



Stability-indicating LC Method for Quantification of Azelnidipine: Synthesis and Characterization of Oxidative Degradation Product

Azelnidipin Miktar Tayini İçin Stabilite Göstergeli LC Yöntemi: Oksidatif Bozunma Ürününün Sentezi ve Karakterizasyonu

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ABSTRACT

Objectives: In the work presented here, the degradation behavior of azelnidipine under diverse forced degradation conditions was studied. A stability-indicating liquid chromatographic method was established which could separate and resolve azelnidipine from its degradation products. Further, chemical kinetics under acidic and alkaline conditions were studied, and validation studies were performed.

Materials and Methods: Using reversed-phase chromatography, azelnidipine and its formed degradants were resolved using phosphate buffer (pH 3.0) and methanol in a mixture of 10:90% v/v as a mobile phase at a flow rate of 1.0 mL/min. All eluents were detected at a wavelength of 256 nm.

Results: Azelnidipine was degraded under acid, alkali, wet heat, and oxidized environment. The pH-dependent rate of hydrolysis of azelnidipine was studied under acidic and alkaline conditions and chemical kinetics were determined. Further, the oxidative degradation product of azelnidipine was synthesized and characterized as 3-(1-benzhydrylazetid-3-yl) 5-isopropyl 2-amino-6-methyl-4-(3-nitrophenyl) pyridine-3,5-dicarboxylate (dehydro-AZD).

Conclusion: The susceptibility of azelnidipine to hydrolysis was attributed to the presence of ester at 3 and 5 positions of 1,4 dihydropyridine. Further, under oxidative conditions, the aromatization of 1,4 dihydropyridine resulted in dehydro-AZD. Azelnidipine followed the first-order reaction under acid and alkali hydrolysis, and was more susceptible to degradation under acidic conditions. The synthesized and confirmed dehydro-AZD was found as one of the metabolites and impurities of azelnidipine. The evaluated validation parameters ascertained the practicality of the method for the quantification of azelnidipine tablets.

Key words: Azelnidipine, chemical kinetics, degradation product, HPLC, method validation, stability-indicating

ÖZ

Amaç: Bu çalışmada, azelnidipinin çeşitli zorlu bozunma koşulları altında bozunma davranışı incelenmiştir. Azelnidipinin stabilitesini gösteren, azelnidipini bozunma ürünlerinden ayırabilen ve çözebilen sıvı kromatografik yöntem geliştirilmiştir. Ayrıca asidik ve alkali koşullar altında kimyasal kinetikler incelendi ve doğrulama çalışmaları yapıldı.

Gereç ve Yöntemler: Azelnidipin ve oluşan bozunma ürünleri, mobil faz olarak kullanılan fosfat tamponu (pH 3,0) ve metanol (%10:90 h/h) ile, 1,0 mL/dk akış hızında ters faz kromatografisi kullanılarak ayrıştırıldı. Tüm eluentler 256 nm dalga boyunda tespit edildi.

Bulgular: Azelnidipin asit, alkali, ıslak ısı ve oksitlenmiş ortam koşullarında degrade edildi. Azelnidipinin pH'ye bağlı hidroliz hızı asidik ve alkali koşullar altında çalışılmış ve kimyasal kinetik belirlenmiştir. Ayrıca, azelnidipinin oksidatif bozunma ürünü olan 3-(1-benzhidrilazetid-3-il) 5-izopropil 2-amino-6-metil-4-(3-nitrofenil) piridin-3,5-dikarboksilat (dehidro-AZD) sentezlendi ve karakterize edildi.

Sonuç: Azelnidipinin hidrolize duyarlılığı, 1,4 dihidropiridin 3 ve 5 pozisyonlarındaki esterin varlığı ile ilişkilendirildi. Ayrıca, oksidatif koşullar altında 1,4 dihidropiridin aromatzasyonu dehidro-AZD eldesi ile sonuçlandı. Azelnidipin için, asit ve alkali hidroliz altında bozunmasının birinci derece kinetiğe uyduğu belirlendi ve asidik koşullar altında bozunmaya daha duyarlıydı. Sentezlenen ve doğrulanan dehidro-AZD, azelnidipinin

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metabolitlerinden ve safsızlıklarından biri olarak bulundu. Değerlendirilen doğrulama parametreleri, azelnidipin tabletlerinin miktar tayini için yöntemin pratikliğini belirlemiştir.

Anahtar kelimeler: Azelnidipin, kimyasal kinetik, bozunma ürünü, HPLC, metot validasyonu, stabilite göstergesi

INTRODUCTION

Stability experiments aim to recognize the likely alterations with drug substances and products with regards to time under various storage conditions. It is anticipated that all the analytical methods used during the study should be stability-indicating. Although any method evaluating changes in the physico-chemical properties of a drug substance or product should indicate stability, chromatographic methods are the most common stability-indicating methods (SIM).¹ A major challenge in SIM development is the generation of stability test samples which are the real-time samples and contain all degradation products which may form under normal storage conditions. For stability evaluation, forced degradation experiments are conducted where the drug substances and products are heated to elevated temperatures under different pH conditions at different intervals, oxidation, dry heat, and photolytic conditions.

Chemically, AZD (Figure 1) is (\pm) 3-(1-benzhydrylazetid-3-yl) 5-isopropyl 2-amino-6-methyl-4-(3-nitrophenyl)-1,4-dihydropyridine (DPH)-3,5-dicarboxylate.² AZD is official in Indian Pharmacopoeia 2014³ and Japanese Pharmacopoeia 2016.⁴

AZD is a third-generation calcium channel antagonist and an effective antihypertensive agent used in patients suffering from hypertension.⁵ It specifically suppresses the L-type calcium channels of smooth muscle cells, and prevents the influx of transmembrane calcium.⁶

A literature review found numerous analytical methods stated for the estimation of AZD, including AZD estimation in pharmaceutical formulations by high performance liquid chromatography (HPLC),⁷⁻¹⁰ ultraviolet (UV) spectroscopy¹¹ in biological fluids by hyphenated LC-mass spectrometry (MS) techniques,^{12,13} enantiomeric separation and estimation of AZD by HPLC¹⁴ and LC-tandem MS.¹⁵ Along with these, an extensive literature exists on SIM for the estimation of AZD, including HPLC^{16,17} and HPTLC.¹⁸ Two SIM have been reported for the

simultaneous estimation of AZD and olmesartan.^{19,20} Further, the degradation of AZD under radical initiator-based oxidative conditions was studied.²¹

Moreover, limited shreds of evidence have been reported regarding the degradation behavior of AZD under different degradation conditions as well as no chemical kinetic study has been performed to date.

To address these unaccounted phenomena, the objectives of the current investigation was to ascertain an LC approach for AZD quantification in bulk and tablets which could separate and resolve the AZD from its degradants, to validate the method to prove the accuracy, precision, robustness, and stability-indicating power of the method. The study was set out to explore the degradation behavior of AZD under different forced degradation conditions and to study the kinetics under acidic and alkaline conditions.

Further, from the literature, it was revealed that 3-(1-benzhydrylazetid-3-yl) 5-isopropyl 2-amino-6-methyl-4-(3-nitrophenyl) pyridine-3,5-dicarboxylate (dehydro-AZD) is the oxidative degradation product of AZD²¹ as well as one of the major AZD metabolites.²² According to the literature 1,4-DHP derivatives oxidize in the liver by cytochrome P-450 into pyridine derivatives (aromatization of 1,4-DHP).²³ This was of high interest and therefore, the study was continued to synthesize and interpret the oxidative degradation product of AZD.

MATERIALS AND METHODS

Chemicals and reagents

A pharmaceutical grade AZD (certified to contain 99.91% w/w on dried basis) was obtained from Precise Pharmaceuticals Ltd, Nashik, India as a gift sample. In the investigation, methanol was of HPLC grade and other chemicals were of Analytical Reagent grade. All chemicals were bought from SDFLC - SD Fine Chem Ltd, Mumbai, India. The varying strengths of hydrochloric acid, sodium hydroxide, and hydrogen peroxide were prepared freshly by diluting appropriately with double distilled water, and were further used after filtering through membrane filter papers (Millipore India Pvt. Ltd., Bengaluru, India). The tablets containing AZD 16 mg were bought from the residential market.

Instrumentation and chromatographic conditions

HPLC system used in the analyses consisted of binary pumps (PU 2080 plus), Jasco Corporation, Tokyo, Japan with 20 μ L sample injector and multi-channel UV-visible detector, UV-2077, Jasco Corporation, Tokyo, Japan. All signals were recorded using Borwin software (version 1.50).

All chromatographic analyses were conducted on the C18 column with dimensions of 250 \times 4.6 mm, 5 μ m using a blend of

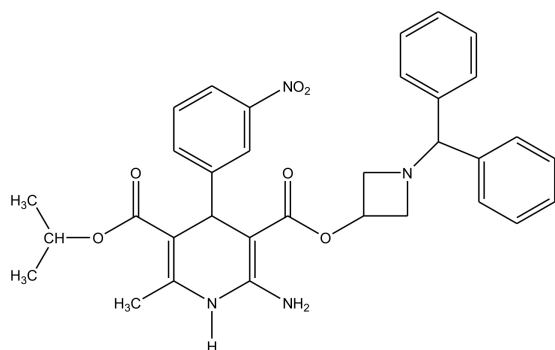


Figure 1. Chemical Structure of AZD

25 mM phosphate buffer (pH 3.0), and methanol (10:90% v/v) at a constant flow of 1.0 mL/min. The detector wavelength was set out at 256 nm, which was the absorbance maxima of the AZD.

Forced degradation studies

Forced degradation trials were carried out on AZD bulk drug sample and on AZD tablets as per ICH Q2A (R1).²⁴ Preliminary experiments were conducted to decide the strength of the stressor used, temperature of exposure, and time of heating. Under acidic and alkaline degradation, AZD was exposed to 0.1 N HCl and 0.1 N NaOH at 70°C for 35 min, respectively. Wet heat degradation was achieved by refluxing the drug into double distilled water for 8 h at 70°C. Further, AZD was exposed to 3% v/v hydrogen peroxide under dark for 24 h. The oxidized sample was heated on water bath to eliminate the leftover hydrogen peroxide. Degradation under dry heat condition occurred by heating the AZD for 6 h in a hot air oven at 70°C and photolytic degradation by exposing the AZD under 7-day cycles to direct sunlight.

After exposure, degradation samples were collected and diluted suitably with the mobile phase. The obtained 10 µg/mL of samples were injected in the LC system. The decrease in the area under curve (AUC) of AZD compared to the standard AZD sample and with the appearance of secondary peaks in the chromatograms were noted as degradation. Appropriate counter blank samples were used to impede errors.

Chemical kinetic studies

To study the chemical kinetics of AZD under acidic and alkaline conditions, 10 mg of AZD was transferred to two separate round bottom flasks, and in each flask, 10 mL of 0.1 N HCl and 0.1 N NaOH were added, respectively. The resulting solutions were heated in a thermostatic water bath at 50°C for 35 min. After 5 min intervals, the appropriate quantity was quenched, diluted with the mobile phase to obtain 10 µg/mL, and injected in the LC system.

Synthesis and characterization of dehydro-AZD

An accurately weighed 1 g quantity of AZD was transferred to a conical flask, to which 20 mL of acetonitrile was added and the solution was stirred for 10 min. To it, 1 g of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone was added. The resulting mixture was again stirred for 20 min and was kept in dark at room temperature for 7 h. The obtained reaction mixture was washed with double distilled water. The resulting product was characterized using MS.

Preparation of standard stock solution, calibration curve standards, and estimation of AZD in tablets

The standard stock solution of 1 mg/mL of AZD was prepared in methanol. The prepared standard stock solution was diluted appropriately with the mobile phase to obtain 10, 20, 30, 40, 50, and 60 µg/mL of calibration curve standards and injected in triplicate. To obtain the calibration curve equation, the recorded AUC at each calibration standard was plotted against respective concentrations, and the regression coefficient (r^2), y-axis intercept, and the slope of the line were determined.

To estimate the AZD in tablets, twenty tablets were weighed and ground to a fine powder. The amount equal to the total weight of one tablet was weighed and moved to a 100 mL volumetric flask. The mixture was sonicated for 10 min after the addition of 70 mL methanol and diluted further to 100 mL with methanol. The obtained solution was filtered, subsequently diluted to obtain 10 µg/mL, and injected in triplicate. The corresponding concentrations and the % label claim were calculated using the calibration curve equation.

Method validation

The developed method was validated as per the ICH Q2 R1 guidelines²⁵ to evaluate the accuracy, precision, detection limit (DL), quantitation limit (QL), robustness, and specificity.

Accuracy and precision were executed by spiking the standard sample of AZD in the tablet solution at 80%, 100%, and 120% levels across the calibration range in triplicate for three successive days. The acceptable accuracy was established by the closeness of the % amount recovered with the % amount added and the precision with low relative standard deviation % (RSD). Further, the obtained data of accuracy and precision were subjected to One-Way ANOVA to ascertain the intermediate precision of the method. DL and QL were determined as DL: (3.3 σ /S) and QL: (10 σ /S), where σ : SD of AUC and S is the slope of the calibration curve, respectively. Robustness of the method was verified by executing minor changes in the flow rate (± 0.2 mL), % methanol ($\pm 10\%$) and the detection wavelength (± 5 nm) and the effects on the system of the AZD peak were observed. To prove the specificity of the method, absolute separation of AZD from its degradation products and the absence of the interfering peaks at the retention times of AZD were evaluated.

Statistical analysis

In the results and discussion section, the statistical analysis was discussed in chemical kinetics and method validation.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

To obtain the adequate retention time of AZD with acceptable system suitability, different mobile phases were tried. Initially, water was tried as an aqueous phase along with acetonitrile and methanol. However, the splitting of the AZD peak suggests a buffer in the mobile phase. Good peak shape and acceptable system suitability parameters (theoretical plates: 8991, asymmetry: 1.10) were obtained when phosphate buffer at pH 3.0 was tried with methanol using Phenomenex Hyperclone ODS (C18) column (250×4.6 mm, 5 µm). The adequate retention time of AZD at 4.703±0.12 min was obtained when 25 mM phosphate buffer was used with methanol in the ratio of 10:90% v/v, respectively. All eluents were detected at 256 nm in an isocratic mode at the flow rate of 1 mL/min.

Forced degradation studies

Under acidic conditions, two degradation products were obtained, whereas, under alkaline conditions, wet heat, and oxidative conditions, one degradation product was obtained.

Inconsiderable decrease in the peak area of AZD or appearance of secondary degradation products were detected in dry heat and photolytic conditions.

The degradation behavior of AZD under different forced degradation conditions is presented in Table 1 and the respective chromatograms are presented in Figure 2.

From the degradation behavior of AZD under different conditions, it was observed that AZD is more susceptible to degrade under acidic and alkaline conditions followed by oxidation and wet heat conditions.

Chemical kinetics

A gradual decrease in the peak area (Figure 3) confirmed that AZD follows the first-order reaction under acidic and alkaline conditions. The rate constant (K), half-life ($t_{1/2}$), and shelf-life (t_{90}) were determined using the following equations, respectively.

$$K = \frac{2.303}{t} \log \frac{C_0}{C} \quad \text{equation (1)}$$

$$t_{1/2} = \frac{0.693}{K} \quad \text{equation (2)}$$

$$t_{90} = \frac{0.104}{K} \quad \text{equation (3)}$$

From Table 2, the K value was found to be higher under acidic condition than alkaline condition which concludes that the rate

Table 1. Forced degradation behavior of AZD

Degradation condition	Degradation %	RT of a drug (min)	RT of degradation products (min)
Acid	21.27	4.82	2.76, 3.18
Alkali	17.88	4.57	2.85
Wet heat	8.59	4.82	3.45
Hydrogen peroxide induced oxidation	10.07	4.82	2.21 (peroxide blank), 3.27

RT: Room temperature

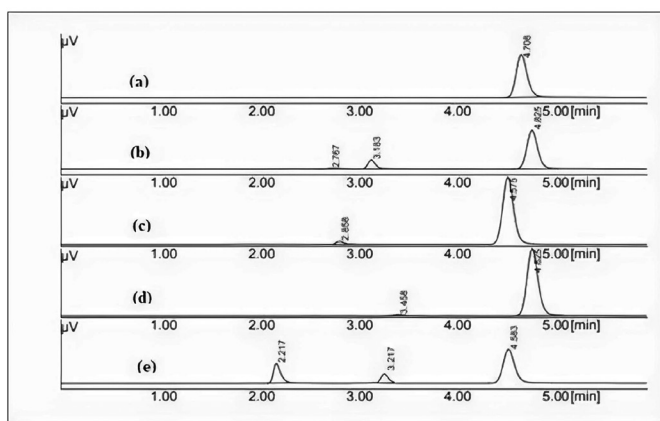


Figure 2. Representative chromatograms of AZD under (a) standard AZD (10 mg/mL), (b) acidic condition, (c) alkaline condition, (d) wet heat degradation (e) hydrogen peroxide induced oxidation

of hydrolysis of AZD is higher in acid as compared to that in alkali. Also, the $t_{1/2}$ and t_{90} values were the lowest for acid and highest for alkali. AZD contains two ester groups and a lactone ring; both groups are susceptible to hydrolysis. However, the rate of hydrolysis depends upon pH, temperature, and substituents.

Characterization of synthesized dehydro-AZD

The MS of the synthesized compound is depicted in Figure 4, where the major fragments identified had an m/z ratio of 581.45 (molecular ion), 342.18, 238.14, and 167.11, respectively. The mass fragmentation pattern is depicted in Figure 5, and it confirmed the synthesis of dehydro-AZD.

Table 2. Summary of AZD acid and alkali hydrolysis kinetics

Degradation condition	K (1/min)	$t_{1/2}$ (min)	t_{90} (min)
Acid degradation	1.10×10^{-2}	63	9.45
Alkali degradation	5.99×10^{-3}	115.69	17.36

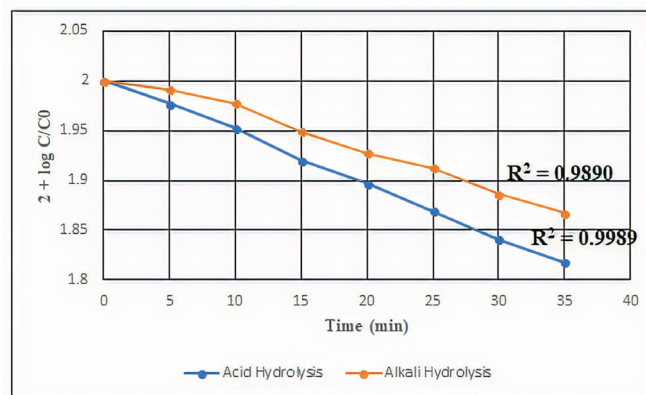


Figure 3. First-order plots of AZD under acidic and alkaline conditions

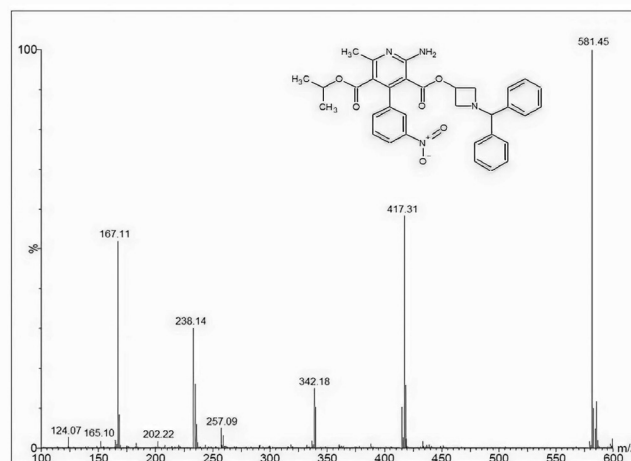


Figure 4. Mass spectrum of dehydro-AZD

Calibration curve and estimation of AZD in tablets

AZD was found to be linear in the range of 10-60 µg/mL with r^2 : 0.9989 with the calibration curve equation, y : 53455x+121119.

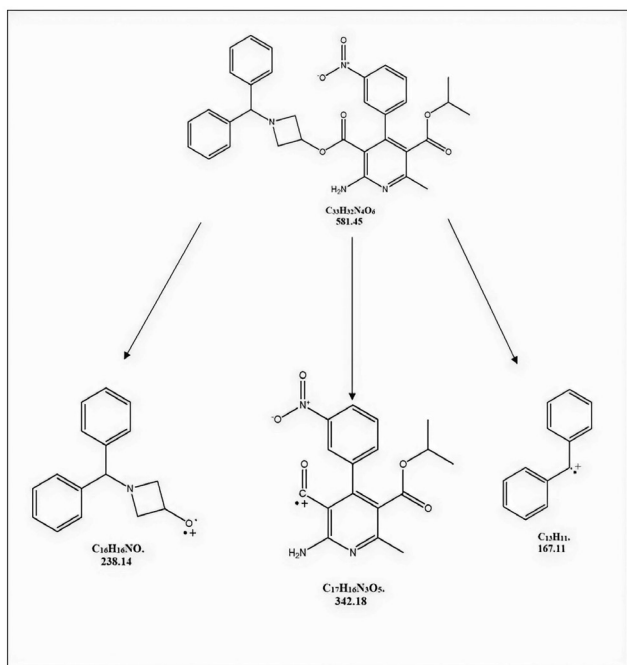


Figure 5. Fragmentation pattern of dehydro-AZD

The calibration curve is depicted in Figure 6. The analysis of the tablet showed 100.54 ± 0.30 of AZD.

Method validation

The results of accuracy and precision studies are presented in Table 3; mean concentration values were close to the spiked concentration of AZD, indicating good recovery. The precision was indicated by the low values of RSD %. When the obtained data of accuracy and precision studies were subjected to ANOVA, the F (observed) at each QC level was lower than the F (theoretical) at 95% confidence interval, indicating insignificant

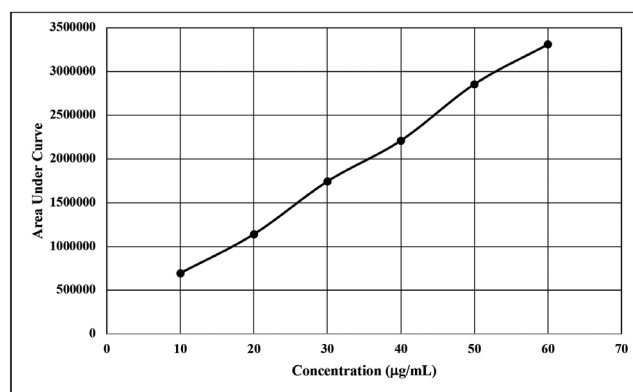


Figure 6. Calibration curve of AZD

Table 3. Summary of accuracy and precision of AZD

Amount added (µg/mL)	Amount found(µg/mL)			Within mean square	Between mean square	F value
	Day 1	Day 2	Day 3			
25+20=45 (80%)	44.78	44.52	44.51	0.0606	0.1541	2.5438
	44.92	44.73	44.85			
	44.92	44.02	44.49			
	Mean	44.87	44.42			
	± SD	0.08	0.36	0.20		
	RSD %	0.18	0.82	0.45		
25+25=50 (100%)	49.33	49.12	49.15	0.0495	0.0825	1.6673
	49.06	49.36	49.46			
	49.14	49.15	49.82			
	Mean	49.18	49.21			
	± SD	0.14	0.13	0.33		
	RSD %	0.28	0.26	0.67		
25+30=55 (120%)	54.77	54.18	54.83	0.2100	0.0546	0.2598
	54.69	55.37	54.80			
	55.05	54.22	54.19			
	Mean	54.83	54.59			
	± SD	0.18	0.67	0.36		
	RSD %	0.34	1.24	0.66		

SD: Standard deviation, RSD: Relative standard deviation

difference of the data of intra- and inter-day precision and proved the intermediate precision. The DL and QL were 0.43 µg/mL and 1.32 µg/mL, respectively. In the robustness experiment, no significant changes were observed in the system suitability parameters for AZD when minor changes were executed in the established chromatographic condition, demonstrating the robustness of the method. AZD was well separated and resolved from its degradation products; the absence of interfering peaks at the retention time of the AZD designated specificity.

CONCLUSION

The main conclusions of this work are drawn together and presented in this section:

- In the present work, LC method was stated for the estimation of AZD in bulk and tablets. The method proved to be simple and economic as the separation was achieved on a C18 column with the mixture of 25 mM phosphate buffer (pH 3.0) and methanol as a mobile phase in the proportion of 10: 90% v/v in an isocratic mode, and all the formed degradation products along with AZD were separated out within less than 10 min of the run time.
- The method proved to be accurate with satisfactory precision. No significant alterations in the system suitability ascertained the robustness of the method. The acceptable specificity proved the stability-indicating nature of the method. The method was linear in the range of 10-60 µg/mL and the assay of the tablet was found to be 100.54%±0.30% of the stated label of AZD.
- The forced degradation trials resulted in the degradation of AZD under acidic, alkaline, wet heat conditions, and peroxide-mediated oxidation.
- Hydrolysis is the major degradation pathway for drug substances with an ester functional group in their structure. The AZD has two ester groups present at 3 and 5 positions of the 1,4-DHP moiety, and hence, AZD may be susceptible to acid, alkali, and wet heat hydrolysis. Further, the pH-dependent rate of hydrolysis of AZD under acid and alkali conditions was determined by the chemical kinetic study, which proved the first-order reaction of AZD under acidic and alkaline conditions, respectively. The $t_{1/2}$ and t_{90} values proved that AZD was more susceptible to degrade under an acidic environment than under an alkaline environment.
- Considering the atomization of 1,4-DHP to pyridine derivative under oxidative conditions, the oxidative degradation product of AZD was synthesized and confirmed using MS, which was found as one of the impurities and metabolites of AZD.

Conflict of interest: No conflict of interest was declared by the authors. The authors are solely responsible for the content and writing of this paper.

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