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Quantitative Study of Impurities in Bedaquiline Fumarate: Identification and Characterization of Its Three Degradation Products Using HPLC, LC/ESI-MS, and NMR Analyses

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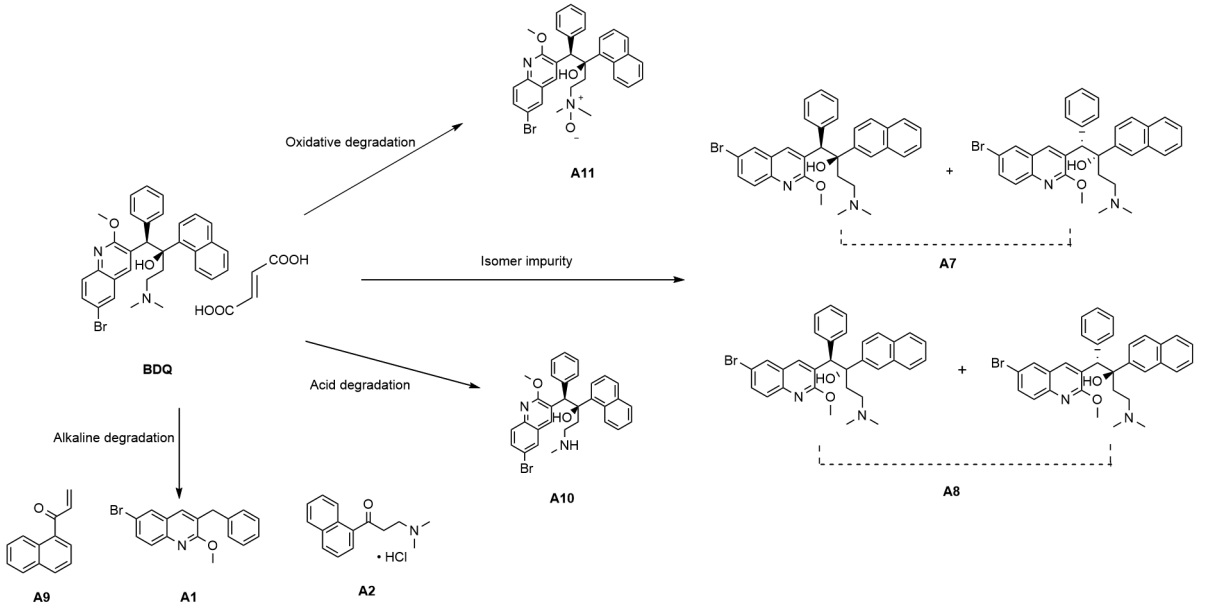

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Abstract

The study aimed to establish a high-performance liquid chromatography (HPLC) method for the quantitative analysis of the related substances of bedaquiline fumarate. Nuclear magnetic resonance and mass spectrometry were used for characterization and assay. A chromatographic method was used for separation. The conditions used were: gradient elution system composed of methanol 0.01mol/L KH_2PO_4 and 0.01 mol/L K_2HPO_4 (pH = 4.1) with a flow rate of 1 mL/min, at 224 nm as the detection wavelength. In this study, three degradation products of bedaquiline fumarate have been disclosed for the first time. The related impurities and degradation products of the drug were well separated. The method provided linear responses within the concentration range, which varied from 0.20 to 10.08 µg/mL with limits of detection of 0.10 µg/mL and limits of quantification of 0.20 µg/mL. The mean percent recovery varied between 91.64 and 105.89%. The method was validated for other parameters such as specificity, stability, and robustness. This method was validated and worked well for the impurity studies and quality control analysis of the laboratory-prepared samples of bedaquiline fumarate.

Keywords

- bedaquiline fumarate
- HPLC
- related substances
- method validation

Introduction

Bedaquiline fumarate (BDQ), chemically named (1*R*, 2*S*)-1-(6-bromo-2-methoxy-3-quinolinyl)-4- (dimethylamino)-2-(1-naphthalenyl)-1-phenyl-2-butanol fumarate, is a diarylquinoline antimycobacterial drug used for the treatment of multi-drug-resistant tuberculosis in adults.[1–3] BDQ was developed by Janssen Therapeutics, approved by the Food and Drug Administration in 2012, and has been marketed in China since 2016.[4] BDQ functions on ATP synthase of *Mycobacterium tuberculosis*, and inhibits ATP production by binding to the F_0 region of ATP synthetase, resulting in the death of *Mycobacterium tuberculosis*.[5–7]

The current synthetic route of BDQ is shown in ►**Fig. 1**.[8] However, analysis methods of BDQ have not been included in the pharmacopeias of any countries, and reported only in a few literatures, such as separation and characterization of its degradation impurities,[9,10] analysis of its related substances by high-performance liquid chromatography (HPLC),[11] separation of its diastereomers by reversed-phase-HPLC,[12] and determination of BDQ in human serum by liquid chromatography-tandem mass spectrometry (LC-MS/MS).[13] Therefore, studies of BDQ quality control, especially the study of BDQ-related substances, could help meet the production goals, and are also important and necessary for further development of the drug.

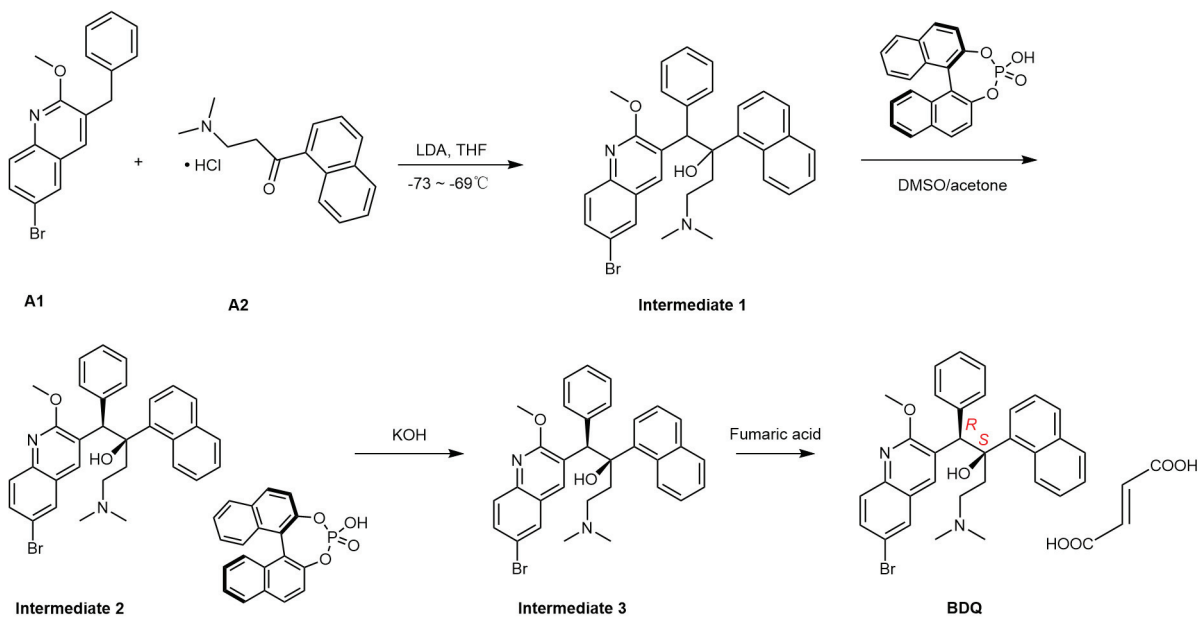


Fig. 1 The synthetic route of BDQ.

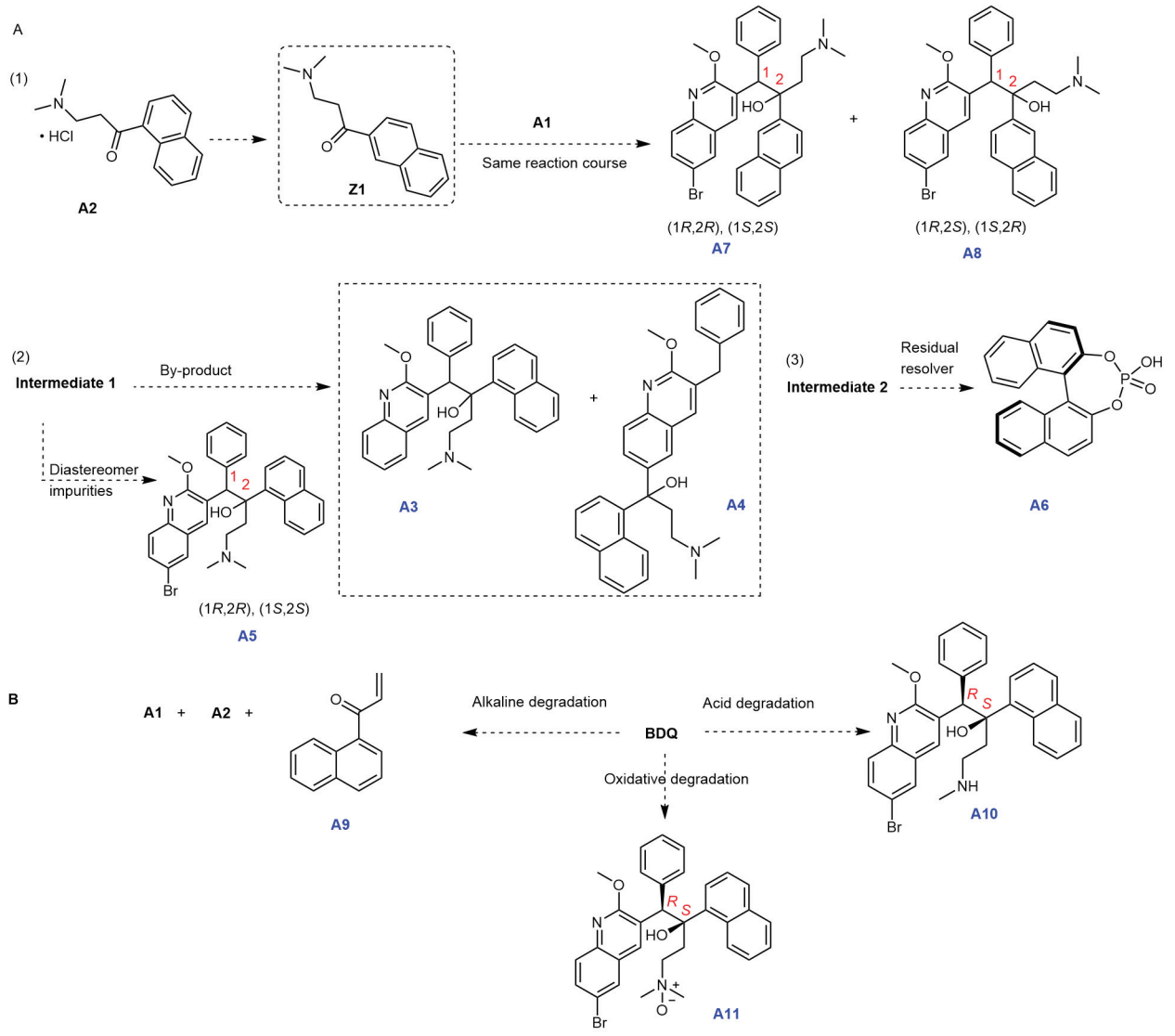


Fig. 2 Proposed mechanistic pathway for the formation of (a) process-related impurities, and (b) degradation impurities of BDQ.

Based on the reported synthesis route[8] and the stress forcing experiments, the impurity profiles of BDQ in bulk drugs as well as 11 potential impurities were analyzed (►**Fig. 2**). These included the residuals of starting materials (**A1**, **A2**), by-products (**A3**, **A4**, **A6**), diastereomer impurities (**A7**, **A8**), and isomer impurities (**A5**), as well as the degradation products (**A9**, **A10**, **A11**). Among them, **A9**, **A10**, and **A11** were characterized and disclosed for the first time. Furthermore, the present research also developed a reversed-phase liquid chromatography (RP-LC) method to simultaneously determine BDQ and its related substances. The method was validated and applied for quality studies of BDQ drug samples.

Materials and Methods

Chemicals and Reagents

Samples of BDQ (purity 99.09%), **A3** (purity 95.68%), **A4** (purity 97.98%), **A5** (purity 99.57%), **A7** (purity 97.91%), **A8** (purity 91.11%), **A9** (purity 84.39%), **A10** (purity 98.05%), and **A11** (purity 93.42%) were synthesized by the Novel Technology Center of Pharmaceutical Chemistry, Shanghai Institute of Pharmaceutical Industry Co., Ltd. (Shanghai, China). **A1** (purity 99.61%) and **A2** (purity 99.71%) were purchased from Shanghai SynFarm Pharmaceutical Technology Co., Ltd. (Shanghai, China). **A6** (purity 99.01%) was purchased from Shanghai Darui Fine Chemicals Co., Ltd. (Shanghai, China). HPLC-grade methanol and acetonitrile were purchased from CINC High Purity Solvents Co., Ltd. (Shanghai, China). Other analytical grade reagents were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Water was purified using a Millipore Milli Q-Plus system (Millipore Corp., Billerica, Massachusetts, United States).

Synthesis of Impurities

Synthesis of Compound A3

Intermediate **3** (5.0 g) was dissolved in tetrahydrofuran (THF) (50 mL), then Pd/C (5%, 6.0 g) was added under N_2. The mixture was heated to 60°C and hydrogen was passed into the mixture for 8 hours. After filtration and concentration,

the crude product was chromatographed on silica gel eluting with methylene dichloride (DCM):methanol (MeOH) = 20:1 to give **A3** (2.6 g, 60.7%) as a white solid.

Synthesis of Compound A4

n-BuLi (in *n*-hexane, 20 mL, 2 mol/L) was diluted with THF (12 mL), and cooled to −76°C under N_2. A solution of **A1** (10.0 g) in THF (24 mL) was added. The mixture was stirred for 1 hour, followed by addition of a solution of **A2** (8.0 g) in THF (12 mL). The reaction was performed at −70°C to −75°C for 5 hours, and quenched with acetic acid. The organic phase was separated and concentrated. The crude product was chromatographed on silica gel (DCM:MeOH = 20:1) to give **A4** (1.3 g, 8.9%) as a white solid.

Synthesis of Compounds A7 and A8

Lithium diisopropylamide (in THF, 20 mL, 2 mol/L) was diluted with THF (12 mL), and cooled to −70°C. A solution of **A1** (10.0 g) in THF (24 mL) was added. The mixture was stirred for 1 hour, followed by addition of a solution of **Z1** (8.0 g) in THF (12 mL). The reaction was stirred for 2 hours and quenched with acetic acid. The organic phase was separated and concentrated to obtain a residue, which was purified by column chromatography (DCM:MeOH = 20:1) to give **A7** (1.1 g, 10.1%) and **A8** (1.8 g, 16.2%).

Synthesis of Compound A9

A2 (5.0 g), dissolved in water, and NaOH aqueous solution (20 mL, 2 mol/L) was stirred at room temperature for 2 hours. The pH of the aqueous solution was adjusted to pH 7 with concentrated hydrochloric acid, followed by extraction with ethyl acetate. The organic phase was separated and concentrated. The crude product was purified by column chromatography (DCM:MeOH = 20:1) to obtain **A9** (150 mg, 3.7%).

Synthesis of Compound A10

Intermediate **3** (3.0 g) was dissolved in acetonitrile (100 mL). Then, concentrated hydrochloric acid (12 mL) was added. The mixture was heated to 60°C, and stirred for 2 days. The crude product was chromatographed on silica gel (DCM:MeOH = 20:1) to give **A10** (0.4 g, 14.7%).

Synthesis of Impurity A11

Intermediate **3** (3.0 g) was added to a solution consisting of acetonitrile and hydrogen peroxide (H_2O_2) aqueous solution (100 mL, v:v = 50%). The reaction mixture was heated to 60°C, stirred for 24 hours, and evaporated. The crude product was purified by column chromatography (DCM:MeOH = 50:1) to give **A11** (84 mg, 2.7%). The synthesis scheme is represented in ►**Fig. 3**.

Structure Elucidation

Nuclear magnetic resonance (NMR) and mass spectrometry (MS) spectra data were used for structure analysis. NMR spectra were obtained using a Bruker 400 MHz spectrometer (AC, AVANCE, Bruker Daltonics GmbH, Fäl-landen, Switzerland) with chloroform-*d* ($CDCl_3$) as a solvent, and tetramethylsilane (TMS) as an internal standard. Qualitative analysis was performed by LC-MS using an Agilent 6420 Triple Quad LC-MSD equipped with an ESI (electrospray ionization) source, which was used in positive ionization mode in full scan and product scan. The spray voltage was set

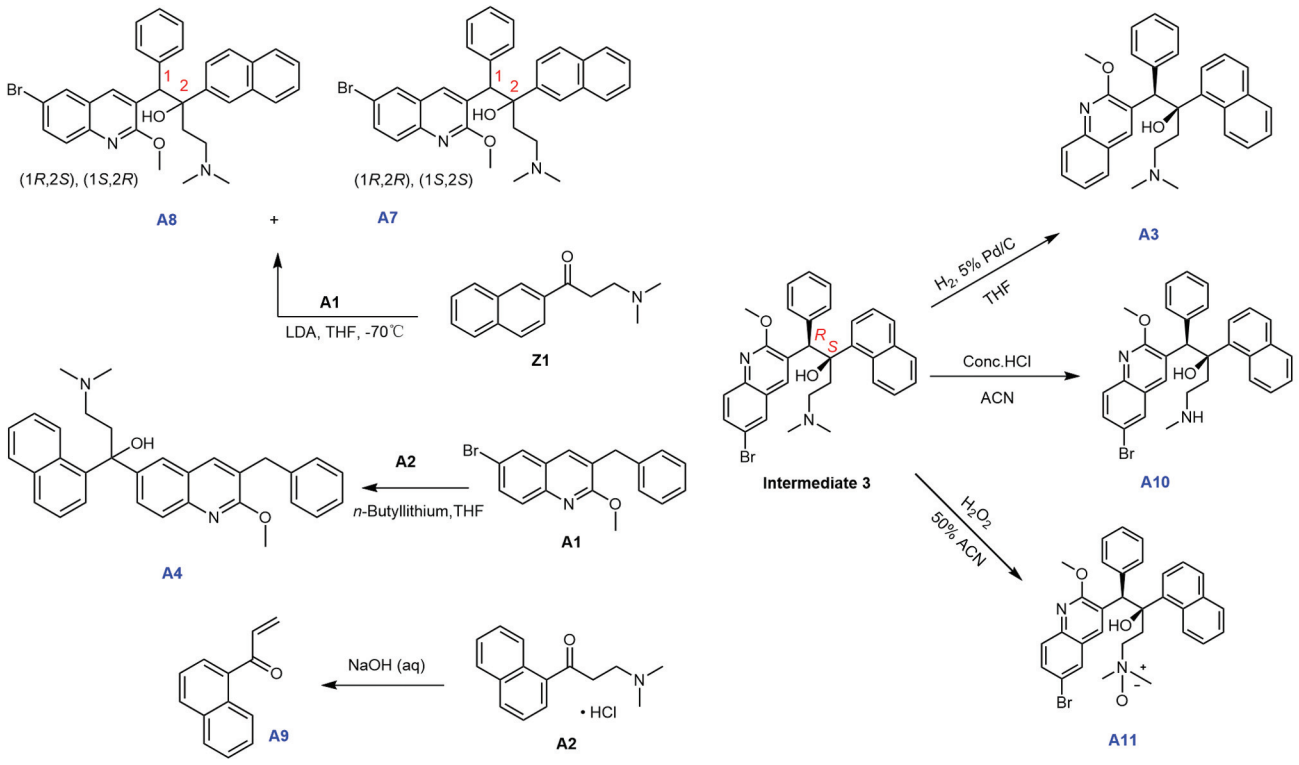


Fig. 3 Proposed synthesis pathway of the impurities.

at 4 kV, and the capillary temperature was maintained at 350°C. Nitrogen was used for nebulization. During full-scan MS analysis, the spectra were recorded in the range of m/z 100–1,100. The fragmentor was set at 135 eV. Analytical data were acquired using the Masshunter software.

Quantitative Analysis of BDQ and Its Impurities

Analyses were conducted using Waters series system equipped with a quaternary pump, a vacuum degasser, an autosampler, a column heater-cooler, and a DAD detector. The data were acquired and processed using the Chromeleon 7 software. Chromatographic separation was achieved on a Waters SunFire C18 column (250 mm × 4.6 mm, 5 µm) (Waters, United States). Water (containing 0.01 mol/L KH_2PO_4 and 0.01 mol/L K_2HPO_4 of pH 4.1)–methanol was used as the mobile phase to control **A1**–**A11**, and BDQ with a gradient program: time (t)/% methanol: 0/40, 10/70, 40/75, 55/90, 65/90, 66/40, 75/40. The flow rate was set at 1.0 mL/min. The column temperature was maintained at 30°C, and the ultraviolet (UV) detection wavelength was set to 224 nm. The sample injection volume was 5 µL.

Sample Preparation

The diluent is a solution of acetonitrile, water, and trifluoroacetic acid, with a volume ratio of 50:50:0.1. For the related substances' assay, the test concentrations of BDQ were 0.5 mg/mL. The system suitability solution was prepared by dissolving BDQ and 11 impurities in the diluent to reach the concentration 0.5 mg/mL for BDQ and 0.5 µg/mL for each impurity.

Forced degradation solutions were analyzed under different stress conditions as per ICH (International Council of Harmonization) requirements. For oxidation degradation, BDQ (1.0 mg/mL) was dissolved in acetonitrile:water (v:v = 1:1), then 10% H_2O_2 (aq) was added in the ratio of 5:1 (v:v). The mixture was kept at 60°C for 48 hours. For basic degradation, BDQ (1.0 mg/mL) was dissolved in acetonitrile:water (v:v = 1:1), then 0.1 mol/L NaOH was added in the ratio of 5:1 (v:v). The mixture was kept at room temperature for 1 hour, and then neutralized with 0.1 mol/L HCl. For acidic degradation, BDQ (1.0 mg/mL) was dissolved in acetonitrile:water (v:v = 1:1), then 1 mol/L HCl was added in the ratio of 5:1 (v:v). The mixture was stored at 60°C for 12 hours, and then neutralized with 1 mol/L NaOH. For thermal degradation, a solution of 1.0 mg/mL BDQ in acetonitrile was kept at 80°C for 48 hours. For photolytic degradation, a solution of 1.0 mg/mL BDQ in acetonitrile was treated with the illumination of 4,500 lux ± 500 lux and kept at room temperature for 7 days.

Method Validation for Quantitative Analysis

The developed stability-indicating LC method was validated as per ICH guidelines with respect to limits of detection (LODs) and limits of quantification (LOQs), linearity and range, stability, precision, accuracy, and robustness. The linearity test was performed at concentrations ranging from LOQ to 10.0 µg/mL. The precision was evaluated as repeatability and intermediate precision. The accuracy was evaluated at three concentration levels (50, 100, and 150% of the impurities' limit concentration). The percentage recoveries were calculated from the calibration curve. The robustness was evaluated by deliberately altering chromatographic conditions, including batches of C18 column, flow rate, column temperature, wavelength, and mobile phase. The resolution between BDQ and its impurities was assessed.

Results

Structural Elucidation of A9, A10, and A11

Analysis of LC-MS full scan of the degradation solution showed that the molecular information of **A9**, **A10**, and **A11** was m/z 183.10 $[M+H]^+$, 541.09 $[M+H]^+$, and 571.16 $[M+H]^+$, respectively. As shown in ►**Table 1**, data of ^1H NMR and ^{13}C NMR confirmed the structure of three novel degradation products as 1-(1-naphthalenyl)-2-propen-1-one for **A9**, (1*R*,2*S*)-1-(6-bromo-2-methoxyquinolin-3-yl)-4-(methylamino)-2-naphthalen-1-yl-1-phenylbutan-2-ol for **A10**, and (1*R*,2*S*)-1-(6-bromo-2-methoxyquinolin-3-yl)-4-(methylamino)-2-naphthalen-1-yl-1-phenylbutan-2-ol *N*-oxide for **A11**.

Optimization of Chromatographic Conditions for Quantitative Analysis

A strong chromogenic group has been found in the structure of BDQ and the related substances, which is suitable for a UV detector. The detection wavelength of 224 nm was selected as suitable by taking into account the absorption intensity. Since pK_a values of BDQ and the related substances are different, and most of them are weakly alkaline and sensitive to pH changes, thus, an acidic mobile phase containing salt was selected to provide a buffer environment. Besides, time gradient with mobile phase, pH, and organic modifiers have been studied to acquire a better resolution and peak shape among the impurities. Based on these results, a HPLC method was established (►**Fig. 4**).

Results of the Forced Degradation

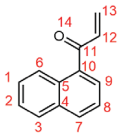
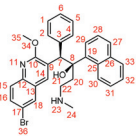
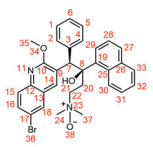
Chromatograms of the stress studies are indicated in ►**Fig. 5**. Our data showed that BDQ was stable under stress conditions such as photolytic and thermal conditions. Under acidic conditions, **A10** was detected; under basic conditions, **A9** was detected; and under oxidation conditions, **A11** was detected. DAD analysis suggested a good peak purity of BDQ among all the samples. The samples had a mass balance between 90 and 110% of BDQ. The assay result of BDQ was unaffected by the presence of the related compounds, confirming its stability during the LC method.

Results of Method Validation

The determination method of the impurities of BDQ was validated systematically and separately. The results of linearity, LOD, LOQ, precision, stability, and accuracy are summarized in ►**Table 2**.

Our data suggested that LODs of BDQ and **A1**–**A11** were approximately 0.1 µg/mL, and their LOQs were approximately 0.2 µg/mL. The test for linearity of BDQ and its impurities was completed by injecting a series of solutions with concentrations of 0.04, 0.08, 0.1, 0.4, 0.8, and 2% of the sample, and the peak

Table 1 NMR assignments for A9, A10, and A11

A9			A10			A11		
								
Position	**δ C (ppm)**	**δ H (ppm)**	**Position**	**δ C (ppm)**	**δ H (ppm)**	**Position**	**δ C (ppm)**	**δ H (ppm)**
1	134.4	7.489–7.593 (m, 3H)	1	128.4	6.881–6.914 (m, 3H)	1	128.7	7.219–7.243 (m, 2H)
2	126.2	7.489–7.593 (m, 3H)	2	129.8	6.881–6.914 (m, 3H)	2	129.8	6.867–6.884 (m, 3H)
3	129.8	7.719 (dd, J = 3.2, 7.2 Hz, 1H)	3	139.6	–	3	139.5	–
4	130.8	–	4	129.9	6.848–6.852 (m, 1H)	4	129.8	6.867–6.884 (m, 3H)
5	128.9	–	5	128.6	6.881–6.914 (m, 3H)	5	128.6	7.219–7.243 (m, 2H)
6	125.4	8.326 (d, J = 7.6 Hz, 1H)	6	128.1	7.581–7.622 (m, 2H)	6	127.9	7.584–7.705 (m, 4H)
7	134.3	7.977 (d, J = 8.4 Hz, 1H)	7	54.4	5.820 (s, 1H)	7	50.4	5.870 (s, 1H)
8	127.0	7.489–7.593 (m, 3H)	8	80.3	–	8	82.8	–
9	129.7	7.883–7.907 (m, 1H)	9	124.6	–	9	124.8	–
10	135.7	–	10	163.4	–	10	163.3	–
11	190.9	–	12	145.7	–	12	145.7	–
12	132.7	6.921 (dd, J = 10.4, 17.6 Hz, 1H)	13	127.6	–	13	127.6	–
13	128.4	6.245 (dd, J = 1.2, 17.6 Hz, 1H) 6.036 (dd, J = 1.2, 17.6 Hz, 1H)	14	132.3	7.874 (d, J = 8.0 Hz, 1H)	14	134.1	7.997 (d, J = 2.0 Hz, 1H)
–	–	–	15	127.3	7.667 (d, J = 8.0 Hz, 1H)	15	127.3	7.858 (d, J = 7.6 Hz, 1H)
–	–	–	16	132.4	8.082–8.126 (m, 2H)	16	132.4	8.555 (d, J = 8.8 Hz, 1H)
–	–	–	17	120.0	–	17	120.1	–
–	–	–	18	130.0	8.723 (d, J = 8.4 Hz, 1H)	18	130.0	8.965 (s, 1H)
–	–	–	19	140.2	–	19	140.2	–
–	–	–	20	43.8	3.627–3.669 (m, 1H) 2.750 (m, 4H)	20	33.8	2.870–3.132 (m, 9H)
–	–	–	21	–	–	21	–	–
–	–	–	22	50.3	3.403–3.433 (m, 1H) 2.014 (m, 1H)	22	66.4	2.446–2.507 (m, 1H) 2.870–3.132 (m, 9H)
–	–	–	23	–	10.300 (s, br, 1H)	23	–	–
–	–	–	24	34.2	2.490 (s, 3H)	24	58.2	2.870–3.132 (m, 9H)
–	–	–	25	134.8	–	25	134.1	–
–	–	–	26	134.7	–	26	134.2	–
–	–	–	27	128.0	7.284–7.334 (m, 2H)	27	128.0	7.412 (t, J = 7.6 Hz, 1H)
–	–	–	28	125.7	7.581–7.622 (m, 2H)	28	125.6	7.584–7.705 (m, 4H)
–	–	–	29	126.9	6.596–6.620 (m, 1H)	29	126.8	6.867–6.884 (m, 3H)
–	–	–	30	125.3	8.082–8.126 (m, 2H)	30	125.2	8.076 (dd, J = 0.8, 7.6 Hz, 1H)
–	–	–	31	126.1	7.284–7.334 (m, 2H)	31	126.4	7.584–7.705 (m, 4H)
–	–	–	32	125.9	7.334–7.373 (m, 1H)	32	125.9	7.584–7.705 (m, 4H)
–	–	–	33	127.7	6.997–7.017 (m, 1H)	33	127.9	7.303 (t, J = 7.6 Hz, 1H)
–	–	–	35	55.2	2.750 (m, 4H)	35	54.4	4.233 (s, 3H)
–	–	–	37	–	–	37	58.2	2.870–3.132 (m, 9H)

area was recorded. As shown in ►**Table 2**, strong linear relationships were obtained with r values ranging between 0.9996 and 0.9999. The precision study (%RSD) values of **A1**–**A11** were all below 5.0%, confirming the high level of precision of the developed LC method. Besides, the stability result showed a 72-hour stability of the system suitability solution at room temperature. The accuracy of the method was evaluated in triplicate at three different concentration levels. As shown in ►**Table 3**, mean recoveries of all the impurities ranged from 90.0 to 110.0%. Through deliberately altering chromatographic conditions (e.g., batches of C18 column, detection wavelength, flow rate, pH, and column temperature), resolution between impurities and BDQ was always greater than 1.5, illustrating the robustness of the method.

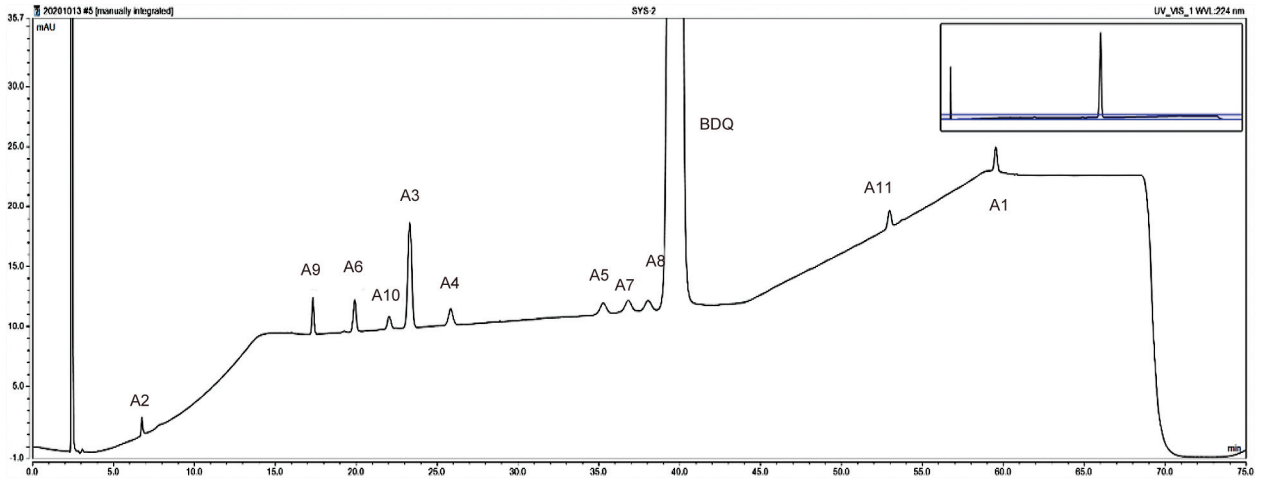


Fig. 4 Typical chromatograms of the separation of **A1–A11**, and BDQ.

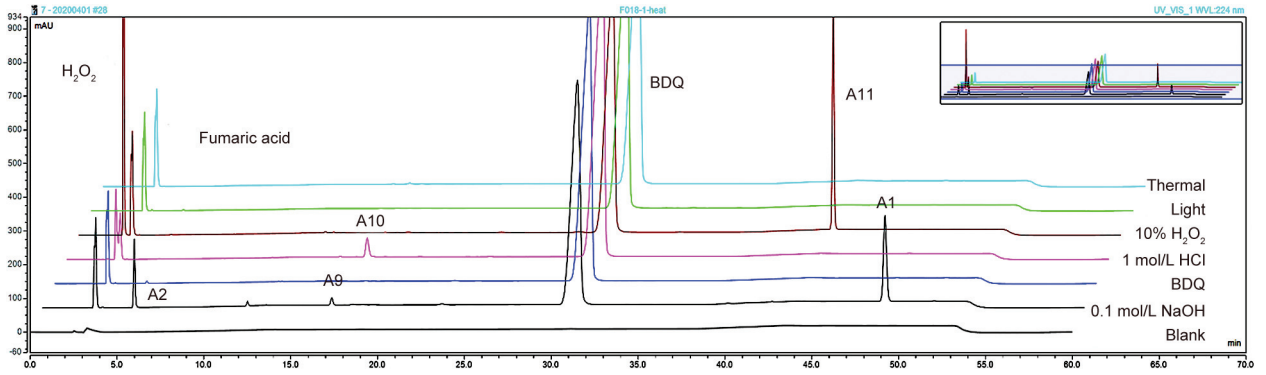


Fig. 5 Typical chromatograms of the stress studies.

Table 2 Summary of method validation of A1–A11 and BDQ

Analytes	Regression equation	Linear range (μg/mL)	*r*	LOD (μg/mL)	LOQ (μg/mL)
BDQ	$A = 0.5324c$-0.0049	0.20–10.08	0.9996	0.10	0.20
A2	$A = 02378c$-0.0173	0.20–10.01	0.9998	0.10	0.20
A9	$A = 0.4539c$-0.0129	0.20–10.07	0.9998	0.10	0.20
A6	$A = 0.8519c$-0.0270	0.20–10.08	0.9999	0.10	0.20
A10	$A = 0.4977c$-0.0130	0.20–10.02	0.9998	0.10	0.20
A3	$A = 0.6047c$-0.0193	0.20–10.00	0.9999	0.10	0.20
A4	$A = 0.5716c$-0.0188	0.20–10.06	0.9998	0.10	0.20
A5	$A = 0.5392c$-0.0234	0.20–10.02	0.9998	0.10	0.20
A7	$A = 0.5854c$-0.0296	0.20–10.04	0.9998	0.10	0.20
A8	$A = 0.5621c$-0.0272	0.20–10.05	0.9998	0.10	0.20
A11	$A = 0.4669c$-0.0159	0.20–10.02	0.9999	0.10	0.20
A1	$A = 0.5212c$-0.0252	0.20–10.00	0.9996	0.10	0.20

Abbreviations: BDQ, bedaquiline fumarate; LOD, limit of detection; LOQ, limit of quantification.

Table 3 Summary of method validation of A1–A11 and BDQ

Analytes	Precision (RSD%)		Stability (RSD%)	Accuracy (%)		
	Intra-day	Inter-day		50%	100%	150%
BDQ	–	–	0.40	–	–	–
A2	0.81	1.44	2.57	95.09	98.26	98.75
A9	0.70	0.70	2.00	102.47	102.67	102.67
A6	0.81	0.70	1.55	104.87	96.08	95.39
A10	1.22	2.06	4.25	104.35	105.76	104.44
A3	2.24	1.97	0.92	105.83	104.43	100.57
A4	1.26	1.32	1.15	102.49	101.48	99.51
A5	1.26	1.68	1.26	99.84	94.13	92.48
A7	1.23	2.10	1.93	107.09	94.08	93.08
A8	1.07	1.66	2.51	91.64	101.33	104.41
A11	2.66	1.48	1.86	94.88	101.73	102.72
A1	0.91	1.27	1.38	105.89	104.43	101.65

Abbreviation: RSD, relative standard deviation.

Application

The detection of related substances was performed on different batches of intermediates and the active pharmaceutical ingredient (API). Our data showed that the detected amount of impurity **A3** in BDQ sample is 0.16% (►**Table 4**), lower than that of impurity **A3** in reference listed drug. Thus, the quality of this batch of API is qualified.

Discussion

In this study, the degradation mechanism of BDQ was postulated based on structural elucidation of degradation impurities and liquid-phase result of degradation solution. The hydrogen on the benzyl group of BDQ is active, and it is easily captured by free radicals under alkaline conditions, resulting in autooxidation and then dehydration to form **A1** and **A2** impurities. On the tertiary amine group of **A2**, there are lone pair electrons that capture reactive oxygen to form nitrogen oxides, followed by cope elimination reaction, and **A9** was obtained. The acid degradation mechanism of BDQ mainly depends on "acid-catalyzed autoxidation." BDQ possesses lone pair electrons on the nitrogen atoms of tertiary amines, which capture reactive oxygen to form nitrogen–oxygen complexes under acidic conditions, and then dealkylation reaction to produce **A10** under the catalysis of an acid. In oxidative degradation experiments, lone pair electrons on nitrogen atoms of tertiary amines in BDQ capture reactive oxygen to form nitrogen–oxygen complexes **A11**. The generation mechanism of BDQ forced degradation products is shown in ►**Fig. 6**.

Conclusion

In this article, five degradation impurities of BDQ were determined by forced degradation test. The generation mechanism of degradation impurities was also analyzed. The structures of three BDQ forced degradation impurities were characterized by NMR and MS analyses. At the same time, a reversed-phase HPLC method was established, which could simultaneously control 11 impurities in the preparation process of BDQ bulk

Table 4 Detection of related substances on different batches of intermediates and active pharmaceutical ingredient

Related Substances	Intermediate 1	Intermediate 2	Intermediate 3	BDQ
A2	0.61%	Not detected	Not detected	Not detected
A9	0.02%	Not detected	Not detected	Not detected
A3	4.28%	1.21%	0.87%	0.16%
A5	0.15%	0.03%	Not detected	Not detected
A7	0.12%	Not detected	Not detected	Not detected
A1	2.11%	Not detected	Not detected	Not detected
Any other impurity	1.09%	0.15%	0.09%	Not detected
The total impurity	10.83%	1.49%	1.03%	0.16%

Abbreviation: BDQ, bedaquiline fumarate.

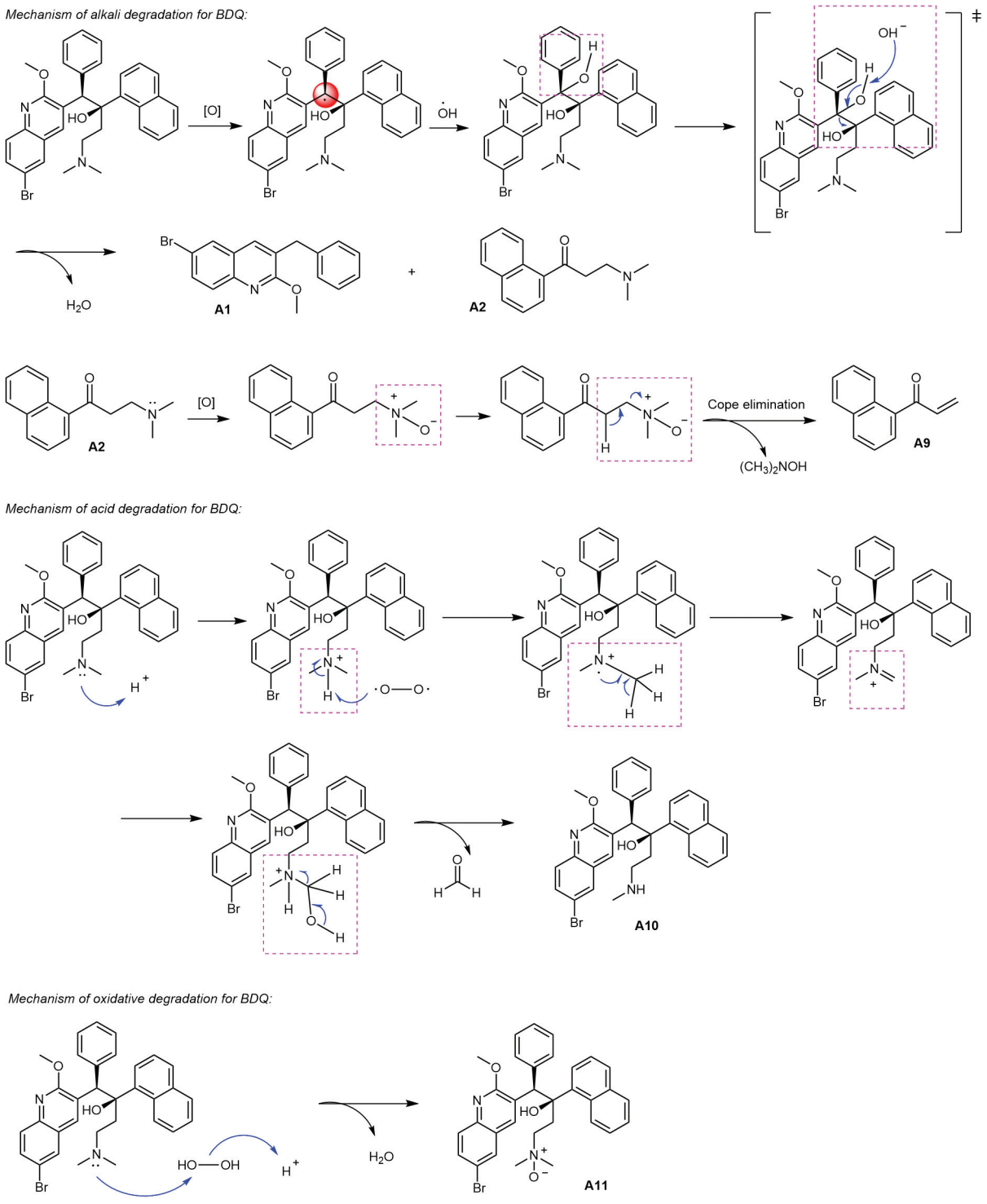


Fig. 6 Mechanism of BDQ forced degradation products.

drug. Particularly, several pairs of diastereomer and isomer impurities with similar structures were even effectively separated by optimizing the pH of mobile phase and improving other chromatographic conditions, such as gradient. The method was validated and proved to be suitable for quality monitoring of laboratory-prepared samples and bulk samples during batch release and stability studies.

Conflicts of Interest

There are no conflicts to declare.

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