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Separation and Characterization of Novel Degradation and Process Related Impurities of Bedaquiline Bulk Drug

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Abstract

Bedaquiline (BDQ) is a new drug approved by United States Food and Drug Administration (USFDA) in 2012 for the treatment of drug-resistant tuberculosis, which has become a major threat globally. The manuscript presents the development of three liquid chromatography (LC) based analytical methods. The first is a stability indicating RP-HPLC (reverse phase-high performance liquid chromatography) method to analyze the BDQ in presence of its degradation products. Another UPLC/ESI–MS (ultra-performance liquid chromatography/electron spray ionization–mass spectrometry) method was developed for the identification of different degradation based and process related impurities and the third, preparative HPLC method was developed for the isolation of major degradation products. Eleven degradation products and one process related impurity were identified using UPLC/ESI–MS whereas preparative HPLC was used to isolate two degradation products and their chemical structure was elucidated using nuclear magnetic resonance, mass and infra-red spectral data.

Introduction

The control of tuberculosis was a challenge in developing countries and emergence of drug-resistant Mycobacterium tuberculosis has forced the scientists to develop new molecules. Bedaquiline (BDQ), a quinolone derivative was first approved by USFDA in 2012 as a new molecule for the treatment of M. tuberculosis. By 2015, BDQ was accepted by all the developing countries and is now being used for the treatment of multi drug-resistant tuberculosis (MDR-TB) either alone or in combination with other antibiotics (1–3). In India, BDQ is available as 100-ng tablets though chemically it is \([(1R, 2S)-1-(6-bromo-2 methoxy-3-quinolinyl)-4-(dimethylamino)-2-(1-naphthalenyl)-1-phenyl-2-butanol]\); Figure 1 shows the chemical structure of BDQ.

Stability is an important study to predict the shelf life of an active pharmaceutical ingredient (API) and is performed as stress degradation or forced degradation study in the laboratory to study the impact of various factors on the quality of API. The factors that may make API unstable are light, moisture, heat, hydrolyzing agent, reductive agent or oxidizing agent. Any reaction of a factor with API would promote formation of degradation product/s (DP). Newly formed DP may or may not exhibit pharmacological effect; but it can definitely make changes in biological activity of API. The regulatory bodies vitally review the stability of molecule and quality of product during product approval, so the results of these studies would be crucial during formulation development to decide the storage conditions and the factors to be avoided during formulation packaging and storage (4). The analytical methods are needed before product development stage so as to analyze the impurities in bulk drug as well as in formulation for routine quality control analysis.

Literature survey for analytical data of BDQ showed that spectrophotometric method (5), forced degradation study (6), simultaneous estimation (7, 8), bio-analytical HPLC/MS methods (9, 10), chiral
analysis (11, 12), pharmacokinetic study (13, 14) and simultaneous bio-analytical analysis (15) have been reported till date. The only one reported stability study (6) does not report the identification of DPs or process related impurities of BDQ.

BDQ dosage is ≤2.0 g/day so as per dosage criterion described in ICH guidelines the impurities ≥0.10% should be identified and qualified (16). In this study, 10 DPs formed during forced degradation study and one process related impurity were identified. BDQ was degraded to >50% to identify the major and minor DPs.

This is the first report, identifying 11 (11 degradation products + one process related impurity) DPs of BDQ by MS data and isolation and characterization of two major DPs by using sophisticated spectral and chromatographic techniques. The present study showed stress degradation and quantification of BDQ, identification of DPs using UPLC/ESI–MS, isolation of major DP using preparative HPLC, and characterization of major DP and process related impurity using spectral data from NMR (1H, 13C and APT) and IR.

Experimental

Instrumentation and reagents

BDQ bulk sample was procured from Dishman Pharmaceuticals and API, Ahmedabad, Gujarat-India. HPLC (high-performance liquid chromatography) grade methanol and OPA (orthophosphoric acid) were purchased from Rankem (Mumbai, India). Sodium dihydrogen ortho phosphate buffer was purchased from Sigma-Aldrich (USA). Highly purified water was prepared using resin filters and deionization by Milli-Q (Millipore Co., USA). UPLC/ESI–MS, isolation of major DP using preparative HPLC, and characterization of major DP and process related impurity using spectral data from NMR (1H, 13C and APT) and IR.

UPLC–MS

Study was completed on Waters Acquity UPLC with quaternary solvent manager with PDA detector, column oven, Acquity ESI performance mass detector and auto sampler.

Mass analysis was carried out on single quad mass spectrometer equipped with Waters jet stream Electron Spray Ionization (ESI) source with positive mode (Waters Corp. USA). MassLynx software was used for the UPLC/ESI–MS-PDA data acquisition and analysis of BDQ and its DPs.

Preparative HPLC

Isolation of major acid stress degradation was completed by preparative HPLC; Shimadzu-LC-20AP, binary, auto sampler and auto fraction collector, high pressure mixing chamber and UV detector. Rheodyne injector (6-mL capacity) was used to load sample in chromatographic system.

NMR

1H NMR, 13C NMR and APT were performed on Bruker 400-MHz NMR spectrometer using deuterated dimethyl sulphoxide (DMSO-d6) as solvent. Chemical shifts were recorded in ppm (δ Scale) and coupling constants in Hertz concerning TMS (0 ppm) as an internal standard. For data analysis Top spin software 3.2 was used.

IR

The Infrared spectroscopy was performed on Shimadzu IR affinity 1S, Miracle ATR-IR instrument, with maximum resolution 0.5 cm⁻¹ in a range of 4000–700 cm⁻¹, analyzed by Lab-Solution IR software.

Methods

RP-HPLC

Waters C8 column (250 × 4 mm, 5 μm) was used for chromatographic separation. Mobile phase consisted of: A) 0.01-M sodium dihydrogen ortho phosphate buffer (pH 2.5 ± 0.5 achieved by OPA) and B) methanol. Detection was carried out at 225 ± 5 nm. Gradient elution was selected to achieve resolution between BDQ and degradation peaks. Gradient procedure was set to; time (min.) / % v/v of B (Methanol), 0/45, 40/80, 50/45 with 1 mL/min. Flow rate, analysis time 50 min.

UPLC/ESI–MS

UPLC/ESI–MS method was developed using Column Acquity BEH C8 (150 × 2.1 mm, 5 μm) Mobile phase A) 0.1% Formic acid in MilliQ water (pH=2.70 ± 0.5) and B) 0.1% Formic acid in MilliQ water: acetonitrile (10:90). Gradient elution program was set to T=0 min (97% A) flow: 0.8 mL/min; T=0.75 min (97% A) flow: 0.8 mL/min; gradient to T=2.7 min (2% A) flow: 0.8 mL/min; gradient to T=3 min (0% A) flow: 1 mL/min; T=3.5 min (0% A) flow: 1 mL/min; to T=3.51 min (97% A) flow: 0.8 mL/min; end of run at T=4 min (97% A), Flow rate: 0.8 mL/min, analysis time 4 min.

Mass probe (Probe temperature 400°C) was set as source for electro spray ionization in positive mode (temp. 120°C) with cone voltage 10 and 30 V and capillary voltage 3.25 kV. Cone gas flow and desolvation (400°C) gas flow was 100 and 800 L/h, respectively.
Column and auto sampler temperature was set to 35° and 5°C, respectively.

**Preparative HPLC**

Isolation of major stress degradation product was achieved using stationary phase X-Bridge (C18, 250 x 19 mm, 5 μm) column set to ambient temperature. Mobile phase consisted of A) water and B) acetonitrile with flow rate 20 mL/min., PDA detection at 225 ± 5 nm. Gradient elution program was set as follows; run time 20 min; T (min) = A) % v/v; T = 0.01(50), T = 15 (30), T = 15.00(0), T = 15.01 (0), T = 18 (50) and stop command after T = 20 (50).

**Analytical sample preparation**

**Method validation**

The developed method was validated in accordance with linearity, range, precision and recovery, limit of detection and limit of quantification.

**Linearity and range.** Accurately weighed 10-mg BDQ was dissolved in methanol by vigorously sonication for 15 min; the final volume was made to the 20 mL with methanol (stock concentration 500 μg/mL). The range selected for linearity was 30.00–180.00 μg/mL. Aliquots of 0.6, 1.2, 1.8, 2.4, 3.0 and 3.6 mL were withdrawn from stock solution and diluted to 10 mL separately with methanol to get desired concentration.

**Precision.** Precision of method was established by intra-day, inter-day and repeatability of concentration 30.00, 60.00 and 90.00 μg/mL. The aliquots of 0.6, 1.2 and 1.8 mL were withdrawn from the stock solution and diluted to 10 mL separately with methanol to get desired concentration. For repeatability aliquot of 1.8 mL was diluted to 10 mL with methanol to get 90.00-μg/mL concentration.

**Recovery study.** The laboratory mixture was prepared for recovery study; equivalent to 10 mg of BDQ was accurately weighed, dissolved in methanol and filtered with Whatman filter paper to remove an undesired or undissolved materials. Final volume was made to 20 mL with methanol. Aliquots of 0.6, 1.2 and 1.8 mL were withdrawn from the prepared stock solution and transferred in separate volumetric flasks. The aliquot of 1.8 mL was withdrawn from bulk drug stock solution and added to each volumetric flask containing synthetic mixture aliquot. The volume of each volumetric flask was made to 10 mL with methanol.

**Stress degradation studies**

BDQ (accurately weighed 500 mg) was dissolved in 10-mL methanol and sonicated for 15 min with occasional shaking; 50-mL final volume was achieved separately using 12% hydrogen peroxide, 1 N HCl, 1 N NaOH and water (final concentration 10 mg/mL). Aliquots of 2 mL were withdrawn at regular time intervals to study the extent of degradation. The samples were kept in dark and diluted to 10 mL with methanol (2 mg/mL), filtered through 0.45 μ Pall syringe filter and injected in chromatographic system for RP-HPLC, UPLC/ESI-MS or preparative HPLC for data acquisition and analytical purpose.

**Major degradation product: Sample preparation for isolation**

About 10 mL aliquot was withdrawn from the stressed sample, kept at 80°C in dark and diluted upto 20 mL with methanol (1 mg/mL). Sample was injected in preparative HPLC following chromatographic conditions described in methods section. The fraction was collected with purity ≥95.0%, combined together to remove organic solvent using rotavapour. The concentrated fraction was again injected in preparative HPLC to collect eluent, was washed with water to remove excess buffer and again concentrated on rotavapour to remove organic solvent (acetonitrile). Final solution was lyophilized using freeze dryer (Virtis Advantage 2XL) to get product of ≥98.0% purity.

**Results**

**Mobile phase optimization**

The isocratic elution was not showing sufficient resolution among various chromatographic peaks, gradient elution was therefore selected for HPLC separation of BDQ and its DPs. Minor DPs were subjected to mass analysis for identification in UPLC system, the efficient separation of peaks was not essential as DPs in a small amount (co-elution or single elution) can be identified by mass spectrum at selected Rt. For isolation of DPs, mobile phase was modified so that major DPs could be resolved from other DPs and BDQ.

**Stress degradation of BDQ**

The stability of BDQ was studied under light, thermal, hydrolytic (acidic and alkaline), oxidative and neutral media (water); it was stable under all the conditions except under acidic and oxidative stress. The sample was kept in 1 N HCl at 80°C for 75 min and in 12% hydrogen peroxide at 80°C for 5 h to degrade BDQ (5.00–80.00%). BDQ was kept in water at 80°C for 24 h, for thermal degradation at 80°C in oven for 21 days and was kept under 1.2 lux hours for 21 days for photolytic degradation. BDQ was stable under these conditions. All peaks were evaluated for their peak purity and found to be well separated from each other. The major DP obtained under oxidative stress condition was isolated, identified and characterized.

**Selection of stressor concentration.** Stress degradation study was started with minimum stressor concentration and under atmospheric conditions; the conditions and concentrations were increased to accelerate the degradation reactions. The hydrolytic degradation study was initially carried out using 0.01 N to 1 N HCl and NaOH separately. The maximum intensity of degradation products were observed in 1 N HCl at 80°C after 75 min though the degradation pattern did not change even at lower concentrations of acid (Figure 2C). While in alkaline condition, BDQ was partially stable as it showed a small peak without peak purity (possibilities of merging of several peaks) and ~18% degradation in harsher condition (2 N NaOH at 80°C after 2 h) but no major degradation product was obtained therefore it was not studied further (Figure 2D). In water, BDQ was highly stable as it was kept in water for 24 h at 80°C but there was no significant degradation observed in it (Figure 2E). For oxidative degradation H2O2 was used in 0.5–12% concentration; the degradation pattern was same in lower to higher concentration but maximum DP peak intensity was observed in 12% H2O2, therefore the chromatogram showing maximum DPs are selected for illustration (Figure 2F).

The stability chromatograms are shown in Figure 2; HPLC-PDA analysis of BDQ under acidic condition showed total eight eluting degradation products (Figure 2C), and major degradation product was eluted at retention time 26.48 ± 2.0 min was named as DP-A8.
and targeted for isolation, identification and characterization. Similarly, HPLC–PDA chromatogram of oxidative condition (Figure 2F) showed three degradation products. Major DP was observed at retention time 32.13 ± 0.5 min, named as DP-O3, targeted for isolation, identification and characterization. The stress degradation study and %degradation of BDQ is shown Table I.

**UPLC/ESI–MS result of BDQ and DPs**

The UPLC chromatogram for mixture of DPs formed under acid condition and oxidative media is shown in Figure 3. The UPLC/ESI–MS data showed that [M+H]+ ion for m/z of BDQ and DPs were obtained in following manner;

The m/z ion for BDQ was 555.0 + 2.0 amu; process related impurity 477.2, acid degradation products (m/z 244.2, 228.1, 154.2/186.1, 463.2 + 1, 543.11 + 2, 477.3/524.9 – 1, 477.2 + 1 and 555.16 + 2 amu for DP-A-1 to A-8, respectively); it is shown in Supplementary File S1 (A–H) and oxidative degradation products (543.15 + 2, 539.05 + 2, 555.0 + 2 and 571.0 + 2 for DP-O1, O2, API and DP-O3, respectively); it is shown in Supplementary File S2 (A–C). The analysis of UPLC/MS data is shown in Table II. The relevant structure elucidation discussion is explained in Discussion section.

**Isolation of major DPs by preparative HPLC**

The preparative chromatographic fractions were collected separately and combined to concentrate the fraction by removing organic solvent on rotavapor and washing with water to remove buffer used in mobile phase. The concentrated sample was again injected in system to check purity and the presence of any other co-eluting substance. The samples when showed purity >98.0% by HPLC were freeze dried to get the solid form. This final product was analyzed for
### Table I. Stress Degradation Behavior of BDQ in HPLC Chromatogram

| Stressor type     | Stressor conc. | Time     | DPs formed with RT | %Deg. of bulk drug | %Deg. in synthetic mixture |
|-------------------|----------------|----------|--------------------|---------------------|---------------------------|
| Acid              | 1 N HCl/80°C   | 75 min   | Peak-1: 2.91       | 74.82%              | 73.82%                    |
|                   |                |          | Peak-2: 3.18       |                     |                           |
|                   |                |          | Peak-3: 13.47      |                     |                           |
|                   |                |          | Peak-4: 25.55      |                     |                           |
|                   |                |          | Peak5: 26.48       |                     |                           |
|                   |                |          | Peak-6: 35.54      |                     |                           |
| Alkali            | 2 N NaOH/80°C  | 120 min  | –                  | 18.86%              | 19.10%                    |
| Neutral           | H2O/80°C       | 24 h     | –                  | 4.35%               | 4.78%                     |
| Oxidative         | 12% H2O2/80°C | 5 h.     | Peak1:3.690        | 63.93%              | 65.08%                    |
|                   |                |          | Peak2: 27.16       |                     |                           |
|                   |                |          | Peak3: 32.13       |                     |                           |
| Photolytic        | –              | 28 days  | –                  | 4.2%                | 4.5%                      |
| Dry thermal       | 80°C           | 28 days  | –                  | 3.8%                | 3.3%                      |
| Process related impurity | –         | –        | –                  | 5.99%              | 4.89%                     |

HCl (Hydrochloric acid) with concentration 1M (Molar) at 80 degree celcius was used as stressor to degrade bulk drug.

UPLC/ESI–MS and NMR \( ^{1}H, ^{13}C \) and APT. The confirmations of isolated DPs were done using RP-HPLC, UPLC and ESI–MS spectra and are shown in Supplementary Files S3, S4 and S5, respectively.

**NMR assignment for major DPs**

The proton, carbon and APT NMR for BDQ, isolated DP-A8 of acid condition and oxidized impurity of BDQ DP-O3 are shown in Supplementary Files S6 and S7 along with analysis table with the assignment of NMR, chemical shift to \( \alpha \)-position of atom number, respectively.

**IR analysis for major DPs**

The IR data for major DPs (DP-A8 for acid and DP-O3 for oxidative condition) were obtained to understand the functional groups present in isolated DPs against the BDQ bulk drug. The IR spectra of BDQ and DPs are shown in Supplementary File S8.
### Table II. UPLC/ESI–MS Data for BDQ and Its DPs

| Acid degradation products | DP No (Rt) | \( m/z \) | Chemical structure | Chemical formula | % of DPs |
|---------------------------|------------|------------|--------------------|------------------|----------|
| DP-A1 (1.273)             | 154.2(*154.14) | ![Chemical structure](image1) | \( \text{C}_{10}\text{H}_{18}\text{O} \) | 10.2%   |
| DP-A2 (1.273)             | 186.13(*186.10) | ![Chemical structure](image2) | \( \text{C}_{13}\text{H}_{14}\text{O} \) |         |
| DP-A3 (1.168)             | 228.1(*228.32) | ![Chemical structure](image3) | \( \text{C}_{15}\text{H}_{18}\text{NO}^- \) | 6.06%   |
| DP-A4 (1.091)             | 244.2(*244.34) | ![Chemical structure](image4) | \( \text{C}_{15}\text{H}_{20}\text{N}_2\text{O} \) | 7.15%   |
| DP-A5 (1.735)             | 463.2       | ![Chemical structure](image5) | \( \text{C}_{31}\text{H}_{31}\text{N}_2\text{O}_2^+ \) | 6.29%   |
| DP-A6                     | 477.2       | ![Chemical structure](image6) | \( \text{C}_{32}\text{H}_{33}\text{N}_2\text{O}_2^+ \) |         |
| DP-A7 (1.917)             | 524.9       | ![Chemical structure](image7) | \( \text{C}_{31}\text{H}_{28}\text{BrN}_2\text{O}^- \) | 5.99%   |

(Continued)
Table II. Continued

| Compound | Molecular Formula | Mass | Percent |
|----------|------------------|------|---------|
| DP-A8    | \( \text{C}_{31}\text{H}_{36}\text{BrN}_{2}\text{O}_{2} \) | 541.3 | 39.13   |
|          | \( 1.853 \)      | \( \leftrightarrow 543.11(\ast 542.50) \) |
| DP-8     | \( \text{C}_{32}\text{H}_{31}\text{BrN}_{2}\text{O}_{2} \) | 555.2 | 25.18   |
|          | \( 2.075 \)      | \( \leftrightarrow 557.16(\ast 555.52) \) |

Oxidative degradation products
Table II. Continued

| Compound | Formula | Molecular Mass | % Identity |
|----------|---------|----------------|------------|
| DP-O1    | C_{31}H_{28}BrN_{2}O_{2} | 539.0 → 541.0 | 9.28% |
| DP-O2    | C_{31}H_{30}BrN_{2}O_{2} | 541.2 → 543.2 (542) | 8.84% |
| DP-O3    | C_{31}H_{20}BrN_{2}O_{4} | 571.0 → 573.0 | 45.8% |
| API      | C_{32}H_{31}BrN_{2}O_{2} | 555.2 | 36.07% |

*Exact molecular mass
$\leftrightarrow m/z [M^+ + N]$
Validation of method
The method was validated as per ICH (Q2) (R1) guidelines (17). The validation parameters results are shown in Table III. Obtained results are in the range of criteria suggested by ICH for analytical method validation. Linearity of BDQ in developed stability indicating method is shown in Supplementary File S9.

Discussion
The reported chromatographic methods (7–10) and stability study method (6) have not reported the identification of DPs of BDQ so the aim of the present study was to develop RP-HPLC stability indicating method and characterization of 11 novel DPs and one process related impurity of BDQ, which is being reported for the first time in public domain.

Optimization of chromatographic conditions
Trials were taken using water and acetonitrile as a mobile phase but system suitability data were not satisfactory. Good system suitability was obtained in water: methanol (10:90%v/v) but the method was non-robust. Changing buffers having pH 3.5–8 could not analyze BDQ as the peak was not ionized in this pH range. Sodium dihydrogen ortho phosphate buffer (pH 2.5 ± 0.5 with OPA) and methanol in gradient elution analyzed BDQ peak with good system suitability and robustness was obtained. Buffer concentration did not affect the chromatogram, the minimum concentration of buffer (0.01 M) therefore was used for analysis. The developed method was tried with two different stationary phases; C18 and C8 column. The data obtained on C8 column were visibly and theoretically acceptable. BDQ is highly polar in nature and needs ionic pH adjustment to be retained on column; C8 is hydrophobic column so it retained well. BDQ and its DPs were well separated with good peak purity on this column. Methanol in HPLC system was preferred over other organic solvent because BDQ is very soluble in it while buffer was selected for ionic pH and good peak efficiency (Figure 2A and B showed the specificity and selectivity of method).

The phosphate buffer is not compatible with MS system, the UPLC/ESI–MS compatible method was therefore developed using formic acid in water (pH 2.7) and acetonitrile with 4-min run time. Low concentration of formic acid did not interfere in ionization and peaks with good intensity were obtained without any adduct formation.

BDQ degradation behavior
In UPLC/ESI–MS experiment, samples with high concentration were used to get good signals and better S/N (Signal to Noise) ratio. UPLC/ESI–MS chromatogram is shown in Figure 3(A) for acid DPs mixture sample and (B) for oxidative DPs mixture sample. UPLC/MS spectra showed peak asymmetry and tailing because high sample concentration to detect small fraction of DPs.

The UPLC/MS spectrometry showed four single eluting peaks [M+] ion without any co-elution of peak while two co-eluting peaks were also observed (S1). Acid stress sample of UPLC/ESI–MS data showed [M+] ion at m/z 244.2, 228.1, 154.2/186.1, 463.2, 543.11, 477.3/524.9, 477.2 and 557.16 for the peak eluting at 1.091, 1.168, 1.273 (Co-elution), 1.735, 1.853 (DP-A-8), 1.917 (Co-elution), 1.957 and 2.075 min (API), respectively. DPs were named as DP-A1 to DP-A8 in increasing order of their molecular weight, respectively (shown in (S1) and data are gathered in Table II. DP-A1 to DP-A8 was identified based on mass spectrometry while DP-A8 was isolated, characterized and structure was elucidated by 1H NMR and 13C NMR and APT.

The UPLC/MS data of oxidative condition showed two eluting peaks [M]+ without any co-elution while BDQ API was co-eluted (S2). Peak elution was obtained at retention time 2.178, 2.228, 2.378-2.434 (Co-elution) minutes for [M]+ ions at m/z 539.0 ↔ 541.0, 541.2 ↔ 543.2, 555.0 (API) and 571.0 ↔ 573.0 named as DP-O1, DP-O2, API and DP-O3, respectively. The DP-O3 was the major DP as it covered the maximum area in HPLC chromatogram of stress degradation study.

Figure 4. Suggested chemical structure of (A) DP-A8 and DP-O3.
**BDQ and DPs analysis by UPLC/ESI–MS**

With the assistance of UPLC/ESI–MS, structures were elucidated for DPs and degradation pathway is discussed in following sections.

**BDQ analysis (UPLC–ESI–MS)**

BDQ contains C–Br⁻ (C–X halogen) group that has two isotopes in natural abundance, which are ⁷⁹ Br and ⁸¹ Br in 50:50% extents; the molecule containing Br⁻ atom might show [M]⁺ ± 2 ion in UPLC/MS analysis with or without same intensity peaks. The UPLC/MS analysis of BDQ showed two m/z values 555.0 and 557.0 in positive ion spectrum matched with chemical formula of C₁₂H₁₃BrN₂O₂ at retention time 2.378 min. UPLC chromatogram for BDQ and process impurity, and ESI/MS spectrum are shown in Supplementary File S10. The NMR data for BDQ bulk drug are shown in Supplementary File S11. The results of ESI–MS are presented in Table I with DP quantity in %. The typical isotope peak pattern for bromine was shown in some fragment peaks.
Structure elucidation for DPs and process related impurity by UPLC/ESI-MS

Structure is elucidated for DPs and process related impurity based on the data obtained from UPLC/MS. Also, the mechanism of formation of DPs and degradation pathways are described in next section.

Acid degradation products

DP-A1 and DP-A2 (m/z 154.2/186.13)

The UPLC/ESI-MS data showed two co-eluting peaks at Rt 1.273 min in positive ion mode with [M] + 154.2 and 186.13 corresponds to chemical formula C_{10}H_{18}O and C_{13}H_{14}O for DP-A1 and DP-A2, respectively. Bromine is not present in DP-A1 and
DP-A2; as indicated by absence of isotope peak. DP-A1 and DP-A2 can be considered as fraction of BDQ formed due to bond breaking at C\textsuperscript{31} and C\textsuperscript{30}. The possible destruction of BDQ in DP-A1 and DP-A2 is shown in scheme-1.

**DP-A3 (m/z 228.1)**

DP-A3 was eluted at Rt 1.168 min with [M]+ 228.1, corresponds to molecular formula C\textsubscript{15}H\textsubscript{18}NO\textsuperscript{−}. Mechanism for formation of DP-A3 is again the result of bond breaking at C\textsuperscript{31} and C\textsuperscript{30} and absence of Br\textsuperscript{−} was confirmed by absence of isotope peak. Bond between C\textsuperscript{31} and C\textsuperscript{30} was broken by catalyst effect of acid and temperature; resulted in DP-A1, A2 and A3.

**DP-A4 (m/z 244.2)**

DP-A4 was corresponding to chemical formula C\textsubscript{15}H\textsubscript{20}N\textsubscript{2}O\textsuperscript{−} with [M]+ 244.2 without isotope peaks. DP-A4 was formed due to removal of benzene ring at C\textsuperscript{31} and naphthalene ring at C\textsuperscript{15}. Br\textsuperscript{−} and methylene (–CH\textsubscript{3}) groups were also removed in acid catalyzed reaction.

**DP-A5 (m/z 464.2)**

DP-A5 was observed at Rt 1.735 min with [M]+ 463.2 and 464.2. M/z value corresponds with chemical formula C\textsubscript{31}H\textsubscript{31}N\textsubscript{2}O\textsuperscript{−}; structure of DP-A5 showed removal of methylene (–CH\textsubscript{3}) group at C\textsuperscript{2} and Br\textsuperscript{−} at C\textsuperscript{21}.

**DP-A6 (m/z 477.2)**

The m/z of 477.2 and 478.2 showed H\textsuperscript{+} ion interchange in DP-A6 at Rt 1.917 match up with chemical formula C\textsubscript{32}H\textsubscript{33}N\textsubscript{2}O\textsuperscript{−}; DP-A6 showed removal of Br atom at C\textsuperscript{21} and H\textsuperscript{+} ion interchange at N\textsuperscript{27}.

**DP-A7 (m/z 524.9)**

DP-A7 was observed at Rt 1.917 along with DP-A6 with [M]+ 524.9 ↔ 523.1 indicated a lone pair of electron included in structure with high peak intensity of m/z of 524.9. Chemical formula for DP-A7 was observed to be C\textsubscript{31}H\textsubscript{28}BrN\textsubscript{2}O. DP-A5, A6, A7 and A8 are formed due to removal of single group from BDQ parent molecule.

**DP-A8 (m/z 541.3)**

DP-A8 was formed in minor quantity while major DP observed was DP-A8. Chemical formula was matched up with C\textsubscript{31}H\textsubscript{30}BrN\textsubscript{2}O\textsuperscript{−} and [M]+ 541.3 ↔ 543.11 indicated presence of Br atom at C\textsuperscript{31} as isotope peaks were observed. BDQ API MS spectra showed m/z of 555.1 ↔ 557.1; M+2 observed due to presence of Br atom isopes. Major DP-A8 was characterized by NMR (\textsuperscript{1}H, \textsuperscript{13}C and APT).

**Oxidative degradation products**

**DP-O1 (m/z 539.05)**

The DP-O1 was eluted at Rt 2.228 min with [M]+ ion at m/z 539.05, which is corresponding to chemical formula C\textsubscript{31}H\textsubscript{28}BrN\textsubscript{2}O\textsubscript{2}, the intense peak in positive mode showed [M]+ + 2 ion with m/z 539.0 and 541.0 indicates presence of Br\textsuperscript{−} atom. DP-O1 could be formed from DP-O2 as a parent molecule with H\textsuperscript{+} atom loss.

**DP-O2 (m/z 543.15)**

DP-O2 was eluted at Rt 2.178 min with [M]+ + 2 ion at m/z 541.1 and 543.1 indicated presence of Br\textsuperscript{−} atom and matches with chemical
formula C₃₁H₂₈BrN₂O₄, the possible reason for formation of DP-O₂ can be the demethylation of BDQ API.

**DP-O₃ (m/z 571.0)**

The [M⁺]+ 2 ion was obtained at m/z 571.0, which is corresponding to molecular formula of C₃₁H₂₈BrN₂O₄. This indicated the oxidation of BDQ at different position. Demethylation of C₃₈ left alkohol group at position 11 (confirmed in proton NMR). Addition of oxygen at C₂₀ and C₂₁ position of benzene ring was confirmed by ¹H NMR. Structure elucidation by NMR is explained briefly in next subsection.

**DP-O₃ structure elucidation by NMR (¹H, ¹³C and APT)**

The UPLC–MS data showed [M]⁺ ion at m/z of 571.0 for DP-O₃, which is 16 amu higher than BDQ, shows addition of oxide (O⁺) in BDQ. The changes were observed in aromatic region and aliphatic region of DP-O₃ ¹H NMR (shown in Scheme-2); demethylation changed the –O–CH₃ group to –OH group, this was confirmed by chemical shift (δ) at 11.234; due to high strong electronegative field generated on –H (Proton) by –O (Oxygen atom). This chemical shift belongs to ester, carboxyl or aldehyde group but presence of alcohol attached to carbon mimics aldehyde group. Suggested chemical composition and molecular weight for this impurity (Oⁿ⁻) were C₃₁H₂₉BrN₂O₄ and 573.49 gm/mol, respectively. Expected changes in proton NMR of DP-O₃ were compared with BDQ API proton NMR and this way it became confirmative way to elucidate the structure. Solvent peaks were observed at 2.5–2.6 ppm for DMSO.

The addition of –OH group to benzene ring at α position 21 and 20, confirmed by chemical shift (δ) of 9.404 and 9.383 ppm in ¹H NMR. Addition of –OH group to quinoline or naphthalene ring lead to chemical shift around 150–155 ppm in ¹³C NMR while absence of this chemical shift confirms –OH group addition at benzene ring. (Elucidated structure is shown in Figure 4).

**Process related impurity**

Process related impurity was found in BDQ at retention time 1.939 min in UPLC–MS spectrum, the [M⁺] ion at m/z was 477.32 amu that is 79.18 amu less than BDQ bulk drug indicates absence of Bromine atom in process related impurity. The impurity was found in 0.6% quantity, as the C–Br⁻ bond is weak ionic bond, BDQ itself lost –HBr and generated impurity. The ESI–MS spectrum is shown in S10 and mechanism for formation of impurity is explained in previous section as a formation of DP-A6 in acid condition. The structure of process related impurity is shown in scheme-1 as a DP-A6; DP-A6 formed in acid condition is relatable with process related impurity although S1 (G) showed presence of process related impurity separately.

**Mechanism for formation of DPs**

**DPs formed under acid condition**

Formation of all the DPs of acid degradation of BDQ can be explained by cleavage of C–C bonds at different position at high temperature and demethylation by acid. DP-A1 and A2 are the fractions generated from DP-A3. Formation of DP-A3 can be explained as cleavage of bond between C₁⁰ and C₁⁴, this cleavage formed DP-A₃; DP-A₃ undergoes demethylation and formed DP-A2. Under the effect of temperature and acid, a benzene ring was lost from naphthalene ring and DP-A₁ was formed. A loss of naphthalene ring at C₁⁵ and benzene ring at C₁¹ generated DP-A₄.

**Conclusion**

Stability studies for BDQ- a novel anti TB drug were performed as per ICH guidelines. BDQ sufficiently degraded in acidic and oxidative medium and formed 8 degradation products in acid media. Although three DPs were formed in oxidative medium, two major DPs were isolated and characterized by sophisticated instruments. BDQ was stable under light, neutral and thermal condition. The DPs formed were analyzed by LC/ESI–MS, whereas major DPs were well separated in HPLC and isolated by preparative HPLC with good purity scale. These DPs were analyzed and characterized by LC/ESI–MS and NMR (¹H, ¹³C and APT). The newly formed degradation products are reported for the first time. The acid DP-A₈ was named as 6-bromo-3[(1R,2S)-4-(dimethylamino)-2-hydroxy-2-(naphthen-1-yl)-1-phentltbutyl]-2-hydroxyquinolin-1-ium. BDQ was stable under light, neutral and thermal condition. The DPs formed were analyzed by LC/ESI–MS, whereas major DPs were well separated in HPLC and isolated by preparative HPLC with good purity scale. These DPs were analyzed and characterized by LC/ESI–MS and NMR (¹H, ¹³C and APT). The newly formed degradation products are reported for the first time. The acid DP-A₈ was named as 6-bromo-3[(1R,2S)-4-(dimethylamino)-2-hydroxy-2-(naphthen-1-yl)-1-phentltbutyl]-2-hydroxyquinolin-1-ium. BDQ was stable under light, neutral and thermal condition. The DPs formed were analyzed by LC/ESI–MS, whereas major DPs were well separated in HPLC and isolated by preparative HPLC with good purity scale. These DPs were analyzed and characterized by LC/ESI–MS and NMR (¹H, ¹³C and APT). The newly formed degradation products are reported for the first time.
(6-bromo-2-hydroxyquinolin-3-yl)-4-((dimethylamino)-2-hydroxy-2-(naphtlen-1-yl) butyl] benzene-1,4-diol and matched with chemical formula C_{31}H_{29}BrN_{2}O_{4}. The process related impurity was also identified and named as 3-((IR, 2S)-4-(dimethylamino)-2-hydroxy-2-(naphtlen-1-yl))-1-phenylbutyl-2-methoxyquinolin-1-ium and matched with chemical formula C_{32}H_{33}N_{2}O_{2}.

The validated method was applied for stability study of BDQ; and the results suggest that during the formulation process or for storage of BDQ, the acidic media/solvent should be avoided. The oxidizing agent can react with BDQ to oxidize it therefore it should be kept away from oxidizing agent. The process related impurity and 11 degradation products are reported for the first time in literature with this study.

Supplementary data

Supplementary data are available at Journal of Chromatographic Science online.

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