Structural Identification and Characterization of potential impurities of Azelnidipine

10.1 Introduction

Azelnidipine (AZL) is a pale yellowish white tablet (16mg) with diameter of 9.2mm and thickness of 3.3mm. It was observed that there are four impurities in the bulk drug of AZL. All the impurities were detected by a gradient high performance liquid chromatographic (HPLC) method. LC-MS was performed to identify the mass number of these impurities. A thorough study was carried out to characterize the impurities. These impurities were identified, characterized and were co-injected with the sample containing impurities and are found to be matching with the impurities present in the sample. Based on the complete spectral analysis (UV, IR, NMR and MS) these impurities were characterized as 1) Azelnidipine stage-I para impurity [impurity 1], whose molecular formula is C_{14}H_{15}NO_{5} and molecular weight is 277.27, 2) Azelnidipine intermediate [impurity 2], whose molecular formula is C_{14}H_{15}NO_{5} and molecular weight is 277.27, 3) 4-nitro azelnidipine [impurity 3], whose molecular formula is C_{33}H_{34}N_{4}O_{6} and molecular weight is 582.65 and, 4) 2-nitro azelnidipine [impurity 4], whose molecular formula is C_{33}H_{34}N_{4}O_{6} and molecular weight is 582.65. The proposed method was validated as per International Conference on Harmonization (ICH) guidelines.

The IUPAC names of the four impurities of AZL are 1) propan-2-yl-2-(4-nitro benzylidene)-3-oxo butanoate [impurity 1]; 2) propan-2-yl 2-(3-nitro benzylidene)-3-oxo butanoate [impurity 2]; 3) 5-propan-2-yl 2-amino-6-methyl-4-(4-nitro phenyl)-1,4-dihydro pyridine-3,5-dicarboxylate [impurity 3]; and 4) 5-propan-2-yl 2-amino-6-methyl-4-(2-nitro phenyl)-1,4-dihydro pyridine-3,5-dicarboxylate [impurity 4]. An increasing number of publications on development of analytical methods for AZL bulk drug analysis indicate the significance of impurities of AZL. The structural formulae of these impurities 1,2,3 and 4 were mentioned in Figures 10 (a), (b), (c) and (d) respectively. The structure of possible impurities related to raw materials or degradants is identified by the various characterization techniques such as UV, IR, NMR and mass spectrography studies. AZL is a white crystalline powder and is used as cardiovascular agent.
Fig 10 (a): Structural formula of para impurity of AZL

Fig 10 (b): Structural formula of azelnidipine intermediate

Fig 10 (c): Structural formula of 4-nitro azelnidipine

Fig 10 (d): Structural formula of 2-nitro azelnidipine
**Literature Survey:** The brand name of AZL is afine or beiqi (China) or calblock (Japan). The therapeutic action of AZL is that it acts as anti-hypertensive agent and also as calcium channel blocker.

Ayre A et al [171] reported that the present review covers various aspects related to the analytical method development for impurity profiling of an active pharmaceutical ingredient. Pawale SS et al [172] developed and adopted the impurity profiling procedure in bulk drugs and in pharmaceutical preparation. Mathieu Verbeken and co-workers [173] have developed stability indicating HPLC–DAD/UV-ESI/MS impurity profiling of the anti-malarial drug lumefantrine. The International Conference on Harmonization (ICH) of technical requirements for registration of pharmaceuticals [174] for human use had published guidelines for validation of methods for analyzing impurities in new drug substances, products, residual solvents and micro biological impurities.

A number of articles have stated guidelines and designed approaches for isolation and identification of process-related impurities and degradation products, using mass spectroscopy (MS), nuclear magnetic resonance (NMR), high performance liquid chromatography (HPLC), fourier transform ion cyclotron resonance mass spectroscopy (FTICR – MS), and tandem mass spectroscopy for pharmaceutical substances. According to International Conference on Harmonization (ICH) guidelines, draft revised guidance on impurities in new drug products [175], impurities Q3C-Guidelines for residual solvents [176] and specifications Q6A-Test procedures and acceptance criteria for new drug substances and new drug products [177] chemical substances, were reported. According to ICH harmonized tripartite guideline [178], the presence of impurities in an active pharmaceutical ingredient (API) can have a significant impact on the quality and safety of the drug product. During the analysis of laboratory batches of AZL, four impurities were observed in HPLC method [179]. Buchi Reddy R et al [180] reported that these impurities are required in pure form to check the HPLC method performance such as specificity, linearity, range, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), robustness, system suitability testing and relative response factor (RRF). An HM and co-workers [181] and Pan YF and co-workers [182] reported for the determination of AZL by HPLC method. Kawabata K et al [183] developed LC-MS
method for the determination of AZL. HPLC-MS-MS method for the determination of AZL was developed by Jia J and co-workers [184]. LC-ESI-MS method for the determination of azelnidipine was reported by Ding L et al [185].

Among all the analytical methods reported, two methods were for the formulation and the remaining for human plasma. No single method was yet reported about the impurity profiling of AZL drug.

10.2 Experimental
10.2.1 Samples and chemicals

Samples of AZL bulk material and its four-related compounds, namely impurity 1, impurity 2, impurity 3 and impurity 4 were received from analytical research and development department of hetero research foundation, Hyderabad, Telangana State, India. KBr, methanol (AR grade) were obtained from SD fine chemicals limited, Mumbai, India. High pure water was prepared by using millipore milli “Q” plus purification system.

10.2.2 Spectroscopic methods

**FT-IR Spectroscopy:** The IR Spectra of AZL impurities were recorded in the solid state as 1% KBr dispersion medium using perkin-elmer spectrum one FT-IR spectrometer. The IR spectra of para impurity, AZL-I, 4-nitro azelnidipine and 2-nitro azelnidipine were shown in Fig 10.1 to 10.4 and their structural assignment have been correlated with the frequencies presented in Tables 10.1 to 10.4 respectively.

**UV Spectroscopy:** The UV Spectra of AZL impurity were recorded with a concentration of 0.01 mg/mL solution in methanol using perkin elmer lambda 35 UV-Visible spectrophotometer. The UV spectra of the four impurities of AZL were recorded and found maximum absorbance of 294, 267, 252 and 252 nm respectively. The UV spectra of para impurity, AZL-I, 4-nitro azelnidipine and 2-nitro azelnidipine were presented in Fig 10.5 to 10.8 and their maximum absorbance were recorded in Tables 10.5 to 10.8 respectively.
NMR Spectroscopy: The $^1$H NMR and $^{13}$C NMR experiments for AZL impurities were performed at 300 MHz and 75 MHz respectively on bruker 300 MHz avance NMR spectrometer. The $^1$H and $^{13}$C chemical shifts were reported on $\delta$ scale in ppm, relative to TMS ($\delta=0.00ppm$) and DMSO-d$_6$ ($\delta=39.50ppm$) as internal standards respectively. The DEPT experiment confirms the methyl and methane carbons. The extra peaks in the data could be due to DMSO-d$_6$ solvent.

The $^1$H NMR, $^{13}$C NMR and DEPT spectra of para impurity, AZL-I, 4-nitro azelnidipine and 2-nitro azelnidipine were presented in Fig 10.9 (a), (b) and (c) to Fig 10.12 (a), (b), (c) and (d) respectively. The extra peaks in the data could be due to solvents or impurities. The list of chemical shift values (in ppm) of these four molecules were tabulated in Tables 10.9 to 10.12 respectively.

Mass Spectrometry (MS): The LC-MS analysis has been performed on agilent 1100 series LC-MSD-TRAP-SL system. The analysis was performed in both ionization modes with turbo ion spray interface with the following conditions. Ion source voltage 5500V, declustering potential 80V, focusing potential 150V, entrance potential 10V, with the nebulizer gas as nitrogen at 60 psi were used for positive ionization mode whereas the negative ionization was performed by switching the polarity of the ion source voltage to -4500V.

The adduct ions at m/z 300 (M+Na)$^+$ and 316 (M+K)$^+$ in positive mode confirms the monoisotopic mass of para impurity as 277 corresponding to molecular formula of C$_{14}$H$_{15}$NO$_5$ and the values were tabulated (Table 13). The mass spectrum of para impurity was presented in Fig 10.13.

The adduct ions at m/z 300 (M+Na) and 316 (M+K) in positive mode confirms the monoisotopic mass of AZL-I as 277 corresponding to molecular formula of C$_{14}$H$_{15}$NO$_5$ and the same values as in Table 10.13 were obtained. The mass spectrum of AZL-I was presented in Fig 10.14.

The protonated molecular ion at m/z 583 (M+1) and deprotonated molecular ion at m/z 581 (M-1) confirms the monoisotopic mass of 4-nitro azelnidipine as 582 corresponding to molecular formula of C$_{33}$H$_{34}$N$_4$O$_6$ and the values were tabulated in
Table 10.14. The mass spectra of positive and negative modes of 4-nitro azelnidipine were presented in Fig 10.15 (a) and (b).

The protonated molecular ion at m/z 583 (M+1) and deprotonated molecular ion at m/z 581 (M-1) confirms the monoisotopic mass of 2-nitro azelnidipine as 582 corresponding to molecular formula of C$_{33}$H$_{34}$N$_4$O$_6$ and the same values as in Table 10.14 were obtained. The mass spectra of positive and negative modes of 2-nitro azelnidipine were presented in Fig 10.16 (a) and (b).

10.3 Applications of impurity profiling

Numerous applications have been sought in the areas of azelnidipine drug designing and in monitoring quality, stability and safety of pharmaceutical compounds, whether produced synthetically, extracted from natural products or produced by recombinant methods. The applications include alkanoids, amines, amino acids, analgesics, antibacterials, anticonvulsants, antidepressants, tranquilizers, antineoplastic agents, local anaesthetics, steroids etc.

10.4 Impurities identification by LC-MS

For identification of the four impurities, the ESI mass spectrum of impurities in positive ion mode showed a molecular ion peaks at m/Z 300, 300, 583 and 583 [(mH)$^+$], indicating the molecular weights of these compounds are 277.27, 277.27, 582.65 and 582.65 respectively. From this data we conclude that these four are intermediates, which were used in the manufacturing process of AZL and these four were also confirmed with photo diode array detector by comparing the spectra's with known standards. These four impurities further were characterized by UV, FT-IR, Mass and NMR ($^{13}$C, $^1$H, DEPT).

ESI mass spectrum of major impurity formed during oxidative stress condition in positive ion mode showed a molecular ion peak at m/Z 583.3 [(mH)$^+$] indicating the molecular weight of the Impurity 3 & 4 as 582.65. This molecular ion mass was higher than that of AZL and this indicates the probability of the formation of Nitro compound. The same impurities were also formed in base, thermal and UV
degradation studies but in smaller quantities. Further these impurities were synthesized and characterized by UV, FT-IR, Mass and NMR ($^{13}$C, $^1$H, DEPT) spectroscopy studies.

10.5 Results and discussion

Impurities 3 & 4 are the potential impurities present in bulk samples of AZL. The main target of chromatographic method is to get the separation of critical closely eluting impurities namely, impurity 1, impurity 2, impurity 3 and impurity 4. These impurities were eluted very closely to each other by using different stationery phases like CN, C$_{18}$, C$_8$ and phenyl and different mobile phases containing buffers like phosphate, sulfate and acetate with different pH values (2.5-5) using organic modifiers like acetonitrile and methanol in the mobile phase. pH of the buffer has played a significant role in achieving the separation between impurity 1, impurity 2, impurity 3 and impurity 4. In the optimized conditions of AZL, these four impurities were well separated with resolution of greater than 3.

A typical analytical HPLC chromatogram of laboratory batch of AZL bulk drug recorded using the LC method was described in these studies. The target impurities under study were marked as impurity 1 (molecular weight 277.27), impurity 2 (molecular weight 277.27), impurity 3 (molecular weight 582.65) and impurity 4 (molecular weight 582.65). The LC-MS compatible method which is used to detect the impurities was described which helps to detect all the impurities of AZL. Batch numbers, molecular formulae, and IUPAC names of these impurities of AZL were shown in Table 10.15. The instruments used for IR, UV, NMR and Mass spectra of AZL drug were shown in Table 10.16.

10.6 Conclusion

Impurity profiling of a substance under investigation gives maximum possible account of impurities present in it. The establishment of guidelines for impurity levels in drug substances and products provides the quality criteria for manufacturers. Beginning with limit tests for impurities, this field of impurity identification and quantitation have progressed. Nowadays, it is mandatory requirements in various
pharmacopoeias to know the impurities present in APIs. Characterization of impurities is required for acquiring and evaluating data that establishes biological safety which reveals the need and scope of impurity profiling of drugs in pharmaceutical research. Keeping in view this regulatory requirement of AZL impurities, the process related impurities and metabolites in AZL bulk drug were identified and characterized using IR, UV, NMR and mass spectral data.

**T A B L E S**

**Table 10.1: Frequencies of IR spectrum of para impurity of AZL**

<table>
<thead>
<tr>
<th>Wave number (cm(^{-1}))</th>
<th>Assignment</th>
<th>Mode of vibration</th>
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<tbody>
<tr>
<td>3112,3088,3051</td>
<td>Aromatic C-H</td>
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<tr>
<td>2986, 2938</td>
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<td>1720, 1701</td>
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</tr>
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</tr>
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<td>C-N</td>
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</tr>
<tr>
<td>1203,1194,1183,1105</td>
<td>C-(C=O)-O</td>
<td>Stretching</td>
</tr>
<tr>
<td>855,765</td>
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</table>

At 3412 cm\(^{-1}\) wave number, the broad spectrum line contains -OH group (keto-enol tautomerism) and the mode of vibration is stretching.
### Table 10.2: Frequencies of IR spectrum of AZL – I

<table>
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<th>Wave number (cm$^{-1}$)</th>
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<td>Bending</td>
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<td>1352</td>
<td>Aromatic (N = O)$_2$</td>
<td>Symmetric stretching</td>
</tr>
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<td>1295, 1227</td>
<td>C-N</td>
<td>Stretching</td>
</tr>
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<td>1212,1101,1039</td>
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<td>826,812,736,678</td>
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### Table 10.3: Frequencies of IR spectrum of 4-nitro azelnidipine

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<td>1215, 1106, 1067</td>
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<td>828, 746, 705</td>
<td>Aromatic C-H</td>
<td>Bending</td>
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Table 10.4: Frequencies of IR spectrum of 2-nitro azelnidipine

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<td>746, 705</td>
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Table 10.5: Absorbance maxima of UV spectrum of para impurity of AZL

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<th>λ (nm)</th>
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<td>202</td>
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<td>294</td>
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Table 10.6: Absorbance maxima of UV spectrum of AZL – I

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<td>267</td>
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Table 10.7: Absorbance maxima of UV spectrum of 4 – nitro azelnidipine

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Table 10.8: Absorbance maxima of UV spectrum of 2 – nitro azelnidipine

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Table 10.9: Chemical shift values of para impurity of AZL

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<th>Position</th>
<th>(^1\text{H})</th>
<th>(\delta) (ppm)</th>
<th>J(\text{Hz})(^1)</th>
<th>(\text{^13C})</th>
<th>DEPT</th>
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<td>1, 5</td>
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<td>d(8.7)</td>
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<tr>
<td>2, 4</td>
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<td>d(9.0)</td>
<td>123.90</td>
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<td>-</td>
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\(^{1}\).\(^{1}\)H-\(^{1}\)H Coupling constants.

Table 10.10: Chemical shift values of AZL – I

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<tr>
<th>Position</th>
<th>(^1\text{H})</th>
<th>(\delta) (ppm)</th>
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<td>1</td>
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<td>13, 13'</td>
<td>6H</td>
<td>1.21</td>
<td>d(6.3)</td>
<td>21.18</td>
<td>CH(_3)</td>
</tr>
</tbody>
</table>

\(^{1}\).\(^{1}\)H-\(^{1}\)H Coupling constants.
### Table 10.11: Chemical shift values of 4 – nitro azelnidipine

![Chemical structure of 4-nitro azelnidipine](image)

<table>
<thead>
<tr>
<th>Position</th>
<th>( ^1\text{H} )</th>
<th>( \delta \text{(ppm)} )</th>
<th>( J\text{(Hz)} )</th>
<th>( ^{13}\text{C} )</th>
<th>DEPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 5</td>
<td>2H</td>
<td>8.18</td>
<td>d(8.7)</td>
<td>129.00</td>
<td>CH</td>
</tr>
<tr>
<td>2, 4</td>
<td>2H</td>
<td>7.45</td>
<td>d(8.7)</td>
<td>123.09</td>
<td>CH</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>145.44</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td>157.22</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>1H</td>
<td>4.75-4.83</td>
<td>m</td>
<td>38.67</td>
<td>CH</td>
</tr>
<tr>
<td>8</td>
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<td></td>
<td>102.59</td>
<td>-</td>
</tr>
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<td>9</td>
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<td></td>
<td>145.23</td>
<td>-</td>
</tr>
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<td></td>
<td></td>
<td>165.92</td>
<td>-</td>
</tr>
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<td>1H</td>
<td>4.75-4.83</td>
<td>m</td>
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<td>CH</td>
</tr>
<tr>
<td>14Ha</td>
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<td>t(6.6)</td>
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<td>CH</td>
</tr>
<tr>
<td>14Hb</td>
<td>1H</td>
<td>3.43</td>
<td>t(7.1)</td>
<td>59.32</td>
<td>CH</td>
</tr>
<tr>
<td>14Ha'</td>
<td>1H</td>
<td>2.40</td>
<td>br</td>
<td>59.88</td>
<td>CH</td>
</tr>
<tr>
<td>14Hb'</td>
<td>1H</td>
<td>3.26-3.33</td>
<td>m</td>
<td>76.93</td>
<td>CH</td>
</tr>
<tr>
<td>15</td>
<td>1H</td>
<td>4.27</td>
<td>s</td>
<td>142.20, 142.30</td>
<td>-</td>
</tr>
<tr>
<td>16, 16'</td>
<td></td>
<td></td>
<td></td>
<td>128.40</td>
<td>CH</td>
</tr>
<tr>
<td>17, 21, 17', 21'</td>
<td>4H</td>
<td>7.14-7.40</td>
<td>m</td>
<td>127.01</td>
<td>CH</td>
</tr>
<tr>
<td>18, 20, 18', 20'</td>
<td>4H</td>
<td>7.14-7.40</td>
<td>m</td>
<td>126.96</td>
<td>CH</td>
</tr>
<tr>
<td>19, 19'</td>
<td>2H</td>
<td>7.14-7.40</td>
<td>m</td>
<td>167.23</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>3H</td>
<td>2.26</td>
<td>s</td>
<td>66.41</td>
<td>CH</td>
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<tr>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td>8.86</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>1H</td>
<td>4.75-4.83</td>
<td>m</td>
<td>21.53, 21.82</td>
<td>CH</td>
</tr>
<tr>
<td>NH(_2)</td>
<td>2H</td>
<td>6.78</td>
<td>br</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NH</td>
<td>1H</td>
<td>8.86</td>
<td>s</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

s-singlet, d-doublet, t-triplet, m-multiplet, broad.

\(^1\text{H}-^1\text{H} \) Coupling constants.
**Table 10.12: Chemical Shift values of 2 – nitro azelnidipine**

![Chemical Structure of 2-Nitro Azelnidipine]

<table>
<thead>
<tr>
<th>Position</th>
<th>$^1$H</th>
<th>δ(ppm)</th>
<th>J(Hz)$^1$</th>
<th>$^{13}$C</th>
<th>DEPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1H</td>
<td>7.15-7.762</td>
<td>m</td>
<td>147.00</td>
<td>CH</td>
</tr>
<tr>
<td>3</td>
<td>1H</td>
<td>7.15-7.762</td>
<td>m</td>
<td>123.84</td>
<td>CH</td>
</tr>
<tr>
<td>4</td>
<td>1H</td>
<td>7.15-7.762</td>
<td>m</td>
<td>126.78</td>
<td>CH</td>
</tr>
<tr>
<td>5</td>
<td>1H</td>
<td>7.15-7.762</td>
<td>m</td>
<td>130.77</td>
<td>CH</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>133.22</td>
<td>CH</td>
</tr>
<tr>
<td>7</td>
<td>1H</td>
<td>4.71-4.83</td>
<td>m</td>
<td>144.18</td>
<td>CH</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>32.77</td>
<td>CH</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>103.38</td>
<td>CH</td>
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<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>144.94</td>
<td>CH</td>
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<tr>
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<td>-</td>
<td>77.47</td>
<td>CH</td>
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<tr>
<td>13</td>
<td>1H</td>
<td>4.71-4.83</td>
<td>m</td>
<td>166.11</td>
<td>CH</td>
</tr>
<tr>
<td>14Ha</td>
<td>1H</td>
<td>2.66-2.71</td>
<td>dd(8.3,6.2)</td>
<td>61.67</td>
<td>CH</td>
</tr>
<tr>
<td>14Hb</td>
<td>1H</td>
<td>3.35-3.40</td>
<td>m</td>
<td>59.32</td>
<td>CH$_2$</td>
</tr>
<tr>
<td>14'Ha</td>
<td>1H</td>
<td>2.57</td>
<td>d(7.1)</td>
<td>59.51</td>
<td>CH$_2$</td>
</tr>
<tr>
<td>14'Hb</td>
<td>1H</td>
<td>2.83</td>
<td>t(6.8)</td>
<td>90.19</td>
<td>CH$_2$</td>
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<tr>
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<td>1H</td>
<td>4.34</td>
<td>s</td>
<td>76.48</td>
<td>CH</td>
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<tr>
<td>16,16'</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>142.33</td>
<td>CH</td>
</tr>
<tr>
<td>17,21,17',21'</td>
<td>4H</td>
<td>7.15-7.762</td>
<td>m</td>
<td>128.31</td>
<td>CH</td>
</tr>
<tr>
<td>18,20,18',20'</td>
<td>4H</td>
<td>7.15-7.762</td>
<td>m</td>
<td>128.31</td>
<td>CH</td>
</tr>
<tr>
<td>19,19'</td>
<td>2H</td>
<td>7.15-7.762</td>
<td>m</td>
<td>128.31</td>
<td>CH</td>
</tr>
<tr>
<td>22</td>
<td>3H</td>
<td>2.24</td>
<td>s</td>
<td>187.2</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>23</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>167.50</td>
<td>CH</td>
</tr>
<tr>
<td>24</td>
<td>1H</td>
<td>4.71-4.83</td>
<td>m</td>
<td>66.22</td>
<td>CH</td>
</tr>
<tr>
<td>25,25'</td>
<td>6H</td>
<td>0.90 &amp; 1.13</td>
<td>d(6.3)&amp;d(6.3)</td>
<td>21.18</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>NH$_2$</td>
<td>2H</td>
<td>6.77-6.85</td>
<td>br</td>
<td>21.46</td>
<td>CH$_3$</td>
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<tr>
<td>NH</td>
<td>1H</td>
<td>8.79</td>
<td>s</td>
<td>-</td>
<td>CH</td>
</tr>
</tbody>
</table>

s-singlet, d-doublet, dd-doublet of doublet, t-triplet, m-multiplet, br-broad.

$^1$H-$^1$H Coupling constants.
Table 10.13: m/z values of para impurity & AZL – I

<table>
<thead>
<tr>
<th>m/z</th>
<th>Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>(M+Na)⁺</td>
</tr>
<tr>
<td>316</td>
<td>(M+K)⁺</td>
</tr>
</tbody>
</table>

Table 10.14: m/z values of 4 – nitro and 2 – nitro azelnidipine

<table>
<thead>
<tr>
<th>m/z</th>
<th>Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>583</td>
<td>(M+H)⁺</td>
</tr>
<tr>
<td>581</td>
<td>(M-H)⁻</td>
</tr>
<tr>
<td>617</td>
<td>(M-H+HCl)⁻</td>
</tr>
<tr>
<td>695</td>
<td>(M-H+CF₃COOH)⁺</td>
</tr>
</tbody>
</table>

Table 10.15: Batch numbers and IUPAC names of impurities of azelnidipine

<table>
<thead>
<tr>
<th>Name of the impurity</th>
<th>Batch No.</th>
<th>Molecular formulae</th>
<th>Molecular weight</th>
<th>IUPAC name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azelnidipine stage-I (para impurity)</td>
<td>AZL-SUN (A-766) 27</td>
<td>C₁₄H₁₅NO₅</td>
<td>277.27</td>
<td>Propan-2-yl 2-(4-nitro benzylidene)-3-oxo butanoate</td>
</tr>
<tr>
<td>Azelnidipine intermediate (AZL-I)</td>
<td>AZL-SUN (A-766) 01 R/C</td>
<td>C₁₄H₁₅NO₅</td>
<td>277.27</td>
<td>Propan-2-yl 2-(3-nitro benzylidene) -3-oxo butanoate</td>
</tr>
<tr>
<td>4-Nitro Azelnidipine (Azelnidipine impurity)</td>
<td>AZL-CSK (A-819) 13</td>
<td>C₃₃H₃₄N₄O₆</td>
<td>582.65</td>
<td>3-[1-(diphenyl methyl) azetidin-3-yl] 5- propan-2-yl 2-amino-6-methyl-4-(4-nitro phenyl)-1,4-dihydro pyridine-3,5-di carboxylate</td>
</tr>
<tr>
<td>2-Nitro Azelnidipine (Azelnidipine impurity)</td>
<td>AZL-CSK (A-819) 23</td>
<td>C₃₃H₃₄N₄O₆</td>
<td>582.65</td>
<td>3-[1-(diphenyl methyl) azetidin-3-yl] 5- propan-2-yl 2-amino-6-methyl-4-(2-nitro phenyl)-1,4-dihydro pyridine-3,5-di carboxylate</td>
</tr>
</tbody>
</table>
Table 10.16: Data showing the instruments used for various spectra of AZL drug

<table>
<thead>
<tr>
<th>Instrument used for</th>
<th>Perkin- elmer spectrum one FT-IR spectrometer using 1 % KBr pellets for all the impurities of AZL</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR spectrum</td>
<td></td>
</tr>
<tr>
<td>Instrument used for</td>
<td>Perkin – elmer lambda 35 UV-Visible spectrophotometer scanned from 200-400 nm with a conc. of 0.01 mg/mL solution in methanol for all the impurities of AZL</td>
</tr>
<tr>
<td>UV spectrum</td>
<td></td>
</tr>
<tr>
<td>Instrument used for</td>
<td>Bruker 300 MHz avance NMR spectrometer, $^1$H and $^{13}$C spectra were recorded in DMSO – d$_6$ at 300 MHz and 75 MHz for all the impurities of AZL</td>
</tr>
<tr>
<td>NMR spectrum</td>
<td></td>
</tr>
<tr>
<td>Instrument used for</td>
<td>Agilent 1100 series LC-MSD-TRAP-SL system</td>
</tr>
<tr>
<td>mass spectrum</td>
<td></td>
</tr>
</tbody>
</table>

F I G U R E S

Fig 10.1: IR spectrum of para impurity of AZL
Fig 10.2: IR spectrum of AZL - I

Fig 10.3: IR spectrum of 4 - nitro azelnidipine
Fig 10.4: IR spectrum of 2-nitro azelnidipine
Fig 10.5: UV spectrum of para impurity of AZL
Fig 10.6: UV spectrum of AZL - I

Fig 10.7: UV spectrum of 4- nitro azelnidipine
Fig 10.8: UV spectrum of 2 - nitro azelnidipine

Fig 10.9: $^1$H NMR spectrum of para impurity of AZL
Fig 10.10: $^{13}$C NMR spectrum of para impurity of AZL

Fig 10.11: DEPT NMR spectrum of para impurity of AZL
Fig 10.12: $^1$H NMR spectrum of AZL - I

Fig 10.13: $^{13}$C NMR spectrum of AZL - I
Fig 10.14: DEPT NMR spectrum of AZL - I

Fig 10.15: $^1$H NMR spectrum of 4 - nitro azelnidipine
Fig 10.16: D$_2$O Exchange NMR spectrum of 4-nitro azelnidipine

Fig 10.17: $^{13}$C NMR spectrum of 4-nitro azelnidipine
Fig 10.18: DEPT NMR spectrum of 4 - nitro azelnidipine

Fig 10.19: $^1$H NMR spectrum of 2 - nitro azelnidipine
Fig 10.20: $\text{D}_2\text{O}$ Exchange NMR spectrum of 2-nitro azelnidipine

Fig 10.21: $^{13}\text{C}$ NMR spectrum of 2-nitro azelnidipine
Fig 10.22: DEPT NMR spectrum of 2-nitro azelnidipine

Fig 10.23: Mass spectrum of para impurity of AZL
Fig 10.24: Mass spectrum of AZL - I

Fig 10.25: Mass spectrum of positive mode of 4 - nitro azelnidipine
**Fig 10.26:** Mass spectrum of negative mode of 4-nitro azelnidipine

**Fig 10.27:** Mass spectrum of positive mode of 2-nitro azelnidipine
Fig 10.28: Mass spectrum of negative mode of 2-nitro azelnidipine
A New and Sensitive UV Spectrophotometric method for the Determination of Belinostat in Dosage Forms

11.A.1 Introduction

Belinostat (PXD101, trade name beleodaq) is a drug under development by topotarget for the treatment of hematological malignancies and solid tumors. The chemical name of belinostat (BLT) is (2E)-N-Hydroxy-3-[3-(phenyl sulfamoyl)phenyl] prop-2-enamide. The molecular formula of BLT is C\textsubscript{15}H\textsubscript{14}N\textsubscript{2}O\textsubscript{4}S and the molecular mass is 318.35 g/mol. The chemical structure of BLT is represented in Fig 11.1.

![Chemical Structure of belinostat]

Beleodaq is histone deactetylase inhibitor indicated for the treatment of patients with relapsed or refractory peripheral T-cell lymphoma (PTCL). Beleodaq for injection is supplied as a sterile lyophilized yellow powder containing 500 mg BLT as the active ingredient. Each vial also contains 100 mg L.Arginine, USP as an inactive ingredient. The pharmacokinetic characteristics of BLT were analysed from pooled data from phase ½ clinical studies that used doses of BLT ranging from 150 to 25,000 ng/mL. The plasma half-life of BLT is 47-86 minutes. BLT can be used in combination with full doses of chemotherapy, and is currently in a pivotal trial within peripheral T-cell lymphoma (PTCL) and phase II in cancer of unknown primary (CUP). BLT inhibits bladder cancer cell growth, especially in 5637 cells which shows accumulation of GO-G1 phase, decrease in S phase and increase in G2-M phase. BLT was genotoxic in a bacterial reverse mutation test (ames assay), in an vitro mouse
lymphoma cell mutagenesis assay, and in an vivo rat micro nucleus assay. BLT is a cancer indication that interferes with the growth and spread of cancer cells in the body. The UV spectrum of BLT is represented in Fig 11.2.

![UV spectrum of BLT](image)

**Fig 11.2: UV spectrum of BLT**

**Literature Survey:** BLT is a three selective inhibitor which protects the β-cells from cytokine induced apoptosis. Brian F Kiesel and co-workers [186] reported a LC-MS/MS assay method for the quantitation of the HDAC inhibitor BLT and five major metabolites in human plasma. Ling-Zhi Wang and co-workers [187] reported a sensitive and specific liquid chromatography-tandem mass spectroscopic method for the determination of BLT in plasma from liver cancer patients. Katherine E Warren et al [188] reported that BLT can be used as plasma and cerebrospinal fluid pharmacokinetics of the histone deacetylase inhibitor in non-human primates. Epigenetic and molecular mechanisms underlying the antileukemic activity of the histone deacetylase inhibitor Belinostat in human acute promyelocytic leukemia cells was reported by Jurate Savickiene and co-workers [189]. No other methods like UV-Visible spectroscopy, UPLC, spectrophotometric, LC-MS/MS etc., for BLT were reported till date.

The present investigation describes a rapid, accurate, precise for the determination of belinostat in bulk and its pharmaceutical dosage forms. The author
developed UV method for belinostat at a wavelength of 238.9 nm, where the absorption maxima of BLT is high.

11.A.2 Experimental

11.A.2.1 Instrumentation

The determination of belinostat was carried out on UV spectrophotometer. Sartorious balance model–TTA225D,ID.No.QC1 017 electronic balance was used for weighing. A shimazdu UV-2450 PC series UV–visible spectrophotometer with slit width 2.0 nm, light source change wave length of 240 nm and wave length range of 200-400nm with sampling interval of 1.2 is used for measuring the absorbance of solutions.

11.A.2.2 Materials and reagents

All experiments were performed with pharmaceutical belinostat. For the estimation of commercial formulation, beleodaq tablets having 500 mg belinostat were procured from M/s Spectrum labs, Hyderabad, Telangana State, India. HPLC grade milli-Q water was used throughout the analysis.

11.A.2.3 Preparation of mobile phase

Measured accurately 1 mL of ortho phosphoric acid and dissolved in a 1000 mL of water. The solution was filtered through 0.45 μm membrane filter and was degassed. A freshly prepared buffer solution and acetonitrile in a ratio of 40:60 %v/v was filtered through 0.05 μm membrane filter and sonicated by using power sonicator and was used as mobile phase.

11.A.2.4 Preparation of buffer solution

Accurately measured 1 mL of orthophosphoric acid in a 1000 mL volumetric flask, 100 mL of milli-Q water was added and final volume make upto 1000 mL with milli-Q water.
11.A.2.5 Preparation of standard solution

Weighed accurately about 10 mg of belinostat and transferred into 100 mL clean, dry volumetric flask, added 7 mL of diluent and the solution was sonicated for 30 minutes and make up to the final volume with diluents to prepare a 1000 µg/mL stock solution. From the above stock solution, 1 mL of standard stock solution was pipetted out into 10 mL volumetric flask and make up to the final volume with diluents. The solution was filtered through 0.45 µ nylon membrane filter paper. Thus, sample BLT concentration of 100 µg/mL was obtained.

11.A.2.6 Preparation of sample solution

One vial of BLT was placed in 500 mL volumetric flask, 300 mL of diluents were added and sonicated for 25 minutes. Further the volume of the solution was made up with diluents and filtered. From the filtered solution, 1 mL was pipetted out into a 10 mL volumetric flask and made up to 10 mL with diluents.

11.A.3 UV method development

For the quantitative determination of BLT, a suitable UV spectrophotometric method was developed. The spectra of diluted solutions of BLT in water were recorded on UV spectrophotometer. The peaks of maximum absorbance were observed. The spectra of BLT showed that a balanced wave length was found to be 238.9 nm.

11.A.4 UV method validation

The present developed UV Spectrophotometric method of BLT is validated for precision, linearity, accuracy, ruggedness and repeatability as per the ICH guidelines. The optical characteristics are given in Table 11.1.
Table 11.1: Optical characteristics of belinostat

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wave length of absorbance measurements (nm)</td>
<td>238.9</td>
</tr>
<tr>
<td>2</td>
<td>Regression equation</td>
<td>Y = 0.005 x - 0.005</td>
</tr>
<tr>
<td>3</td>
<td>Slope (S)</td>
<td>0.005</td>
</tr>
<tr>
<td>4</td>
<td>Intercept</td>
<td>0.005</td>
</tr>
<tr>
<td>5</td>
<td>Correlation coefficient (r²)</td>
<td>0.9994</td>
</tr>
<tr>
<td>6</td>
<td>Beer's limits (µg/mL)</td>
<td>25 - 150</td>
</tr>
<tr>
<td>7</td>
<td>Standard deviation</td>
<td>82.31</td>
</tr>
<tr>
<td>8</td>
<td>LOD (3.3 σ/S) µg/mL</td>
<td>0.051</td>
</tr>
<tr>
<td>9</td>
<td>LOQ (10 σ/S) µg/mL</td>
<td>0.154</td>
</tr>
</tbody>
</table>

11.A.4.1 Precision

The precision of the method is evaluated by conducting inter day determinations and intraday determinations under identical conditions of the homogeneous solution. The results of inter day and intraday precision of BLT were given in Table 11.2.

Table 11.2: Results of precision for BLT

<table>
<thead>
<tr>
<th>S.No</th>
<th>Level</th>
<th>Concentration of solution (µg/mL)</th>
<th>Intraday RSD</th>
<th>Inter day RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50%</td>
<td>16</td>
<td>0.847</td>
<td>0.484</td>
</tr>
<tr>
<td>2</td>
<td>100%</td>
<td>20</td>
<td>0.834</td>
<td>0.478</td>
</tr>
<tr>
<td>3</td>
<td>150%</td>
<td>24</td>
<td>0.798</td>
<td>0.443</td>
</tr>
</tbody>
</table>

11.A.4.2 Linearity

Linearity studies are used to know whether the response of the instrument (absorbance) is proportional to the concentration of the analyte or not. Linearity of the method is evaluated by measuring absorbance of six different concentrated
solutions of belinostat at 238.9 nm wavelength. A calibration graph was constructed between concentration of belinostat and absorbances, which was shown in Fig 11.3.

![UV linearity plot of belinostat](image)

The amounts of belinostat present in the linear solutions and their absorbance at 238.9 nm were given in Table 11.3.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Level of the solution</th>
<th>Amount of the drug(µg/mL)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25%</td>
<td>14.0</td>
<td>0.007</td>
</tr>
<tr>
<td>2</td>
<td>50%</td>
<td>16.0</td>
<td>0.014</td>
</tr>
<tr>
<td>3</td>
<td>75%</td>
<td>18.0</td>
<td>0.021</td>
</tr>
<tr>
<td>4</td>
<td>100%</td>
<td>20.0</td>
<td>0.027</td>
</tr>
<tr>
<td>5</td>
<td>125%</td>
<td>22.0</td>
<td>0.035</td>
</tr>
<tr>
<td>6</td>
<td>150%</td>
<td>24.0</td>
<td>0.043</td>
</tr>
</tbody>
</table>

11.A.4.3 Accuracy

Accuracy gives the difference between the experimental results and true value. The accuracy of the test method is evaluated by the % recovery. The accuracy
of the present method is evaluated by the addition of standard APIs of belinostat to the pre-analysed standard solution and recovery of the total assay present in the solution by the present method. Known amount of belinostat is added to the pre-analysed sample of BLT standard solution and the resulting solutions are analyzed by the present method and %RSD is calculated. The lower value of %RSD indicates the accuracy of the method. The results of accuracy studies are given in Table 11.4.

Table 11.4: Results of accuracy studies

<table>
<thead>
<tr>
<th>S.No</th>
<th>Level</th>
<th>Amount taken µg/mL</th>
<th>Amount of API added µg/mL</th>
<th>Total amount present µg/mL</th>
<th>Amount recovered µg/mL</th>
<th>%Recovery</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50%</td>
<td>10</td>
<td>8</td>
<td>18</td>
<td>17.912</td>
<td>99.51</td>
<td>0.055</td>
</tr>
<tr>
<td>2</td>
<td>100%</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td>19.861</td>
<td>99.31</td>
<td>0.285</td>
</tr>
<tr>
<td>3</td>
<td>150%</td>
<td>10</td>
<td>12</td>
<td>22</td>
<td>21.903</td>
<td>99.56</td>
<td>0.241</td>
</tr>
</tbody>
</table>

11.A.4.4 Ruggedness

The ruggedness of the method is determined by the analysis of same sample under different conditions i.e by different analysts, different instruments, in different laboratories etc,. The ruggedness of the present method is evaluated by conducting the analysis by different analysts under identical conditions. The results of ruggedness for BLT were given in Table 11.5.

Table 11.5: Results of ruggedness

<table>
<thead>
<tr>
<th>S.No</th>
<th>Amount taken(µg)</th>
<th>Analyst-1(µg)</th>
<th>%RSD</th>
<th>Analyst-2(µg)</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>9.912</td>
<td>0.107</td>
<td>9.898</td>
<td>0.158</td>
</tr>
</tbody>
</table>

11.A.4.5 Determination of assay of the sample

The absorbance of six identical sample solutions are measured and by statistical methods the %RSD value is determined. The results of pharmaceutical dosage form beleodaq were given in Table 11.6.
Table 11.6: Results of pharmaceutical form beleodaq

<table>
<thead>
<tr>
<th>S.No</th>
<th>Amount of beleodaq taken in mg</th>
<th>Amount found in mg</th>
<th>% of assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>9.94</td>
<td>99.4</td>
</tr>
</tbody>
</table>

11.A.5 Results and discussion

Belinostat shows maximum absorbance at 238.9 nm wavelength. In the present UV-Spectrophotometric method, belinostat obeys Beer's law in the concentration range of 25 - 150 µg/mL. Inter-day and intra-day determinations were conducted under identical conditions and the precision of the method was determined. The % RSD values of inter-day and intra-day precision were less than 2, hence the method was precise. In the linearity studies, solutions of 25 to 150 % of concentration were prepared, their absorbance's are measured at 238.9 nm and a graph was drawn between concentration of belinostat and the absorbances, then a linear calibration graph was obtained. The solutions followed Beer’s law in the concentration range 25 - 150 µg/mL and from the slope and standard deviation of the plot, LOD and LOQ values were calculated and their values are within the limits. The accuracy is evaluated by the addition of standard APIs of belinostat to pre-analyzed solutions and subsequent recovery studies by the present method. The % recoveries are in the limit 100 ± 1, hence the method was accurate. The %RSD of the ruggedness studies is less than 2, and hence the method was rugged. By using the present UV-spectrophotometric method, the assay of the sample is determined, the %assay of sample is in the limit of 100± 1. Hence, the present method could be used for the rapid, accurate, precise and economic for the determination of belinostat in bulk and formulations.

11.A.6 Conclusion

The developed method was validated completely as per the ICH guidelines. High values of correlation coefficients and small values of intercepts validated the linearity of the calibration plots and were in obedience to Beer's laws. The RSD
values, the slopes and intercepts of the calibration graphs indicate the high reproducibility of the developed method. The low values of LOD and LOQ indicate that the method can be employed over a wide concentration range for linearity.

The UV-Spectrophotometric method presently developed is a simple, accurate, precise, economic and sensitive for the determination of belinostat in bulk and dosage forms without any interference from the excipients present. Hence this method could be used for the routine analysis of belinostat in quality control laboratories.