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ABSTRACT
Objectives: In the work presented here, the degradation behavior of azelnidipine under diversified 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 acid and alkali 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 under acidic and alkaline conditions was studied and chemical kinetics were determined. Further, the oxidative degradation product of azelnidipine was synthesized and characterized as 3-(1-Benzhydrylazetidin-3-yl) 5-isopropyl 2-amino-6-methyl-4-(3-nitrophenyl) pyridine-3,5-dicarboxylate (dehydro-AZD).

Conclusions: The susceptibility of azelnidipine to hydrolysis was associated with the presence of ester at 3 and 5 positions of 1,4 dihydropyridine. Further, under oxidative conditions, the aromatization of 1,4 dihydropyrridine could form dehydro-AZD. Azelnidipine follows the first-order reaction under acid and alkali hydrolysis and found more susceptible to degrade under acidic conditions. The synthesized and confirmed dehydro-AZD was found as one of the metabolites and impurities of azelnidipine.

Keywords: azelnidipine, chemical kinetics, degradation product, HPLC, method validation, stability-indicating

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INTRODUCTION:
Stability experiments aim to recognize the likely alterations with drug substances and products with regards to the time at various storage conditions. It is anticipated that all the analytical methods used during the study should be stability-indicating. Although, any method which is used to evaluate changes in the Physico-chemical property of a drug substance or product should be stability-indicating, however, the most commonly used stability-indicating methods (SIM) are the chromatographic methods 1. A major challenge in developing any SIM is the generation of stability test samples which is the real-time samples and contains all degradation products which may form under normal storage conditions. To forecast it, forced degradation experiments are conducted where the drug substance and product are heated to elevated temperatures in different pH conditions at a different time, to oxidation, to dry heat, and to photolytic condition.
Chemically, AZD (Figure 1) is (±) 3-(1-Benzhydrylazetidin-3-yl) 5-isopropyl 2-amino-6-methyl-4-(3-nitrophenyl) -1,4-dihydropyridine-3,5-dicarboxylate 2. It was found official in Indian Pharmacopoeia 2014 3 and Japanese Pharmacopeia 2016 4.

![Figure 1. Chemical Structure of AZD](image)

AZD is a third-generation calcium channel antagonist, an effective antihypertensive agent used in patients suffering from hypertension5. It specifically suppresses the L-type calcium channels of smooth muscle cells and prevents the influx of transmembrane calcium 6.
A review of the literature undertaken found numerous analytical methods stated for the estimation of AZD includes, estimation of AZD in pharmaceutical formulations by HPLC 7-10, by UV spectroscopy 11, in biological fluids by hyphenated LC-MS techniques 12,13. An enantiomeric separation and estimation of AZD by HPLC 14 and by LC-tandem mass spectrometry 15. Along with this, there exists an extensive literature on stability-indicating methods for estimation of AZD, these includes the estimation of AZD by HPLC 16,17 and by HPTLC 18. Two stability-indicating methods have been reported for the simultaneous estimation of AZD with Olmesartan 19,20 Further, the degradation of AZD under radical initiator-based oxidative conditions was studied 21.
Despite the mentioned literature, there are limited shreds of evidence reported regarding the degradation behavior of AZD under different degradation conditions as well as no chemical kinetic study was performed to date.
To address these previously unaccounted phenomena, the objectives of the current investigation was to ascertain an LC approach for quantification of AZD in bulk and tablets which could separate and resolve the AZD from its degradation products, 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 acid and alkali conditions.

Further, from the literature, it was revealed that 3-(1-Benzhydrylazetidin-3-yl) 5-isopropyl 2-amino-6-methyl-4-(3-nitrophenyl) pyridine-3,5-dicarboxylate (dehydro-AZD) is the oxidative degradation product of AZD as well as one of the major metabolites of AZD. This has been extensively sightseen in the literature that the 1,4 dihydropyridine (1,4-DHP) derivate oxidize in the liver by cytochrome P-450 to pyridine derivatives (aromatization of 1,4-DHP). 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 used were of AR grade. All chemicals were bought from SDFLC – S D 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 was 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 L sample injector, and multi-channel UV-Vis detector, UV-2077, Jasco Corporation, Tokyo, Japan. All signals were recorded using Borwin software (version 1.50).

All chromatographic analyses were conducted on C18 column with dimensions of 250 × 4.6 mm, 5 μm using a blend of 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 as well as on AZD tablets as per ICH Q2A (R1). Preliminary experiments were conducted to decide the strength of stressor used, temperature of exposure, and time of heating. Under acid and alkali 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 a water bath to eliminate the leftover hydrogen peroxide. Degradation within dry heat condition was made by heating the AZD for 6 h in a hot air oven at 70°C and photolytic degradation by exposing the AZD for 7-day cycles to direct sunlight.

After exposure, degradation samples were collected and diluted suitably with the mobile phase. The obtained 10 g/mL of samples were injected in the LC system. The decrease in the area under curve (AUC) of AZD to the standard AZD sample and with the appearance of secondary peaks in 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 acid and alkali conditions, 10 mg of AZD was transferred to two separate round bottom flasks, in each flask, 10 mL of 0.1 N HCl, and 0.1 N NaOH was added, respectively. The resulting solutions were heated on a thermostatic water bath at a temperature of 50°C for 35 min. Every after 5 min of the interval, the appropriate...
quantity was quenched, diluted with the mobile phase to obtain 10 g/mL, and injected in the LC system.  

**Synthesis and characterization of dehydro-AZD**  
An accurately weighed 1 g quantity of AZD was transferred in a conical flask, to it, 20 mL of dichloromethane was added and the solution was stirred for 10 min. To it, 1 g of 2,3-dichloro-5,6-dicyano-1,4-benzoquinon (DDQ) was added. The resulting mixture was again stirred for 20 min and was kept at room temperature under dark for 7 h. The obtained reaction mixture was washed with double distilled water and with cyclohexane. The resulting product was characterized using mass spectrometry.  

**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 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 g/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 ICH Q2 R1 guidelines 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 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 % 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 /S), and QL = (10 /S), where S = standard deviation (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 (± 10 %) and the detection wavelength (± 5 nm) and its effects on the system suitability 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 interfering peaks at the retention times of AZD were evaluated.  

**RESULTS AND DISCUSSION**  

**Optimization of 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 the use of 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 m). 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 alkali, wet heat, and oxidative conditions, one degradation product was obtained, respectively. No considerable 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.
Table 1. Forced degradation behavior of AZD

<table>
<thead>
<tr>
<th>Degradation Condition</th>
<th>% Deg.</th>
<th>RT of a drug (min)</th>
<th>RT of degradation products (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid</td>
<td>21.27</td>
<td>4.82</td>
<td>2.76, 3.18</td>
</tr>
<tr>
<td>Alkali</td>
<td>17.88</td>
<td>4.57</td>
<td>2.85</td>
</tr>
<tr>
<td>Wet heat</td>
<td>8.59</td>
<td>4.82</td>
<td>3.45</td>
</tr>
<tr>
<td>Hydrogen Peroxide induced oxidation</td>
<td>10.07</td>
<td>4.82</td>
<td>2.21 (peroxide blank), 3.27</td>
</tr>
</tbody>
</table>

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

From the degradation behavior of AZD under different conditions, it was observed that AZD is more susceptible to degrade under acid and alkali conditions followed by oxidation and wet heat conditions.
Chemical Kinetics
A gradual decreased in the peak area (Figure 3) confirmed that AZD follows the first-order reaction under acidic and alkaline conditions, respectively. The rate constant (K), half-life (t½), and shelf-life (t90) were determined using the following equations, respectively.

\[ K = \frac{2.303}{t} \log \frac{C_0}{C} \]  
Equation 1

\[ t_{1/2} = \frac{0.693}{K} \]  
Equation 2

\[ t_{90} = \frac{0.104}{K} \]  
Equation 3

![Figure 3. First-order plots of AZD under acid and alkali conditions](image)

**Table 2. Summary of AZD acid and alkali hydrolysis kinetics**

<table>
<thead>
<tr>
<th>Degradation Condition</th>
<th>K (1/min)</th>
<th>t½ (min)</th>
<th>t 90 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid degradation</td>
<td>1.10 × 10⁻²</td>
<td>63</td>
<td>9.45</td>
</tr>
<tr>
<td>Alkali degradation</td>
<td>5.99 × 10⁻³</td>
<td>115.69</td>
<td>17.36</td>
</tr>
</tbody>
</table>

From Table 2, the K value was found to be higher under acidic condition than under alkaline condition which concludes that the rate of hydrolysis of AZD is more in acid as compared to alkali. Also, the t½ and t90 values were found lowest under acid and highest for alkali. AZD contains two ester groups and a lactone ring; both groups are susceptible to the hydrolysis. However, the rate of hydrolysis depends upon pH, temperature and on the substituents.

**Characterization of synthesized dehydro-AZD**
The mass spectrum of the synthesized compound is depicted in Figure 4, where the major fragments identified were m/z: 581.45 (molecular ion), 342.18, 238.14, and 167.11. The mass fragmentation pattern is depicted in Figure 5, confirmed the synthesis of dehydro-AZD.
Figure 4. Mass spectrum of dehydro-AZD
AZD was found linear in the range of 10-60 g/mL with $r^2 = 0.9989$ with calibration curve equation, $y = 53455x + 121119$. The calibration curve is depicted in Figure 6. The analysis of tablet shows $100.54 \pm 0.30$ of AZD.
Method Validation
The results of accuracy and precision studies are presented in Table 3, mean values of concentration found were close to the spiked concentration of AZD, indicates good recovery. The precision was proved with low values of % RSD. When the obtained data of accuracy and precision studies were subjected to ANOVA, the F (observed) at each QC level were lower than the F (theoretical) at 95 % confidence interval, indicates no significant difference of the data of intra- and inter-day precision and proved the intermediate precision. The DL and QL was found 0.43 g/mL and 1.32 g/mL, respectively. In the robustness experiment, no significant changes were observed in system suitability parameters for AZD when minor changes were executed in the established chromatographic condition, demonstrate the robustness of the developed method. AZD was well separated and resolved from its formed degradation products, the absence of interfering peaks at the retention time of the AZD designates specificity.
Table 3. Summary of Accuracy and Precision of AZD

<table>
<thead>
<tr>
<th>Amount Added (g/mL)</th>
<th>Amount Found (g/mL)</th>
<th>Within Mean Square (WMS)</th>
<th>Between Mean Square (BMS)</th>
<th>F Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
<td></td>
</tr>
<tr>
<td>25 + 20 = 45 (80 %)</td>
<td>44.78</td>
<td>44.52</td>
<td>44.51</td>
<td>0.0606</td>
</tr>
<tr>
<td>Mean ± SD % RSD</td>
<td>44.87</td>
<td>44.42</td>
<td>44.62</td>
<td>0.08</td>
</tr>
<tr>
<td>25 + 25 = 50 (100 %)</td>
<td>49.33</td>
<td>49.12</td>
<td>49.15</td>
<td>0.0495</td>
</tr>
<tr>
<td>Mean ± SD % RSD</td>
<td>49.18</td>
<td>49.21</td>
<td>49.48</td>
<td>0.14</td>
</tr>
<tr>
<td>25 + 30 = 55 (120 %)</td>
<td>54.77</td>
<td>54.18</td>
<td>54.83</td>
<td>0.2100</td>
</tr>
<tr>
<td>Mean ± SD % RSD</td>
<td>54.83</td>
<td>54.59</td>
<td>54.61</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>0.34</td>
<td>1.24</td>
<td>0.66</td>
<td></td>
</tr>
</tbody>
</table>

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

- In the present work, an LC method was stated for the estimation of AZD in bulk and tablets. The developed 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 separate out less than 10 min of run time.

- The method showed 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 g/mL and the assay of a tablet found 100. 54 % ± 0.30 of the stated label of AZD.

- The forced degradation trials proved the degradation of AZD under acidic, alkaline, wet heat conditions, and to the peroxide mediated oxidation.

- Hydrolysis is the major degradation pathway for drug substances having 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, respectively, 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 obtained t1/2 and t90 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 condition, the oxidative degradation product of AZD was synthesized and confirmed using mass spectrometry which was found as one of the impurities and metabolites of AZD.
REFERENCES