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ABSTRACT

Objectives: Forced degradation study of the drug product and drug substance is very much important in drug development and drug discovery to establish the intrinsic stability and understand its behaviors towards different stress conditions. In the present work, stress testing of neostigmine bromide (NBr) was carried out as per ICH guidelines to identify and characterize the degradation product (DP) formed. Materials and Methods: According to ICH guidelines, drug substance was subjected to various degradation conditions. Waters BEH (Ethylene Bridge Hybrid) C18 column (1.7 m, 100 mm 2.1 mm) having the composition of mobile phase Eluent A: 0.01M KH2PO4 in water pH-2.5 with orthophosphoric acid and Eluent B: Acetonitrile utilizing 220 nm wavelengths provided the best separation of DP and drug component. Throughout the analysis, the injection volume (5 µL) and flow rate (0.3 mLmin⁻¹) were kept constant. Results: The limits of detection (LOD) and quantitation (LOQ) were set at 25 ngmL⁻¹ and 50 ngmL⁻¹, respectively. The method showed excellent linearity over a concentration range of 25-250 ngmL⁻¹ with a regression coefficient (R²) value of 0.9999. The results showed that significant degradation was observed in base and oxidative degradation conditions whereas found in neutral, acidic, photolytic and thermal degradation conditions. Conclusion: The DP was identified and characterized using a sophisticated HRMS/MS/TOF approach for accurate mass measurement using the ESI positive mode of ionization. In the present study, the establishment of the degradation pathway of drug substance and fragmentation pathway of DP-I were explained which was never reported in any literature.

Keywords: Neostigmine bromide, RP-UPLC, Stability-indicating, Stress study, Validation.

INTRODUCTION

Stability-indicating HPLC method for determination of process and degradation-related impurities in the drug substance. Degradation study of the drug substance can help to find out the probable DPs, molecule stability, elucidate degradation pathways, establish the intrinsic stability of drug molecule, and also validate the stability, selectivity, and specificity of the analytical procedures followed. It also provides evidence on how the quality of a drug substance varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light, which are necessary for the recommendation of storage conditions, retest periods, and shelf-life establishment. These DPs can be separated and identified by developed and validated stability-indicating methods. Forced degradation studies can be helpful to determine the appropriate packaging to minimize or avoid the
formation of DPs. Typical degradation studies include acidic and alkaline hydrolysis, oxidative degradation, photolytic degradation, and thermal degradation.\textsuperscript{1-7} NBr, a US FDA-approved drug, chemically known as \([3-(\text{dimethylcarbamoyloxy})\text{ phenyl}-\text{trimethylazanium} \text{ bromide having molecular formula } C_{12}H_{18}BrN_{2}O_{3}\] and molecular weight 303.20 g mol\(^{-1}\).\textsuperscript{8} It is used as a competitive cholinesterase inhibitor. It decreases the breakdown of acetylcholine in the synaptic cleft, thus, increasing the levels of the same. This acetylcholine competes for the binding sites as a nondepolarizing neuromuscular blocking agent and reverses the neuromuscular blockade. This intensifies the nicotinic and muscarinic effects. It activates the skeletal muscles. This is clinically used for the treatment of myastenia gravis, treatment or prevention of postoperative and nonobstructive abdominal distention, and reversal of nondepolarizing neuromuscular blocking agents. As an anticholinesterase, it was reported that NBr has a narrow margin of safety, with a small therapeutic index.\textsuperscript{9} In large doses by intravenous injection clinically, atropine was utilized in combination with NBr to prevent or reduce its adverse effects. The structure of NBr is shown in Figure 1.

Looking into the emergent applications of a competitive cholinesterase inhibitor, we decided to execute stress degradation studies of NBr and to establish a highly sensitive and fast LC-HRMS method for the identification and characterization of its DP. A very few HPLC and LC-MS methods were available for identification and characterization of NBr and its DP.\textsuperscript{10-24} Separation, identification and characterization of DPs of few antineoplastic drugs using the UPLC-MSMS technique have been reported.\textsuperscript{25-34} Based on the recommendations of ICH guidelines Q3A (R2) and Q3B (R2), identification and characterization of all the DPs or process-related impurities of dabrafenib have been carried out.\textsuperscript{35-36} But to date, no study is reported on the degradation and stability of drugs under varied conditions such as hydrolytic, oxidative, thermal and photolytic. Herein we report (i) A comprehensive forced degradation study of NBr under hydrolytic, oxidative, thermal and photolytic conditions as prescribed by ICH guidelines; (ii) to separate the DP by UPLC (iii) structural characterization of DP through HR-MS-TOF; and (iv) most plausible mechanisms of NBr degradation.

**MATERIALS AND METHODS**

**Chemicals and Reagents**

Pure NBr drug was generously procured from AET Laboratories Pvt. Ltd., Hyderabad, India, as a gift sample. Ultra-pure LC-MS grade acetonitrile (CH\(_2\)CN) and methanol (CH\(_3\)OH) were procured from JT Baker (Bangalore, India). Sodium hydroxide (NaOH) pellets and formic acid (HCOOH) of Analytical Reagent (AR) grade were procured from Merck (Mumbai, India). Finar chemicals (Hyderabad, India) provided the analytical grade Hydrochloric acid (HCl, 37%). Hydrogen peroxide (H\(_2\)O\(_2\), 30%), Potassium dihydrogen phosphate (KH\(_2\)PO\(_4\)) and orthophosphoric acid were procured from S.D Fine chemicals (New Delhi, India). Water (HPLC grade) was taken from Milli-Q-water and was used throughout the analysis from Millipore India. All the solution preparation was filtered before injecting into the system using 0.22 mm Filter paper (Millipore, India).

**Equipment**

The Waters ACQUITY H Class UPLC system with a quaternary solvent manager (QSM), sample manager, and photodiode array (PDA) detector was used. All weighing processes were performed using an analytical balance (Mettler Toledo, Switzerland). Other instruments used in the study were Photostability chamber (Thermo lab scientific instruments, India), hot air oven (Bionics Scientific Technologies, India) and digital pH meter (Metrohm, India). Empower PRO 2.0 software was used for monitoring and integrating chromatographic peaks.

**UPLC chromatographic conditions**

Various parameters were taken into consideration while developing and optimizing the chromatographic separation conditions including mobile phase, stationary phase, flow rate and detector wavelength. Chromatographic separation was attained with ACQUITY BEH C\(_{18}\) (100 × 2.1) mm, 1.7 μm (waters) at 40°C (column temperature) having 0.01M KH\(_2\)PO\(_4\) in water pH-2.5 with orthophosphoric acid (Eluent-A) and acetonitrile (Eluent-B) as mobile phase using gradient mode of elution (T/%B) = 0/30, 5/80, 7.9/80 and 8/30 with 3 min equilibration time. The flow rate and injection volume were set at 0.3 mL/min and 5 μL respectively throughout the analysis. PDA detector was
used for monitoring the absorbance between 190-400 nm and for quantitative analysis, 220 nm wavelength was used. Eluent-A and Eluent-B were filtered through 0.22 µm filter paper before analysis. All the analysis including the integration of all chromatographic peaks was carried out using Empower 2.0 software. Table 1 represents the optimized chromatographic conditions.

### Forced Degradation Studies

Force degradation studies help in the evaluation of chemical stability, the pathway of degradation and DPs. In these studies, the drug substance was exposed to several conditions (such as hydrolytic, photolytic, oxidative and thermal degradation conditions). The capability of the method in terms of specificity and stability was depicted by these studies. According to International Conference on Harmonization (ICH) guidelines, NBr was exposed to various stress environments. PDA detector was used to assure peak purity of NBr and its DPs formed during the stress study. 1 mg mL⁻¹ stock solution of the drug substance was prepared initially to perform stress studies. Neutralization of acid hydrolyzed sample with sodium hydroxide and alkaline hydrolyzed sample with hydrochloric acid was carried out. Before performing analysis, these solutions were filtered through 0.22 µm filter paper.

### Hydrolytic Degradation

Hydrolytic degradation was carried out on a wide range of pH values. Various functional groups like Esters, amides, alcohol, amylamines, carbamates, etc. may undergo hydrolysis. The sample was treated individually with 5mL of 1M HCl, 5mL of 1M NaOH and water at 80°C for 8 hr. After the desired time interval, the treated sample was allowed to room temperature and the neutralization was done with acid and base. And then, the final concentration of 100 µg mL⁻¹ was achieved by diluting the above-neutralized sample with diluent.

### Photolytic Degradation

When the drug was exposed to UV or fluorescent light, photolytic stability was utilized to assess the impact of light. Functional groups such as N-oxide, carbonyl, aryl chloride, nitroaromatic, weak O-H and C-H bonds, polyenes and sulfides are more likely to cause drug light sensitivity. In the photostability chamber, enough drug substance was placed in a petri-dish and subjected to fluorescent irradiation of 1.2 million lx hr and an integrated near-ultraviolet (UV) energy of 200 W h/m². The substance was then taken out from the photostability chamber and diluted to a final concentration of 100 µg mL⁻¹.

### Thermal Conditions

Thermal degradation tests were performed following ICH Q1A guidelines. The Arrhenius equation is used to investigate the effects of temperature on a substance’s thermal degradation. In a Petri dish, enough amount of drug substance is placed and kept at 105°C for 24hr in a hot air oven (dry oven). After sufficient time, the sample was removed from the dry oven and the final concentration of 100 µg mL⁻¹ was prepared with diluents.

### UPLC and LC-MS Sample Preparation

1 mg/mL of stock solution was prepared with diluents. In a volumetric flask, 10mg of drug substance was taken and the diluent was used to make up the final volume. This stock solution was further diluted to obtain a final concentration of 100µg/mL⁻¹. Again, the solution was diluted to perform the various parameters such as precision, linearity, accuracy, Limit of Detection (LOD) and Limit of Quantitation (LOQ). These solutions were filtered using 0.22 µm nylon filter paper before
being injected into the UPLC system. In every stress degradation condition, the blank was also prepared the same way without using a sample to nullify any peak due to the stressed blank. To compare % age of degradation without treating any stressful conditions, a control sample was also injected. DP generated during the degradation study was mixed with NBr as a specificity solution and optimization and best separation were achieved using this specificity solution for method validation (UPLC) and characterization to established degradation pathway (LC/MS/MS).

**HRMS and LC-HRMS Studies**

The LC-MS/MS experiments were carried out using a Shimadzu Nexera series liquid chromatography system with a 2777 sample management, 1525 binary pump, a TCM/CHM column oven, and a 2489 UV–visible detector, and a 4600 series triple TOF mass spectrometer with an ESI source. Acquity BEH C$_{18}$ (100*2.1) mm, 1.7 μm column was used for LC separation and identification. A mobile phase composed of Eluent A (0.05% Formic acid in water) and Eluent B (acetonitrile), with a gradient program of $(T/ \text{ percent B}) = 0/30, 5/80, 7.9/80$, and $8/30$, with a flow rate of $0.3 \text{ mL min}^{-1}$. Nitrogen gas was used as desolvation and nebulizing gas and the collision gas was argon. The detection wavelength was 220 nm, and the injection volume was 5 μL.

**Method Validation**

As per ICH guidelines, method validation was performed including linearity, accuracy, specificity, robustness and stability. The validation parameters and their acceptance standards are given in Table 2 according to the ICH recommendations.

**System Suitability**

System suitability is the most critical parameter while performing the analytical method validation. NBr (standard solution) was injected six times at a concentration of $150 \text{ ng mL}^{-1}$. System suitability was verified by calculating parameter like USP tailing factor, % RSD (relative standard deviation) USP resolution and theoretical plate count. With the help of an area of six replicate injection analyses, the % RSD was calculated.

**Specificity and Selectivity**

The capability of the analytical procedure to estimate the response of drug in the existence of potential impurities and DPs formed during stress study is referred as specificity. The specificity was assessed by analyzing mixtures of the acidic, basic and oxidative DPs with known concentrations of NBr. Specificity parameter was accomplished by establishing the desired USP resolution between the NBr peak and DP formed during the degradation study. PDA detector was used to verify the peak purity of NBr peak and DPs peak to check the method selectivity.

**Linearity**

This is a very important parameter to assure that response is linear from a low level to a high level. Response and concentration of NBr standards were used to plot the calibration curve. Seven points of different concentrations in triplicate were used for covering the complete linearity ranging from $25-250 \text{ ng mL}^{-1}$ with constant injection volume. The correlation coefficient $(R^2)$, y-intercepts value and slope of NBr were estimated.

**Precision**

System precision was checked in terms of reproducibility and repeatability. A precision study was performed by injecting three different concentrations $(50 \text{ ng mL}^{-1}, 150 \text{ ng mL}^{-1}, \text{ and } 250 \text{ ng mL}^{-1})$ in triplicate in RP-UPLC on the same day and the next day. The value of % RSD and standard deviation (SD) was checked for both intra-day and inter-day precision.

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**Table 2: Method validation parameters and acceptance criteria.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Experiment</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>System precision</td>
<td>Six duplicate-injections of neostigmine bromide standard preparation (100g mL$^{-1}$)</td>
<td>The RSD should not exceed 2.0 percent.</td>
</tr>
<tr>
<td>Method precision</td>
<td>Three different concentrations of sample (QL, 100 percent, and 150 percent) were produced and evaluated in triplicate according to the established procedure.</td>
<td>The RSD should not exceed 2.0 percent.</td>
</tr>
<tr>
<td>DL and QL</td>
<td>DL and QL are calculated using the slope of linearity.</td>
<td>The correlation-coefficient $(r^2)$ must be more than 0.999.</td>
</tr>
<tr>
<td>Recovery</td>
<td>In triplicate, a control-sample was spiked with QL, 100%, and 150 percent concentrations.</td>
<td>98-102%</td>
</tr>
<tr>
<td>Robustness</td>
<td>Changing the flow rate or the temperature of the column or changing the B percent composition</td>
<td>There should be no less than 2.0 USP resolution between closely eluted impurities.</td>
</tr>
<tr>
<td>Solution stability</td>
<td>Stability of the solution over time intervals of 0, 12, and 24 hr</td>
<td>The RSD should not exceed 2.0 percent.</td>
</tr>
</tbody>
</table>
Accuracy/Recovery

The precision of an analytical method is measured by how close the test results are when compared with the true values. The accuracy of the method was determined by performing recovery studies for the drug using a standard addition approach. A known concentration of 150ng/ml of drug material was added to three concentration levels (QL, 100 and 150%). The percent recovery was calculated at each level after the control and recovery samples were injected in triplicate.

Limits of Detection (LOD) and Limit of Quantitation (LOQ)

The sensitivity of the method is demonstrated by LOD and LOQ. The calibration curve method was used to estimate LOD and LOQ. To determine the LOD and LOQ values, a series of a dilute solution (known concentration) was injected. The S/N ratio was calculated for LOD (detection limit) and LOQ (quantitation limit). At the QL concentration level, the percent RSD of the peak area was calculated.

\[
\text{LOD} = \frac{3 \times \text{standard deviation of } y - \text{intercept}}{\text{slope of calibration curve}}
\]

\[
\text{LOQ} = \frac{10 \times \text{standard deviation of } y - \text{intercept}}{\text{slope of calibration curve}}
\]

Robustness

The measurement of the capacity of the method of analysis to remain unaffected by little but deliberate changes in developed experimental conditions is called robustness. In a robustness experiment, the flow rate, column temperature, and initial mobile phase composition were investigated. Specificity solution containing drug substance and its DP was injected in actual condition as well as in all variable conditions to check the robustness of developed method conditions. Effects on system suitability parameters like USP resolution between closely related compounds and USP tailing were observed in the initial condition and in each deliberate variation.

Solution Stability

At room temperature, solution stability was established. These solutions at particular time travel were injected sequentially and this solution stability was assessed for 24 hr. Thereafter, these were compared and evaluated with fresh preparation.

RESULTS AND DISCUSSION

Chromatogram of NBr as control (without treatment) shown in supplementary Figure S1. LC-MS method was used to identify and characterize the DP formed during the stress study. The purity angle was less the purity threshold for both drug substances as well as DP indicated its peak purity. DP-I was formed by treating the drug with alkaline hydrolysis. UV spectra of NBr is shown in Supplementary Figure S8.

Method Development

Development and optimization of UPLC chromatographic conditions

The main objective of stability-indicating method development was to obtain the separation and estimation of NBr and its impurities (process-related or degradation impurities). The absorbance maximum of the drug substance and its DP is 220 nm. Therefore, the detection was evaluated at a wavelength of 220 nm. Depending upon the physicochemical properties, solubility, molecular weight and structure of NBr, different systematic trials such as selection of stationary phase, selection of buffer, organic modifier and different pH conditions were taken for the development of robust, linear, precise and accurate UPLC method for estimation of NBr.

UPLC Column Selection

Various columns of UPLC (stationary phase) are attempted to separate the NBr peak from its DP. Depending on several factors (such as resolution between closely related compounds, tailing and plate count, sharp peak shape and short run time), ACQUITY UPLC BEH C_{18}(100 mm × 2.1 mm, 1.7 µm) was chosen as an appropriate column.

pH and Buffer Selection

Many different buffers like formate, phosphate, acetate, and ammonia covering pH having range from acidic to basic were tried based on the pKa value. It was observed that in neutral and basic pH conditions, peak shape
was not symmetrical and also separation between drug substance and DP was not achieved. Best separation was observed at acidic pH (pH-2.5) with phosphate buffer.

**Organic Modifier Selection**

Acetonitrile and methanol have both been assessed as organic solvents for the mobile phase. In comparison to methanol, acetonitrile delivers good resolution and peak shapes. That is why; acetonitrile was used as an organic modifier for the analysis. The polarity of the mobile phase was changed to acquire a better peak shape and reduce the retention time ($R_t$) of the drug.

**Optimization of Chromatographic Conditions**

Various experimental conditions were considered to attain the best peak shape and separate all DPs from NBr in a shorter run time. Various column temperature measurements and flow rates were also tested for obtaining desired peak shape and resolution. The optimal column temperature for achieving the ideal peak shape and separation between closely eluted peaks was 40°C with a flow rate of 0.3 mL/min. The final UPLC method with DP-I retention times was approximately 6.04 min. The optimized method was able to separate DP-I from NBr and also able to evaluate NBr at a very low level.

**Method Validation**

**System Suitability**

The average theoretical plate count of six injections was 100352 and the average USP tailing of six injections was 1.2 (<1.8) and the % RSD of the area of NBr peak in six replicate injections of system suitability solution was 0.10 (<5.0). The method was suitable for use as all the parameters were within the limit. The results of the system suitability parameter were summarized in Table 3.

**Linearity**

Method linearity was analyzed with a series of seven different concentrations of standard solution ranging from 25 to 250 ngmL$^{-1}$. 111.1, 0.9999, and -63.94 are the values of the slope, coefficient of correlation ($R^2$) and intercept respectively. Linearity data information is given in Table 4. The calibration curve of NBr is shown in Figure 2.

**Precision**

The % RSD value for the intra-day precision study was 0.10% - 0.46% and for inter-day, it was 0.10 - 0.55. Table 5 represents the intra-day and inter-day precision study of the UPLC method of NBr.

**Accuracy/Recovery**

The % recovery was found to be in the range of 100.6% - 102.8%. The recovery results indicate that the method is precise and also found that there was no intervention due to the presence of DP. The results are shown in Table 6.
Robustness

The robustness study was accomplished by making small but deliberate modifications to the flow rate (0.02 mL/min), temperature (5°C), and starting buffer composition in the gradient, all of which were determined to have no effect. The results are shown in Table 7.

**LOD and LOQ**

The LOD and LOQ of NBr were obtained as 25ngmL\(^{-1}\) and 50ngmL\(^{-1}\), respectively. For LOD, the signal-to-noise ratio had been greater than 3, and for LOQ, it was greater than 10. Tables 8 and 9 show the results of the LOD and LOQ tests, respectively.

**Solution Stability and Mobile Phase Stability**

% RSD was less than 1.0% with negligible difference in peak area indicating solution stability of NBr for 24 hr.

**Forced Degradation Studies**

**Specificity and selectivity**

In optimized UPLC techniques, all the DPs formed were separated efficiently with drug substance revealing that it is a specific method. To make sure peak purity, a PDA detector was utilized. The purity angle of DP including NBr was less than the purity threshold. No additional DP peak, impurity or matrix was found to interfere selectivity of the developed method. To perform the
mass balance study, an assay study of stressed samples was carried out. The specificity chromatogram of NBr and its one DP is shown in Figure 3. The results of method specificity and peak purity are presented in Table 10.

### Degradation in Neutral Condition

At various time intervals such as 0 min, 1 hr, 4 hr, and 8 hr, neutral degradation study was performed. At each interval, the sample was filtered with a 0.22 µm filter before injection. The results are shown in Table 10 and the chromatogram of the neutral degradation condition is shown in Supplementary Figure S2.

### Degradation in Acidic Condition

The acid degradation study was conducted at several time intervals, including 0 min, 1 hr, 4 hr, and 8 hr. Before analysis, stressed samples were neutralized with 1M NaOH and filtered with a 0.22 µm filter. During acid hydrolysis, no DP was formed. The observations are presented in Table 10 and the chromatogram of acidic degradation condition is shown in Supplementary Figure S3.

### Degradation in Basic Condition

Alkaline degradation study was taken at different time intervals as in acidic conditions. Before analysis, each stressed sample was neutralized with 1M HCl and filtered through a 0.22 µm filter. During basic hydrolysis, an 11.56% DP (Rt 6.042 min) was obtained. The results are shown in Table 10 and the chromatogram of the alkaline degradation condition is shown in Supplementary Figure S4. UV spectra of DP-I is shown in Supplementary Figure S9.

### Oxidative Degradation

Oxidative degradation study was executed at different time intervals. Before analysis, the stressed sample was neutralized with 1M NaOH and filtered with a 0.22 µm filter. During acid hydrolysis, no DP was formed. The observations are presented in Table 10 and the chromatogram of oxidative degradation condition is shown in Supplementary Figure S5.

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**Table 10: Forced degradation study of NBr.**

<table>
<thead>
<tr>
<th>Stress Condition</th>
<th>Assay of Neostigmine bromide (%)</th>
<th>Observation</th>
<th>Mass Balance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Sample</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral degradation</td>
<td>99.28</td>
<td>There was no significant degradation found.</td>
<td>99.36</td>
</tr>
<tr>
<td>Acid degradation</td>
<td>99.52</td>
<td>There was no significant degradation found.</td>
<td>99.39</td>
</tr>
<tr>
<td>Basic degradation</td>
<td>88.44</td>
<td>One unknown DP of 11.56% was formed.</td>
<td>99.25</td>
</tr>
<tr>
<td>Oxidative degradation</td>
<td>82.57</td>
<td>One unknown DP of 17.43% was formed.</td>
<td>99.60</td>
</tr>
<tr>
<td>Photolytic degradation</td>
<td>99.52</td>
<td>There was no significant degradation found.</td>
<td>99.56</td>
</tr>
<tr>
<td>UV degradation</td>
<td>99.55</td>
<td>There was no significant degradation found.</td>
<td>99.87</td>
</tr>
<tr>
<td>Thermal degradation</td>
<td>99.48</td>
<td>There was no significant degradation found.</td>
<td>99.85</td>
</tr>
</tbody>
</table>

* Mass balance =% Assay +% Sum of all impurities +% Sum of all degradants.
filtered through a 0.22 μm filter. A DP (R<sub>t</sub> 6.038 min) of 17.43% was obtained during oxidative degradation. The results are presented in Table 10 and the chromatogram of oxidative degradation condition is shown in Supplementary Figure S5.

**Photolytic Degradation**

On exposure of NBr drug to photostability chamber (1.2 million lux h) and UV light, the stability of drug substance was found. No DP was formed. The results are shown in Table 10 and the chromatogram of the photolytic degradation condition is shown in Supplementary Figure S6.

**Thermal Degradation**

The thermal degradation analysis was carried out using two Petri dishes containing 100-100 mg of material each. For 24 hr, one was held at 105°C in a hot air oven (dry oven), while the other was kept in controlled conditions. Stressed and control samples were diluted to 100 μg/mL with diluents and then filtered through filter paper before analysis. In thermal stress conditions (105°C for 24 hr), NBr was shown to be stable. The results are presented in Table 10 and the chromatogram of the thermal degradation condition is shown in Supplementary Figure S7.

**Stress Decomposition Behavior**

The DP formed during the stress study was well separated from the drug in optimized UPLC techniques, demonstrating that the approach is specific. To assure peak purity, a PDA detector was being used. To carry out a mass balance analysis, an assay analysis of stressed samples was conducted. In every stressed condition, the assay values were greater than 98.0%. Each basic and oxidative degradation condition formed one main DP. In acidic, neutral, photolytic, and thermal degradation conditions, the drug was determined to be stable.

**Mass Fragmentation pattern of Drug**

Figure 4 shows the LC-MS/MS line spectra of NBr. The neostigmine bromide likely fragmentation process is depicted in and illustrated in Figure 5, the expected degradation pathway of NBr resulted in five fragments. The fragment with m/z 208.12 was obtained by removing one methyl group from the trimethylamine substituted on the benzene ring. Further dimethyl amine
loss led to the formation of a new fragment with a m/z of 164.07. The loss of N,N-dimethylformamide from the drug molecule resulted in the production of the m/z 151.09 fragments. Following that, the hydroxyl ion was eliminated, resulting in the production of m/z 135.10. With the elimination of N,N,N-trimethylbenzenaminium from the drug molecule, the N,N-dimethylformamide with m/z 72.04 was also discovered as a fragment. The NBr structure was verified by all fragmentation data. Table 11 depicts the interpretation of MS/MS data of NBr fragments. Competitive Fragmentation Modeling (CFM) software was used for interpretation of tandem mass spectra (MS/MS) for the purpose of automated DP identification.40

**HR-MS/MS Studies of Stressed Sample**

Figure 6 shows the DP-I line spectra obtained during the stress degradation analysis of NBr. Table 12 lists the most likely molecular formulae, theoretical mass, experimental mass, ring double bond value, and main fragments, as well as chemical formulae for DP-I.

**Identification/Characterization of DP**

**Characterization of DP-I (m/z 152.10)**

The mass of DP-I was discovered to be 152.10 as [M+H]+, as seen in the LC-MS/MS line spectra in Figure 6, which was formed by the loss of N,N-dimethylformamide from the drug molecule under basic conditions. Figure 7 depicts the suggested DP-I fragmentation process. DP-I elutes with a retention period of 6.04 min due to its polar nature. The fragment
Table 12: LC-MS/MS data of DP along with its major fragments and possible molecular formulae.

<table>
<thead>
<tr>
<th>DP</th>
<th>Experimental mass</th>
<th>Best possible molecular formulae</th>
<th>Theoretical mass</th>
<th>RDB*</th>
<th>Major fragments (Chemical formulae)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP-I</td>
<td>152.10</td>
<td>C₃H₇NO⁺</td>
<td>152.10</td>
<td>3.5</td>
<td>137.08 (C₆H₁₀NO⁺), 121.08 (C₅H₁₀N⁺), 91.05 (C₄H₆N⁺), 60.08 (C₃H₄N⁺)</td>
</tr>
</tbody>
</table>

* RDB: ring plus double bonds.

The drug under investigation was shown to be more sensitive to alkaline, and oxidative degradation conditions, as it degraded by 11.56% and 17.43%, respectively. NBr was observed to be stable when treated to neutral hydrolysis, photolytic, acidic, and thermal degradation conditions.

HRMS/MS technique was used for the identification and characterization of NBr and its DP (DP-I) in ESI positive mode. In the current study, the degradation pathway of DP-I was discussed and outlined. The current research could be highly valuable in identifying process-related impurities and other potential DPs that may exist in bulk pharmaceuticals at trace quantities. This type of analysis can be used to estimate QC samples for regular and stability investigations. The degradation pathway and mechanism were established for NBr and DP-I.

**CONCLUSION**

A linear, accurate, highly sensitive, reliable and stability indicating UPLC method was developed. Different stressed conditions were subjected to NBr for stressful degradation and a validation method was done for determining the drug NBr and all it's one degraded product. It is a fast and highly sensitive UPLC method capable of separating NBr and its DP up to nanogram level.

**ACKNOWLEDGEMENT**

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**ABBREVIATIONS**

AIBN: 1,1’-azobis(cyclohexane-carbonitrile); °C: Degree Celsius; ACN: Acetonitrile; AU: Arbitrary Unit; BEH: Ethylene Bridged Hybrid; CFM: Competitive Fragmentation Modeling; Da: Dalton; HPLC: High-Performance Liquid Chromatography; HCl: Hydrochloric acid; H₂O₂: Hydrogen peroxide; hr: Hour; ICH: International Council for Harmonisation of Technical Requirements for Pharmaceuticals
for Human Use; K$_2$HPO$_4$; Potassium dihydrogen phosphate; LC-MS: Liquid chromatography coupled with mass spectrometry; min: Minute; mL: Milliliter; MS: Mass Spectrometry; NaOH: Sodium hydroxide; OPA: Orthophosphoric acid; PDA: Photodiode array; QSM: Quaternary Solvent Manager; RP: Reverse-Phase; RSD: Relative standard deviation; RT: Retention time; UPLC: Ultra Performance Liquid Chromatography; UV: Ultra Violet.

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**PICTORIAL ABSTRACT**

Forced degradation study of Neostigmine bromide was examined by exposing the drug substance under various stressed conditions as per ICH guidelines. This study optimized the stability-indicating method using RP-HPLC as per ICH guideline and evaluated the stability of Neostigmine bromide under various forced degradation conditions, including acidic, alkaline and oxidative conditions (H₂O₂, AIBN and FeCl₃), thermal and photolytic. The drug substance is sensitive to alkaline and oxidative degradation conditions whereas it is stable in neutral, acidic, thermal, and photolytic degradation conditions. Developed a very sensitive UPLC method capable of separating and quantification of neostigmine bromide and its one DP down to the nanogram level. All the degraded samples were analyzed first by RP-UPLC technique and the newly formed DP was identified and characterized with the help of ultra-high performance liquid chromatography which is interlinked with tandem quadrupole time-of-flight mass spectrometry. The method was validated over a linearity range of 25 ng mL⁻¹ to 250 ng mL⁻¹. The method is useful for routine quality control testing.

**SUMMARY**

Dr. Parul Grover has completed her Ph.D from Punjabi University, Patiala. She is presently serving as Assistant Professor in KIET School of Pharmacy, KIET Group of Institutions, Ghaziabad, Uttar Pradesh. She has more than 10 years of experience in teaching and research. She is positive thinker, scientific and self-determined research professional. She has more than 45 publications in journals of high repute, out of which 25 are SCI/Scopus indexed. She has presented and attended various research papers at national and international conferences. She has published 3 books and 5 book chapters. She has published 8 patents. She received Young Scientist Award-2014 for best research paper publication by Biotechnology Society of India and has been honoured with prestigious C V Raman Award in recognition of Best Researcher at Institute level in 2021. She has an ongoing consultancy project on preformulation and docking studies. She is Co-PI in AICTE RPS project. She is lifetime member of APTI. Her areas of interest include Pharmaceutical Analysis, Natural Product Chemistry, Carbohydrate Chemistry and Biocatalysis.

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