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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF UNIVARIATE SPECTROPHOTOMETRIC METHODS FOR QUANTIFYING BINARY DRUG COMBINATION IN HYPERTENSIVE CARE

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ABSTRACT

The objective of present research work was to develop a novel, precise, and accurate UV spectrophotometric method based on four approaches: Ratio Subtraction Coupled with Extended Ratio Subtraction Method (RS-ERSM), Ratio Subtraction Coupled with Constant Multiplication Method (RS-CM), Ratio subtraction coupled with Spectrum Subtraction (RS-SS) and the Absorption Factor method (AFM) for simultaneous estimation of Azelnidipine and Olmesartan medoxomil in fixed dose combination. The method was validated according to ICH guideline Q2 (R2). For RS-ERSM, RS-CM and RS-SS, the absorbance of Azelnidipine was measured at 258.8nm, and that of Olmesartan medoxomil was measured at 255nm. For AFM, Azelnidipine was measured at 342.2nm, whereas Olmesartan medoxomil was measured at 255nm. The coefficients of correlation were found to be 0.9997 and 0.9995 for Azelnidipine and OLME, respectively, for the RS-ERS, RS-CM AND RS-SS method and for AFM it was found to be 0.999 and 0.9995 for Azelnidipine and Olmesartan medoxomil respectively. Precision studies indicated % RSD values of less than 2% for both intra-day and inter-day measurements. The % recovery was found to be within the accepted criteria, i.e., 98-102%. The assay results met the acceptable pharmacopeial limits

of 90-110%. These methods were further evaluated for their greenness using two assessment tools, namely Agree and complex Gapi.

KEYWORDS: Azelnidipine, Olmesartan medoxomil, Ratio Subtraction Coupled with Extended Ratio Subtraction Method, Ratio Subtraction Coupled with Constant Multiplication Method, Ratio subtraction coupled with Spectrum Subtraction, Absorption Factor Method, ICH Q2 (R2)

INTRODUCTION

AZELNIDIPINE

Azelnidipine is chemically 3-[1-(Diphenylmethyl)-3-azetidinyl] 5-isopropyl 2-amino-6-methyl-4-(3-nitrophenyl)-1,4-dihydro-3,5-pyridinedicarboxylate. It is a long-acting calcium channel blocker of the dihydropyridine class, prescribed mainly for hypertension. By inhibiting calcium influx in vascular smooth muscle, it promotes vasodilation, decreases peripheral resistance, and reduces cardiac afterload. Its gradual onset of action ensures sustained blood pressure control with minimal reflex tachycardia, thereby improving both hemodynamic stability and long-term cardiovascular outcomes. It is freely soluble in acetone, soluble in ethyl acetate, slightly soluble in methanol, sparingly soluble in water.

OLMESARTAN MEDOXOMIL

Olmesartan medoxomil (5-methyl-2-oxo-1,3-dioxol-4-yl) methyl 5-(2-hydroxypropan-2-yl)-2-propyl-3-[[4-[2-(2*H*-tetrazol-5-yl) phenyl] phenyl] methyl] imidazole-4-carboxylate. It is an angiotensin II receptor blocker that is converted in vivo to its active form, Olmesartan. By selectively blocking AT_1 receptors, it prevents angiotensin II–mediated vasoconstriction and aldosterone release, leading to arterial relaxation, reduced vascular resistance, and sustained blood pressure control. It is slightly soluble in methanol, ethanol, practically in-soluble in water.

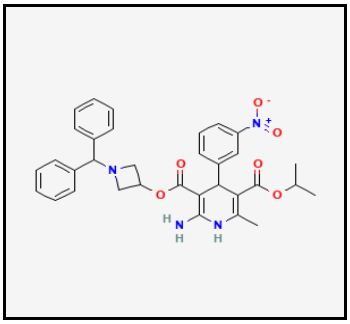

Fig. 1: Chemical structure of Azelnidipine.

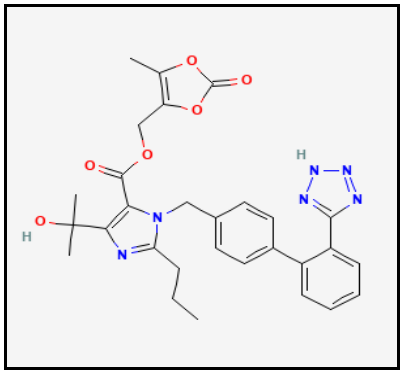

Fig. 2: Chemical structure of Olmesartan medoxomil.

A review of the literature indicates that numerous analytical methods have been established for Azelnidipine[5,11] both as a single drug and in combination with Olmesartan medoxomil[12,19] or other agents. The reported spectrophotometric approaches include UV[20,23] and RP-HPLC[24,26] HPTLC,[27] LC-ESI-MS-MS,[28] UPHPLC,[29] LC MS-MS.[30]

MATERIAL AND METHOD

Instrumentation

A UV visible double beam spectrophotometer (SHIMADZU, model UV- 2700) equipped with a pair of matched quartz cuvettes (1 cm path length) was used for the analysis (spectral bandwidth of 1 nm, scan speed of 400 nm/min and data interval of 0.5nm). All weighing procedures were carried out using Wensar electronic balance (model MAB220). Throughout the study only calibrated glassware was utilized.

Reagents and chemicals

Azelnidipine (AZE) having percent purity (100.37%) and Olmesartan medoxomil (OLME) having percent purity (99.99%) were received as gift samples from Mascot Health Series Pvt.Ltd., Janakpuri, New Delhi and MSN laboratories Pvt. Ltd., Sangareddy, Telangana respectively. HPLC-grade methanol was purchased from Merck Life Science Pvt Ltd. (Mumbai-400 013).

Marketed formulation

"Olmezest-Az 20" tablet manufactured by Sun Pharma Ltd was purchased from local pharmacy. Each tablet contains AZE 8mg and OLME 20mg.

Preparation of solution

(a) Standard Stock Solution of AZE (1000 µg/mL)

A standard stock solution of AZE was prepared by accurately weighing 100 mg of AZE and transferring it into a 100 mL volumetric flask. To this 50 mL of methanol was added to facilitate complete dissolution of the drug. Same solvent was used to make the volume up to the mark, resulting in a solution containing 1000 µg/mL of AZE.

(b) Working Standard Solution of AZE (100 µg/mL)

From the standard stock solution of AZE (1000 µg/mL), 10 mL of aliquot was precisely transferred into a 100 mL volumetric flask. The volume was then made up to the mark with methanol to yield a working standard solution with a concentration of 100 µg/mL.

(c) Standard Stock Solution of OLME (1000 µg/mL)

A standard stock solution of OLME was prepared by accurately weighing 100 mg of OLME and transferring it into a 100 mL volumetric flask. To this 50 mL of methanol was added to facilitate complete dissolution of the drug. Same solvent was used to make the volume up to the mark, resulting in a solution containing 1000 µg/mL of OLME.

(d) Working Standard Solution of OLME (100 µg/mL)

From the standard stock solution of OLME (1000 µg/mL), 10 mL of aliquot was precisely transferred into a 100 mL volumetric flask. The volume was then made up to the mark with methanol to yield a working standard solution with a concentration of 100 µg/mL.

METHOD 1: RATIO SUBTRACTION AND EXTENDED RATIO SUBTRACTION METHOD (RS-ERS)[31]

In a binary mixture of OLME and AZE with overlapping spectra, where AZE shows a more extended spectrum than OLME. The λ_{max} of OLME and AZE were measured at 255 nm and 258.8 nm, respectively, in a 70:30 water–methanol mixture.

For determination of OLME by Ratio Subtraction (RS)

$$\textit{Step 1: } \frac{OLME + AZE}{AZE'} = \frac{OLME}{AZE'} + \frac{AZE}{AZE'} = \frac{OLME}{AZE'} + constant$$

$$\textit{Step 2: } \frac{OLME}{AZE'} + constant - constant$$

$$\textit{Step 3: } \frac{OLME}{AZE'} \times AZE' = OLME(Recovered\ spectra)$$

Determination of OLME by RS METHOD

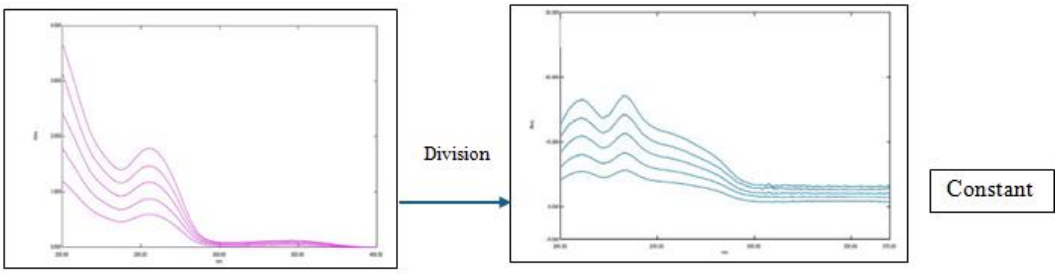

Fig. 3: Zero order spectra of different laboratory prepared mixtures of OLME (10-30µg/mL) and AZE (4-12µg/mL).

Fig. 4: Ratio spectra of different laboratory prepared mixtures of OLME (10-30µg/mL) and AZE (4-12µg/mL) using AZE 8µg/mL as a divisor (Y').

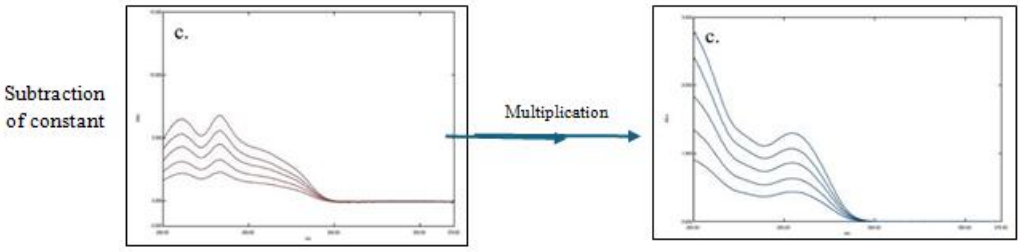

Fig. 5: Ratio spectra of different laboratory prepared mixtures of OLME and AZE after subtraction of constant.

Fig. 6: Zero order spectra of different concentration of OLME (10-30µg/mL) after multiplication using AZE' (8µg/mL) as a divisor.

For determination of AZE by Extended

Ratio Subtraction,

$$\textit{Step 1: } \frac{OLME\,(Recovered\ Spectra)}{OLME'} = constant$$

$$\textit{Step 2: } \frac{OLME + AZE}{OLME'} = \frac{OLME}{OLME'} + \frac{AZE}{OLME'} = \frac{AZE}{OLME'} + constant$$

$$\textit{Step 3: } \frac{AZE}{OLME'} + constant - constant = \frac{AZE}{OLME'}$$

$$\textit{Step 4: } \frac{AZE}{OLME'} \times OLME' = AZE\,(Recovered\ Spectra)$$

Determination of AZE by ERS METHOD

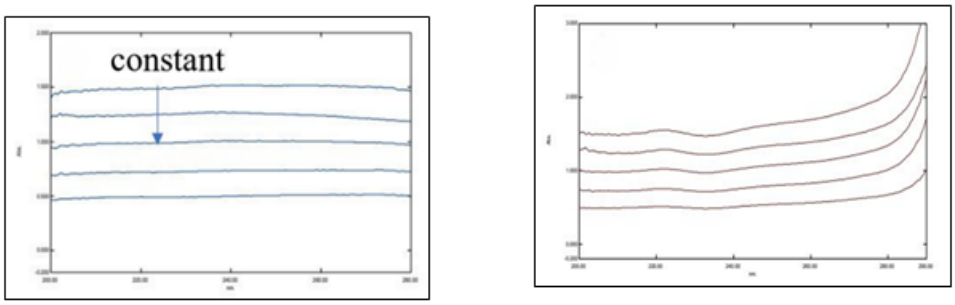

Fig. 7: Ratio spectra of different recovered OLME (10-30µg/mL) using OLME 20µg/mL as a divisor for determination of constant.

Fig. 8: Ratio spectra of different laboratory prepared mixtures of OLME (10-30µg/mL) and AZE (4-12µg/mL) using OLME 20µg/mL as a divisor.

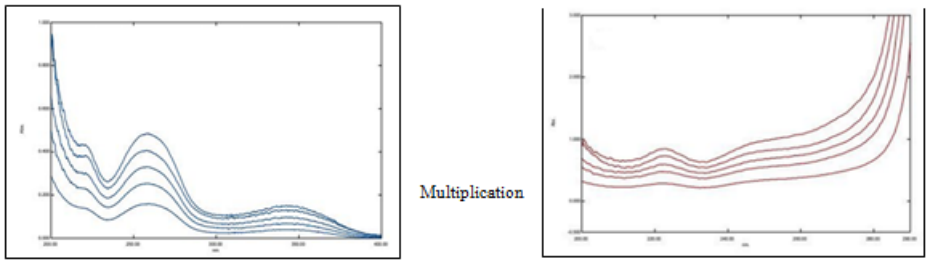

Fig. 9: Zero order spectra of different concentration of AZE (4-12µg/mL) after multiplication using OLME' (20µg/mL) as a divisor.

Fig. 10: Ratio spectra of different laboratory prepared mixtures of OLME and AZE after subtraction of constant

METHOD 2: RATIO SUBTRACTION METHOD COUPLED WITH CONSTANT MULTIPLICATION METHOD (RS-CM)[32]

The RS-CM method is applied to binary mixtures of OLME and AZE, where AZE shows a more extended spectrum. The λmax of OLME and AZE were measured at 255 nm and 258.8 nm, respectively, in a 70:30 water–methanol mixture.

For determination of AZE,

$$\textit{Step 1: } \frac{OLME + AZE}{AZE'} = \frac{OLME}{AZE'} + \frac{AZE}{AZE'} = \frac{OLME}{AZE'} + constant$$

$$\textit{Step 2: } Constant \times AZE' = AZE(\,Recovered\ Spectra)$$

For determination of OLME

Step 1: $(OLME + AZE) - AZE(\ Recovered\ Spectra\ ) = OLME$

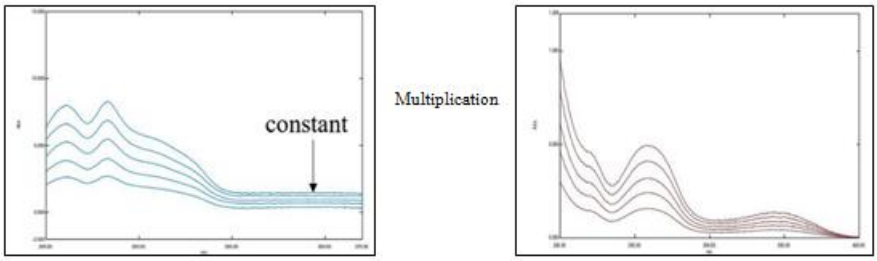

Fig. 11: Ratio spectra of different laboratory prepared mixtures of OLME ((10-30µg/mL) and AZE (4-12µg/mL) using AZE' (8µg/mL) as divisor to determine the constant.

Fig. 12: Zero order spectra of different concentration of AZE9 (4-12µg/mL) obtained after multiplication of constant value.

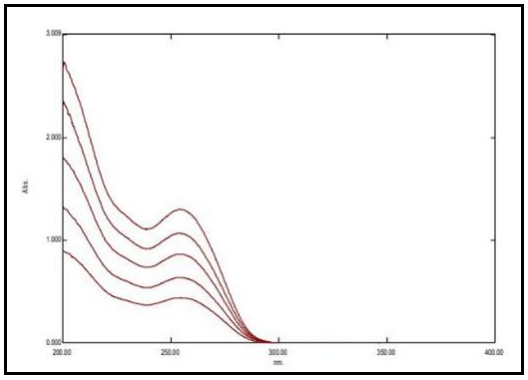

Fig.13: Zero order spectra of different concentration of OLME (10-30µg/mL) obtained after subtraction of recovered AZE spectra.

METHOD 3: RATIO SUBTRACTION COUPLED WITH SPECTRUM SUBTRACTION (RS-SS)[33-35]

This spectrophotometric method is applied to binary mixtures of OLME and AZE, where AZE has a more extended spectrum. The λmax of OLME and AZE were measured at 255 nm and 258.8 nm, respectively, in a 70:30 water–methanol mixture.

OLME is determined using the ratio subtraction approach as mentioned

For determination of AZE,

Step 1*:* $(OLME + AZE) - OLME(\ Recovered\ Spectra\ ) = AZE$

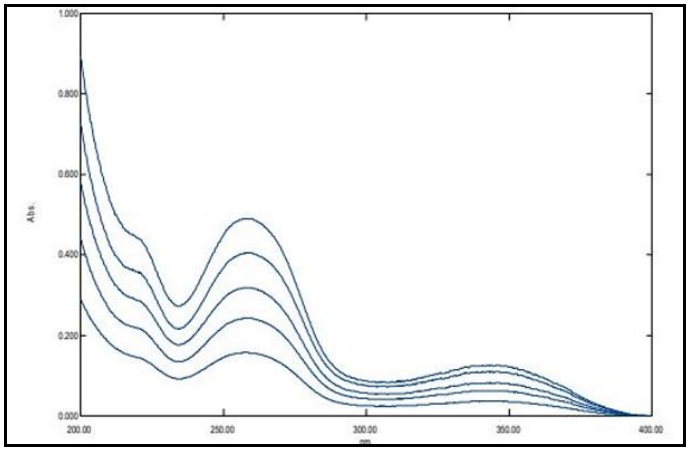

Fig. 14: zero order spectra of different concentration of AZE (4-12µg/mL) obtained after subtraction of recovered OLME spectra by the RS method.

METHOD 4: ABSORPTION FACTOR METHOD (AFM)[34-35]

This method is used for the spectroscopic analysis of a binary mixture of OLME and AZE with overlapping spectra. AZE interferes at the maximum absorption wavelength of OLME (λ_1), while OLME does not interfere at another wavelength (λ_2). The absorption factor of AZE is calculated as the ratio of its absorbance at λ_1 to that at λ_2. Since the absorbance of the mixture (OLME + AZE) at λ_2 is equal to that of pure AZE, due to the absence of OLME interference at this wavelength, the absorbance of OLME at λ_1 can be calculated using the following equation.

$$Absorption\ value\ of\ OLME\ at\ \lambda 1 = Abs\ \lambda 1(OLME + AZE) - \frac{Abs1}{Abs2} \times Abs\ (OLME + AZE)$$

Where, $\frac{Abs1}{Abs2}$ is absorption factor of pure AZE (Abs at λ_1/ Abs at λ_2

Absorbance λ_1 is absorbance of the mixture of (OLME+AZE) at λ_1

Absorbance λ_2 is absorbance of the mixture of (OLME+AZE) at λ_2

The concentrations of OLME and AZE are then calculated using their respective calibration curves obtained by plotting absorbance at λ_1 and λ_2 against concentration.

ANALYSIS OF TABLET FORMULATION

Twenty 'Olmezest AZ-20' tablets were precisely weighed and the average tablet weight was calculated. A powdered sample equivalent to 8 mg of AZE (containing 20 mg of OLME) was transferred into 100 mL volumetric flask, followed by extraction using sonication for 20 min in 50 mL methanol. The volume was then made up to the mark with methanol to obtain a

final concentration of 80 µg/mL of AZE and 200 µg/mL of OLME. The solution was centrifuged, and the clear supernatant was collected for further analysis.

ANALYTICAL METHOD VALIDATION

As per the ICH guideline Q2 (R2), the developed analytical method was validated by assessing key parameters, including linearity and range, specificity, precision, accuracy, and assay.

RESULTS AND DISCUSSION

Standard solutions of OLME (20 µg/mL) and AZE (8 µg/mL) were scanned between 200 and 400 nm using a UV spectrophotometer. The absorbance maxima for OLME and AZE were found to be 255 nm and 258.8 nm, respectively, and were used for the RS-ERS, RS-CM, and RS-SS methods, while for the AFM method, absorbance maxima for OLME and AZE were measured at 255 nm and 342.2 nm, respectively.

VALIDATION STUDIES OF THE DEVELOPED ANALYTICAL METHOD

SPECIFICITY

Specificity was confirmed for all four methods (RS-ERS, RS-SS, RS-CM, and AFM) for AZE and OLME at 8 µg/mL and 20 µg/mL, respectively. The spectral analysis showed that the spectra obtained from the marketed formulation closely overlapped with the spectra obtained from the lab-prepared AZE and OLME mixture, indicating minimal interference. Moreover, the excipients used in tablet formulation did not negatively affect spectral properties.

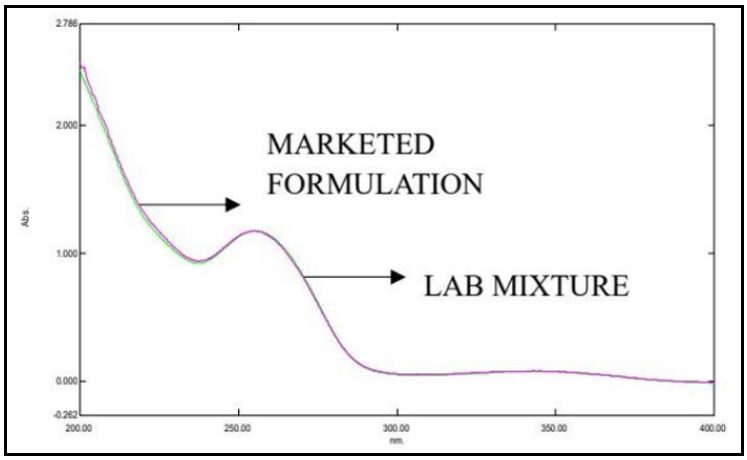

Fig. No. 15: Overlain spectra of AZE (8 µg/mL) and OLME (20 µg/mL).

LINEARITY

The linearity of AZE and OLME was evaluated across specific concentration ranges. Five different concentrations were prepared: 4-12 µg/mL.[4,6,8,10,12] for AZE and 10-30 µg/mL.[10,15,20,25,30] for OLME. Spectral data were recorded using a UV-Visible spectrophotometer within the 200-400 nm range. For RS-ERS, RS-CM and RS-SS methods, absorbance of AZE and OLME was measured at 258.8 nm and 255 nm, respectively. For AFM, AZE and OLME was measured 342.2 nm and 255 nm, respectively. Calibration curves were constructed by plotting absorbance against concentration, and the regression equation was determined for all methods.

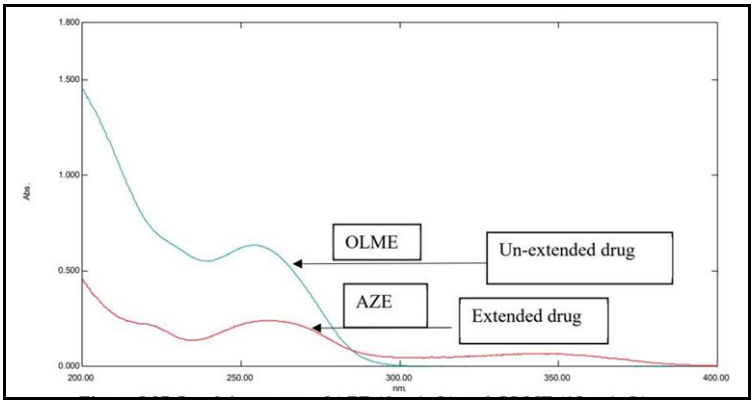

Fig. 16: Overlain spectra of laboratory prepared mixture and tablet formulation of AZE (8 µg/mL) OLME (20 µg/mL) for RS-ERS, RS-CM, RS-SS and AFM method.

TABLE 1: LINEARITY STUDY OF AZE AND OLME FOR RS-RES, RS-CM AND RS-SS.

Parameter	AZE	OLME
Linearity range	4-12 µg/mL	10-30 µg/mL
Regression equation	y=0.0418x-0.0103	y = 0.0434x-0.0071
Coefficient of correlation R^2	0.9997	0.9995
Slope	0.0418	0.0434
Intercept	-0.0103	-0.0071

TABLE 2: LINEARITY STUDY FOR AZE AND OLME FOR AFM METHOD.

Parameter	AZE	OLME
Linearity range	4-12 µg/mL	10-30 µg/mL
Regression equation	y=0.0112x-0.0051	y = 0.0434x-0.0052
Coefficient of correlation R^2	0.9992	0.9996
Slope	0.0112	0.0434
Intercept	-0.0051	-0.0052

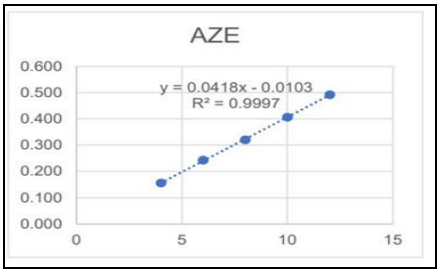

Fig. 17: Calibration curve of AZE for RS-ERS, RS-CM and RS-SS.

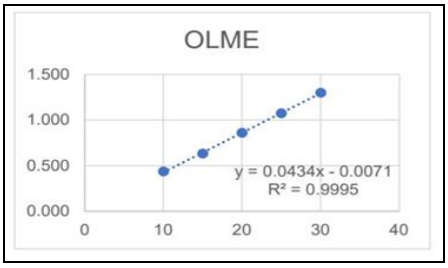

Fig. 19: Calibration curve of OLME for AFM method.

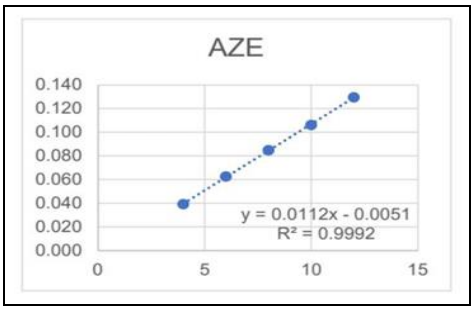

Fig. 20: Calibration curve of AZE for AFM method.

PRECISION

Intra-day and inter-day precision at the assay concentration level (AZE 8 µg/mL and OLME 20 µg/mL) was assessed for all four methods (RS-ERS, RS-CM, RS-SS, and AFM). For RS-ERS, RS-CM, and RS-SS, AZE was evaluated using OLME (20 µg/mL) as a divisor at 258.8 nm, whereas OLME was determined using AZE (8 µg/mL) as a divisor at 255 nm. In the AFM method, AZE and OLME were directly measured at 342.2 nm and 255 nm, respectively. The percentage relative standard deviation (%RSD) for both compounds was found to be below 2%, confirming the reliability and accuracy of the methods. The results are summarized in the table below.

TABLE 3: PRECISION STUDY OF AZE AND OLME.

Method	Parameters	AZE		OLME	
		Mean Abs. ± SD	%RSD	Mean Abs. ± SD	%RSD
RS-ERS	Intra-day	7.962 ± 0.067	0.836	20.060 ± 0.066	0.331

	Inter-day	7.946 ± 0.079	0.997	19.995± 0.161	0.807
RS-CM	Intra-day	8.010± 0.045	0.559	20.002 ± 0.132	0.660
	Inter-day	7.970 ±0.076	0.957	19.964 ± 0.182	0.911
RS-SS	Intra-day	7.962 ±0.066	0.926	20.060 ± 0.066	0.331
	Inter-day	7.934 ±0.094	1.186	19.995 ± 0.161	0.807
AFM	Intra-day	7.969± 0.068	0.913	20.041 ± 0.101	0.502
	Inter-day	7.940 ± 0.131	1.655	20.025 ± 0.126	0.630

ACCURACY

The accuracy of the proposed method was evaluated using the standard addition technique at 80%, 100%, and 120% of the assay concentration for both AZE and OLME. Standard additions from working solutions (100 µg/mL) were made to achieve the desired concentration levels. In the RS-ERS, RS-CM, and RS-SS methods, OLME was quantified using AZE (8 µg/mL) as the divisor at 255nm, while AZE was determined using OLME (20 µg/mL) as the divisor at 258.8nm. For the AFM method, AZE and OLME were measured at 342.2 nm and 255 nm, respectively. The recoveries were calculated in triplicate, and the result were found to be within 98–102% with %RSD values below 2%, which confirms the accuracy of the method.

TABLE 4: ACCURACY STUDY OF AZE AND OLME FOR RS-ERS AND RS-CM METHOD.

Parameters	**Acceptance criteria**	**Method**			
		RS-ERS		**RS-CM**	
		(n=3) Recovery (%): Mean ± SD, RSD (%)			
		AZE	**OLME**	**AZE**	**OLME**
80%	98 – 102%	99.958±0.010, 0.328	101.125±0.047, 0.058	99.958±0.01, 0.328	101.125±0.047, 0.058
100%	98 – 102%	99.958±0.029, 0.751	98.566±0.057, 0.579	99.958±0.029, 0.751	98.566±0.057, 0.579
120%	98 – 102%	100.138±0.020, 0.427	99.944±0.023, 0.239	101.25±0.033, 0.150	99.583±0.108, 0.907

TABLE 5: ACCURACY STUDY OF AZE AND OLME FOR RS-SS AND AFM METHOD.

Parameters	**Acceptance criteria**	**Method**			
		RS-SS		**AFM**	
		(n=3) Recovery (%): Mean ± SD, RSD (%)			
		AZE	**OLME**	**AZE**	**OLME**
80%	98 – 102%	99.958±0.328, 1.482	101.125±0.047, 0.058	101.100±0.004, 0.145	98.980±0.058, 0.058

100%	98 – 102%	99.080±0.057, 1.457	100.677±0.057, 0.567	99.410±0.037, 0.940	99.060 ±0.037, 0.377
120%	98 – 102%	100.138±0.049, 1.016	99.972±0.028, 0.239	100.800±0.081, 1.686	99.194±0.089, 0.752

ASSAY

The assay was performed by diluting the tablet sample solution to achieve final concentrations of AZE (8 µg/mL) and OLME (20 µg/mL) using a 70:30 v/v mixture of distilled water and methanol. The prepared solution was then scanned over the 200–400 nm range using a UV-visible spectrophotometer. In the RS-ERS, RS-CM and RS-SS methods, OLME was measured at 255 nm with AZE (8µg/mL) as the divisor, whereas AZE was determined at 258.8 nm using OLME (20 µg/mL) as the divisor. For the AFM method, AZE and OLME were quantified at 342.2 nm and 255 nm, respectively. All measurements were carried out in triplicate, and the percent relative standard deviation (%RSD) was calculated to confirm the consistency of the results. The validated RS-ERS, RS-CM and RS-SS method was successfully applied for the simultaneous estimation of AZE and OLME in a marketed formulation.

TABLE 6: ASSAY STUDY OF AZE AND OLME FOR RS-ERS, RS-CM, RS-SS AND AFM METHOD.

Method	**Parameter**	**Acceptance criteria**	**AZE**	**OLME**
	Assay	**(n=3)**	**Average ± SD**	
RS-ERS	**% Content**	AZE 90-110% OLME 90-110%	99.378 ± 0.813	100.156 ± 0.290
RS-CM			100.617± 0.309	99.651± 0.533
RS-SS			99.896±1.285	100.231±0.420
AFM			100.619± 1.611	100.565±0.854

GREENNESS ASSESSMENT

These methods were evaluated for their greenness using two greenness evaluation tools, namely, Agree and complex Gapi.

AGREE

RS-ERS, RS-CM, RS-SS, and AFM successfully met the sustainability criteria, achieving a central score of 0.68 as illustrated in Fig.21

COMPLEX GAPI (COMPLEMENTARY GREEN ANALYTICAL PROCEDURE INDEX)

RS-ERS, RS-CM, RS-SS, and AFM achieved an E-factor of 0.02, achieving confirming their compliance with green chemistry standards as illustrated in Fig.22 This systematic evaluation ensures environmentally responsible analytical methodologies while maintaining their efficiency and scientific integrity.

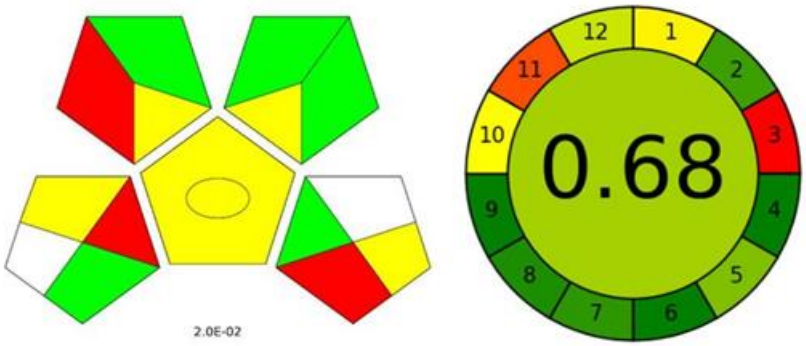

Fig. 21: Greenness Assessment by AGREE.

Fig. 22: Greenness Assessment by Complex GAPI.

CONCLUSION

Four new UV spectrophotometric methods were developed for the quantification of AZE and OLME in a fixed-dose combination. The results complied with the label claims and provided an alternative method for the accurate determination of these drugs in fixed-dose combination. The developed methodologies effectively address the challenge of quantifying target components in pharmaceutical formulations, even in the presence of interfering components. Compared to other analytical techniques, such as chromatography, these spectrophotometric methods offer a simpler, cost-effective, and reliable alternative that does not require extensive sample preparation or high-end instrumentation.

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