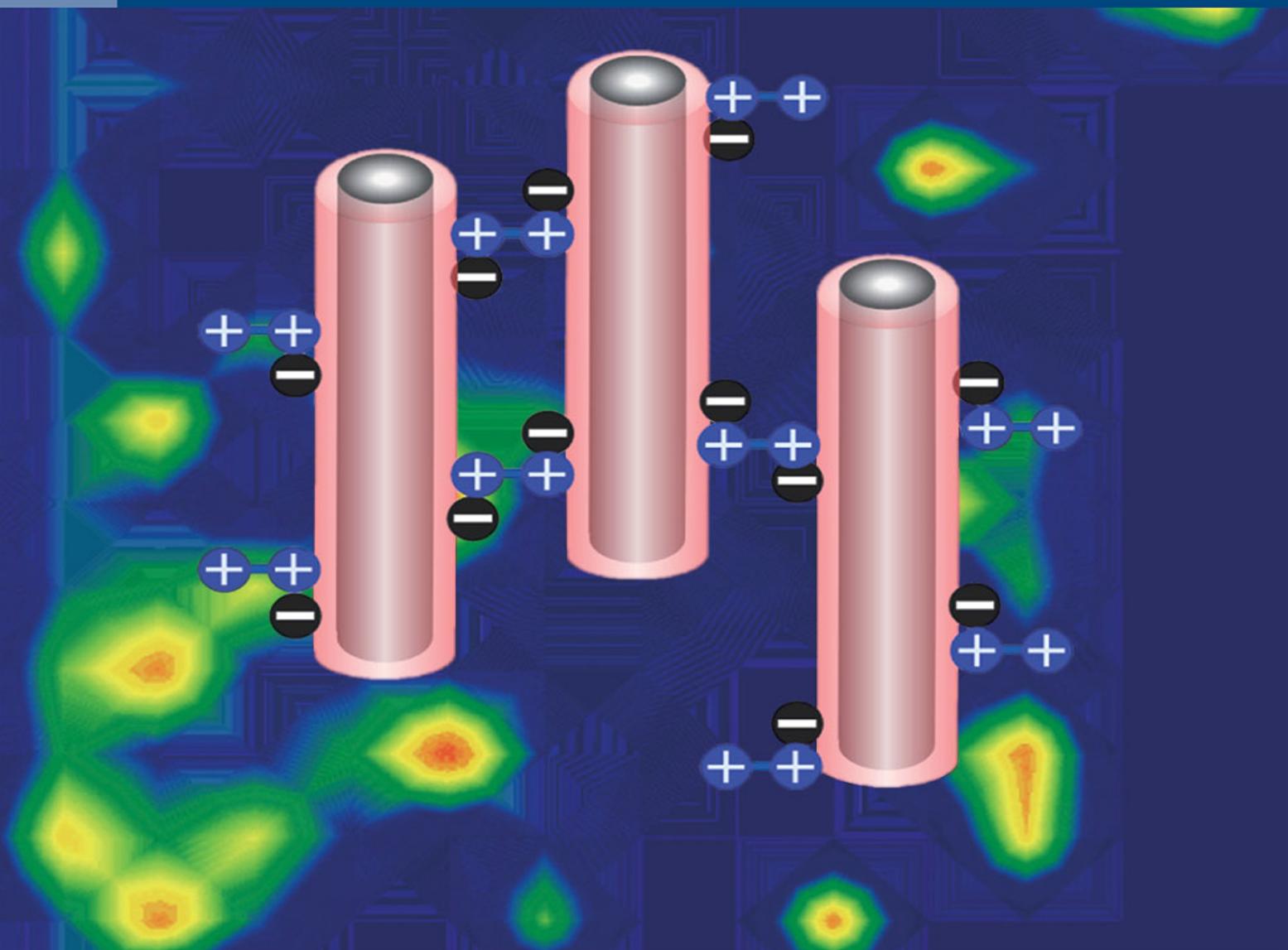


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Research Article

Selective separation and characterization of the stress degradation products of ondansetron hydrochloride by liquid chromatography with quadrupole time-of-flight mass spectrometry

Ondansetron hydrochloride was subjected to forced degradation studies under various conditions of hydrolysis (acidic, basic, and neutral), oxidation, photolysis, and thermal as prescribed by International Conference on Harmonisation guideline Q1A (R2). A simple, selective, precise, and accurate high-performance liquid chromatography method was developed on a Waters Xterra C₁₈ (150 × 4.6 mm id, 3.5 μm) column using 10 mM ammonium formate (pH 3.0)/methanol as a mobile phase in gradient elution mode at a flow rate of 0.6 mL/min. The method was extended to liquid chromatography quadrupole time-of-flight tandem mass spectrometry for identification and structural characterization of stress degradation products of ondansetron. The drug showed significant degradation in base hydrolytic and photolytic stress conditions in the liquid state, while it was found to be stable in neutral, acidic, thermal, and oxidative stress conditions. A total of five degradation products were characterized and most probable mechanisms for the formation of degradation products have been proposed on the basis of a comparison of the fragmentation of the [M + H]⁺ ions of the drug and its degradation products. Finally, the developed method was validated in terms of specificity, linearity, accuracy, precision, and robustness as per International Conference on Harmonisation guideline Q2 (R1).

Keywords: Degradation products / Mass spectrometry / Ondansetron hydrochloride

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1 Introduction

Ondansetron (OND) is used to prevent nausea and vomiting caused by cancer drug treatment (chemotherapy), surgery and radiation therapy [1, 2]. Its chemical name is (RS)-9-methyl-3-[(2-methyl-1H-imidazol-1-yl)methyl]-2,3-dihydro-1H-carbazol-4(9H)-one. The drug works by blocking the serotonin binding to its 5-HT₃ receptor, which is one of the body's natural substances (serotonin) that causes the nausea [3]. The drug has modest effect on vomiting caused by motion sickness [4] and does not have any effect on dopamine

receptors or muscarinic receptors. It has been prescribed to all cancer patients; hence it is on the World Health Organization's List of Essential Medicines, a list of the most important medication needed in a basic health system (<http://www.who.int/medicines/publications/essentialmedicines/en/index.html>).

Stability study is a significant aspect of the early drug development process to optimize new chemical entities. Over time, a drug undergoes degradation, which may result in a loss of drug activity and generates the toxic degradation products (DPs) that are one of the main contributors for adverse effects to human. The International Conference on Harmonization (ICH) guideline entitled "Stability Testing of New Drug Substances and Products" (Q1A, R2) stated that stability indicating assay method (SIAM) needs to be developed to elucidate the inherent stability of the active substance by applying different stress conditions [5, 6]. Stress

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Abbreviations: DP, degradation product; OND, ondansetron

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testing of drugs includes the effect of temperature, humidity, photolysis, oxidation, and hydrolysis. Based on the potencies and maximum daily dosages of the drugs, ICH guidelines require that the impurities and/or degradants of a drug should be structurally elucidated, once they exceed certain thresholds, which are typically between 0.05 and 0.5%, relative to the drug substances [7]. Therefore, sensitive and powerful analytical methodologies such as LC–MS/MS have been increasingly used for structural characterization of DPs or impurities of drugs formed under various stress conditions [8–18].

Various analytical and bioanalytical methods have been developed for the determination of OND alone as well as in combination with other drugs. It includes UV spectrometric methods [19, 20], high-performance TLC method for sublingual tablets [21, 22], RP-HPLC method for solid dosage form and injection [23–25], RP-HPLC method for human plasma [26–28], and LC and LC–MS/MS method for rat plasma [29–31]. In spite of the presence of OND in the generic market for considerable period of time, inadequate information exists in the literature on the identification and characterization of its DPs. Even the drug substance monograph of OND in British Pharmacopoeia and United State Pharmacopoeia have listed eight (A–H) and four (A–D) impurities, respectively [32, 33]. These impurities have not been classified as DPs. Therefore, it becomes essential to know about complete degradation profile of the drug under stress conditions. Moreover, the official monographs show the separation of the related substances (impurities) using nonvolatile buffer (phosphate) that cannot be compatible for MS. This has prompted us to develop a method using volatile buffer that can be compatible for LC–MS/MS for structure elucidation of the DPs. Hence, the present work is an endeavor to (i) conduct the stress degradation study on OND under the ICH prescribed conditions to identify the possible DPs arising under various stress conditions, (ii) characterize the DPs through LC–Q-TOF-MS/MS, (iii) establish degradation pathways and the intrinsic stability characteristics of the drug, and (iv) finally validation of stability-indicating HPLC method.

2 Materials and methods

2.1 Drug and reagents

Pure OND was obtained as a gift sample from Symed Labs, Hyderabad, India. All analytical reagent grade reagents such as ammonium formate, ammonium acetate, formic acid, hydrochloric acid (HCl), sodium hydroxide (NaOH), and acetic acid were purchased from S.D. Fine Chemicals (Mumbai, India). Analytical reagent grade H₂O₂ was purchased from Merck (Mumbai, India). HPLC grade water was obtained by using Milli-Q Gradient system (Millipore, Bedford, MA, USA) and was used to prepare all solutions. For LC–MS analysis, LC–MS CHROMASOLV® grade methanol (MeOH) and acetonitrile (ACN) were procured from Sigma–Aldrich (Bangalore, India).

2.2 Instrumentations and chromatographic conditions

An Alliance e2695 series HPLC system (Waters, Milford, MA, USA) equipped with integral autosampler and quaternary gradient pump with an in-line degasser was used. The column compartment having temperature control and a photodiode array detector (model 2998) was employed throughout the analysis. Chromatographic data were acquired using Empower software. The final optimized chromatographic condition was achieved on a Waters Xterra C₁₈ (150 × 4.6 mm id, 3.5 μm) column using 10 mM ammonium formate (pH 3.0): methanol as a mobile phase in gradient elution mode at a flow rate of 0.6 mL/min. The gradient program was as follows: percentage of methanol from 35 to 65% v/v for 14 min as linear gradient. The injection volume and detection wavelength were set at 10 μL and 247 nm, respectively.

LC–MS studies were performed on an Agilent 1200 series LC instrument (Agilent Technologies, Santa Clara, CA, USA) attached to a Q-TOF mass spectrometer (Q-TOF LC-MS 6540 series, Agilent Technologies) coupled with a dual ESI source to minimize the mass error. The data were acquired using Mass Hunter Workstation software. The typical operating source conditions for MS scan of OND and its DPs in positive ESI mode were optimized as follows: the fragmentor voltage was set at 150 V, the capillary at 4000 V, the skimmer at 60 V, and nitrogen was used as the drying (325°C, 10 L/min) and nebulizing (45 psi) gas. Ultrahigh purity nitrogen gas was used as collision gas and 15–20 eV energy was used for MS/MS studies. The chemical formula of all DPs and its product ions were generated based on accurate mass measurements using Agilent Mass Hunter Qualitative Analysis B.06.00 software. Accurate mass was derived through true isotopic patterns and restricted number of elements. During the data processing, four elements, C, H, O, and N were selected with maximum limits of 30, 120, 5, and 5, respectively. The maximum limits of selected elements were decided based on the parent structure. All DPs were giving hits in the range of 2–10 and correct formula was selected based on the maximum score and minimum error. The thermal stress degradation study was carried out using a hot air oven (Oswald Scientific, Mumbai, India). The stress photodegradation was carried out in a photostability chamber (Oswald OPSH-G-16-GMP series, Oswald scientific, Mumbai, India). The chamber was equipped with illumination bank made of light source as described in the ICH guideline Q1B [5]. An ultrasonicator from Power Sonic-405 (Hwashin Technology, Seoul, Korea) and pH meter from pH tutor (Eutech Instruments, Singapore) were used.

2.3 Stress degradation study

Stress studies were carried out on the bulk drug according to the regulatory guideline, ICH Q1A (R2). Stress studies were performed on 500 μg/mL solution of OND for all the conditions. Neutral, acidic, and basic hydrolysis were carried out

in water, 2 N HCl, and 1 N NaOH at 80°C, for 72, 48, and 24 h, respectively. The 1 N NaOH solution was prepared by dissolving NaOH in equal volume of water and methanol. Oxidative degradation was carried out in 30% H₂O₂ for 24 h. A photolytic stress study was carried out in solution and solid sample at 40°C by exposing to a total dose of 200 Wh/m² of UV illumination and 1.2 × 10⁶ lux h of fluorescent light for two days. During photolytic stress study, the drug was dissolved in water only as neutral solution. Thermal degradation study was carried out in solid state by exposing pure drug in a petri plate in a very thin layer to dry heat at 80°C for five days.

2.4 Sample preparation

Acidic and neutral stress samples were individually prepared by dissolving accurately weighed 10 mg of the pure drug in 20 mL of 1 N HCl and 20 mL of water, respectively. The drug was insoluble in 1 N NaOH solution. The organic cosolvents are common to use for increasing the solubility of drug substances [34]. Therefore, methanol was used as cosolvent for solubilization. The acidic and basic stressed degradation samples were neutralized with NaOH and HCl, respectively and diluted with mobile phase. All stress samples were diluted with mobile phase and filtered through a 0.22 μm membrane filter before LC and LC–MS/MS analysis.

3 Results and discussion

3.1 Chromatographic separation

To attain an adequate separation of OND and its DPs in stress samples, the components of the mobile phase, their proportions, pH, flow rate, and column temperature were appropriately optimized. A Waters Xterra C₁₈ column (150 × 4.6 mm, 3.5 μm) was found to be suitable for this analysis after trying with different RP columns such as Grace C₁₈, Phenomenex C₁₈, and Phenomenex C₈. During optimization process on this column, several isocratic chromatographic conditions were used during initial scouting such as MeOH/water and ACN/water in different proportion. It was observed that methanol was found to be better in terms of peak shapes as compared to acetonitrile. Therefore, methanol was used as an organic modifier for optimization of the method. However, a few degradants showed asymmetrical peak shape due to absence of buffer.

In an attempt to improve peak symmetry and resolution on Xterra C₁₈ column, TFA (0.05%) was used. But severe tailing (>2) was observed for the drug. Hence, ammonium formate (10 mM, pH 3) buffer with gradient program was tried to improve the resolution and peak symmetry. The resolution was improved (>2) using gradient analysis. The final optimized linear gradient program was obtained using methanol from 35 to 65% for 14 min, and the flow rate was 0.6 mL/min.

Further studies were carried out to test the effect of pH (3, 4, and 5) with optimized gradient program on retention time, resolution, and peak tailing of analytes (Supporting Information Fig. S1, Table S1). It was found that OND and its DPs (DP3 and DP4) were coeluted and broader peaks observed at pH 5. In case of pH 4, degradants (DP3 and DP4) and the drug were not separated ($R_s < 1.5$) from each other and tailing factor of the drug was >2. In the mobile phase pH 3, peak shape, and resolution of all DPs were improved and all other chromatographic parameters were well within the limits (Supporting Information Table S2). Therefore, pH 3 was finalized to get most favorable separation with optimized gradient program. The retention of all analytes was not affected by changing the buffer strength.

3.2 Validation of the method

The stability indicating HPLC assay method of OND was validated with respect to specificity, linearity, accuracy, precision, and robustness as per ICH guideline Q2 (R1) [35]. Specificity of method was found out by evaluating the peak purity angle and purity threshold of OND using a photodiode array detector and it was found that purity threshold was greater than purity angle, suggesting that the drug peak is pure and the developed method is specific. The linearity was performed by analyzing OND at six different concentrations (i.e., 50, 100, 150, 200, 300, and 400 μg/mL) level from 25 to 200% of its specific concentration of 200 μg/mL. Each concentration solution was analyzed triplicate. Standard calibration curve was built by taking average peak area of each concentration on the y-axis and nominal concentrations of the drug on the x-axis. The data were subjected to statistical analysis using a linear regression model; the linear regression equation and correlation coefficient (r^2) were $y = 11\ 018x - 10\ 517$, and 0.999, respectively. The system suitability testing was evaluated by injecting five replicate of 200 μg/mL solution of OND. The tailing factor (average: 1.12) and theoretical plate (average: 16116) were found well within the limits indicating that system is suitable to use. Accuracy of the standard drug was evaluated in triplicate at three levels, 50% (100 μg/mL), 100% (200 μg/mL), and 150% (300 μg/mL) of its specific concentration of 200 μg/mL ($n = 3$). The percentage recovery range and SD values were found to be 100.0–101.0 and 0.6, respectively (Supporting Information Table S3). Similarly, the intra- and interday precision were determined at the concentrations of 100, 200, and 300 μg/mL on the same day ($n = 3$) and on consecutive days ($n = 3$), respectively. Additionally, interday precision was evaluated for different columns and analyst. The %RSD values for intraday and interday precision studies were <2% (Supporting Information Table S4), which confirms that the method is precise. Robustness of method was evaluated by deliberately varying flow rate (± 0.1 mL/min), column temperature ($\pm 5^\circ\text{C}$), and pH of mobile phase (± 0.2). No major change in assay value of OND was observed by changing the chromatographic conditions, which confirms that the developed method is robust.

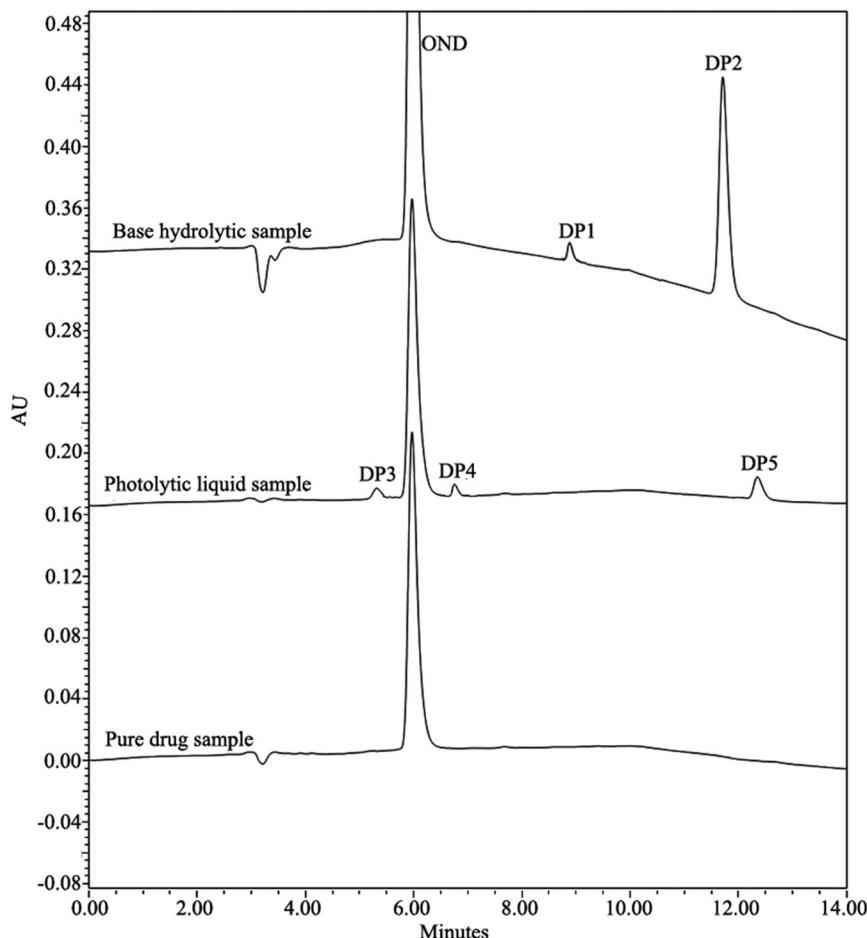


Figure 1. The overlay of HPLC chromatogram of base hydrolytic stress sample, photolytic liquid stress sample, and pure drug.

3.3 Degradation behavior of OND

The degradation behavior of OND was studied using HPLC under various stress degradation conditions. The drug was found to be degraded in base hydrolytic and photolytic stress conditions, while it was stable in neutral, acidic, oxidative, and thermal stress conditions. The overlay of HPLC chromatograms of base hydrolytic, photolytic stress degradation samples and pure drug are monitored at 247 nm (Fig. 1).

The drug was refluxed in 1 N NaOH at 80°C for 24 h. It decomposed into two DPs that are denoted as DP1 and DP2. Three DPs (DP3–DP5) were generated in solution after exposure to the stress photolytic conditions, whereas the drug was found to be stable in photo solid sample. Elemental formulas of proposed structures of all DPs are generated using accurate mass measurements (Supporting Information Table S5).

3.4 LC-ESI-Q-TOF-MS/MS study of OND and its DPs

3.4.1 MS/MS of OND

The fragmentation pathway of protonated OND was established using LC-ESI-MS/MS combined with accurate mass

measurements. The ESI-MS/MS spectrum shows the product ions at m/z 212 (loss of $C_4H_6N_2$ from m/z 294), m/z 184 (loss of CO from m/z 212), m/z 170 (loss of C_2H_2O from m/z 212), m/z 169 (loss of $\cdot CH_3$ from m/z 184), m/z 168 (loss of C_2H_4O from m/z 212), and m/z 143 (loss of C_2H_2 from m/z 169) (Fig. 2, Supporting Information Scheme S1). The formation of the product ion at m/z 212 can be explained by the loss of neutral 2-methyl-1H-imidazole with the retention of charge on the methylene attached to 2,3-dihydro carbazole moiety. The fragment ion at m/z 184 formed by the loss of CO group from the ion at m/z 212. The m/z 212 ion appears to undergo a ring expansion followed by loss of ketene to generate the ion at m/z 170.

3.4.2 Characterization of DPs using LC-ESI-MS/MS

Initially, LC-ESI-MS/MS analysis was tried in both positive and negative mode. All the degradants showed intense $[M + H]^+$ peaks in positive-ion mode and very low intensity peaks in negative mode. Thus, the analysis of samples was carried out in positive ionization mode. The MS/MS spectra of all the DPs were collected in positive mode (Figs. 2 and 3). Most plausible structures have been proposed for all the DPs based on the m/z values of their $[M + H]^+$ ions and the MS/MS

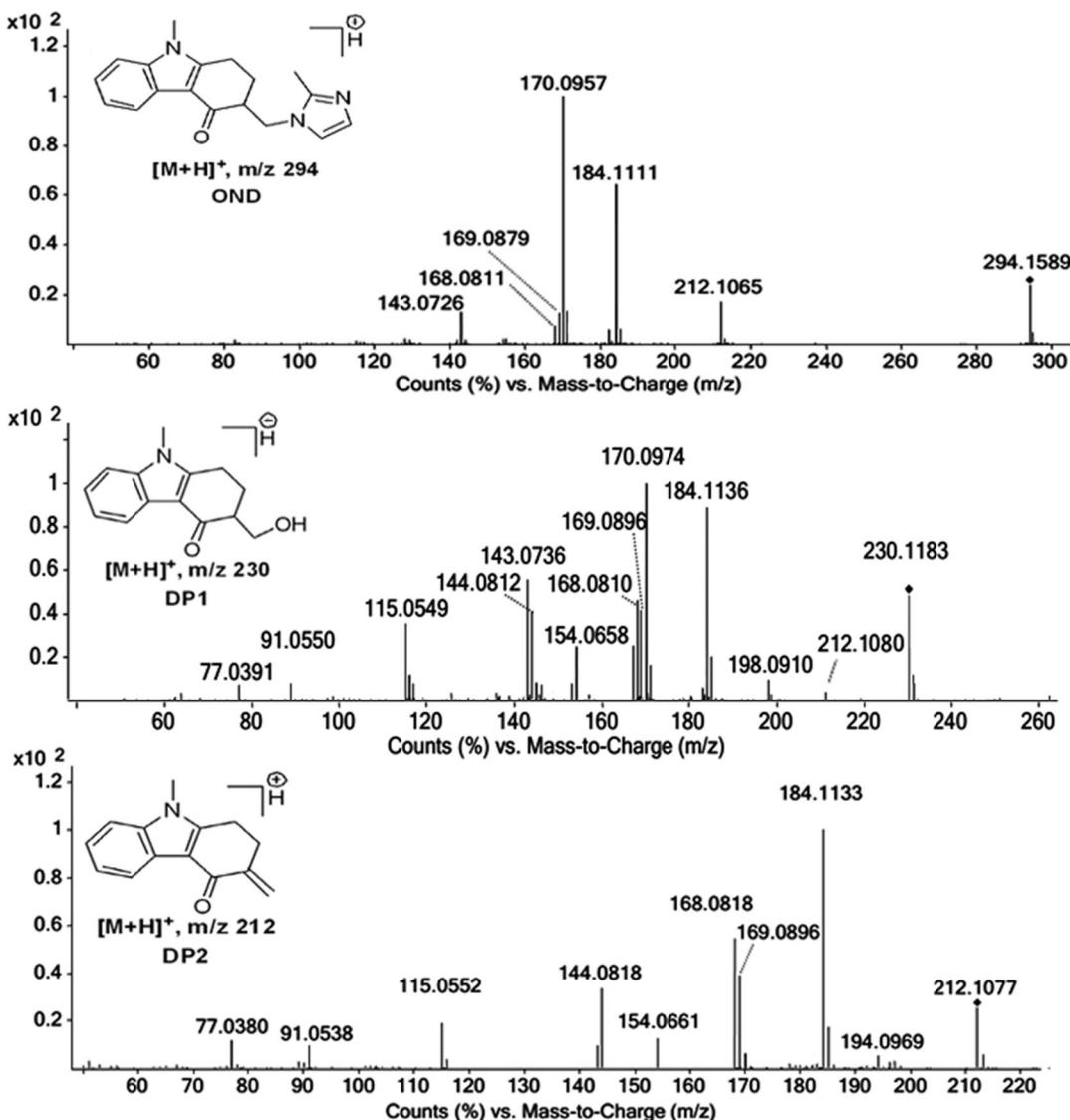


Figure 2. Proposed protonated structure along with LC-ESI-MS/MS spectrum of $[M + H]^+$ ions of the drug (OND, m/z 294) at 20 eV, DP1 (m/z 230) at 15 eV and DP2 (m/z 212) at 20 eV.

data in combination with elemental compositions derived from accurate mass measurements (Supporting Information Table S6). Comprehensive characterization of all the DPs is discussed below.

The LC-ESI-MS/MS spectrum of $[M + H]^+$ of DP1 (m/z 230, $C_{14}H_{16}NO_2^+$, $R_t = 8.85$ min) shows the product ions at m/z 212, 198, 184, 170, 169, 168, 154, 143, 144, 115, 91, and 77 (Fig. 2). Some of these product ions were also observed in the MS/MS of the $[M + H]^+$ of OND. Based on the structure of OND and the characteristic fragment ions in combination with accurate mass data, the 3-(hydroxymethyl)-9-methyl-2,3-dihydro-1H-carbazol-4(9H)-one structure is proposed for DP1. A most plausible mechanism for the formation of DP1 under base hydrolytic condition may involve the cleavage of a C–N bond followed by addition of OH group (Scheme 1).

The intense DP2 at m/z 212 ($[M + H]^+$; $C_{14}H_{14}NO^+$) was eluted at 11.72 min. Its ESI-MS/MS spectrum shows product

ions at m/z 194 (loss of H_2O from m/z 212), m/z 184 (loss of CO from m/z 212), m/z 169 (loss of CH_3 from m/z 184), m/z 168 (loss of C_2H_4O from m/z 212), m/z 154 (loss of C_3H_6O from m/z 212), m/z 144 (loss of C_3H_4 from m/z 184), m/z 143 (loss of C_2H_2 from m/z 169), m/z 115 (loss of C_3H_4 and CNH_3 from m/z 184), m/z 91 (loss of C_3H_3N from m/z 144), and m/z 77 ($C_6H_5^+$) (Fig. 2). The base peak at m/z 184, also observed in the protonated drug spectrum, indicates that 2,3-dihydro carbazole moiety of the drug was intact and 2-methyl imidazole group was eliminated from the drug resulting in the generation of DP2. The fragment ions of DP2 are structure indicative ions and highly compatible with structure, 9-methyl-3-methylene-2,3-dihydro-1H-carbazol-4(9H)-one (Supporting Information Scheme S1). The formation of DP2 under base hydrolytic stressed condition can be explained by the abstraction of hydrogen of methylene by –OH group to form

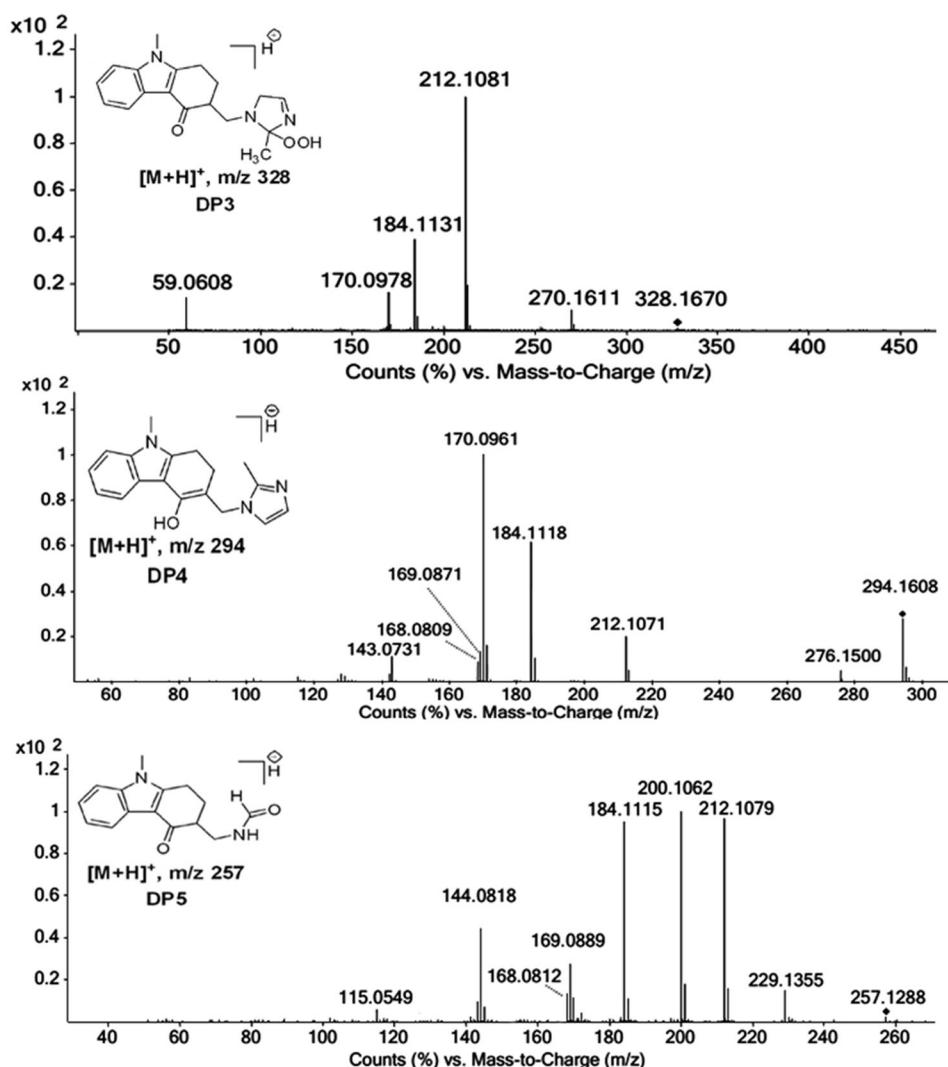


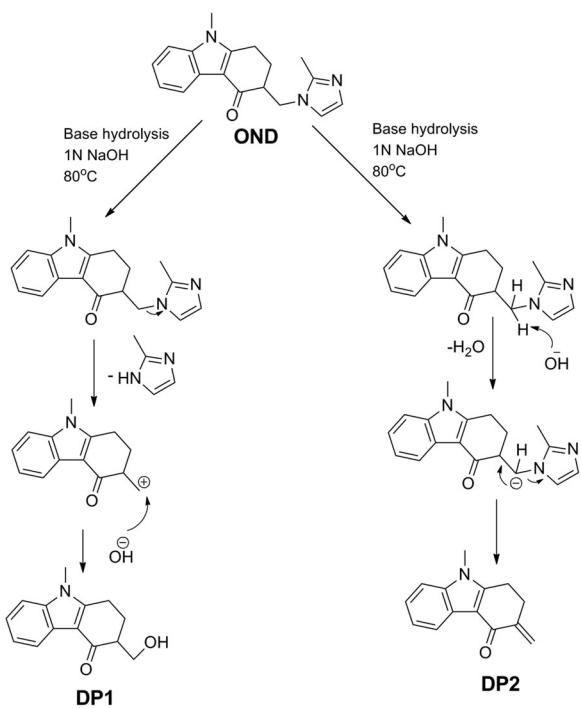
Figure 3. Proposed protonated structure along with LC-ESI-MS/MS spectrum of $[M + H]^+$ ions of DP3 (m/z 328) at 20 eV, DP4 (m/z 294) at 20 eV and DP5 (m/z 257) at 20 eV.

a carboanion followed by elimination of 2-methyl imidazole (Scheme 1).

The LC-ESI-MS/MS spectrum of $[M + H]^+$ of DP3 ($R_t = 5.35$ min, m/z 328.1670, $C_{18}H_{22}N_3O_3^+$) is given in Fig. 3. The elemental composition indicates an addition of two oxygen atoms to the drug molecule. The MS/MS spectrum shows the structure indicative fragment ions at m/z 270, 212, 184, 170, and 59. The fragment ions such as m/z 212, 184, and 170 are characteristic of the 2,3-dihydro carbazole structure. This suggests that two oxygen atoms are probably attached on the imidazole group of the drug. The characteristic product ion at m/z 270 is most likely formed by two consecutive steps involving breakage of the C-N bond of imidazole, formation of amide bond followed by elimination of ethanone. Moreover, it is reported that the singlet oxygen generated by photosensitization adds onto the five-membered imidazole ring to form a bicyclic 2,5-endoperoxide intermediate and endo peroxide decomposes to hydroperoxide [36] (Scheme 2). All these data are highly compatible with the proposed structure,

3-[(2-hydroperoxy-2-methyl-2,5-dihydro-1H-imidazol-1-yl)methyl]-9-methyl-2,3-dihydro-1H-carbazol-4(9H)-one (Fig. 3).

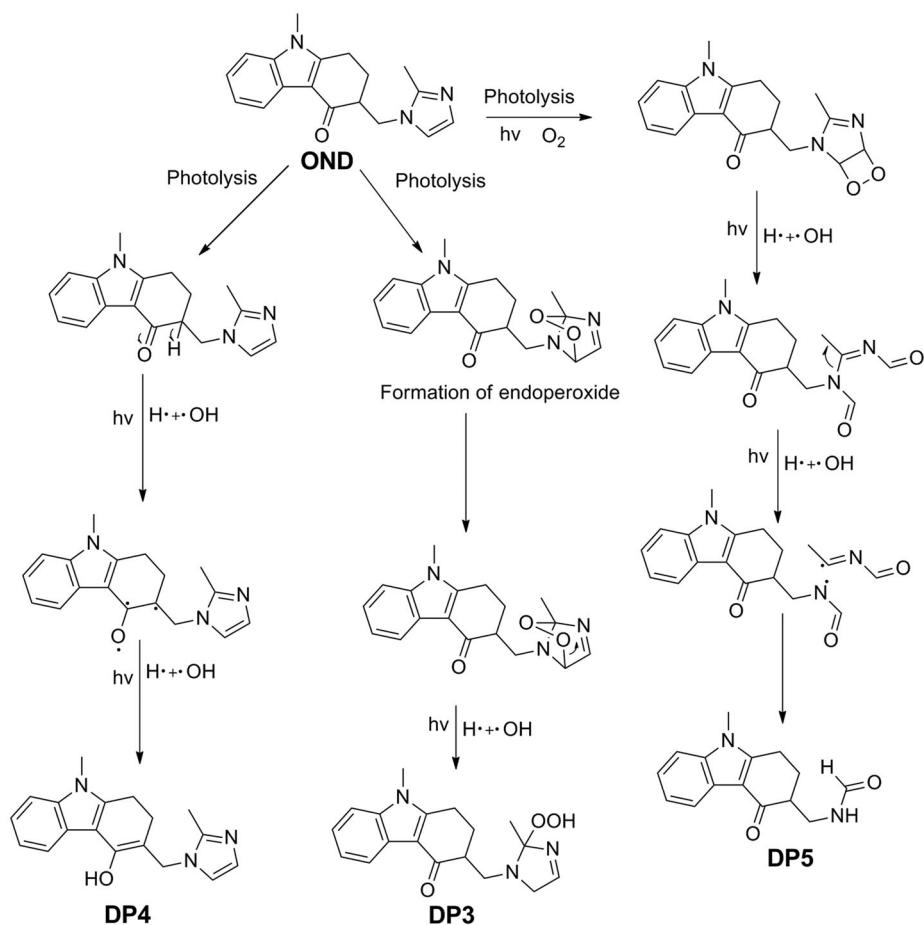
The $[M + H]^+$ of DP4 ($R_t = 6.70$ min) at m/z 294 with an elemental composition, $C_{18}H_{20}N_3O^+$ suggests that DP4 and the drug are isomeric structures. The ESI-Q-TOF-MS/MS spectrum shows almost similar product ions at m/z 212, 184, 170, 169, 168, and 143 (except that the product ion at m/z 276 was observed only in DP4) (Fig. 3). The fragment ion at m/z 276 was formed by the loss of H_2O (18 Da), suggesting the presence of hydroxyl group in DP4 in place of the keto group in the drug. Moreover, photolytic degradation of the drug in the solution, probably leads to keto-enol tautomerization resulting in the generation of hydroxyl group containing degradant, DP4. The most plausible proposed structure is 9-methyl-3-[(2-methyl-1H-imidazol-1-yl)methyl]-2,9-dihydro-1H-carbazol-4-ol (Supporting Information Scheme S2). The probable mechanism for the formation of DP4 under photolytic stress conditions is depicted in Scheme 2. The elemental compositions of the



Scheme 1. Probable mechanism of the formation of DP1 and DP2 under base hydrolytic stressed conditions.

product ions have been confirmed by the accurate mass measurements (Supporting Information Table S6).

The ESI-Q-TOF-MS/MS spectrum of $[M + H]^+$ ion of DP5 (m/z 257; $C_{15}H_{17}N_2O_2^+$, $R_t = 12.33$ min) formed under photolytic stressed condition, is shown in Fig. 3. The elemental composition indicates that three carbons are eliminated and one additional oxygen is attached to the drug. The MS/MS spectrum shows the structure indicative fragment ions at m/z 229, 212, 200, 184, 169, 168, 144, and 115. The fragment ion at m/z 212 is characteristic of 2, 3-dihydro carbazole structure. It suggests that the imidazole group of the drug was altered under photolytic conditions. The product ions at m/z 229 and 212 were formed by the loss of CO (28 Da) from DP5 and the loss of NH_3 (17 Da) from m/z 229, suggesting the presence of a formamide group in DP5. The formation of m/z 200 could be explained by the McLafferty type of rearrangement involving a 1,5-H migration followed by loss of formylmethylenamine. The fragmentation pathway of DP5 is shown in Supporting Information Scheme S2. The elemental composition of all the product ions were confirmed by the accurate mass measurements (Supporting Information Table S6). All these data are highly consistent with the structure, N -[(9-methyl-4-oxo-2,3,4,9-tetrahydro-1H-carbazol-3-yl)methyl] formamide. A plausible mechanism for the formation of DP5 is shown in Scheme 2.



Scheme 2. A probable mechanism for the formation of DPs (DP3–DP5) under photolytic liquid state conditions.

4 Concluding remarks

A selective validated stability indicating HPLC method was developed to study the degradation behavior of OND under hydrolysis (acid, base, and neutral), oxidation, photolysis, and thermal stress conditions and determined the inherent stability of the drug. A total of five hitherto unknown DPs were characterized unambiguously using LC–ESI–MS/MS experiments combined with accurate mass measurements. Out of five degradants, two DPs (DP1 and DP2) were formed under base hydrolytic stress conditions and three degradants (DP3–DP5) were generated under photolytic liquid state. The proposed structures of the DPs have been rationalized by appropriate mechanisms.

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