

Stability-indicating RP-LC method for determination of azilsartan medoxomil and chlorthalidone in pharmaceutical dosage forms: application to degradation kinetics

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Abstract

A RP-LC method was developed and validated for simultaneous determination of the active components, azilsartan medoxomil (AZL) and chlorthalidone (CLT), in their novel antihypertensive combined recipe. The chromatographic separation was achieved on an Eclipse XDB-C18 (4.6 × 150 mm, 5 μm) column using a mobile phase consisting of methanol/potassium hydrogen phosphate buffer (pH 8, 0.05 M) (40:60, v/v) in isocratic mode. The flow rate was maintained at 0.8 mL min⁻¹ at ambient temperature. Detection was carried out at 210 nm. The method was validated according to the ICH guidelines. Linearity, accuracy, and precision were satisfactory over the concentration range of 5.0–50.0 and 2.5–25.0 μg mL⁻¹ for AZL and CLT, respectively ($r^2=0.9999$). LODs for AZL and CLT were 0.90 and 0.32 μg mL⁻¹, whereas LOQs were 2.72 and 0.98 μg mL⁻¹, respectively. Both drugs were subjected to forced degradation studies under hydrolysis (neutral, acidic, and alkaline), oxidative, and photolytic extensive stress conditions. The proposed method is stability indicating by the resolution of the investigated drugs from their degradation products. Moreover, the kinetics of the acidic degradation of AZL as well as the kinetics of the alkaline degradation of CLT were investigated. Arrhenius plots were constructed and the apparent first-order rate constants, half-life times, shelf-life times, and the activation

energies of the degradation processes were calculated. The method was successfully applied for the determination of the studied drugs simultaneously in their coformulated tablet. The developed method is specific and stability indicating for the quality control and routine analysis of the cited medications in their pharmaceutical preparations.

Keywords Stability-indicating · Liquid chromatography · Degradation kinetics · Azilsartan medoxomil · Chlorthalidone · Pharmaceutical preparations

Introduction

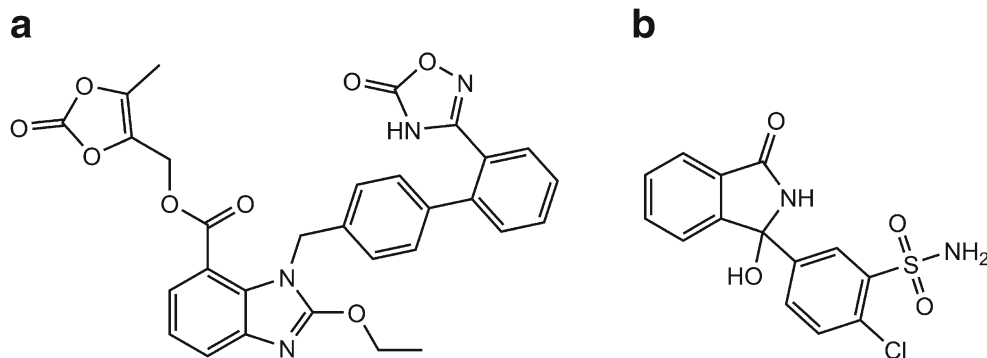
Azilsartan medoxomil (AZL) is chemically designated as ((5-methyl-2-oxo-1,3-dioxol-4-yl) methyl 2-ethoxy-1-((2'-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl) biphenyl-4-yl) methyl)-1H-benzimidazole-7-carboxylate) (Fig. 1a). Azilsartan, the active metabolite of AZL, is a novel nonpeptide angiotensin II type 1 (AT₁) receptor blocker (ARB) that was recently approved for treatment of hypertension [1]. It has a superior ability to control systolic blood pressure relative to other widely used ARBs. Greater antihypertensive effects of AZL might be due in part to its unusually potent and persistent ability to inhibit binding of angiotensin II to AT₁ receptors [2]. Preclinical studies have indicated that AZL may also benefit cellular mechanisms of cardiometabolic disease and insulin-sensitizing activity [3]. Because AZL is a new product and was recently introduced into the market, it is not yet official in any of the pharmacopeias. There is only one reported method [4] for determination of AZL only and it was applied to its analysis in plasma. Chlorthalidone (CLT) (2-chloro-5-(1-hydroxy-3-oxoisindolin-1-yl)benzenesulfonamide), (Fig. 1b), is an orally taken thiazide-like diuretic for controlling hypertension and

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Fig. 1 The structural formulae of the studied drugs; **a** azilsartan medoxomil (AZL) and **b** chlorthalidone (CLT)



edema, including that associated with heart failure [5]. CLT has been determined in different matrices either alone or in combination with other medications using liquid chromatography (LC) [6, 7], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [8], high-performance thin layer chromatography (HPTLC) [9, 10], chemiluminometry [11], spectrophotometry [12], and capillary electrophoresis (CE) [13] techniques. Binary combination therapies of AZL and CLT proved to induce significant reductions in systolic and diastolic blood pressure in patients with mild to severe hypertension [14]. Regarding simultaneous determination of CLT and AZL, there is only one reported RP-LC method and it was performed on a symmetry C18 column with a mobile phase consisting of methanol/water/acetonitrile/0.1 % o-phosphoric acid (30:35:15:5, v/v/v/v) at λ of 251 nm [15]. To the best of our knowledge, the present study is the first reported stability-indicating LC method for the determination of AZL and CLT in tablets. The parent drug stability test guideline Q1A (R2) issued by the International Conference of Harmonization (ICH) [16] suggests that stress studies should be carried out on a drug to establish its inherent stability characteristics. Accordingly, the aim of the present study was to establish the inherent stability of AZL and CLT through stress studies under a variety of ICH recommended test conditions and to develop the first stability-indicating assay method for their determination in their commercially available tablets. In the present work, AZL and CLT were subjected to forced degradation studies under an extensive stress testing. These studies included the effects of hydrolysis (neutral, acidic, and alkaline), oxidation, and photolysis. The currently developed LC method effectively separated the two investigated drugs from their degradation products (DPs). Furthermore, the proposed LC method was applied to study the degradation kinetics of AZL and CLT under acidic and alkaline degradation stress conditions, respectively. Arrhenius plots were constructed and the apparent first-order rate constants, half-life times, shelf-life times, and the activation energies of the degradation processes were calculated. The described method demonstrates its novelty and pros by

combining several advantages in one method—the limit of detections for AZL and CLT were as low as 0.9 and 0.3 $\mu\text{g mL}^{-1}$, respectively. The method was able to simultaneously determine the two drugs in their pharmaceutical preparation. Moreover, the present work was designed to detect and analyze AZL and CLT in presence of their potential DPs using a stability-indicating accurate, affordable, and simple procedure. Another added advantage of the current method resides in its ability to study the kinetics of degradation processes of AZL and CLT which were obtained by subjecting them to acidic and alkaline stress conditions, respectively.

Experimental

Instruments

Separations were performed using an Agilent high-performance liquid chromatography (HPLC) system 1,200 chromatograph (USA) equipped with a quaternary pump G1311A and a Rheodyne injector valve with a 20 μL loop and a G1315D MWD detector. Mobile phases were degassed using a G1322A solvent degasser. Agilent Chemstation for LC systems [Rev.B.03.01-SR1 (317)] PC program was used for instrument control, data analysis, and acquisition. A SympHonly (SB20) pH-meter (Thermo Orion, MA, USA) was used for pH adjustment. Nanopure water was prepared using a Barnstead Nanopure Diamond Analytical ultrapure water system (Fischer Scientific, NJ, USA). Fisher Biotech UV fluorescent lamp, model FBVLS-80 dual wavelength (254/366), was used in the photostability studies (Fisher Scientific, PA, USA). The LC-MS studies was performed using an Agilent 1,200 ultrahigh-pressure liquid chromatography system (USA) consisting of a binary pump (G1312A) with a degasser, autosampler (G1329A), and a DAD (Agilent 1100, G1315B) using a mobile phase composed of water and methanol (20:80, v/v) coupled to a 3200 Q TRAP mass spectrometer (AB Sciex; CA, USA) fitted with a TurboV ion source operating in negative and positive electrospray ionization (ESI) mode. The data were acquired and processed using

Analyst® 1.6.1 Software (AB Sciex; Ontario, Canada). MS source parameters were set at desolvation temperature (TEM) (200 °C; high voltage, -4,500 V (ESI -), 4,500 V (ESI +)), curtain (CUR) and nebuliser (GS1 and GS2) gases (nitrogen 20, 40, and 50 psi, respectively), and nitrogen collision gas pressure (5 mTorr).

Materials and reagents

All the chemicals used were of analytical reagent grade, and the solvents were of HPLC grade. AZL (certified to contain 98 %) was supplied by D-L Chiral Chemicals, LLC., NJ, 08540, USA. CLT (certified to contain 98 %) was supplied by Ark Pharm. Inc., IL, 60048, USA. Edarbyclor® 40–25 mg tablets, batch #NDC 64764-0994-30 (Takeda Pharmaceutical Company Limited, Tokyo, Japan), each labeled to contain 42.68 mg of azilsartan kamedoxomil (equivalent to 40 mg AZL) and 25 mg CLT, were purchased from commercial sources in the local market. Sodium hydroxide, potassium hydroxide, acetic acid and dipotassium hydrogen orthophosphate were obtained from J.T. Baker, NJ, USA. Orthophosphoric acid (85 %), hydrochloric acid, and ammonium acetate (Fisher Scientific, NJ, USA) were also used. Methanol and hydrogen peroxide (30 % w/v) (Sigma-Aldrich, MO, USA) were used as well.

Chromatographic conditions

A mixture of methanol/dipotassium hydrogen phosphate buffer (PBS) (pH 8±0.1, 0.05 M) (40:60, v/v) was prepared. Flow rate was maintained at 0.8 mL min⁻¹. All analyses were performed at ambient temperature. The system was equilibrated and saturated with the mobile phase for 30 min before the injection of the solutions. Quantification was achieved with UV detection at 210 nm based on peak area. Twenty microliters of the solutions were injected with a 50- μ l Agilent analytical syringe in triplicates.

Standard solutions

Ten milligram of each of AZL and CLT were accurately weighed and transferred in to 10 mL volumetric flasks separately. Then, they were dissolved and made up to volume with methanol to give final concentrations of 1,000 μ g mL⁻¹ for each. The solutions were stable for at least 14 days without alteration when kept in the refrigerator at 4 °C and protected from light.

Procedures

Construction of the calibration graphs

Accurate aliquots from the standard solutions were transferred separately into a series of 10 mL Fisherbrand disposable tubes. Then, the volumes were completed with the mobile phase so that the final concentrations were in the range of 5.0–50.0 and 2.5–25.0 μ g mL⁻¹ for AZL and CLT, respectively. Aliquots of 20 μ L were injected (triplicate) and eluted with the mobile phase under the optimum chromatographic conditions using UV detection at 210 nm. The average peak area versus the final concentration of the drug in μ g mL⁻¹ was plotted. Alternatively, the corresponding regression equations were derived.

Preparation of the laboratory-prepared mixtures

Accurate aliquots of AZL and CLT standard solutions were transferred into a series of 10 mL Fisherbrand disposable tubes keeping the medicinally recommended ratio of 1.6:1 for AZL/CLT mixture to give the final concentrations of 8, 16, and 24 μ g mL⁻¹ for AZL and 5, 10, and 15 μ g mL⁻¹ for CLT. The above procedure described under “[Construction of the calibration graphs](#)” section was then applied. The recovery percentage was calculated using the corresponding regression equation.

Analysis of the studied drugs in their pharmaceutical preparation

Ten tablets of Edarbyclor® 40–25 mg were weighed, finely pulverized, and thoroughly mixed. After calculating the average tablet weight, amounts of powder equivalent to 40 and 25 mg of AZL and CLT, respectively, were accurately weighed and transferred into a 25-mL measuring flask. Twenty milliliters of methanol were added and the solution was sonicated for 30 min then completed to volume with the same solvent. The solution was filtered through a Whatman filter paper and then filtered again using 0.2 μ m a Whatman inorganic membrane filter. For analyses, different aliquots from the prepared sample solutions were chromatographed using the same procedure described under “[Construction of the calibration graphs](#)” section. The recovery percentage was calculated by referring to the corresponding regression equation.

Procedures for extensive stress stability studies

Stress degradation by hydrolysis under acidic and alkaline conditions Aliquots of AZL and CLT standard solutions equivalent to 500 and 400 μ g, respectively, were transferred separately into Fischerbrand disposable tubes, treated with 2 mL of 0.1 M NaOH or 0.1 M HCl, and heated at 80 and 100 °C for 1 h, respectively. After the specified time, the

contents of each tube were cooled and neutralized to pH 7.0 with 0.1 M HCl or 0.1 M NaOH, respectively. The solutions were then transferred into 10 mL volumetric flasks. Five milliliters of methanol were added to each flask and completed to the volume with nanopure water. Two milliliters of the resulting solutions were then transferred into Fischerbrand disposable tubes and diluted to 4 mL with the mobile phase (final concentrations were 25 and 20 $\mu\text{g mL}^{-1}$ as equivalent to AZL and CLT, respectively, before degradation) and filtered before injection into the column. The above procedure described under “Construction of the calibration graphs” section was then applied.

Procedure for oxidative- and wet heat-induced degradations Aliquots of AZL and CLT standard solutions equivalent to 500 and 400 μg , respectively, were transferred separately into Fischerbrand disposable tubes; 2 mL aliquots of 6 % H_2O_2 or nanopure water were added and heated at 80 and 100 $^\circ\text{C}$ for 1 h for AZL and CLT, respectively. After the specified time, the resulting solution was cooled and transferred into 10 mL volumetric flasks. Five milliliters of methanol were added to each flask and completed to the volume with nanopure water. Two milliliters of the resulting solutions were then transferred into Fischerbrand disposable tubes and diluted to 4 mL with the mobile phase (final concentrations were 25 and 20 $\mu\text{g mL}^{-1}$ as equivalent to AZL and CLT, respectively, before degradation) and filtered before injection into the column. The above procedure described under “Construction of the calibration graphs” section was then applied.

Procedure for photolytic degradation The photodegradation study was performed by subjecting 4 mL from each of AZL (100 $\mu\text{g mL}^{-1}$) and CLT (80 $\mu\text{g mL}^{-1}$), prepared in methanol,

and placed in 1 cm stoppered quartz cells to direct UV radiation (UV lamp set at 254 nm) at room temperature for 24 h at a distance of 10 cm. Protected samples, wrapped in aluminum foil in order to protect them from light, were submitted to identical conditions and used as control. One milliliter of the resulting solutions were then transferred into Fischerbrand disposable tubes and diluted to 4 mL with the mobile phase (final concentrations 25 and 20 $\mu\text{g mL}^{-1}$ as equivalent to AZL and CLT, respectively, before degradation) and filtered before injection into the column. The above procedure described under “Construction of the calibration graphs” section was then applied.

Kinetic investigation

Study of the acidic-induced degradation of AZL at different temperatures Aliquots of AZL standard solution (equivalent to 500 μg) were transferred into Fischerbrand disposable tubes. Two milliliters of 0.1 M HCl were added for each tube. The tubes were heated at different temperatures (50, 60, 70, and 80 $^\circ\text{C}$) for different time intervals (15–60 min). At the specified time interval, the contents of each tube were cooled and neutralized to pH 7.0 with 2 mL 0.1 M NaOH. The solutions were then transferred into 10 mL volumetric flasks. Five milliliters methanol were added to each flask and completed to the volume with nanopure water. Two milliliters of the resulting solutions were then transferred into Fischerbrand disposable tubes and diluted to 4 mL with the mobile phase (final concentration was 25 $\mu\text{g mL}^{-1}$ as equivalent to AZL before degradation) and filtered before injection into the column. The above procedure described under “Construction of the calibration graphs” section was then applied. The

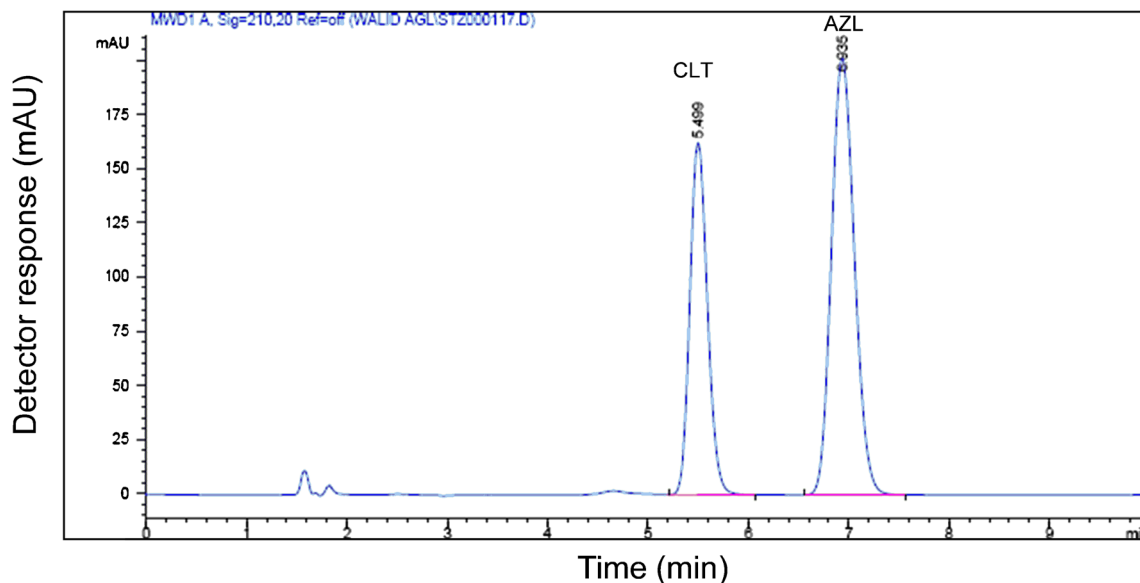


Fig. 2 Typical LC chromatogram for Edarbyclor[®] tablets—24 $\mu\text{g mL}^{-1}$ AZL (6.9 min) and 15 $\mu\text{g mL}^{-1}$ of CLT (5.5 min) under the described chromatographic conditions

concentration of the remaining drug was calculated for each temperature and time interval. Data were further processed and degradation constants were calculated for each temperature to construct the Arrhenius plot. An Arrhenius plot was constructed and the apparent first-order rate constants, half-life times, shelf-life times, and the activation energy of the degradation process were then calculated. All solutions were filtered through 0.2 μm membrane filters and degassed for 5 min before injection to the LC system.

Study of the alkaline-induced degradation of CLT at different temperatures Aliquots of CLT stock solutions (equivalent to 400 μg) were transferred into Fischerbrand disposable tubes. Two milliliters of 0.1 M NaOH were added for each tube. The tubes were heated at different temperatures (70, 80, 90, and 100 $^{\circ}\text{C}$) for different time intervals (30–120 min). At the specified time interval, the contents of each tube were cooled

and neutralized to pH 7.0 with 2 mL 0.1 M HCl. The solutions were then transferred into 10-mL volumetric flasks. Five milliliters of methanol were added to each flask and completed to the volume with nanopure water. Two milliliters of the resulting solutions were then transferred into Fischerbrand disposable tubes and diluted to 4 mL with the mobile phase (final concentration was 20 $\mu\text{g mL}^{-1}$ as equivalent to CLT before degradation) and filtered before injection into the column. The above procedure described under “Construction of the calibration graphs” section was then applied. The concentration of the remaining drug was calculated for each temperature and time interval. Data were further processed, and degradation constants were calculated for each temperature to construct the Arrhenius plot. An Arrhenius plot was constructed and the apparent first-order rate constants, half-life times, shelf-life times, and the activation energy of the degradation process were then calculated. All solutions were filtered

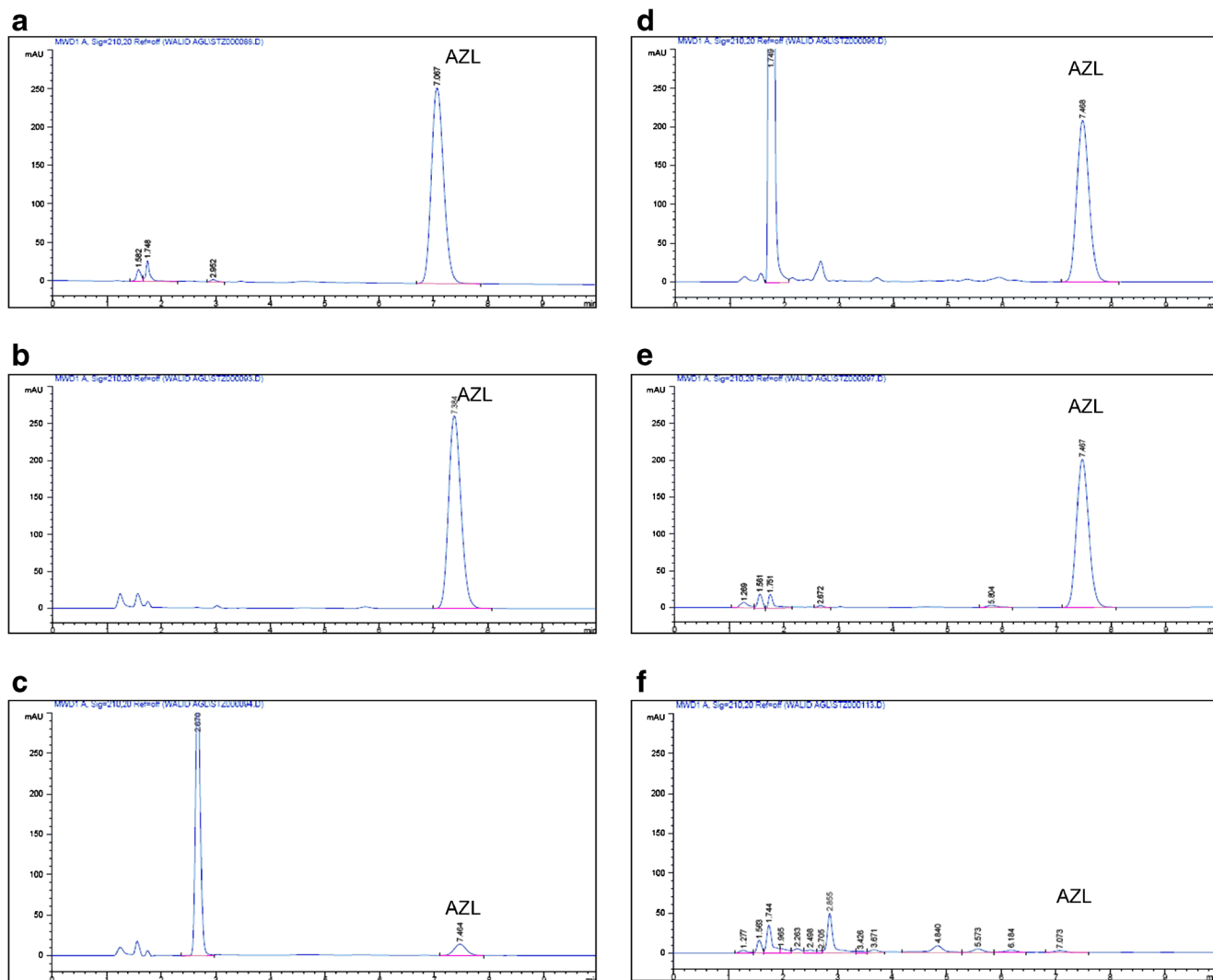


Fig. 3 LC chromatograms of 25 $\mu\text{g mL}^{-1}$ AZL under different stress degradation conditions—**a** control, **b** alkaline (0.1 N NaOH), **c** acidic (0.1 N HCl), **d** oxidative (6 % H_2O_2 , w/v), **e** thermal (wet heat), and **f** photolytic (254 nm) using the described degradation and chromatographic conditions

through 0.2 μm membrane filters and degassed for 5 min before injection to the LC system.

Results and discussion

The proposed method permitted the separation and quantitation of the studied drugs in a short time (<9 min). Furthermore, the suggested procedure was applied for quality control of the commercial dosage form (Fig. 2). The proposed LC method allows the separation of AZL and CLT from all possible degradation products. Figure 3b–f as well as Fig. 4b–f show good resolution of AZL and CLT, respectively, from each of its alkaline, acidic, oxidative, thermal (wet heat), and photolytic degradation products. The stability studies for the acidic degradation kinetics of AZL as well as the alkaline degradation kinetics of CLT were successfully conducted.

Optimization of experimental conditions

Several trials were carried out to achieve the best chromatographic conditions for the simultaneous determination of AZL and CLT as well as their separation from their degradation products. Three columns were tested for performance investigation, including Ultrasphere ODS column (4.6 mm \times 250 mm, 5 μm particle size; Beckman, Fullerton, CA, USA), Eclipse XDB-C18 column (4.6 \times 150 mm, 5 μm ; Agilent, East Windsor, NJ, USA), and Spherex 10 phenyl column (250 mm \times 2.0 mm i.d.; Phenomenex, CA, USA). The experimental studies revealed that the second column was the most suitable one since it produced symmetrical peaks with high resolution and within a reasonable analytical run time. AZL is practically insoluble in water, freely soluble in methanol, dimethylsulfoxide, soluble in acetic acid, slightly soluble in acetone, and acetonitrile, and sparingly soluble in tetrahydrofuran and 1-

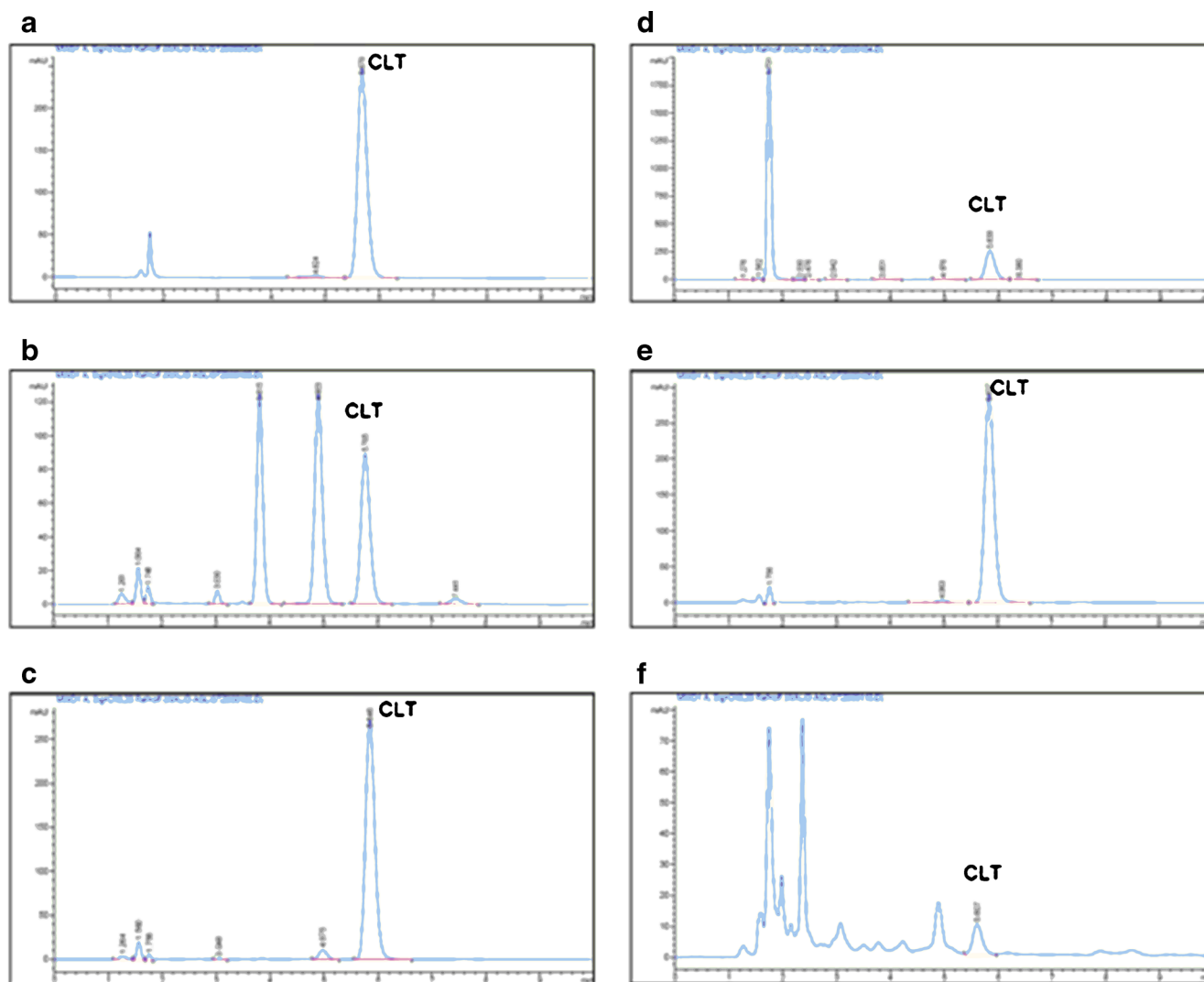


Fig. 4 LC chromatograms of $20 \mu\text{g mL}^{-1}$ CLT under different stress degradation conditions—**a** control, **b** alkaline (0.1 N NaOH), **c** acidic (0.1 N HCl), **d** oxidative (6 % H_2O_2 , w/v), **e** thermal (wet heat), and **f** photolytic (254 nm) using the described degradation and chromatographic conditions

octanol [4]. Methanol and acetonitrile were examined as the organic modifiers, and acetonitrile was found to cause splitting for the peak of AZL due to its poor solubility in that solvent so methanol was chosen as the organic modifier in this study. Water, 1 % acetic acid, ammonium acetate buffer (pH 5.5; 0.05 M), and PBS (pH 8; 0.05 M) were tested as the aqueous component in the mobile phase. The use of buffer is necessary in this method in order to enhance the ionization of the analytes, obtain proper separation, and increase the sharpness of peaks. Both ammonium acetate buffer and PBS are able to achieve proper separation. PBS was selected as it was able to produce sharper and more symmetric peaks than ammonium acetate buffer. Then, the effect of pH on the separation of the analytes was studied. The higher the pH of the used buffer, the lower the retention time with sharp and symmetric peak for AZL so pH 8 was selected as it achieved satisfactory separation at a reasonable run time (<9 min) and with satisfactory resolution between all of the analytes (Fig. 2). The ratio of aqueous to organic component in the mobile phase system was also studied and a ratio of (60:40, v/v) was selected. Increasing the ratio of the aqueous component will produce significant delay and broadening of peaks; meanwhile, increasing the ratio of the organic component will cause overlap between the studied analytes. Different flow rates were studied as it affects both resolution and shape of peaks, and flow rate of 0.8 mL min⁻¹ was selected. Proper choice of the detection wavelength is crucial for sensitivity of the method. Quantitation was achieved with UV-detection at 210 nm based on good peak area for each of the analyzed drugs. System suitability tests were used to verify that the conditions of the chromatographic system were adequate for the separation and hence for the analysis [17] (Table 1).

Method validation

The validity of the proposed LC method was assessed according to ICH guidelines in terms of linearity, range, limit of detection, limit of quantification, accuracy, precision, robustness, specificity, and stability [17].

Table 1 System suitability data for the RP-LC method

Item	AZL	CLT
<i>N</i>	4,422	4,185
<i>K'</i>	1.22	0.76
<i>T</i>	1.19	1.22
<i>R</i>	3.88	
α	1.26	

Linearity and range

Under the above described experimental conditions, linear relationships were established by plotting the peak area versus the corresponding concentration in $\mu\text{g mL}^{-1}$. The graphs were found to be rectilinear over the concentration ranges cited in Table 2. Statistical analysis of the data gave high value of the correlation coefficient (*r*) of the regression equation; small values of the standard deviation of residuals ($S_{y/x}$), intercept (S_a), and slope (S_b); and small value of the percentage relative standard deviation and the percentage relative error (Table 2). These data indicated the linearity of the calibration graphs.

Limit of quantification and limit of detection

Limits of quantification (LOQs) and limits of detection (LODs) were calculated according to ICH Q2 (R1) recommendations using the following equation [17]: $\text{LOQ}=10 S_a/b$ and $\text{LOD}=3.3 S_a/b$, where S_a =standard deviation of the intercept and b =slope of the calibration curve. Results are given in Table 2.

Accuracy and precision

The satisfactory recovery results for the assay of AZL and CLT in their laboratory-prepared mixtures indicate the accuracy of the method (Table 3). Repeatability (intraday) and intermediate precision (interday) were assessed using three concentrations and three replicates of each concentration. The relative standard deviations were found to be very small indicating reasonable repeatability and intermediate precision of the proposed method (Table 4).

Table 2 Characteristics and results of the proposed RP-LC method

Item	AZL	CLT
Limit of detection, LOD ($\mu\text{g mL}^{-1}$)	0.90	0.32
Limit of quantitation, LOQ ($\mu\text{g mL}^{-1}$)	2.72	0.98
Intercept (a)	-189.71	-72.54
Slope (b)	175.78	193.75
Correlation coefficient (r)	0.9999	0.9999
S.D. of residuals ($S_{y/x}$)	61.09	24.24
S.D. of intercept (S_a)	47.84	18.99
S.D. of slope (S_b)	1.71	1.36
% Error	1.02	0.83
% RSD	2.27	1.86
Linearity range ($\mu\text{g mL}^{-1}$)	5.00–50.00	2.50–25.00
Mean %recovery \pm S.D.	100.83 \pm 2.29	99.48 \pm 1.85

Table 3 Assay results for the determination of the studied drugs in their laboratory-prepared mixtures and dosage forms

Ratio	Compound	Laboratory-prepared mixtures			Dosage form		
		Amount taken ($\mu\text{g mL}^{-1}$)	Amount found ($\mu\text{g mL}^{-1}$)	% Found	Amount taken ($\mu\text{g mL}^{-1}$)	Amount found ($\mu\text{g mL}^{-1}$)	% Found
(AZL:CLT) (1.6:1.0)	AZL	8.00	8.10	101.25	8.00	8.22	102.75
		16.00	15.98	99.88	16.00	16.19	101.19
		24.00	23.93	99.71	24.00	24.10	100.42
	Mean recovery%			100.28			101.45
	$\pm\%$ RSD			0.84			1.17
	CLT	5.00	5.04	100.80	5.00	5.06	101.20
		10.00	10.03	100.30	10.00	9.99	99.90
		15.00	14.60	97.33	15.00	15.28	101.87
	Mean recovery%			99.48			100.99
	$\pm\%$ RSD			1.88			0.99

Each result is the average of three separate determinations

Stability of standard solutions and mobile phase

Stability of the standard solutions of AZL and CLT, stored at 4 °C, were evaluated at various time points over 1 month. The concentrations of freshly prepared solutions and those aged for 1 month were calculated by the proposed method, and the difference between them was found to be nonsignificant (% RSD <2 %). Similarly, the stability of the mobile phase was checked for 2 weeks. The results obtained in both cases proved that the samples solutions and mobile phase used during the assay were stable for the specified durations, when kept in the refrigerator at 4 °C.

Robustness of the method

Minor deliberate changes in different experimental parameters such as flow rate ($\pm 0.1 \text{ mL min}^{-1}$), pH (± 0.1), and ratio of mobile phase components ($\pm 2\%$) did not significantly affect resolution of the analyzed compounds indicating that the proposed method is robust (Table 5).

Specificity

The specificity is the ability of the analytical method to assess unequivocally the analyte in the presence of components which may be expected to be present. The specificity of the method was investigated by observing any interference encountered from common tablet excipients, and it was confirmed that the signals measured was caused only by the analytes. The inactive ingredients in Edarbyclor[®] are mannitol, microcrystalline cellulose, fumaric acid, sodium hydroxide, hydroxypropyl cellulose, crospovidone, magnesium stearate, hypromellose 2910, talc, titanium dioxide, ferric oxide red, polyethylene glycol 8,000, and printing ink gray F1. It was found that the excipients did not interfere with the results of the proposed method. As shown in Fig. 2, the chromatograms of the combined formulations did not show any additional peaks when compared with the chromatograms of the laboratory-prepared mixtures which confirm the specificity of the developed method. Specificity of the method was also confirmed by its ability to measure AZL and CLT in the

Table 4 Accuracy and precision data for the RP-LC method

Conc. of AZL ($\mu\text{g mL}^{-1}$)	AZL		Conc. of CLT ($\mu\text{g mL}^{-1}$)	CLT	
	Intraday assay Recovery % $\pm\%$ RSD ^a	Interday assay Recovery % $\pm\%$ RSD ^a		Intraday assay Recovery % $\pm\%$ RSD ^a	Interday assay Recovery % $\pm\%$ RSD ^a
12.50	100.48 \pm 0.96	101.50 \pm 1.25	7.50	100.65 \pm 0.75	101.59 \pm 0.88
25.00	98.87 \pm 1.22	100.17 \pm 1.25	12.50	98.67 \pm 1.08	99.61 \pm 1.75
40.00	100.32 \pm 0.94	101.55 \pm 1.05	20.00	98.12 \pm 0.61	99.03 \pm 0.96
Mean recovery % $\pm\%$ RSD	99.89 \pm 0.89	101.07 \pm 0.77	Mean recovery % $\pm\%$ RSD	99.15 \pm 1.34	100.08 \pm 1.34

^a Mean and relative standard deviation of three determinations

Table 5 Results of the robustness study

Parameter	Drug	Standard method	Flow rate (mL min ⁻¹)		pH		Mobile phase composition	
			0.7	0.9	7.9	8.1	PBS/methanol (62:38)	PBS/methanol (58:42)
Retention time (min)	AZL	7.0	8.1	6.4	7.3	6.8	5.7	8.8
	CLT	5.6	6.4	5.0	5.7	5.5	4.8	6.5
Resolution	CLT/AZL	3.7	3.8	3.7	3.9	3.7	2.7	4.9

presence of all of their degradation products, as revealed by the results of the stability study (Figs. 3b–f and 4b–e).

Application of the proposed method for quality control of AZL and CLT in their combined commercial dosage form

The proposed method was applied to determine AZL and CLT in their commercially available tablets. Satisfactory recoveries were obtained, and the results are given in Table 3.

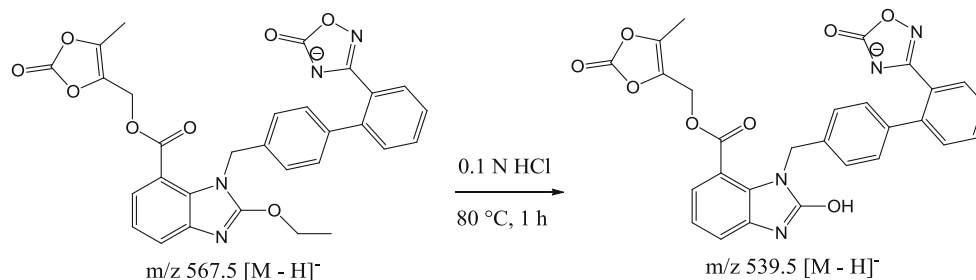
Forced degradation studies

AZL and CLT were subjected to forced decomposition by alkali, acidic, wet heat, light, and oxidation as suggested in the ICH Q1A (R2) and (Q1B) guideline [16] (Electronic supplementary material (ESM) Fig. S1).

Alkaline-induced degradation

AZL showed high stability to alkaline degradation. Only 3.1 % degradation was observed as evidenced by the decrease in the parent drug peak area upon the addition of 2 mL 0.1 M NaOH to the drug and boiling at 80 °C for 1 h (Fig. 3b). On the contrary, CLT was found to undergo alkaline degradation very readily after addition of 2 mL 0.1 M NaOH to the drug and boiling at 100 °C for 1 h. A 69.0 % degradation was observed as evidenced by the decrease in the parent drug peak area, and the appearance of two major DPs with peaks at 3.8 and 4.9 min (Fig. 4b).

Scheme 1 The proposed structure for the acid-induced degradation product of AZL



Acid-induced degradation

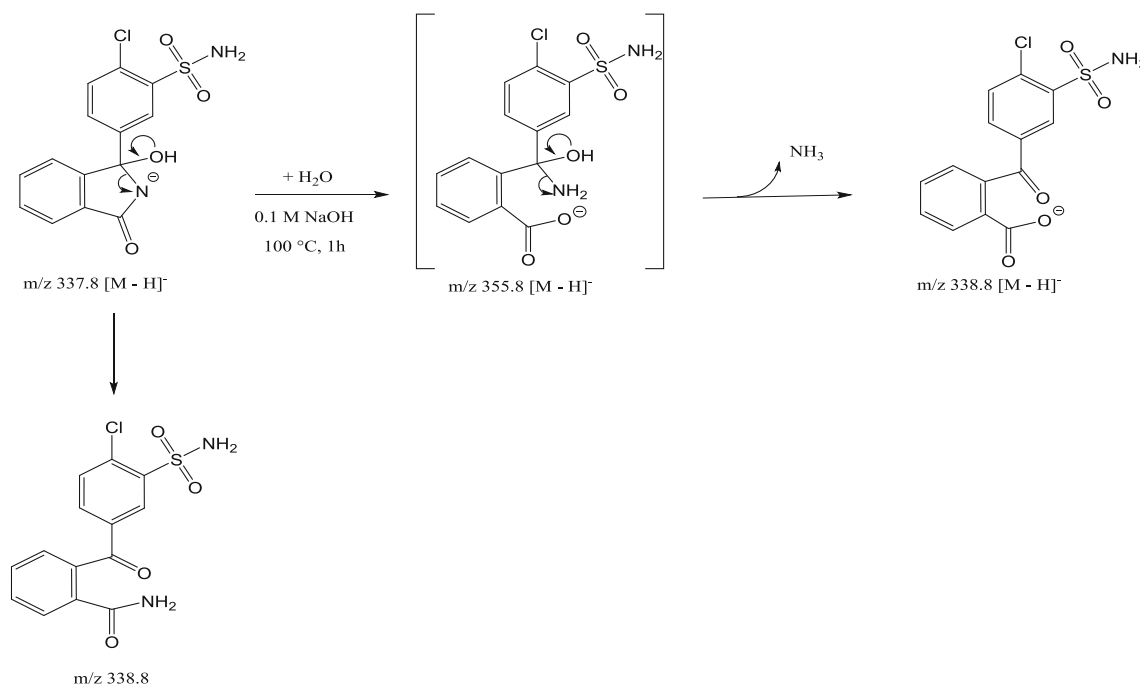
AZL was found to undergo acid degradation very readily upon the addition of 2 mL 0.1 M HCl to the drug and boiling at 80 °C for 1 h. A 94.7 % degradation was observed as evidenced by the decrease in the peak area of the parent drug and the appearance of one major DP peak at 2.7 min (Fig. 3c). On the contrary, CLT was found to show high stability to acidic degradation upon the addition of 2 mL 0.1 M HCl to the drug and boiling at 100 °C for 1 h. Only 3.6 % degradation was observed as evidenced by the decrease in the parent drug peak area (Fig. 4c).

Hydrogen peroxide and thermal (wet heat)-induced degradation

Oxidative and thermal (wet heat) degradation of AZL and CLT were also studied. AZL was found to show certain stability to oxidative and thermal (wet heat) degradation upon the addition of 2 mL of 6 % H₂O₂ or water, respectively, to the drug and boiling at 80 °C for 1 h. Only 19.2 and 2.6 % degradation, respectively, were observed as evidenced by the decrease in the parent drug peak area (Fig. 3d, e). Also, CLT showed certain stability to oxidative and thermal (wet heat) degradation upon the addition of 2 mL of 6 % H₂O₂ or water, respectively, to the drug and boiling at 100 °C for 1 h. Only 7.7 and 0.7 % degradation respectively, were observed as evidenced by the decrease in the parent drug peak area (Fig. 4d, e).

Photolytic degradation

The effect of UV light on the stability of AZL and CLT were studied. It was found that 99.1 and 96.1 % degradation



Scheme 2 The proposed structures for the alkaline-induced degradation products of CLT

were observed after exposure of the drug to 254 nm light for 1 day at room temperature, respectively (Figs. 3f and 4f).

Identification of the potential acid degradation product for AZL

As shown in Scheme 1 and in the ESM Fig. S2, the LC-MS total ion chromatograms (negative mode) for the intact AZL and its acidic-induced degradation after 1 h boiling at 80 °C, a predominant DP was found to be present with a molecular ion at m/z 539. It is postulated that the drug degraded through cleavage of the ether linkage attached to the benzodiazole moiety. This DP showed a fragment at m/z 427 due to cleavage of the ester bond. Then, further fragmentation occurred through opening of the oxadiazole ring and losing CO_2 molecule to give a fragment at m/z 383. This anticipation was supported by the disappearance of the peaks at $\delta=1.3$ and 4.6 of the ethyl group in the proton nuclear magnetic resonance (^1H NMR) spectrum (400 MHz, DMSO) of AZL DP and by the appearance of new peaks at $\delta=5.5$ and 11.4 of the resonating iminol group in the benzodiazole ring (ESM Fig. S3).

Identification of the potential alkaline degradation product for CLT

Figure S4 in the ESM shows the LC-MS total ion chromatogram (negative mode) for the intact CLT and

its alkaline degradation after 1 h boiling at 100 °C. This chromatogram revealed some distinguishable molecular ion peaks at m/z 375, 339, 356, 294, and 258 from that of the ion chromatogram of the intact drug. Also, it

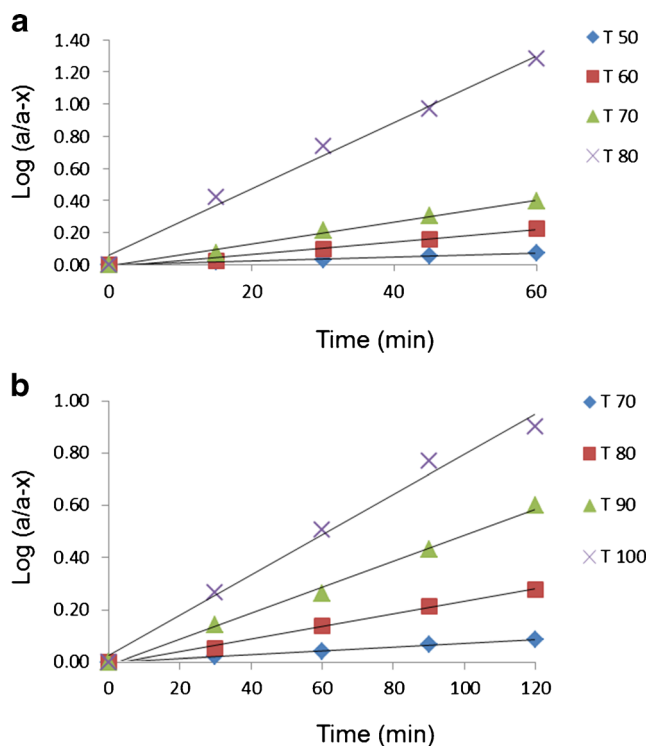


Fig. 5 Effect of different heating times with 0.1 M HCL and 0.1 M NaOH on the rate of degradation of **a** AZL ($25\ \mu\text{g mL}^{-1}$) and **b** CLT ($20\ \mu\text{g mL}^{-1}$), respectively

Table 6 Kinetics data of AZL and CLT degradation

Drug	Temperature (°C)	$K \times 10^{-4}$ (min ⁻¹)	$t_{1/2}$ (min)	t_{90} (min)	E_a (k cal mol ⁻¹)
AZL in 0.1 N HCl	50	27.64	250.8		
	60	89.82	77.2		
	70	158.91	43.6		
	80	476.72	14.5		
	Value at 25 °C	1.96	–	537.8	35.34
CLT in 0.1 N NaOH	70	16.12	429.9		
	80	55.27	125.4		
	90	115.15	60.2		
	100	177.33	39.1		
	Value at 25 °C	0.22	–	4,834.9	20.39

showed many peaks for dimer adducts. As demonstrated in Scheme 2, there are two anticipated pathways for the alkaline-induced degradation of CLT. The first one is through hydrolytic cleavage of the amide bond in the isoindole ring with a molecular ion at m/z 356 followed by removal of the ammonia group to form a stable benzophenone derivative with m/z at 339. Further fragmentation of that DP was proposed to be via decarboxylation to give a fragment at m/z 294. The second pathway is through direct opening of the isoindole ring to form amido benzophenone derivative at m/z 339. Further fragmentation of that DP was through removal of sulphonamide moiety to form a fragment at m/z 258. The second anticipated pathway was supported by the disappearance of the peaks at $\delta=8.1$ and 9.4 of the hydroxyl and the secondary amino groups in the ¹H NMR spectrum (400 MHz, DMSO) of CLT DP and by the appearance of a new peak at $\delta=6.1$ of the amide group (ESM Fig. S5).

Degradation kinetics for AZL and CLT

The experimental investigation of the possible breakdown of the drugs is usually studied to produce more stable preparations and to assist the pharmacists and physicians regarding the proper storage and handling of the medicinal agents. Degradation reaction rates are affected by temperature, as suggested by the Arrhenius equation [18]:

$$\text{Log } K = \text{Log } A - \frac{E_a}{2.303} \frac{1}{RT}$$

Where K =specific reaction rate at temperature T , A =Arrhenius factor (collision factor) constant, E_a =energy of activation (the minimum kinetic energy a molecule must possess in order to undergo reaction), R =gas constant, and T =absolute temperature in Kelvin.

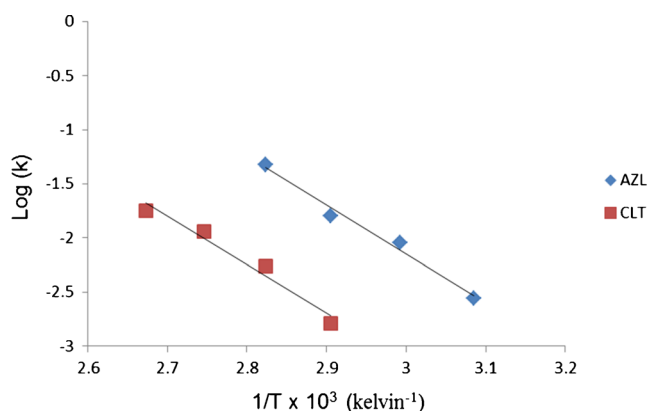
In this study, the constant A and E_a were evaluated by determining K for the acid degradation of 25 $\mu\text{g mL}^{-1}$ of

AZL in 0.1 M HCl at different temperatures (50, 60, 70, and 80 °C) as well as for the alkaline degradation of 20 $\mu\text{g mL}^{-1}$ of CLT in 0.1 M NaOH at different temperatures (70, 80, 90, and 100 °C). As shown in Fig. 5, the degradation was found to be temperature dependent.

The apparent first-order degradation rate constant, half-life time ($t_{1/2}$), shelf-life (t_{90}), and the activation energy E_a were calculated (Table 6). By plotting $\log K_{\text{obs}}$ values versus $1/T$, Arrhenius plot was obtained (Fig. 6) and the resulting lines were extrapolated at room temperature to obtain K_{25}^o , which is used as a measure of the stability of the drug under ordinary shelf conditions. Arrhenius equations [18] were found to be: ($\text{Log } K=11.482+4.5441 \times 10^3/T$) for AZL as well as ($\text{Log } K=10.235+4.4562 \times 10^3/T$) for CLT. Where K is the specific reaction rate and T is the absolute temperature.

Conclusion

The present study represents the first report describing a stability-indicating LC method for determination of AZL and CLT in their commercially available tablets. The proposed method has the advantages of being simple,

**Fig. 6** Arrhenius plots for AZL (25 $\mu\text{g mL}^{-1}$) and CLT (20 $\mu\text{g mL}^{-1}$)

specific, accurate, and selective, so that it was applied to analysis of the studied drugs in its pharmaceutical dosage forms. Moreover, the suggested method permits separation of the studied drugs from their alkaline, acidic, oxidative, thermal (wet heat), or photolytic degradation products within a reasonable time. Furthermore, the proposed method was successfully applied to study the kinetics of the acidic degradation processes in the case of AZL as well as the kinetics of the alkaline degradation processes in the case of CLT, and to derive the first-order degradation rate constant, half-life time ($t_{1/2}$), shelf life (t_{90}), and the activation energy (E_a) of the investigated drugs.

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References

- Baker WL, White WB (2011) Azilsartan medoxomil: a new angiotensin II receptor antagonist for treatment of hypertension. *Ann Pharmacother* 45:1506–1515. doi:10.1345/aph.1Q468
- Kurtz TW, Kajiya T (2012) Differential pharmacology and benefit/risk of azilsartan compared to other sartans. *Vasc Health Risk Manag* 8:133–143
- Kusumoto K, Igata H, Ojima M, Tsuboi A, Imanishi M, Yamaguchi F, Sakamoto H, Kuroita T, Kawaguchi N, Nishigaki N, Nagaya H (2011) Antihypertensive, insulin-sensitising and renoprotective effects of a novel, potent and long-acting angiotensin II type 1 receptor blocker, azilsartan medoxomil, in rat and dog models. *Eur J Pharmacol* 669:84–93
- Vekariya PP, Joshi HS (2013) Development and validation of RP-HPLC method for azilsartan medoxomil potassium quantitation in human plasma by solid phase extraction procedure. *ISRN Spectrosc* 2013:6. doi:10.1155/2013/572170
- Sweetman S (2009) Martindale: the complete drug reference, 36th edn. The Pharmaceutical Press, London
- Singh B, Patel DK, Ghosh SK (2009) A reversed-phase high performance liquid chromatographic method for determination of chlorthalidone in pharmaceutical formulation. *Int J Pharm Sci* 1:24–29
- Mhaske RA, Sahasrabudhe S, Mhaske AA, Garole DJ (2012) RP-HPLC method for simultaneous determination of atorvastatin calcium, olmesartan medoxomil, candesartan, hydrochlorothiazide and chlorthalidone - application to commercially available drug products. *Int J Pharm Sci Res* 3:793–801
- Woo H, Kim JW, Han KM, Lee JH, Hwang IS, Kim J, Kweon SJ, Cho S, Chae KR, Han SY (2013) Simultaneous analysis of 17 diuretics in dietary supplements by HPLC and LC-MS/MS. *Food Addit Contam, Part A* 30:209–217. doi:10.1080/19440049.2012.738939
- Youssef RM, Maher HM, El-Kimary EI, Hassan EM, Barary MH (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. doi:10.5740/jaoacint.11-347
- Salem H (2004) High-performance thin-layer chromatography for the determination of certain antihypertensive mixtures. *Sci Pharm* 72:157–174
- Ciborowski M, Icardo MC, Mateo JV, Martinez Calatayud J (2004) FI-chemiluminometric study of thiazides by on-line photochemical reaction. *J Pharm Biomed Anal* 36:693–700. doi:10.1016/j.jpba.2004.08.020
- Parmar KE, Mehta RS (2013) First order derivative spectrophotometric method for simultaneous estimation of telmisartan and chlorthalidone in bulk and pharmaceutical dosage form. *Int Res J Pharm* 4:224–228. doi:10.7897/2230-8407.04348
- Lu M, Li X, Feng Q, Chen G, Zhang L (2010) Analysis of diuretics by capillary electrochromatography using poly(1-hexadecene-co-TMPTMA) monolithic column. *Sepu* 28:253–259. doi:10.3724/sp.j.1123.2010.00253
- Cheng JWM (2013) Azilsartan/chlorthalidone combination therapy for blood pressure control. *Integr Blood Press Control* 6:39–48. doi:10.2147/ibpc.s34792
- Kasimala MB, Kasimala BB (2012) Reverse phase-HPLC method development and validation for the simultaneous estimation of azilsartan medoxomil and chlorthalidone in pharmaceutical dosage forms. *Jamonline* 2:117–126
- ICH (1996) International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use. ICH Harmonised Tripartite Guideline Q1A(R2) and (Q1B), Stability testing of new drug substances and products, 2003 and stability testing: photostability testing of new drug substances and products. ICH: Geneva, Switzerland
- ICH (2013) Harmonized tripartite guideline, validation of analytical procedures: text and methodology, Q2(R1), current step 4 version, parent guidelines on methodology. Dated November 6 1996, Incorporated in November 2005. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1_Guideline.pdf. Accessed April 23
- Sinko PJ (2011) Martin's physical pharmacy and pharmaceutical sciences: physical chemical and biopharmaceutical principles in the pharmaceutical sciences. 6th ed. Lippincott, Philadelphia, PA