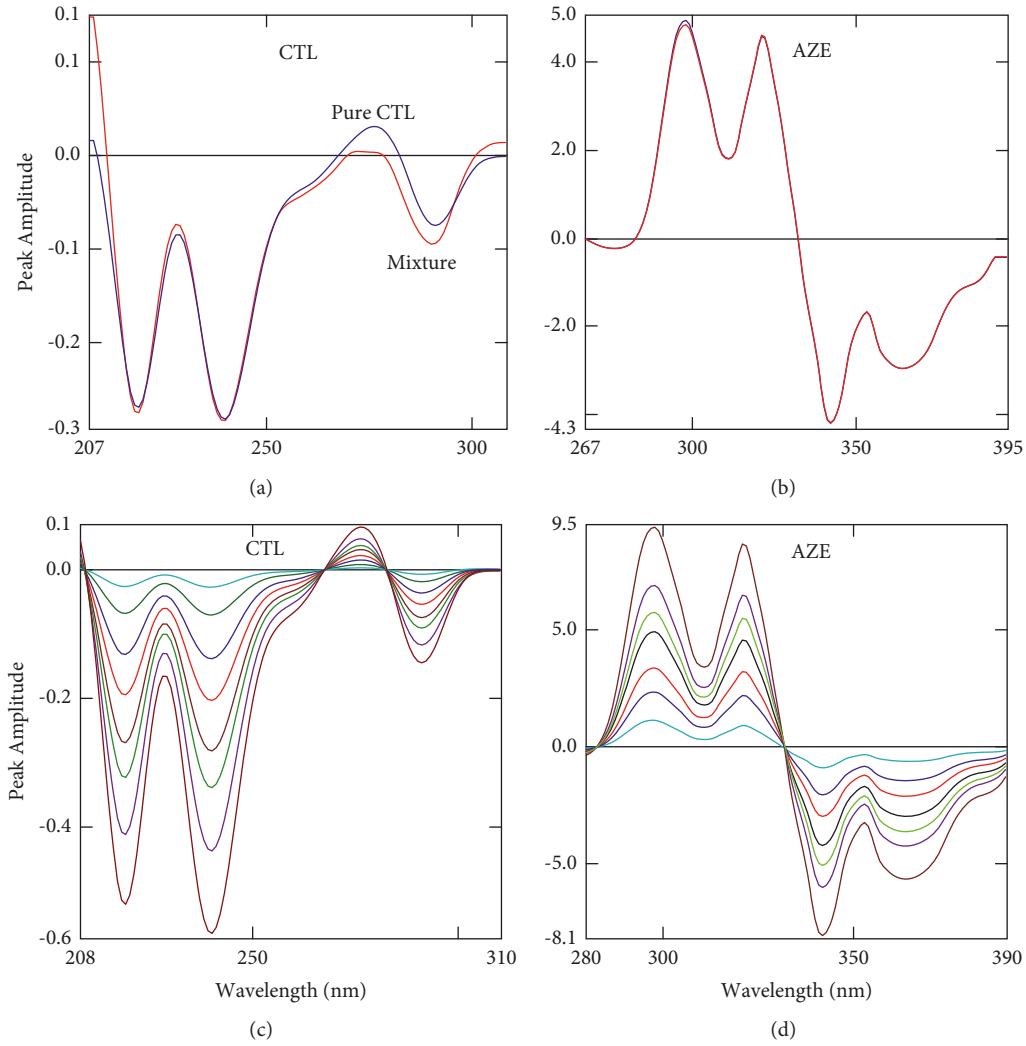


Figure 4: (a) Ratio first derivate spectra of pure CTL (blue) and mixture (red), (b) Ratio first derivate spectra of pure AZE (blue) and mixture (red). (c) Ratio first derivative spectra of CTL (1–15 μg/mL). (d) Ratio first derivative spectra of AZE (1–15 μg/mL).

three different days to construct the calibration curves. The linearity concentration of both the analytes was selected based on the amount of analytes in the formulation. Good linearity was obtained by all four methods with good regression coefficients ($R^2 > 0.999$). The wavelength used for measuring the peak amplitude, linearity range, regression equations, and coefficient values are tabulated in Table 1.

3.5.2. Sensitivity. The sensitivity of the method confirms the minimum quantity that can be detected and quantified by the proposed methods in terms of limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were determined from the calibration curve parameters using 3.3 times d/s and 10 times d/s, respectively, where d is the standard deviation of the response and s is the slope of the calibration curve. The LOQ was found to be below 1 μg/mL for both the analytes by all four methods (Table 1).

3.5.3. Accuracy. The accuracy of the assay results obtained by the proposed methods was evaluated by analyzing the three different concentrations of both the analytes in the calibration range in triplicate. The concentration of the analytes was calculated from the respective regression equations. Then the percentage recovery and percent relative error were calculated at three different concentrations and the mean percentage recovery was computed as shown in Table 1. The mean percentage recovery was found to be in the range of 98.95%–100.24% and 98.37%–100.38% with less than ±2 relative standard error for CTL and AZE, respectively, by all the four methods.

3.5.4. Precision. The precision of the proposed four methods was expressed as intraday and interday precision. For interday precision, three different concentrations of both the analytes were analyzed in triplicates by all four methods. For the intraday precision, the same solutions were analyzed for three consecutive days. The

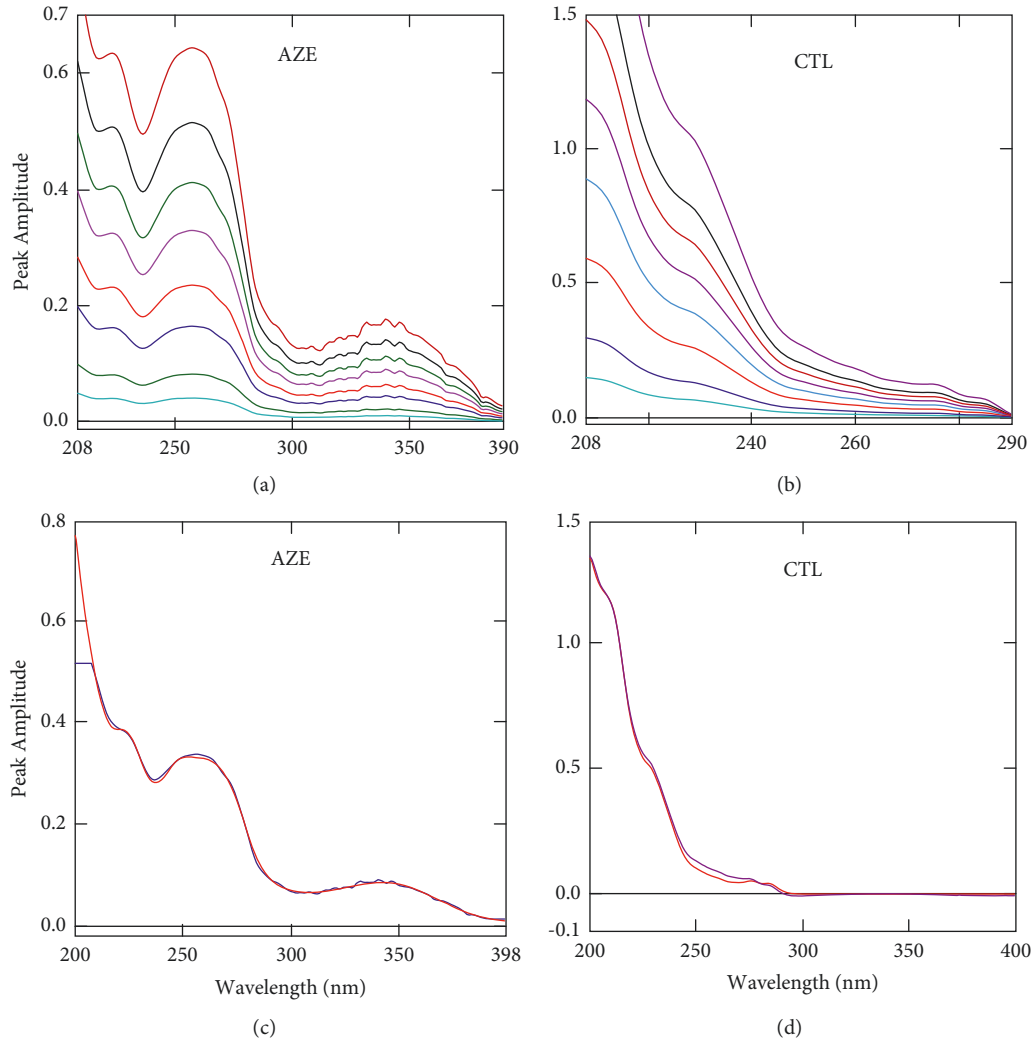


Figure 5: (a) Zero-order spectra of CTL (1–15 μg/mL). (b) Zero-order spectra of AZE (1–15 μg/mL). (c) Comparison of zero-order spectra of pure CTL (red) and mixture (blue). (d) Comparison of zero-order spectra of pure AZE (red) and mixture (blue).

Table 1: Validation parameter results of the proposed spectroscopic methods for the simultaneous determination of CTL and AZE.

Validation parameters	Chlorthalidone				Azelnidipine			
	^{1}DD	RAD	R^{1}DD	RCS	^{1}DD	RAD	R^{1}DD	RCS
Wavelength (nm)	238.5	211.8–267.2	239.8	226.9	272.1	328.4–286.1	342.1	257.3
Linearity range (μg/mL)	1–15	1–15	1–15	1–15	1–15	1–15	1–15	1–15
Slope	0.3025	1.0924	0.0367	0.0684	0.1129	16.441	0.4986	0.0432
Intercept	−0.0772	−0.3106	−0.0091	−0.0063	−0.0129	8.8035	0.1193	−0.0089
Regression coefficient (r^2)	0.9996	0.9998	0.9998	0.9997	0.9999	0.9997	0.9997	0.9995
LOD (μg/mL)	0.305	0.267	0.408	0.306	0.128	0.301	0.186	0.328
LOQ (μg/mL)	0.924	0.768	0.806	0.928	0.387	0.933	0.599	0.995
Accuracy (mean % ± RSE)	100.24 ± 0.92	98.95 ± 1.04	99.47 ± 1.34	99.27 ± 1.61	98.37 ± 1.48	99.46 ± 0.54	100.34 ± 1.49	100.38 ± 1.41
Precision (%RSD)								
Intraday	1.053	0.945	1.438	0.893	1.232	0.715	1.758	1.422
Interday	1.127	0.846	0.807	1.803	1.459	0.397	0.931	1.087

RSE: Relative standard error; RSD: Relative standard deviation.

percentage relative standard deviation calculated to express the precision of the proposed methods was recorded in Table 1.

3.5.5. Selectivity. The major issue with the quantification of multicomponent formulation by UV spectroscopic method is the selectivity due to high UV absorption in the range of

Table 2: Assay results of the laboratory mixed solutions of CTL and AZE.

Laboratory prepared mixture (μg/mL)		Chlorthalidone (% recovery)				Azelnidipine (% recovery)			
CTL	AZE	^{1}DD	RAD	R^{1}DD	RCS	^{1}DD	RAD	R^{1}DD	RCS
1	15	98.26	100.67	99.27	100.45	99.66	100.37	99.15	99.34
8	8	99.18	99.24	98.69	98.46	100.57	99.08	101.26	99.17
15	1	101.34	99.47	101.33	101.34	99.28	100.64	98.45	100.78
4	12	100.79	98.39	98.92	99.94	98.71	101.18	99.44	98.66
12	4	99.28	100.08	101.04	100.47	100.83	100.38	100.83	100.93
Across mean		99.77	99.57	99.85	100.13	99.81	100.33	99.83	99.78
Standard deviation		1.26	0.86	1.24	1.06	0.88	0.77	1.18	1.02

Table 3: Assay results of the formulation and the standard addition method results.

Formulation concentration		Chlorthalidone (mean % ± SD)				Azelnidipine (mean % ± SD)			
CTL	AZE	^{1}DD	RAD	R^{1}DD	RCS	^{1}DD	RAD	R^{1}DD	RCS
6.25 mg	8 mg	100.28 ± 0.54	98.79 ± 1.04	100.49 ± 0.75	99.39 ± 1.34	100.92 ± 0.75	99.67 ± 1.71	100.32 ± 0.62	99.44 ± 0.67
12.5 mg	8 mg	99.47 ± 1.42	98.76 ± 0.94	99.76 ± 1.81	100.43 ± 0.88	99.53 ± 1.66	98.85 ± 0.98	100.54 ± 0.84	101.24 ± 1.78
Standard addition method									
Amount added (μg/mL)		Chlorthalidone (% recovery)				Azelnidipine (% recovery)			
3.125	2	100.49	99.75	100.64	101.39	100.34	99.37	100.08	100.49
6.25	4	99.78	101.66	99.45	99.34	99.42	98.55	99.36	99.78
9.375	6	100.24	99.46	98.76	98.71	101.84	100.42	98.7	100.24
Across mean		98.71	100.17	100.29	99.62	99.81	100.53	99.45	99.38
%RSD		0.36	1.19	0.95	1.40	1.22	0.94	0.69	0.36

SD: standard deviation.

200 nm to 400 nm. Hence, selectivity was established by analyzing the different ratios of both the analytes in the concentration just above and below the formulation amount, within the range of the calibration curve. The mean percentage recovery calculated from the three determinations confirmed the selectivity of the methods (Table 2). Further percentage of relative standard deviation is within the acceptable range. Additionally, selectivity and accuracy were confirmed by the recovery study of both the analytes by the standard addition method. The previously analyzed sample solution (3.125 μg/mL of CTL + 4 μg/mL of AZE) was mixed with three different concentrations (50% 100 and 150%) of standard analytes solutions and analyzed in triplicate. The recovery was calculated by determining the percent assay of an added amount of both the analytes. The mean percentage recovery from all the four methods was found to be in the range of 98.71%–100.29% and 99.38%–100.53% for CTL and AZE, respectively, with less than 2 standard deviations (Table 3). It confirmed the absence of interference from the excipients and the analytes.

3.6. Assay of Pharmaceutical Preparation. The fixed-dose combination of CTL and AZE prepared in two different strengths was analyzed simultaneously using the proposed UV derivative spectroscopic methods. (Supplementary file Figures S1 and S2). The amount of both the analytes were in agreement with the amount claimed in the label by all four methods (Table 3). Further, the selectivity and accuracy of the proposed methods were confirmed by an assay of laboratory-mixed solutions and by the standard addition method, indicating no interference from the formulation excipients. Hence, the prosed UV derivative spectroscopic methods can be used for quality control studies of formulations consisting of CTL and AZE without prior separation.

3.7. Evaluation of Greenness and Whiteness. Green analytical chemistry involves the development of environmentally safe analytical methods to protect animals and plants and save energy. There are several approaches to evaluate the eco-friendly nature of the analytical process; here we adopted two recently proposed greenness assessment methods and one whiteness evaluation method.

3.7.1. Analytical Greenness Matric Approach (AGREE). Analytical Greenness Matric Approach (AGREE) is a comprehensive greenness evaluation matric based on the 12 principles of green analytical chemistry [36]. In AGREE the 12 principles of GAC are converted into scores. AGREE is a flexible matrix that allows us to select the different weights for the 12 principles, depending upon their importance, and transferred them into a score in the range of 0–1. The outcomes are represented as an easily readable clocklike pictogram. It is also color shaded from red-yellow-green, full

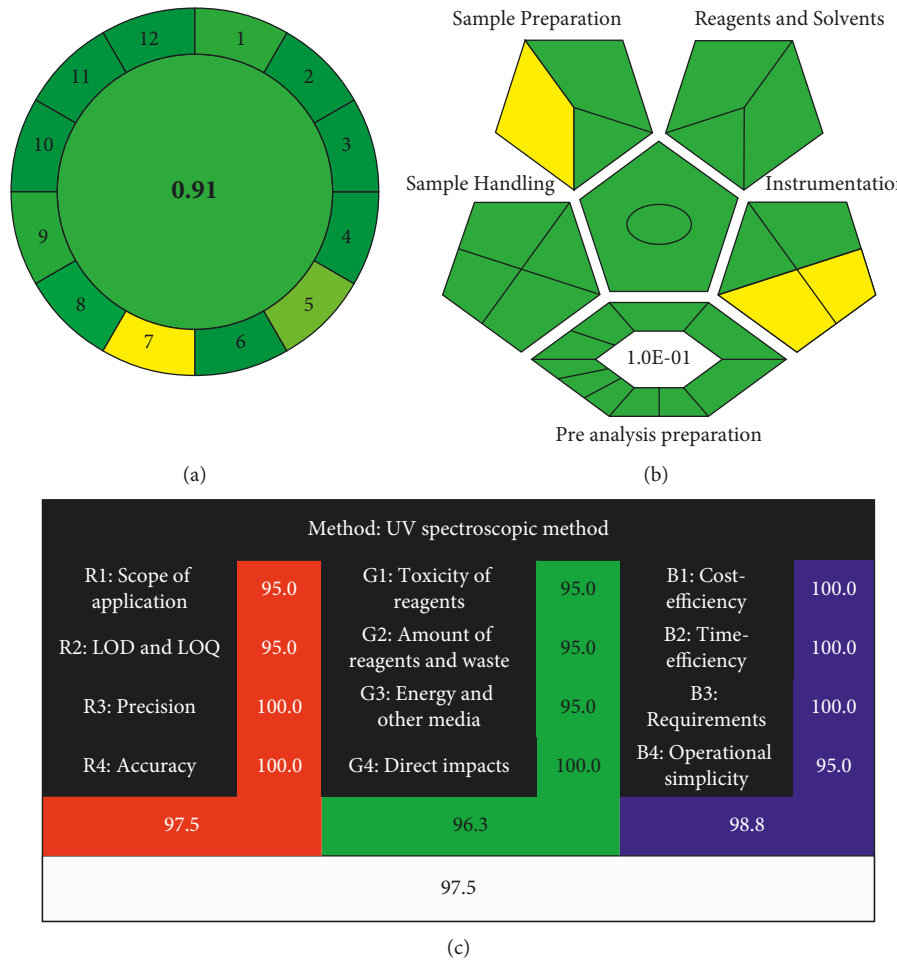


Figure 6: Green evaluation results of UV spectroscopic method by (a) AGREE, (b) ComplexGAPI, and (c) white analytical chemistry.

red for 0 scores, and dark green for 1 to represent the bad analytical procedure to environment-friendly greener method, respectively. The final score of the analytical procedure will be shown in the middle of the circular pictogram with color variation, and if the score is close to 1, dark green indicates the greenness of the analytical procedure. All the 12 principles of GAC starting from the sample size, sample treatment, site of measurements, energy consumption, automation, use of safe or toxic chemicals, generation and management of waste, and safety of the analyst were calculated for the proposed method using the easy to handle and simple software. An overall score of 0.91 with dark green color (Figure 6(a)) confirmed the greenness of the proposed UV derivative methods for simultaneous quantification of CTL and AZE from the formulation (Supplementary file Figure S3).

3.7.2. Complimentary Green Analytical Procedure Index (ComplexGAPI). The green analytical procedure index (GAPI) is a well-established greenness evolution tool used since 2018 [37]. In this method, each step of the analytical procedure is evaluated using terms of four major categories with 15 parameters, covering the sample collection, transportation, preservation, preparation, reagents, and compounds used, and instrumentation along with the purpose of the method qualitative or quantitative. GAPI has been represented as a color-coded pictogram with two or three levels of evolution for each step. Complex GAPI is a modified/extended GAPI matric with an additional presentation tool at the bottom of the GAPI pictogram representing the summary of GAPI evolution results along with the effect of the preanalysis process on the environment [38]. The additional tool has been presented as a hexagonal field with subdivisions representing selectivity and experimental conditions, economy, effects of reagents and solvents, instrumentation workup, and purification. It also covers the waste generated by calculating the e-factor, the higher the factor (close to 1), higher the waste generated, the greater the undesirable effect on the environment. The GAPI pictogram (Figure 6(b)) developed for the proposed UV spectroscopic method showed yellow color for only three parameters (Supplementary file Figure S4). Because the method is not solvent-free, the waste has been generated and no waste treatment has been developed. However, a small amount of ethanol has been used as a solvent, which is a green solvent, the waste generated is less than 10 mL, and the waste is biodegradable. Further, the e-factor is less than 0.1, because

there is no preanalysis preparation except powdering the tablets and dissolving them in the ethanol, confirming the complete ecofriendly nature of the proposed analytical method.

3.7.3. White Analytical Chemistry (WAC). White analytical chemistry is an extended matric of the red, green, and blue (RGB) model based on the 12 WAC principles [39]. WAC principles cover all the 12 GAC principles along with the quality of the analytical method. In addition, WAC displays the whiteness of the analytical method by combining the effect of all three groups (RGB) by mixing all three colors. The whiteness of the analytical method represents the consistency and combined effect of the analytical procedure and environmental and practical features. The 12 WAC principles are divided into three groups and three primary colors (RGB) with four parameters in each group. The validation parameters such as LOQ, LOD accuracy, precision, and scope of the analytical method related to analytical performance were evaluated under the red group. The parameters responsible for the safety and ecofriendly nature of solvents and reagents were assessed under the green group, which includes toxicity of reagents and waste generated, amount of reagents, solvents, and energy consumption. The practical efficiency and productivity including time and cost efficiency, simplicity, training, and maintenance of instruments were estimated under the blue category. The proposed method is accurate and precise with good sensitivity and scope. The method is eco-friendly as safer solvent ethanol has been used as a solvent, less waste is generated which is biodegradable and had no direct impact on the environment. The spectroscopic method is simple, rapid, and economical compared to other sophisticated instruments such as HPLC making the method white with an overall score of 97.5% (Figure 6(c)) (Supplementary file Table S1).

4. Conclusion

A simple green and white four UV spectroscopic methods were established for simultaneous quantification of CTL and AZE from a fixed-dose combination formulation. Proposed methods were based on simple mathematical manipulation of scanned UV spectra to resolve the overlapped spectra of two analytes and quantify them simultaneously without prior separation. Further, UV spectroscopic methods were successfully applied to formulation and laboratory mixed solution, and the results were in agreement with the expected concentrations. In addition, a small amount of nontoxic solvent ethanol has been used throughout the experiments. Finally, the greenness and whiteness of the proposed methods were evaluated by using three recently developed evaluation methods. Proposed methods satisfy all green-and-white analytical principles, making the method environment friendly.

Data Availability

The data supporting the conclusion of this work have been provided in the article and as supplementary files.

Conflicts of Interest

The authors declare that they have no conflicts of interest or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Materials

Supplementary Materials: Figure S1. Normal UV spectra of formulation (a), first derivative spectra (b), ratio spectra of AZE (c) First derivative of ratio spectra of AZE (d). Figure S2. Ratio spectra of CTL (a), First derivative of ratio spectra of CTL (b), Zero order spectra of CTL (c) and AZE (d). Figure S3. Analytical Greenness report sheet. Figure S4. ComplexGAPI for spcetroscopic method. Table S1. White analytical chemistry Evaluation. (*Supplementary Materials*)

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