CHAPTER 4
STRESS DEGRADATION STUDY AND IMPURITY PROFILING OF BEDAQUILINE

4.1 Selection of Drug [1-5]

Research for tuberculosis vaccine is still going on and new molecules for treatment are needed for patients of TB as *Mycobacterium tuberculosis* bacteria is becoming resistant to existing drugs. Patients who have TB associated with other morbid situations are more susceptible and difficult to treat, requires special treatment. Bedaquiline (BDQ) is new molecule approved by U.S FDA and is prescribed to patients who do not respond to treatment regimen of anti bacterial drug combination for pulmonary TB. BDQ is recommended in patients of pulmonary TB and or patients with multi drug resistance. The BDQ is prescribed in combination with other suitable anti bacterial drugs under the guidance of doctor. World Health Organization (WHO) included BDQ in list of WHO recommended drug for anti-TB in 2013 and CDSCO approved BDQ in 2015.[1] BDQ targets *Mycobacterium* Adenosine tri phosphates (ATP) synthase enzyme which is required for energy supply to *Mycobacterium.*[2] It has strong bactericidal activity. Dosing frequency for BDQ is divided in 2 phase, in phase 1 400mg/day for week 1-2, phase 2 during week 3-24 600mg/week (200mg three days in week), after week 26 other regimen only recommended, BDQ is not prescribed. [3] BDQ is a novel molecule and there is no literature describing stability, impurity and degradation behavior of bedaquiline to the best of our literature survey so it was topic of interest to carry out analytical and impurity studies.

4.2 Drug Profile [6]

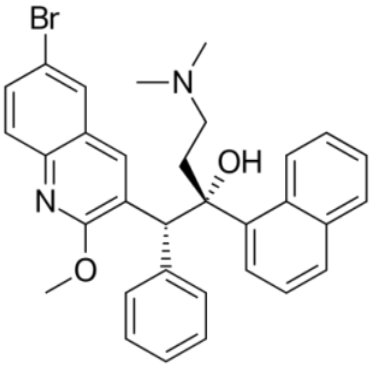
Drug property	Inference
CDSCO Approval	January, 2015.
Drug Category	Anti Tuberculosis
Mechanism of action	BDQ acts on *Mycobacterium* Adenosine 5'-triphosphates (ATP) synthase enzyme which is required for energy supply to *Mycobacterium,* energy supply stopped thus BDQ exhibits bactericidal activity.
Marketed Formulation	Sirturo® (Janseen Pharmaceuticals, US) contains 100mg of Bedaquiline fumarate.
Chemical structure[4]	
IUPAC Name	(1R-2S)-1-(6-Bromo -2-methoxyquinolin-3-yl)-4-(dimethylamino)-2-(napthelen-1-yl)-1-phenylbutan-2-ol
Molecular Weight	555.505g/Mol
Molecular Formula	$C_{34}H_{31}BrN_2O_2$
Physical Appearance	White solid amorphous powder
Solubility	Water insoluble, very soluble in alcohol.
Pka	13.61 (strongest acid), 8.91 (strongest basic)
Log P	6.37

Table 4.1 Drug Profile of Bedaquiline

4.3 Literature Review [7-11]

Bedaquiline is new molecule entity approved to treat MDR-TB after 40years since last molecule approved. There are five methods reported, UV method [7, 8] and HPLC method [8,9] in human serum and plasma, LC/MS/MS method [10, 11], stability study in dissolution media[12], Chiral separation [13,14], Pharmacokinetic study [15], simultaneous estimation [16] There were no stability, impurity or degradation products data reported till date from our extensive literature survey for BDQ. It was thought of interest, therefore to carry out stability study and to identify impurities in BDQ.

4.4 Bulk Drug Identification

BDQ was received as gift sample from Dishman Pharmaceuticals, Ltd, Gujarat, India. Obtained sample was analyzed for Infrared spectroscopy (IR), Differential Scanning Calorimetric (DSC) and UV spectroscopy to confirm the identification of BDQ.

4.4.1 IR spectroscopic study[17]

Approximately 1mg of bulk drug was used for IR spectrum analysis for identification of chemical groups present in BDQ (Fig.4.1). Wave numbers in IR spectrum of BDQ and associated chemical groups for inference of IR spectrum are shown in Table 4.2.

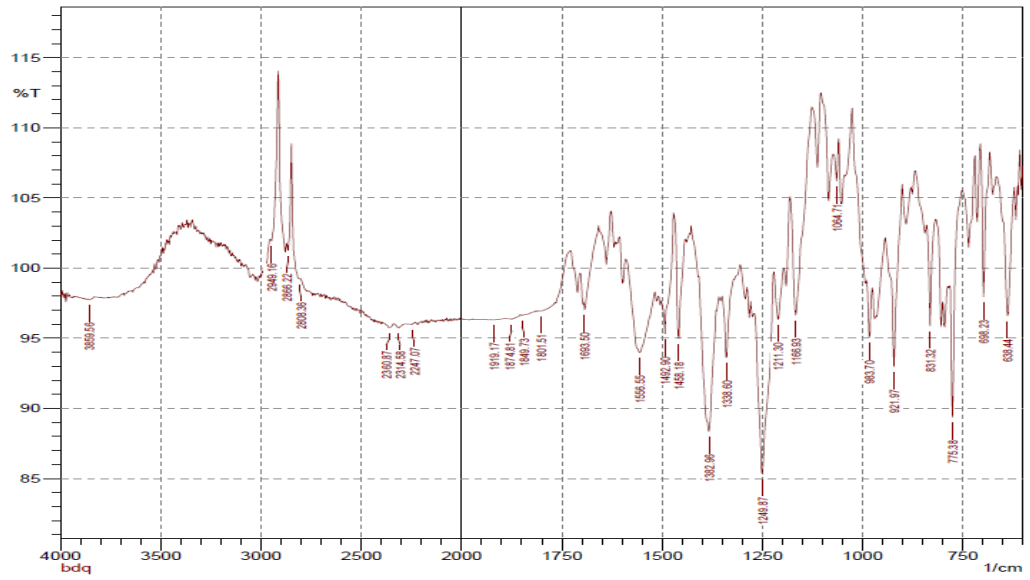


Fig. 4.1 IR spectrum of BDQ

Group	**Obtained wave number (Cm^{-1})**	**Assigned wave number (Cm^{-1})**
Halo compound (C-Br stretching)	638,693	690-515
C-H bending (Strong)	775	800-760
C=C bending	831	840-790
C-O stretching	1064	1085-1050
C-N stretching	1249	1250-1020
O-H bending	1382	1390-1310
C=O stretching	1693	1710-1680
C-H stretching (Alkane)	2866,2949	3000-2840

Table 4.2: Inferences of IR Graph for BDQ.

There was no reference IR spectrum of BDQ available in literature therefore identification of BDQ was carried out using inference obtained from IR spectrum and it was compared with the chemical groups present in chemical structure of BDQ. As it can be seen in Table 4.2 the prominent groups in BDQ structure are seen in IR spectrum analysis, so IR analysis identified the sample as BDQ.

4.4.2. Melting point study[18]

The melting point range is an important identification test for sample purity as trace amount of impurity leads a drastic change in melting point range. The melting point test was carried out using Differential Scanning Calorimetric (DSC) instrument. Approximately 2mg of BDQ was utilized for DSC study; the thermogram obtained is shown in Fig. 4.2.

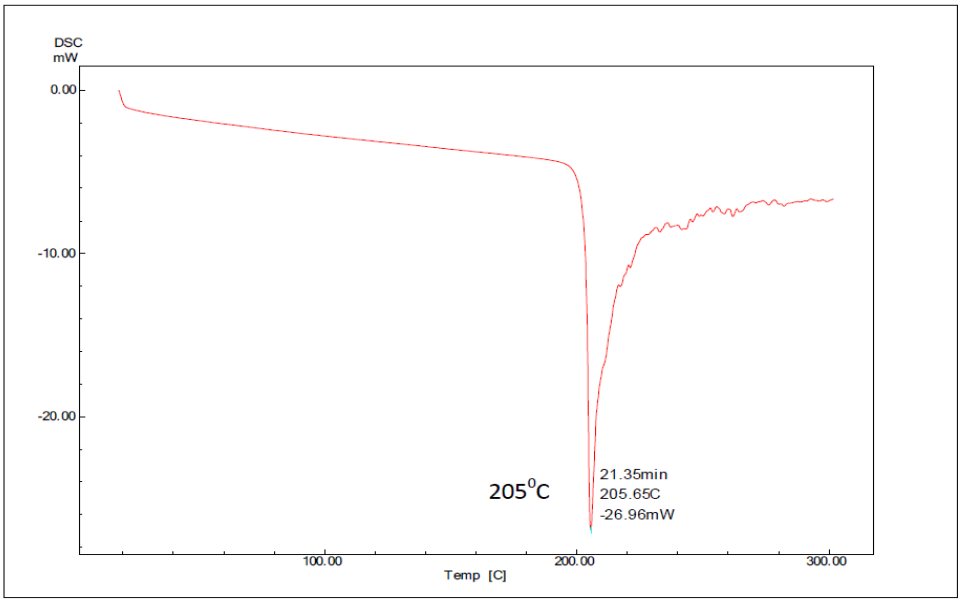


Fig.4.2 DSC thermogram of BDQ

The DSC thermogram was started at temperature 30^0C with 10^0C/min rate and 300^0C hold temperature, it showed intense endothermic peak at 205^0Cindicateing melting of substance; the reported melting point range for BDQ is 205-210^0C. The melting point test identified the sample as BDQ.

4.4.3 Solubility study

The solubility test was performed by evaluating the quantity of BDQ required to dissolve in 1ml solvent. The study showed that BDQ is very soluble in alcohol (methanol, ethanol, propanol etc.) (>1mg/ml); methanol was chosen as the organic solvent for studies as it is easily available and suitable for chromatographic studies.BDQ is practically insoluble in water.

4.4.3 UV Spectrophotometric study [18]

Another identification test for sample is UV Spectrophotometric test; as each substance has its unique $\lambda_{max.}$ To perform UV Spectrophotometric test for BDQ, accurately weighed 10mg BDQ was dissolved in 10ml methanol to produce 1mg/ml stock solution. From stock solution 0.5ml aliquot was diluted in 10ml methanol to produce 50µg/ml sample;this solution was scanned in 200-400nm to get UV Spectrum (Fig. 4.3.)

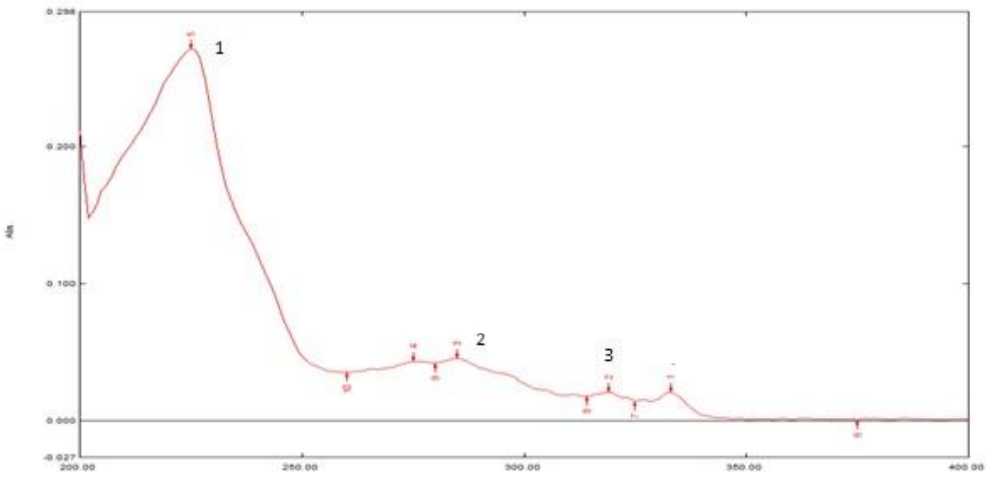


Fig. 4.3 UV Spectrophotometric graph for BDQ (50µg/ml)

The UV spectrum showed λ_{max} at 225nm, and small peaks at 285nm, 320nm and 333nm; all these λ_{max} are reported for BDQ in literature ref. The UV Spectrophotometric test confirms the sample as BDQ.

Result and discussion:

The identification tests; IR, melting point and UV Spectrophotometric test results were matched with BDQ reported data or inference from obtained data; the results of identification tests together confirm the sample as BDQ and further studies were started with RP-HPLC.

PART- A

4.5 DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD AND STRESS DEGRADATION STUDIES FOR BDQ

4.5.1 Experimental

4.5.1.1 Chemicals and Reagents

Bedaquiline API was obtained as gift sample from Dishman pharmaceuticals LTD., Ahmadabad, Gujarat. HPLC grade methanol, acetonitrile and OPA (Orthophosphoric acid) were purchased from Rankem Chemicals, Mumbai, India. Sodium dihydrogen phosphate (NaH_2Po_4) was purchased from Loba Chemicals Pvt. Ltd, Mumbai, India.

Unless and otherwise specified, all solutions were filtered through a 0.2µm Nylon 6,6 membrane filter, Ultipore® N66® From Pall Life Sciences, USA; prior to use. Analytical grade Hydrochloric acid (HCl) and Sodium hydroxide (NaOH) were procured from SD Fine Chem. Ltd., Mumbai. Hydrogen peroxide (H_2O_2) was procured from Fischer Ltd., India.

4.5.1.2.Equipment and chromatographic conditions

Equipment was same as described in section 3.5.1.2.

UV Spectrophotometer: Same as described in section 3.5.1.2.

Liquid Chromatographic system: Same as described in section 3.5.1.2.

The chromatographic separation was accomplished on a Thermo scientific Inertsil RP-C_8 column (250×4.6mm, 5µm) at wavelength 225nm. The analysis was performed at ambient temperature with injection volume of 20µL. The mobile phase was filtered through 0.2µm disposable filters from Ultipore®, PALL Life sciences, and degassed with ultra sonicator prior to use.

Stability indicating method: Stability indicating method was developed to separate degradation products from drug peak using mobile phase **A)** 0.01M sodium dihydrogen ortho phosphate pH 2.5 buffer using ortho phosphoric acid (OPA) and **B)** methanol as mobile phase ran in gradient mode. (Table 4.3)

Time	%A	Flow rate
0	55	1
40	14	1
50	55	1

Table.4.3. Scheme for Gradient elution

4.5.1.3.Analytical sample preparation

Preparation of Stock, Sample and Buffer solutions

Sample for method validation: Stock solution of BDQ was prepared by dissolving 10 mg of API in 2ml methanol and solution was made up to 10ml with methanol to produce 1mg/ml. The working standards for linearity were prepared by taking 0.3, 0.6, 0.9, 1.2,1.5 and 1.8ml aliquot in 10ml vol. flask ; the aliquots were diluted in separate vol. flask to 10ml with methanol to produce 30.0-180.0 µg/ml.

Buffer preparation: 0.01M sodium dihydrogen phosphate pH 2.5 buffer using ortho phosphoric acid (OPA) was prepared by dissolving 1.19 gm of sodium dihydrogen phosphate buffer in a 900ml of double distilled water and adjusted to pH 2.5 using OPA, remaining volume was added to make final volume of 1Litre, which was finally filtered with 0.2 µm nylon membrane filter and degassed by ultra sonicator for 5 minutes.

Preparation of Stress Degradation Samples

A preliminary stability of BDQ was evaluated in organic solvent and for solid bulk drug at room temperature to gather some basic information about the stability of the API at room temperature and in selected organic solvent. Two samples were generated for every stress condition samples;

i) The blank solution(Without API) subjected to stressed condition in the same manner as the API
ii) The API solution (with degradant) subjected to stressed condition.

BDQ was stressed to maximum condition where 5-100% decrease in peak area of BDQ API occurred. Marketed formulation of BDQ was not available in India at the time of study, so that BDQ synthetic mixture was prepared (described in section 4.5.1.4). The same degradation study was performed with synthetic mixture to determine formation of any DPs due to drug excipient interaction.

Sample for stress degradation study: BDQ (accurately weighed 500mg) was dissolved in 10ml methanol and sonicated for 15minutes with provisional shaking; 50ml final volume was achieved using 12% hydrogen peroxide, 1N HCl, 1N NaOH and water (final concentration 100.00mg/ml) individually. At regular time interval, aliquot of 2ml was withdrawn from the 0minute to till the stability study was completed, sample was kept in dark and diluted to 10ml with methanol (200.00µg/ml); filtered through 0.45 µ Pall syringe filter (procedure was carried out in dark to partial darkness) and injected in chromatographic system described in section 4.5.1.2 for RP-HPLC for data acquisition and analytical purpose.

The stress degradation samples for specific conditions are as follow:

Hydrolytic degradation (Acidic/Alkali/Neutral)

Hydrolytic degradation studies were performed in acidic, alkali and neutral conditions if required with heating and refluxing API in HCl, NaOH and H_2O. The stability conditions are as described below;

For acid hydrolysis, BDQ stress degradation sample prepared in 1N HCl was kept at 80^0C in dark for 75minutes.

For alkali hydrolysis, BDQ stress degradation sample prepared in 2N NaOH was allow to stand in dark at 80^0C for 120min.

For neutral hydrolysis, BDQ stress degradation solution was kept at 80^0C in dark for 2days.

Peroxide induced (Oxidative) Degradation

For oxidative degradation study, BDQ stress degradation sample prepared in 12% H_2O_2 was kept at 80^0C in dark for 5 hours.

Photolytic Degradation

For light induced degradation, BDQ bulk drug in solidstate spread in approximately 1mm thickness on a petri plate and exposed to 5382 LUX and 144UW/cm^2 in photo stability chamber for 28 days.

Dry heat induced (Thermal) Degradation

For thermal degradation, BDQ bulk drug in solid state spread on a petri dish with approximately 1mm thickness and placed in oven at 80^0C for 28 days under dry heat condition in dark.

The above described stress degradation conditions showed 5-75% degradation of BDQ in HPLC chromatogram.

4.5.1.4 Preparation of Synthetic mixture [14]

The BDQ formulation was not available in market at the time of the study. A laboratory mixture was prepared by taking the excipient described in patent for BDQ formulation (Sirturo™) [14]. The composition of formulation is shown in Table 4.4.

As per BDQ formulation patent formulation contains 100mg of BDQ fumarate in 120mg of total drug weight.

Content	% of Content
BDQ fumarate	80%
Lactose monohydrate	12%
Maize Starch	2%
Hypromellose	0.05%
Polysorbate 20	0.1%
Microcrystalline cellulose	0.8%
Crosscarmellose sodium	0.05%
Silica, colloidal anhydrous	5%

Table: 4.4. The laboratory mixture of BDQ

Solution for recovery and degradation study was prepared by dissolving equivalent to 10mg weight of BDQ API in 2ml of methanol and remaining 8ml volume was made up by methanol to produce 1000.00µg/ml.

4.5.1.5 Method Development

Preliminary investigations

Preliminary investigations were executed to study the effect of various parameters on chromatographic separation and method development.

Choice of organic solvent: organic solvent selection for dilution or dissolving purpose as well as for chromatographic condition depends on the solubility of drug substance in that organic solvent. As per literature report (Table 4.1) and practical experience, BDQ is very soluble in alcohol (methanol, ethanol) and insoluble in water indicates that BDQ is non-polar and hydrophobic in chemical nature; as a result methanol was a choice of organic solvent. The standard solution of BDQ was prepared in methanol before stress study to dissolve the bulk drug. As DPs formed were polar and non polar in nature and API has limited solubility, methanol was preferred for mobile phase in terms of DP resolution, to optimize R_t, sensitivity and theoretical plates.

Detection wavelength and Polarity: as seen in identification test (Fig. 4.3) BDQ showed better sensitivity at 225nm hence detection was carried out at this wavelength. BDQ is non polar in nature with pK_avalue of 8.31. DPs are polar and non polar in nature, so gradient volume of buffer and organic solvent was used to optimize retention time (Rt) and resolve the DPs.

Buffer and pH selection: The insolubility of BDQ in water did not allow the use of water in chromatographic conditions or sample preparation and led toward the use of buffer for the same conditions. The pKa of BDQ is 8.31, so the range for buffer selection should be ±2.0 pH from pKa but BDQ remains insoluble as a solid material in the pH range ±7.0. The buffers in the range of pH 3 to 4.7 were tried for BDQ method development in RP-HPLC; the intensity of the peak was very low and a broad peak was obtained. Lower pH was tried using water but BDQ bulk drug was eluted at Rt 2.0minutes that was practically not feasible for stability studies; water was replaced by buffer sodium dihydrogen phosphate, an intense symmetric peak of BDQ was acquired. The pH of buffer (2.5) showed a high impact on drug peak shape while it showed less effect on DPs' Separation. Lower buffer pH 2.5 to 3 contributed to sharp bulk drug peak, while increasing pH provided peak broadening, more increase in pH leads to drug peak splitting. pH had no effect on the separation of degradation products.

After all these trials, the final obtained chromatographic conditions are described in section 4.5.2.2.

4.5.1.6 Method Validation as per ICH Q2 (R1) guideline [20]

Procedure for the validation of method is described in section 3.5.1.5.

4.5.2. Results and discussion

4.5.2.1.Determination of suitable wavelength

The UV spectrum of BDQ is shown in figure 4.3. The spectrum indicates that BDQ showed peaks at four different ʎ.The UV Spectrum of BDQ (linearity and range 10.0-70.0µg/ml) were extracted from 200-400nm and it is shown in figure 4.4.The spectrum indicates that all ʎ showed good linearity and good sensitivity for the BDQ bulk drug but during stress degradation studies chromatogram was recorded at 225nm as at this wavelength BDQ as well as DPs showed sufficient absorption.

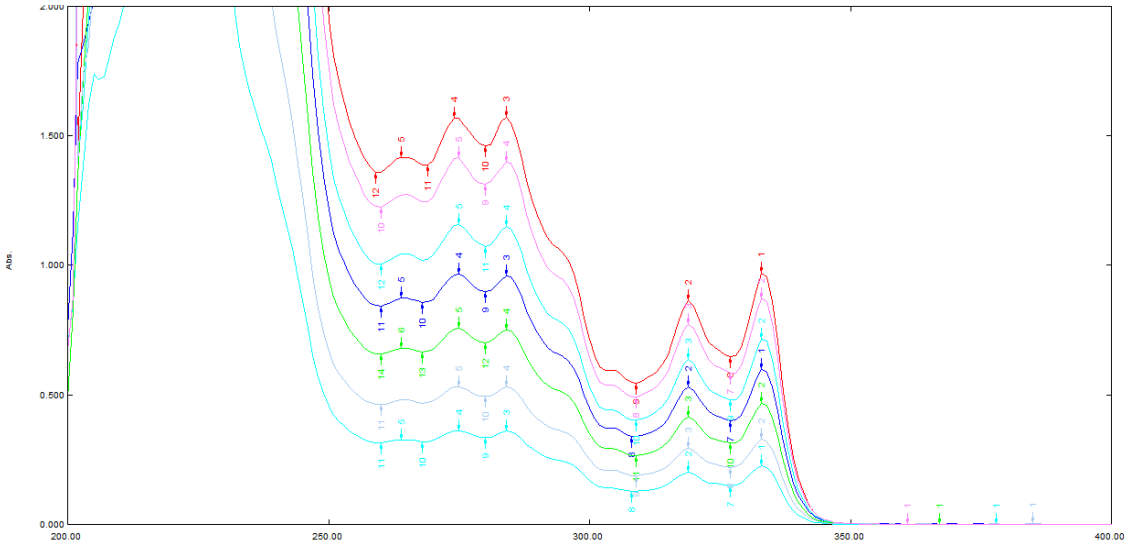


Fig.4.4 Linearity and range of UV spectra for BDQ bulk drug (10.0-70.0µg/ml)

4.5.2.2 Method development and optimization

Based on BDQ polarity and pK_a value initial trials were carried out on 100% methanol as drug is insoluble in water. Several trials were carried out to get good system suitability parameters (discussed in section 4.5.1.5.) Final method was optimized in 0.01M sodium dihydrogen phosphate buffer pH 2.5 buffer using ortho phosphoric acid (OPA) and methanol in gradient program at a flow rate of 1.0 ml/min. Some trials for optimization of method are shown in Table 4.5.

Mobile Phase	Ratio	Result
1. Methanol	100%	Peak at 2.0min.
2. Water: methanol	10:90	No repeatability of results.
3. 0.01M sod. dihydrogen phosphate pH 2.5: methanol	50:50	Short Rt, needed long run time.
4. 0.01M sod. dihydrogen phosphate pH 2.5: methanol	10:90	Good peak shape and short run time.(Final method was developed in gradient scheme)

Table: 4.5. Optimization of HPLC method for BDQ

4.5.2.2. Stress degradation studies

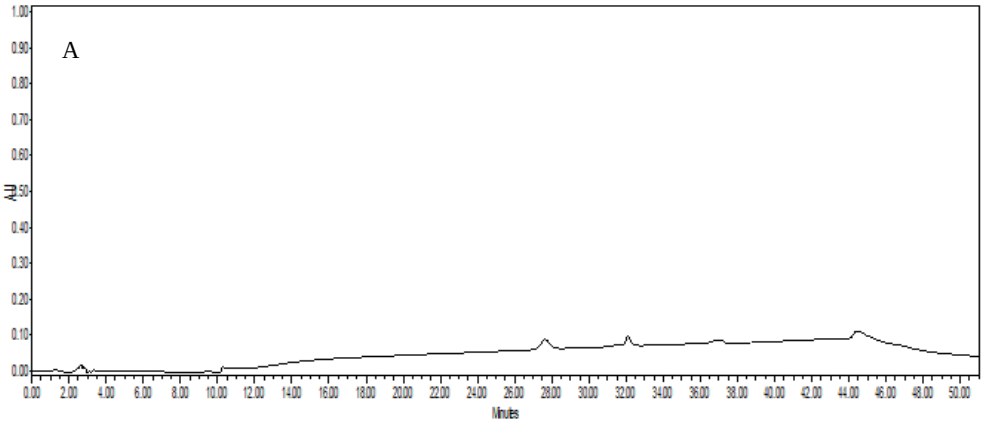
The stress degradation studies were carried out in hydrolytic (acid, base and neutral) condition, oxidative condition, under the UV light, thermal and moisture conditions. When stressed conditions samples were analyzed in LC-PDA, DPs had formed under different stressed conditions. The summary of forced degradation condition with %degradation (%deg), DPs formed with Rt in various stress condition is presented in Table 4.6.

The chromatogram of stress degradation studies are shown in Fig. 4.5, the analysis of chromatogram showed that BDQ was sufficiently degraded under acidic and oxidative condition to identify and characterize the DPs formed in these conditions. While in neutral, alkaline condition, under UV light, thermal and humid condition BDQ showed slight degradation or no degradation. The blank chromatogram showed that method was BDQ specific.

Stressor Type	**Stressor Conc.**	**Time**	**DPs formed with RT**	**%Deg (API)**	**%Deg. (Synthetic mixture)**
Acid	1N HCl/80^{0}C	75 minutes	Peak 1: 2.91 Peak 2:3.18 Peak 3: 13.47 Peak 4: 25.55 **Peak 5 (Major DP): 26.48** API: 31.80 Peak 6 : 35.54	74.82%	73.82%
Alkali	2N NaOH/ 80^{0}C	120 minutes	-	18.86%	19.10%
Neutral	H_2O/80^{0}C	2days	-	4.35%	4.78%
Oxidative	12% H_2O_2/ 80^{0}C	5hrs.	Peak 1:3.690 Peak 2: 27.16 API: 31.60 **Peak 3 (Major DP): 32.13**	63.93%	65.08%
Photolytic	-	28 days	-	4.2%	4.5%
Dry thermal	80^{0}C	28 days	-	3.8%	3.3%

*Bold face indicates major DP peak in chromatogram

Table: 4.6Summary of stress degradation study of BDQ bulk drug and synthetic mixture.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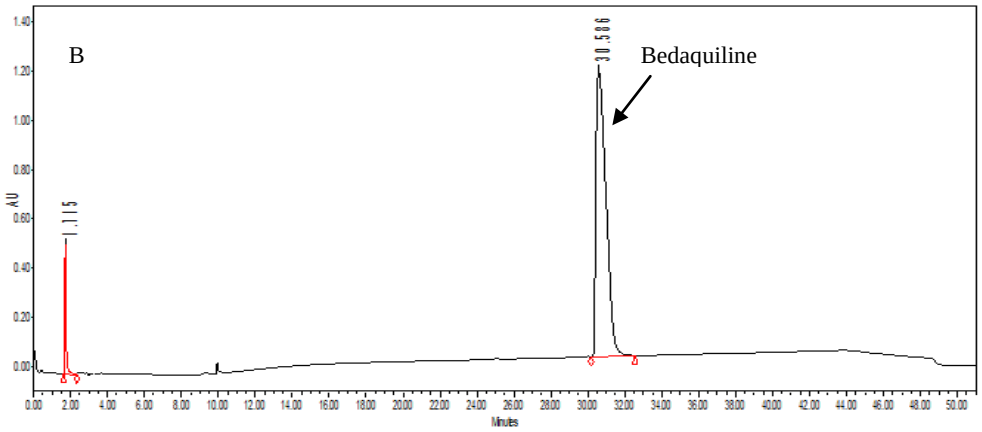
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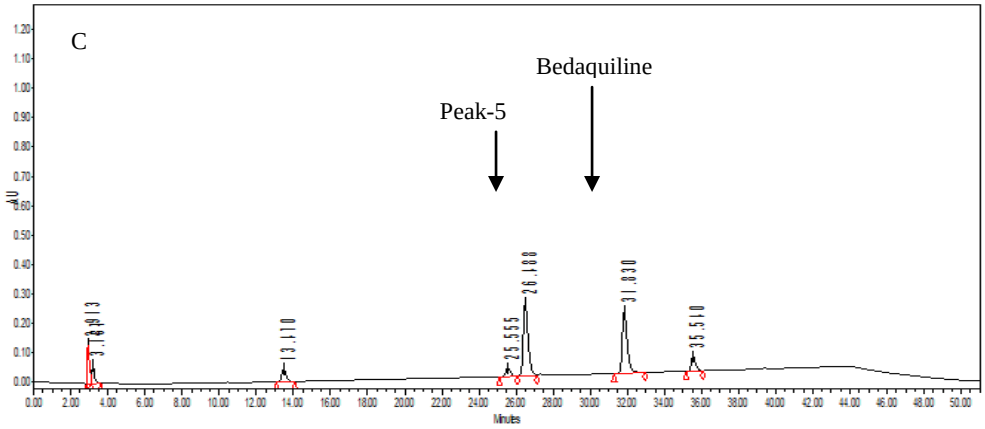
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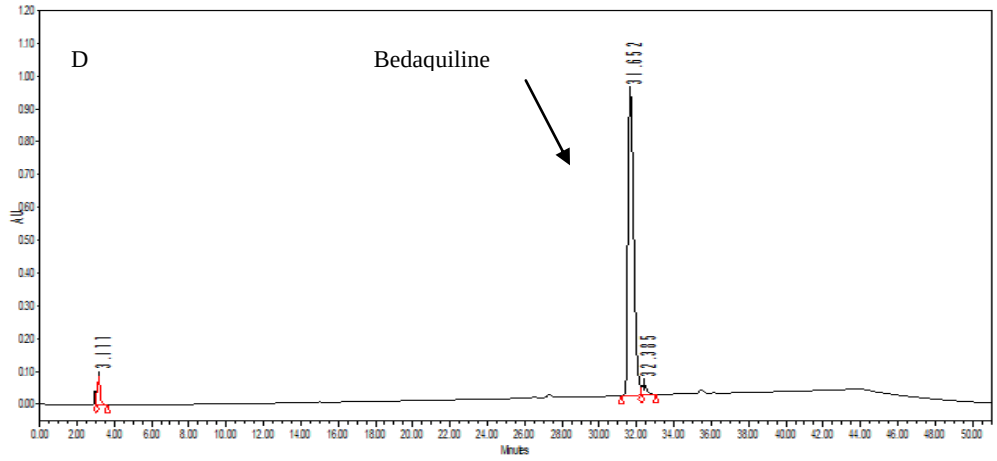
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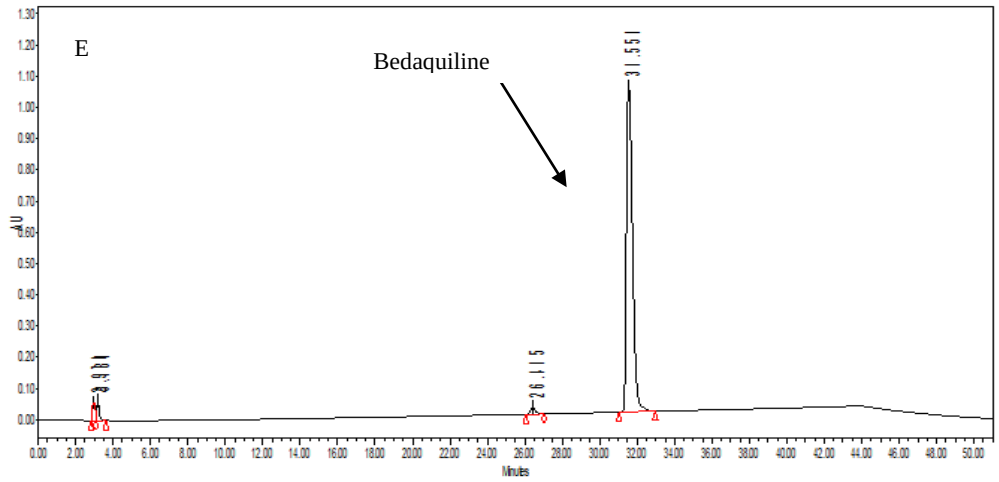
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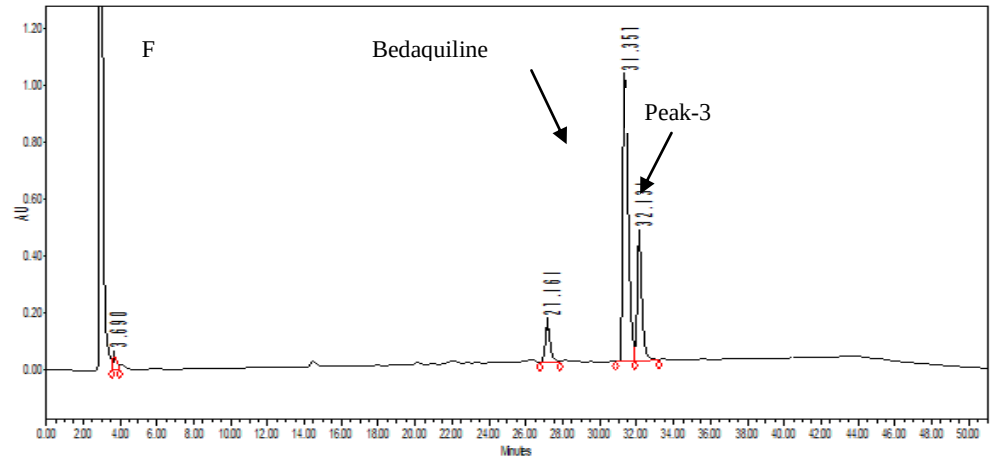
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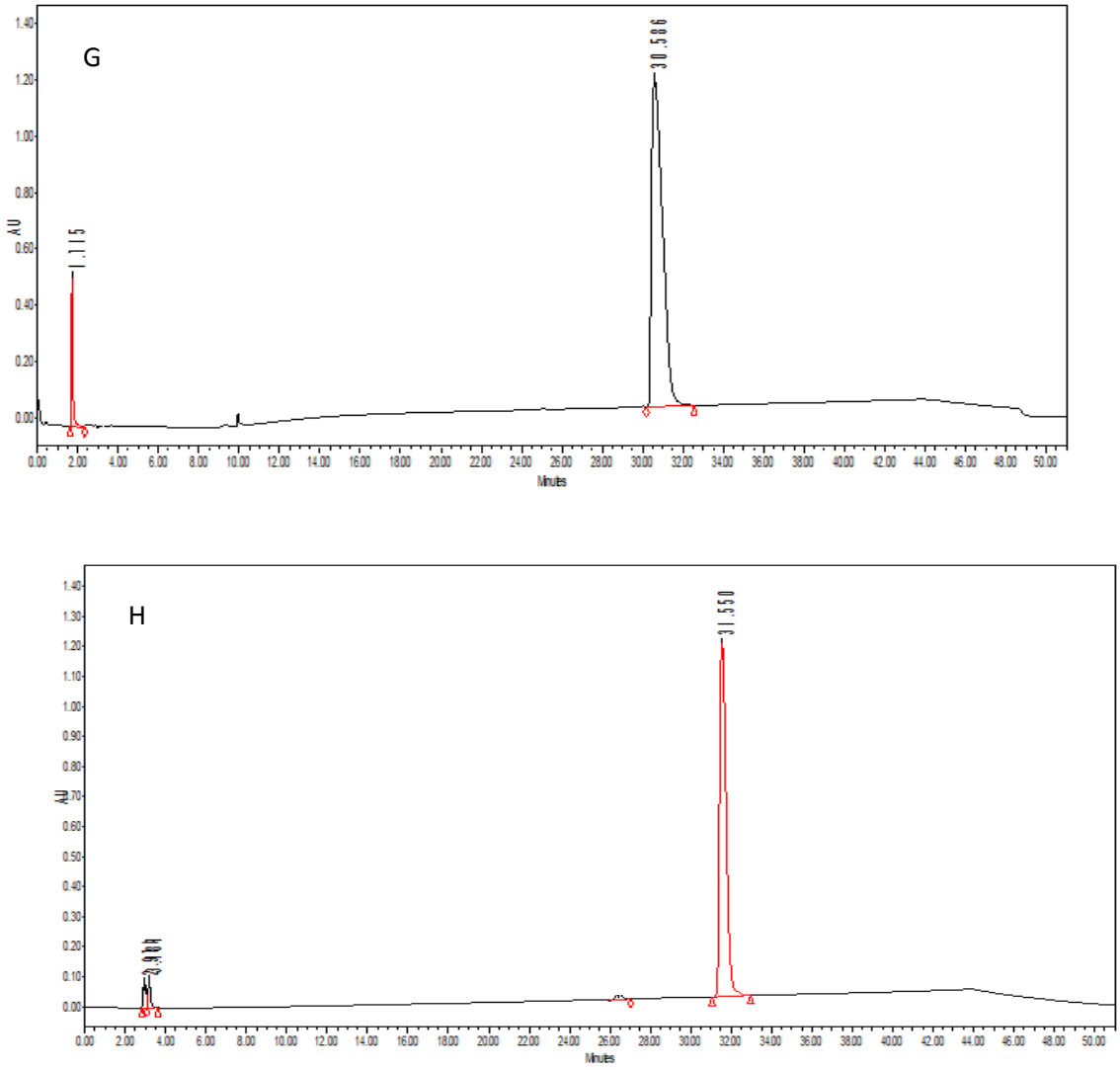


Fig. 4.5 Stress degradation study by RP-HPLC for BDQ A) method specificity B)method selectivity for BDQ C) Acid stress degradation D)Alkali stress degradation E)Neutral stress degradation F)Oxidative stress degradation of BDQ G) thermal degradation and H)photolytic degradation of BDQ.

Chromatograms for thermal and photolytic degradation were not showing any DP formation and the peak pattern was same as the bedaquiline bulk drug chromatogram. The DPs fell in the classification for reporting, identification and quantification were analyzed for the same by LC/ESI/MS, IR and NMR (^{1}H, ^{13}C NMR and Attached Proton Test).

Peak Purity test: Peak purity tests were performed for the DPs classified under to be identified and /or characterized. The peak purity tests were performed to know whether any co-eluting DP with bulk drug peak or DP peak is obtained or not. It was performed for

chromatogram showing Fig.4.5 (B), (C) and (F). The result for peak purity is shown in table 4.7 with purity angle which should not be exceeding the purity threshold.

DPs and R_t	**Purity angle**	**Purity threshold**	**Pass/Fail**
(B) BDQ bulk drug chromatogram			
Peak1: 1.71	0.319	0.321	Pass
API-30.88	0.180	0.304	Pass
(C) BDQ acid degradation sample chromatogram			
Peak 1: 2.91	3.02	0.321	Co-elution
Peak 2:3.18	6.25	0.483	Co-elution
Peak 3:13.47	0.257	0.465	Pass
Peak 4: 25.55	0.304	0.418	Pass
Peak 5:26.48	0.137	0.285	Pass
API:31.80	0.194	0.303	Pass
Peak 6:35.54	0.364	0.420	Pass
(F) BDQ oxidative degradation sample chromatogram			
Peak 1:3.69	0.233	0.303	Pass
Peak 2: 27.16	1.23	1.48	Pass
API:31.75	0.222	0.381	Pass
Peak 3: 32.13	1.22	1.44	Pass

Table: 4.7 Peak purity test for BDQ bulk drug and degraded (acid and oxidative medium) sample chromatogram

Peak purity test for Fig. 4.5 (B) BDQ bulk drug chromatogram is shown in Fig. 4.6. The chromatogram showed process related impurity and BDQ bulk drug peak; both with peak purity and non-co eluting peak. The process related impurity was identified in Part-D.

A **B**

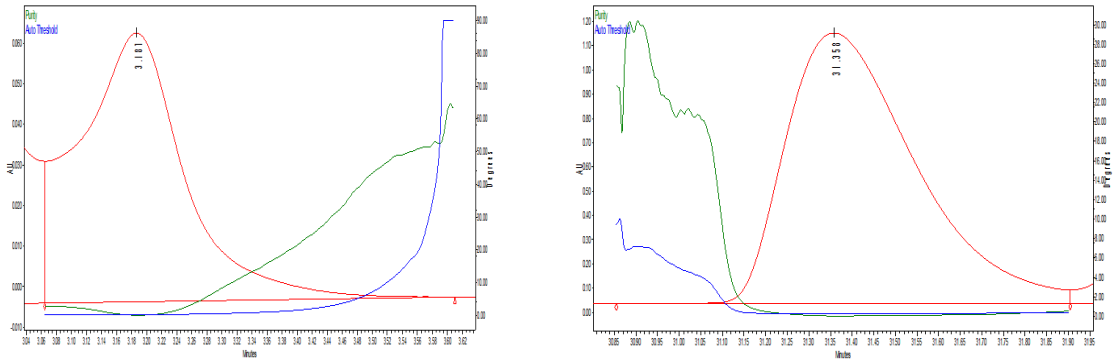
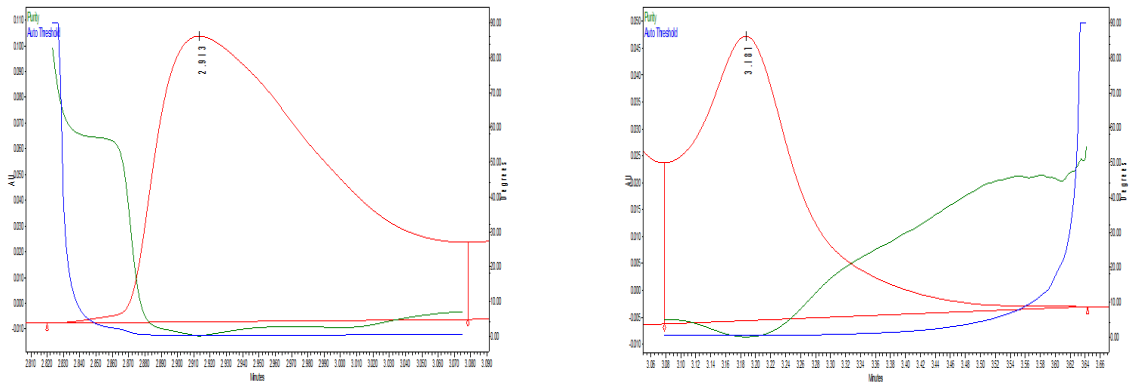


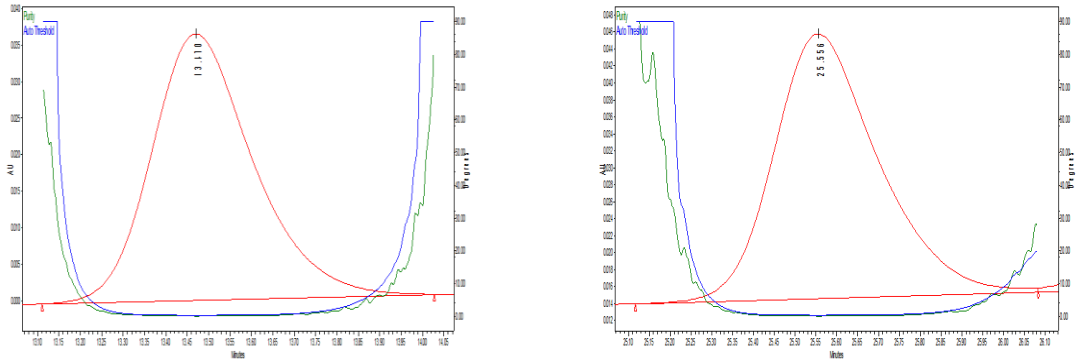
Fig.4.6 Peak purity test for BDQ bulk drug chromatogram A) Process related impurity B) BDQ bulk drug

Peak purity angle for Fig. 4.5(C) acid degraded sample chromatogram is shown in Fig.4.7. Peak-1 and 2 were co-eluting peaks, so it showed the angle exceeding purity threshold while Peak-3, 4, 5, 6 and bulk drug peaks were passed for the purity.

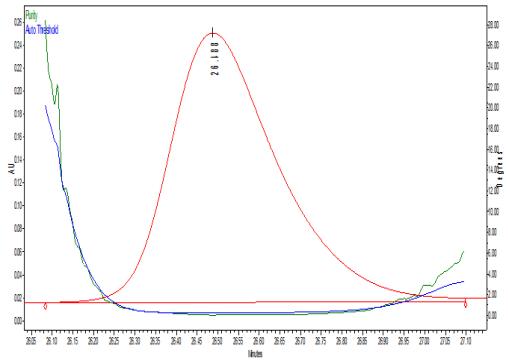
A **B**



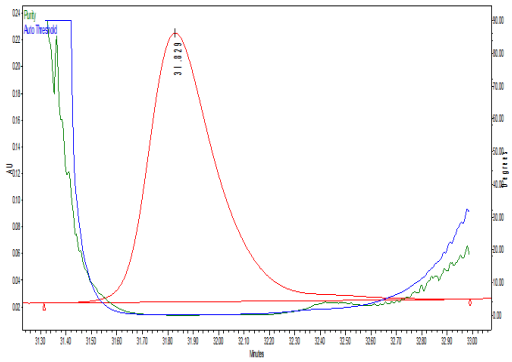
C **D**



E



F



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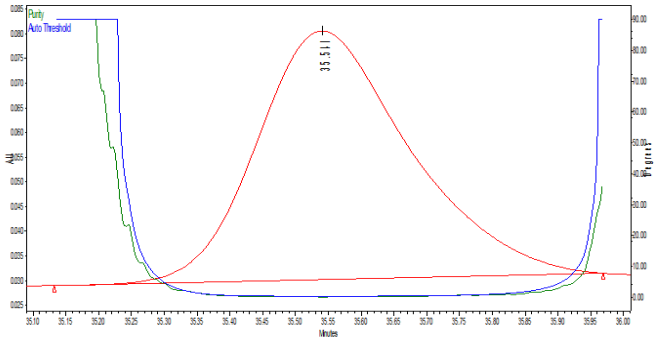


Fig.4.7 Peak purity test for acid degraded sample of BDQ chromatogram A) Peak-1 B) Peak-2C)Peak-3 D) Peak-4 E) Peak-5 F) API G) Peak-6

The sample degraded in presence of oxidizing agent was containing 3 DPs and API (Fig.4.5 (F)); peak purity angle for the chromatogram is shown in Fig. 4.8. All the peaks obtained in chromatogram were passed purity test without co-elution.

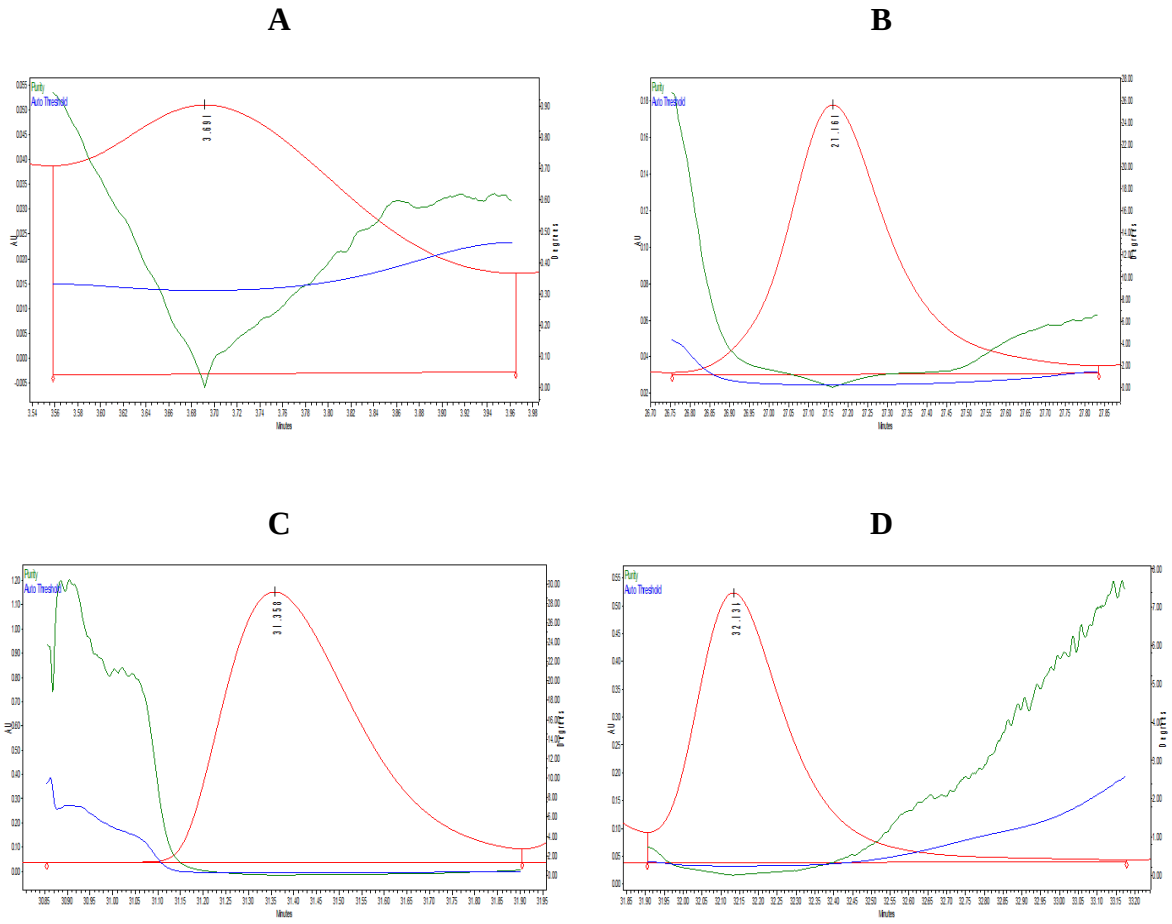


Fig. 4.8 Peak purity test for peroxide degraded sample of BDQ chromatogram A) Peak-1 B) Peak-2 C) API D) Peak-3

The Peak-5 of acid degraded sample and Peak-3 of peroxide degraded sample were identified as major DPs of BDQ. Identification, isolation and characterization of those DPs are discussed in section-D.

4.5.2.3. Validation of stability-indicating method [20]

The stability-indicating method was validated as per ICH Q2 (R1) guidelines. The validation was completed taking consideration of linearity, range, precision, accuracy, LOD (Limit of Detection) and LOQ (Limit of Quantification) to confirm method susceptibility for changes.

Linearity and Range

Analytical method development was significant for selection of range, pH and stability study. Initial stability studies were performed on isocratic developed method but in isocratic method DPs were merged with each other so it was needed to develop stability indicating method which separated DPs from each other and helped to identify major DPs as seen in Fig. 4.5

and in purity plots shown in Fig. 4.6, 4.7 and 4.8. Stability indicating method was developed using gradient mode of HPLC. (Table 4.3)

Linearity test solutions for BDQ assay method were prepared from bulk drug stock solution of 1mg/ml in methanol at concentration level 30.0-180.0 µg/ml in triplicate. The calibration curve was constructed by plotting concentrations versus peak area of BDQ. The regression equation was calculated and was found to be linear in the selected concentration range with r^2 value 0.998 and regression equation y = 16446x + 2E+06. Overlay chromatogram and calibration curve showing linearity of BDQ is shown in Fig. 4.9.

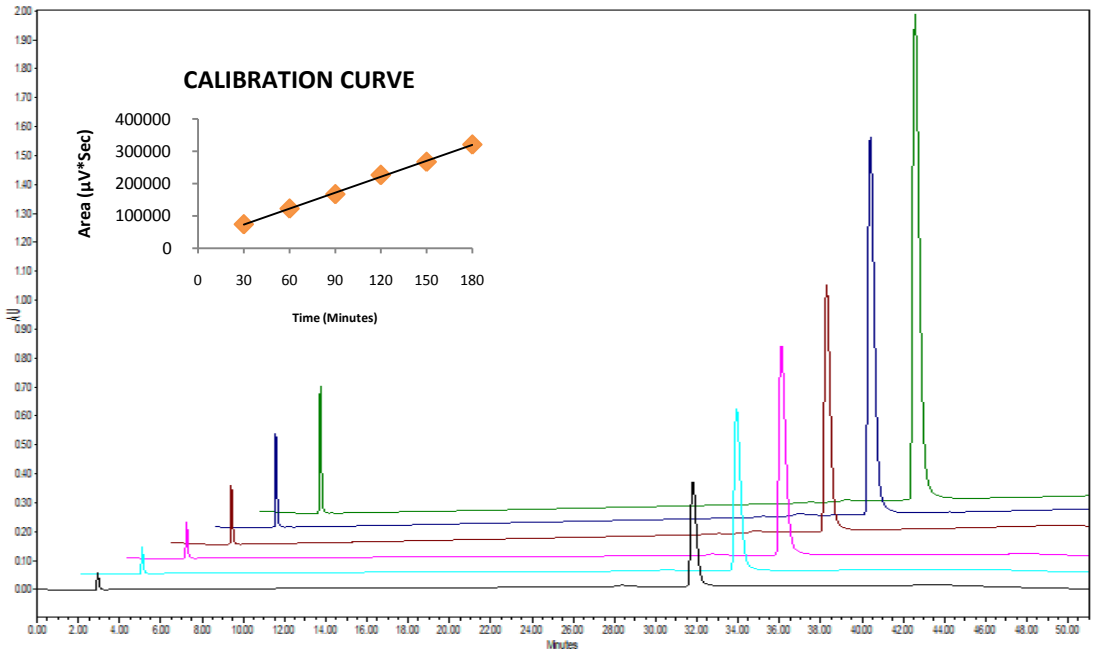


Fig. 4.9 Overlay chromatogram for linearity and range of BDQ (30.0-180.0µg/ml)

The good co relational coefficient (0.998) showed that the method was eligible to generate linear results in a given range. The mean area versus concentration (30.0-180.0µg/ml) plot is shown in Table 4.8.

Conc. (µg/ml)	*Mean peak area (µV*S) ± RSD
30.00	7468266± 0.0013
60.00	12310371± 0.0016
90.00	16773324± 0.0019
120.0	22669169± 0.0021
150.0	26801180± 0.0020
180.0	32131642± 0.0020

*Mean peak area of three replications

Table 4.8 Mean peak area versus concentration table for linearity of BDQ

Precision

The RSD value for intermediate and repeatability precision was less than 2.0%; indicates method was precise to regenerate the chromatogram. The average area, standard deviation of area (SD) and Relative Standard Deviation (RSD) for precision study is shown in Table 4.9.

Repeatability			
Conc. (µg/ml)	***Avg. Area (µV*S)**	**SD**	**% RSD**
60.00	12415412	22347	0.0018
Inter day precision			
30.00	7478560	11965	0.0016
60.00	11900854	23801	0.0020
90.00	16579323	36474	0.0022
Intraday precision			
30.00	7470271	10458	0.0014
60.00	12510513	18765	0.0015
90.00	15978329	31956	0.0020

*Average of three replication for intermediate precision and six replications for repeatability

Table: 4.9. Result of intermediate precision and Repeatability

Accuracy

The recovery study was completed using laboratory mixture and standard addition method. The average recovery was more than 98.9% and RSD was less than 2.0% indicates that good recovery was obtained. (Shown in Table 4.10)

Level (%)	Conc. from formulation (µg/ml)	Std Conc. Spiked (µg/ml)	Mean % con. recovered ± RSD
50	60.00	30.00	98.9 ±0.0196
100	60.00	60.00	99.7 ± 0.0213
150	60.00	90.00	99.4 ± 0.0270

*Mean of three replications

Table: 4.10 Result of accuracy study for BDQ bulk drug and laboratory mixture

LOD and LOQ

The LOD and LOQ for developed method were found to be 8.22µg/ml and 24.91µg/ml, respectively. This indicates that the developed method had enough sensitivity to analyze API in laboratory mixture.

Synthetic mixture assay

The synthetic mixture was analyzed for assay study and 99.8% sample was recovered indicated that excipient did not interfere in detection of BDQ in HPLC. (Limit for assay recovery is 95-105% for synthetic mixture).

Robustness

Small but deliberate changes in the flow rate, organic ration in mobile phase, pH and detection wavelength were made to study the robustness of the developed method and were determined in the form of %RSD. Table 4.11 represents the results of robustness study, showing the effect of variation on area of analyte, Rt, tailing factor and number of theoretical plates. Robustness study was carried out using 60.00µg/ml solutions.

Chromatographic Changes	Observations*			
	Area (%RSD)	**Rt (%RSD)**	**Tailing factor (%RSD)**	**Theoretical Plates (%RSD)**
Flow rate ± 0.1 (ml/min)				
0.9	0.0016	0.00055	0.034	0.0139
1.0	0.0016	0.00056	0.032	0.0139
1.1	0.0016	0.00056	0.030	0.0143
pH ± 0.5				
2.0	0.0020	0.00059	0.042	0.0130
2.5	0.0016	0.00056	0.032	0.0139
3.0	0.0032	0.00061	0.056	0.0129
Detection wavelength ± 10 (nm)				
215	0.0016	0.00056	0.032	0.0139
225	0.0016	0.00056	0.032	0.0139
235	0.0018	0.00056	0.032	0.0139

*Result of three replications

Table: 4.11 Results of robustness study for BDQ

4.5.2.4. Application of developed stability indicating assay method for analysis of BDQ in bulk drug and laboratory mixture

The developed method was used to analyze stress degraded samples of synthetic mixture containing BDQ. Stress degradation was carried out under same condition as specified for API and analyzed in the same way by same chromatographic condition. The degradation products were discernible and well separated. As represented in Table 4.6 the same degradation pattern was observed for API as well as in synthetic mixture depicted that there was no interference of excipient in degradation of BDQ. The developed stability indicating method can be applied for routine analysis in clinical and pre-clinical studies of BDQ. The method has detection limit of 8.22µg/ml indicates that method can detect low concentration of BDQ. The average tailing factor for peak was ≤ 2.0. The stability study results can be applied during formulation development, shelf life study, and for suggested storage condition of BDQ formulation.

PART- B

4.6 DEGRADATION KINETICS STUDY FOR BEDAQUILINE

The degradation kinetics was studied for acid, alkali and oxidative conditions, as BDQ was susceptible for these conditions. The factors that can alter the degradation rate (temperature, concentration of degradants and time) were selected for kinetic studies. Kinetic parameters were calculated and analyzed to study their effect on degradation rate of BDQ.

4.6.1. EXPERIMENTAL

4.6.1.1. Chemicals and Reagents

The Chemicals and reagents utilized in present section were same as described in section 3.5.2.1.

4.6.1.2. Equipment and Chromatographic conditions

The instrument and mobile phase utilized for the present section were same as described 4.5.2.2. Gradient conditions were changed to reduce run time to save utilization of time and solvent. Gradient sequence is shown in table 4.12.

Time	%A
0	35
13.6	86
16	35

%A: % of sodium dihydrogen ortho phosphate buffer

%B: % of methanol

Table: 4.12 gradient sequences for degradation kinetic study of BDQ

4.6.1.3. Preparation of stock, sample and buffer solution

The stock preparation and buffer preparation were same as described in **section 4.5.1.3.**The stock was prepared in different degradant concentration and kept at different temperatures to study the degradation kinetics. The 2ml aliquot was withdrawn prescribed time interval, neutralized with respective HCl/NaOH concentration for acid and/or alkali degradation study;diluted with methanol to get 10ml volume (concentration 200 µg/ml), filtered through 0.2µm membrane filter before HPLC analysis. The solution (Initial concentration 200µg/ml) was injected using the chromatographic conditions of the HPLC gradient method described in table 4.12 using the same column.

The degradation rate kinetics were determined using linear regression analysis by plotting %Drug versus Time (for Zero order reaction), Log of %Drug versus Time (for First Order reaction) and 1/%Drug versus Time(for second order reaction). Experiments were done in triplicate and average values were considered for the analysis. Also, the Arrhenius plots were constructed to study the effect temperature on the rate of hydrolysis and oxidation. The rate constant (K), half life (t_{50}), shelf life (t_{90}) and activation energy (E_a) were also calculated from the slope of lines at each temperature for acid, alkali and oxidation. The enthalpy of activation ($\Delta H^{\updownarrow}$) and entropy of activation ($\Delta S^{\updownarrow}$) were calculated using equations; equation 1 and equation 2, respectively.

To study degradation kinetics of BDQ, different concentration of degradant ($HCl/NaOH/H_2O_2$) wereselected based on stability study. At different time interval sample was withdrawn(2ml aliquot) and neutralized(in case of acid and base) with respective N concentration of base/acid, diluted if required, filtered and injected in RP-HPLC system using gradient method described in table 4.12. Area obtained was utilized to calculate %drug which is further requiredforcalculation to find out order of reaction. Regression coefficient values were compared for zero order, first order and second order to get order of reaction. Degradation kinetic parameters were calculated once order of reaction is confirmed; degradation kinetic parameters were calculated by equation as well as graphical method.

4.6.2. Results and Discussion.

4.6.2.1.Hydrolytic degradation kinetics.

The %drug versus time, *ln C* versus time and 1/C versus time plot was constructed and correlation coefficients were compared. For hydrolytic (acid and alkali) degradation kinetic study of BDQ *ln C* versus time plot showed strongest correlation coefficients indicates that degradation reaction followed first order kinetics.

Acid degradation kinetic study

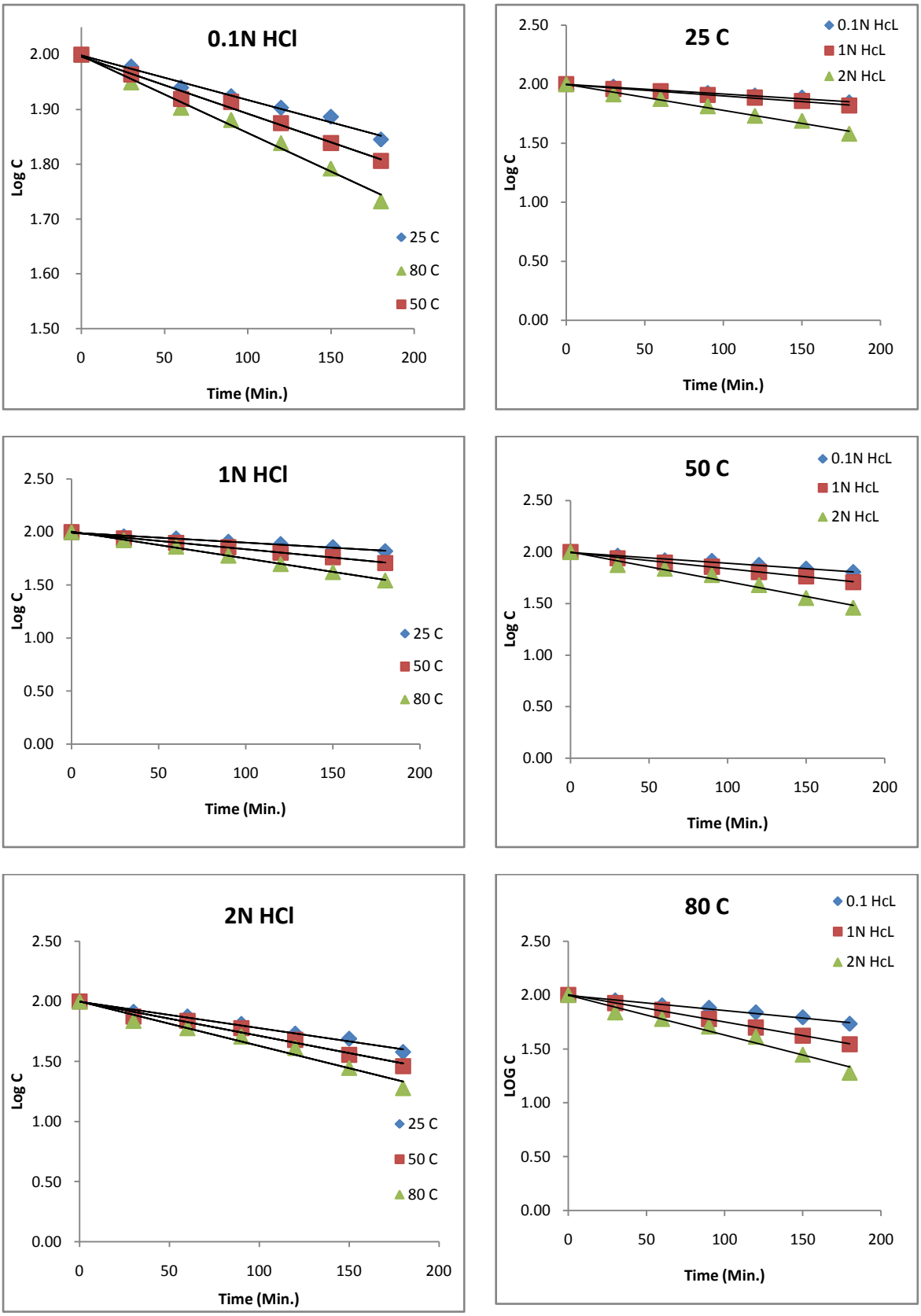
The 74.82% degradation of BDQ was observed in 1N HCl at 80^0C in 75min therefore three level of concentration of HCl (1N HCl ± 1.0), temperature (50^0C ± 25) and time (0 to 180 minutes, 7points) were selected for study. Regression equation and correlation coefficient for zero, first and second order of reaction in acidic medium is shown in table 4.13;value ≥ 0.900 indicated linear and continues process of degradation was observed. The strongest correlation coefficient was observed in first order plots. The slope of regression equation was used for calculation of kinetic parameters.

Conc. HCl	Temp	r^2			Regression Equation		
		Zero Order	**First Order**	**Second Order**	**Zero Order**	**First Order**	**Second Order**
0.1N	25	0.981	**0.983**	0.982	y = -0.158x + 98.96	y = -0.0008x + 1.9984	y = 0.0002x + 0.5000
	50	0.981	**0.986**	0.985	y = -0.192x + 98.07	y = -0.0010x + 1.9962	y = 0.0003x + 0.5003
	80	0.988	**0.989**	0.983	y = -0.241x + 97.46	y = -0.0014x + 1.9975	y = 0.0004x + 0.4992
1N	25	0.989	**0.993**	0.991	y = -0.178x + 98.07	y = -0.0009x + 1.9955	y = 0.0003x + 0.5005
	50	0.986	**0.997**	0.995	y = -0.261x + 96.57	y = -0.0016x + 1.9946	y = 0.0005x + 0.4996
	80	0.983	**0.998**	0.990	y = -0.359x + 95.78	y = -0.0025x + 2.0047	y = 0.0008x + 0.4936
2N	25	0.981	**0.987**	0.972	y = -0.325x + 95.39	y = -0.0022x + 1.9989	y = 0.0007x + 0.4962
	50	0.969	**0.980**	0.958	y = -0.371x + 93	y = -0.0029x + 1.9997	y = 0.0010x + 0.4921
	80	0.951	**0.970**	0.922	y=-0.412x +91	y = -0.0037x + 1.9997	y = 0.0014x + 0.4838

Table: 4.13 Regression equations and r^2 value for acid degradation kinetic

Effect of temperature and stressor concentration on degradation

The effect of temperature and stressor (acid) concentration on degradation rate of BDQ was studied by plot of *ln C* versus time at different stressor concentration and at different temperatures. Fig. 4.10 (A) showed the effect of temperature keeping the concentration constant, as temperature increased, the degradation of BDQ was also increased; while (B) showed the effect of stressor concentration keeping the temperature constant, it has inverse relation on degradation rate of BDQ. The degradation of BDQ can be differentiated at 80^0C in different acid concentrations.



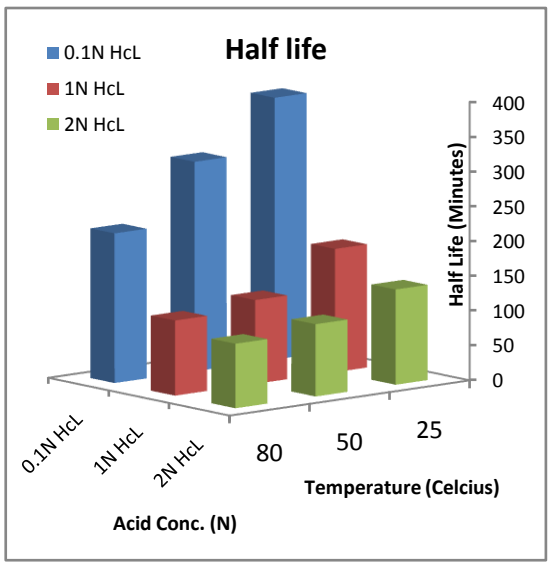
A **B**

Fig. 4.10 first order reaction: **A)** Effect of temperature **B)** Effect of stressor concentration

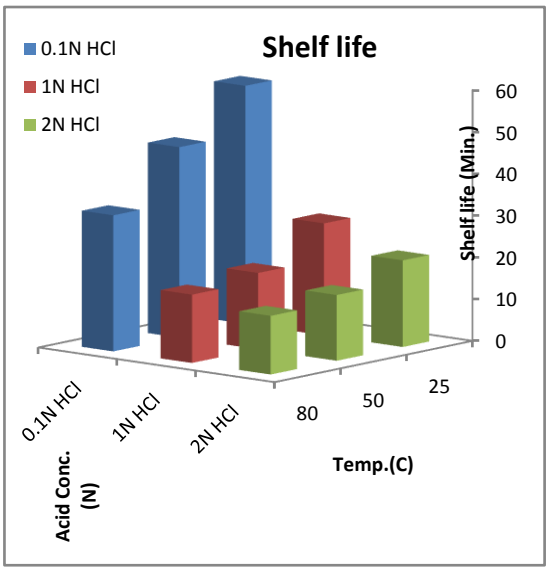
Effect of temperature and stressor concentration on kinetic parameters

The degradation kinetic parameters were calculated for first order reaction; the parameters were rate constant (k), half life (t_{50}), shelf life (t_{90}), and activation energy (E_a), enthalpy of activation ($\Delta H^{\ddagger}$) and entropy of activation ($\Delta S^{\ddagger}$). The effects of temperature and stressor concentrations were studied on these parameters. It is shown in Fig. 4.11; (A) a 3D plot shows the effect of temperature and acid concentration simultaneously on half life of BDQ in acid medium. The longest half life was observed in 0.1N HCl at 25^0C, as the temperature and concentration of acid increased the half life of BDQ was decreased in acid medium. (B) Shelf lifeof BDQ decreased in solution as the temperature and concentration of acid was increased. (C) Rate Constantwas calculated by equation 4 and 5, equation and graphical method respectively. Degradation rate constant increased with temperature and stressor concentration indicates that reaction rate was increased with increment in acid concentration and temperature. The reaction was temperature and acid- catalyzed. (D) Energy of activation:Activation energy of reaction was calculated from slope (m) of regression equation of Arrhenius Plot (*ln k* versus 1/t (K)) using equation 3.

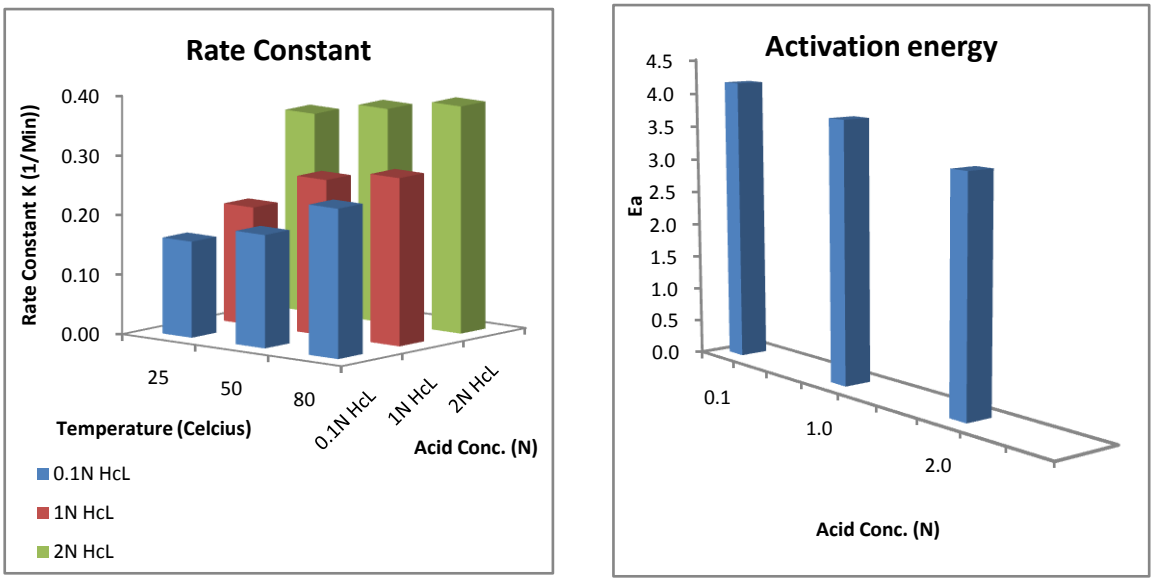
The Activation energy of the reaction decreased with increase in these two factors indicates that temperature and stressor concentration provided external energy that reduced the internal energy of reaction.



A



B



C **D**

Fig.4.11 Effect of temperature and stressor concentration on**A)**Half life**B)**shelf life**C)** Rate constant, and**D)** Activation energy of BDQ acid degradation kinetics

Arrhenius Plot and activation parameters for degradation of BDQ

Arrhenius plot (Fig.4.12) was constructed for *ln k* versus 1/Temperature (unit Kelvin) to study the effect of temperature, energy of activation and degradation rate of BDQ. A straight line indicates that reaction was thermally activated and can be used to calculate energy of activation for the reaction.

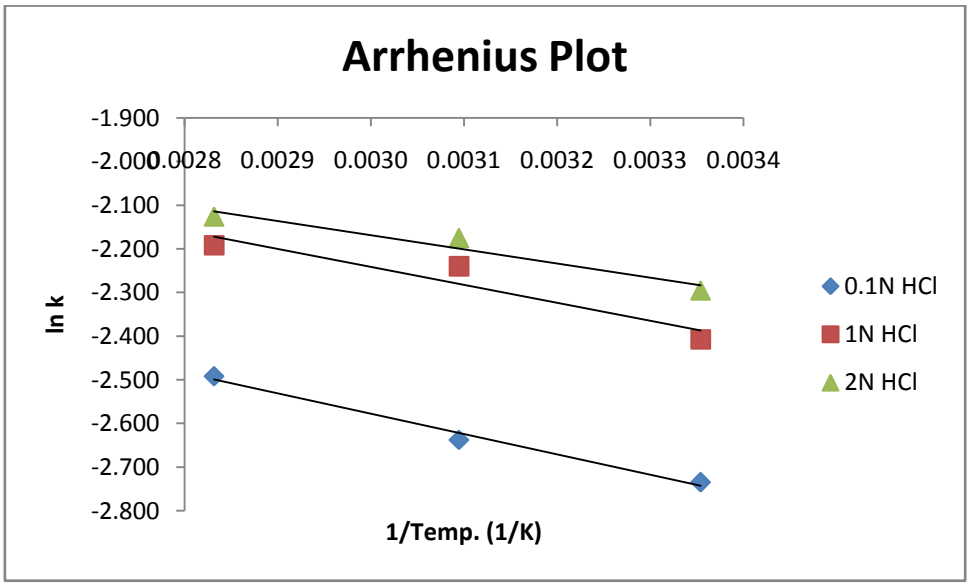


Fig. 4.12 Arrhenius plot for degradation kinetics of BDQ

The activation parameters were calculated from Arrhenius plot, the activation parameters were; enthalpy of activation ($\Delta H^{\updownarrow}$), entropy of activation ($\Delta S^{\updownarrow}$) and energy of activation for reaction were calculated using equation 1, 2 and 3 respectively and shown in Table 4.14. The reaction in system brings out rearrangement of molecules and leads disarrangements in system, this disarrangement of system at start and end of reaction can be measured by entropy. The negative value of entropy shows less disarrangement in system. The linear equations of Arrhenius plot has correlation coefficient r>0.90 for the range of temperature 25-80^0C, slope of the equations in Arrhenius equation (Ea/R) were large this indicated BDQ molecule was stable at lower pH (acid) and this statement is reliable in concern of effect of pH on stability of bedaquiline. The enthalpy values are positive indicating that the reaction was endothermic and absorbed the externally applied energy to speed up the reaction as system enthalpy was less than the environmental enthalpy.

Acid	**Activation**	**r**	**Ea(kj/mol)**	**$\Delta H^{\updownarrow}$**	**$\Delta S^{\updownarrow}$**
0.1N	*ln* k= -500.4x - 1.181	0.98	4.16	6.43	-54.93
1.0N	*ln k*= -457.3x - 1.007	0.90	3.80	6.07	-53.62
2.0N	*ln k*= -400.1x - 1.196	0.94	3.33	5.60	-51.89

Table 4.14 Arrhenius Plot and activation parameters of bedaquiline in different concentration of acid

Degradation kinetic parameters

The degradation kinetic parameters were calculated using equations for first order of reaction. The rate constant was calculated using equation 4 and 5 for equation and graphical method, the half life was calculated using equation 6 and shelf life was calculated using equation 7. The effect of temperature and concentration of acid on kinetic parameters are discussed in section *Effect of temperature and stressor concentration on kinetic parameters*. The kinetic parameters are shown in Table 4.15.

			From Graph		From Equation			
Conc. (N HCl)	**Temp. (^{O}C)**	**Slope**	**k**	**Log k**	**k**	**Log k**	**t_{50} [Min]**	**t_{90} [Min]**
0.1	25	0.00080	0.00184	-2.735	0.00198	-2.703	376.14	56.99
	50	0.00100	0.00230	-2.638	0.00232	-2.634	300.91	45.59
	80	0.00140	0.00322	-2.492	0.00322	-2.493	214.94	32.57
1	25	0.00170	0.00392	-2.407	0.00402	-2.396	177.01	26.82
	50	0.00250	0.00576	-2.240	0.00582	-2.235	120.36	18.24
	80	0.00279	0.00643	-2.192	0.00650	-2.187	107.85	16.34
2	25	0.00220	0.00507	-2.295	0.00488	-2.312	136.78	20.72
	50	0.00290	0.00668	-2.175	0.00666	-2.177	103.76	15.72
	80	0.00325	0.00748	-2.126	0.00751	-2.125	92.59	14.03

Table: 4.15 Acid degradation kinetic parameters for BDQ

Alkaline degradation kinetics of BDQ

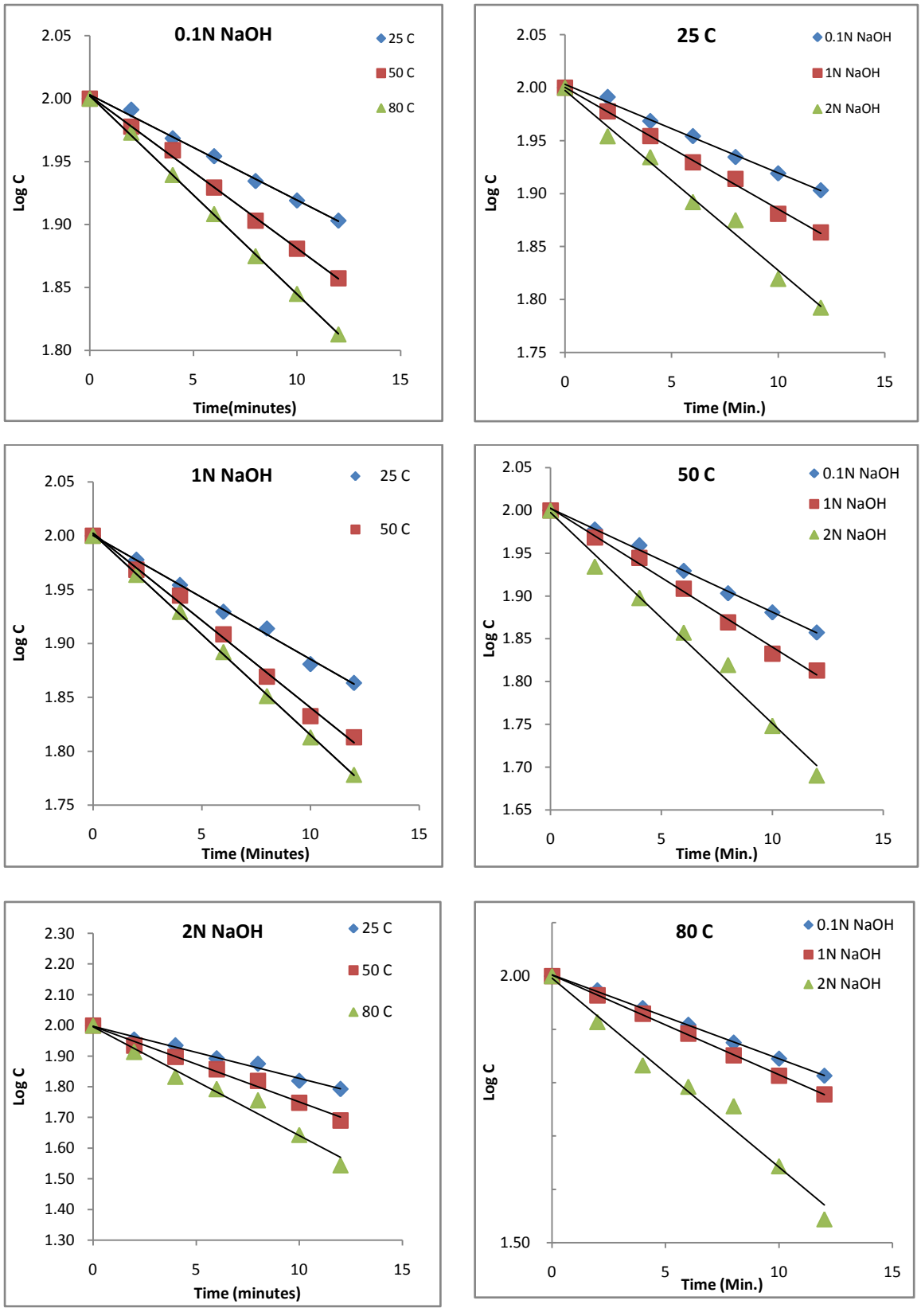
As shown in Table 4.6, 18.86% BDQ degraded in alkaline condition (2N NaOH) at 80^{0}C after 120minutes. It shows that BDQ was stable in harsh conditions of high temperatures and high alkali concentrations, although the presence of DPs indicatedtowards the study of the degradation kinetics in alkaline medium. The harsh conditions (high temperature and high NaOH concentration) as well as long time interval (0 to 12 hrs, 7 points) were used to study alkaline degradation kinetic study of BDQ. As it can be seen in Table 4.16 the strongest correlation coefficients were observed in *ln C* versus time plot indicates that BDQ degradation followed first order kinetics in alkaline medium.

		R^2 Value			Regression equation		
Conc. [N NaoH]	**Temp. (^{0}C)**	**Zero order**	**First order**	**Second order**	**Zero order**	**First order**	**Second order**
	25	0.994	**0.995**	0.994	y = -0.115x + 100.3	y = -0.0006x + 2.0032	y = 0.0001x + 0.4990
0.1	50	0.996	**0.997**	0.996	y = -0.158x + 99.82	y = -0.0008x + 2.0023	y = 0.0002x + 0.4990
	80	0.996	**0.999**	0.998	y = -0.196x + 99.39	y = -0.0010x + 2.0022	y = 0.0003x + 0.4987
	25	0.995	**0.996**	0.995	y = -0.151x + 99.46	y = -0.0008x + 2.0004	y = 0.0002x + 0.4995
1	50	0.992	**0.994**	0.992	y = -0.201x + 99.39	y = -0.0011x + 2.0025	y = 0.0003x + 0.4986
	80	0.995	**0.999**	0.997	y = -0.223x + 98.85	y = -0.0012x + 2.0017	y = 0.0003x + 0.4985
	25	0.986	**0.988**	0.986	y = -0.206x + 98.10	y = -0.0011x + 1.9974	y = 0.0003x + 0.4998
2	50	0.987	**0.988**	0.980	y = -0.269x + 96.78	y = -0.0016x + 1.9975	y = 0.0005x + 0.4985
	80	0.969	**0.977**	0.958	y = -0.335x + 94.21	y = -0.0024x + 1.9959	y = 0.0008x + 0.4961

Table: 4.16 Regression equations and r^2 value for alkali degradation kinetics

Effect of temperature and alkali concentration on BDQ degradation

There was not any noticeable degradation of BDQ in lower stressor concentration (0.1N and 1N NaOH) and at lower temperatures (25^0 and 50^0C). The visible representation is shown in Fig. 4.13 (A) and (B). Temperature of 80^0 C shows well the effect of stressor concentration on BDQ degradation. The highest degradation was observed in 2N NaOH at 80^0C after 12hours (35% degradation of BDQ).

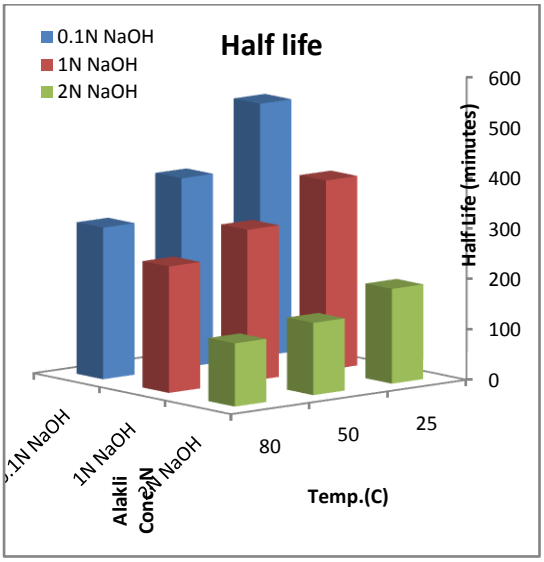


A **B**

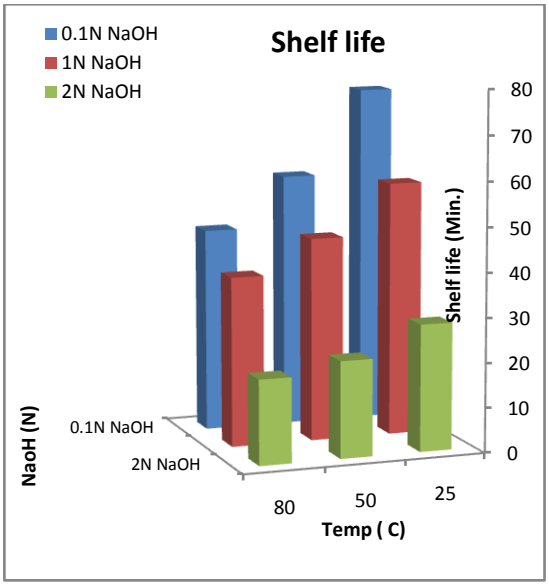
Fig.4.13**A)** Effect of temperatures and **B)** Effect of stressor concentration BDQ degradation

Effect of temperature and stressor concentration on degradation kinetic parameters

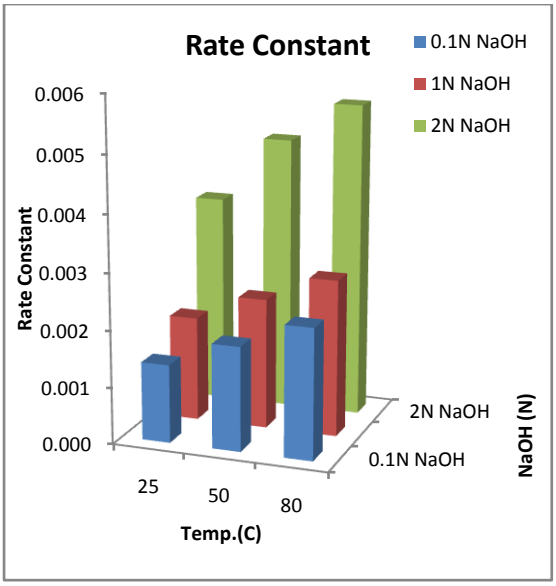
The BDQ was stable under lower temperatures and lower alkaline conditions; it degraded to remarkable extent while kept for longer duration in harsh condition (2N NaOH at 80^0C for 12hrs). This is illustrated in 3D in Fig. 4.17 (A), (B),(C) and (D). The intense peaks are seen for 0.1N and 1N NaOH at $25^0$and 50^0C for half life and shelf life in Fig. 4.14 (A) and (B), respectively. The degradation rate (C) is noticeably high in 2N NaOH indicates the degradation was higher in that condition. The activation energy (D) was higher for 0.1N NaOH indicates that the energy required for the reaction was high and for that reason, degradation of BDQ in lower temperature and lower stressor concentration was the negligible extent.



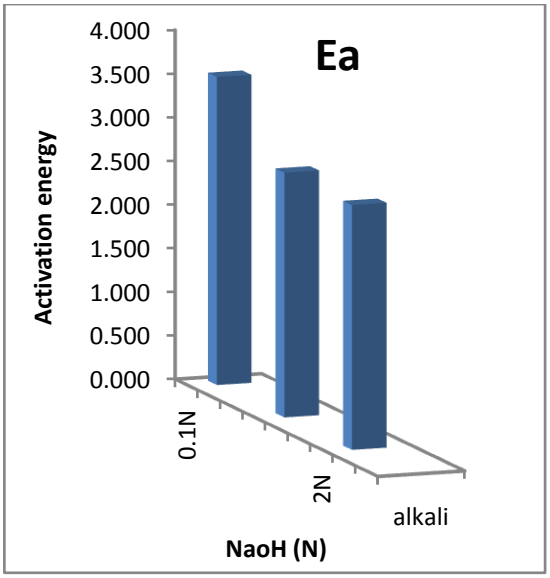
A



B



C



D

Figure: 4.14 Effect of temperature and stressor concentration on **A)**Half life**B)**shelf life**C)** Rate constant **D)** Activation energy for alkali degradation kinetics of BDQ

Arrhenius Plot and activation parameters for BDQ alkaline degradation

Arrhenius plot was constructed for *ln k* versus 1/temperature (K) to calculate activation energy using equation 3. The linear straight line of Arrhenius plot shows the linear degradation and eligibility of plot for using slope to calculate the energy of activation. (Fig.4.15)

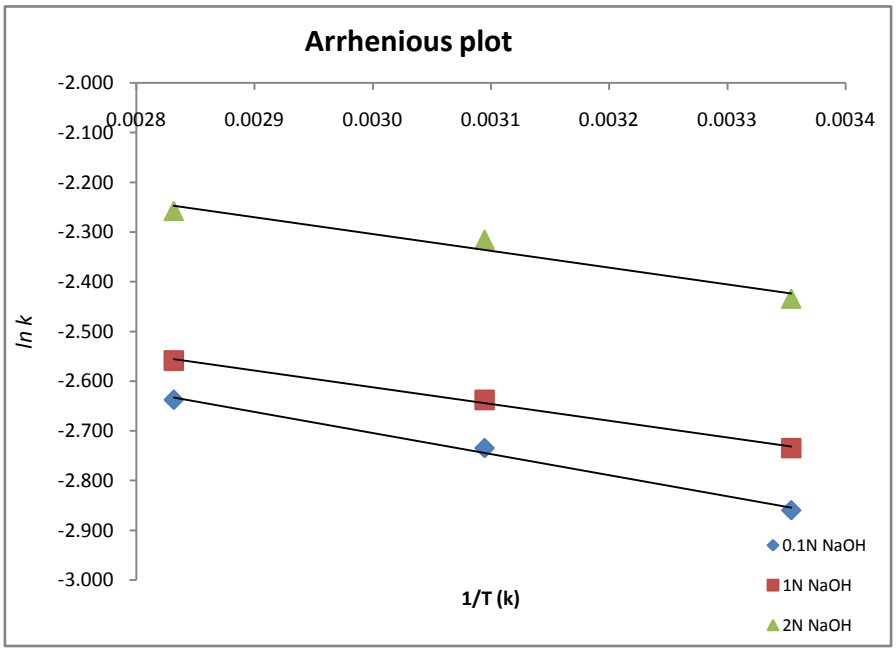


Fig. 4.15 Arrhenius plot for alkali degradation kinetics of BDQ

The activation parameters were calculated (entropy, enthalpy and energy of activation) using equation 1, 2 and 3. The system enthalpy was higher than environmental enthalpy indicated by positive value of enthalpy in Table 4.17. The disarrangement of molecule in reaction was at less extent indicated by negative value of entropy. The difference in activation energy of three concentration of alkali was less shows that reaction was conducted at linear utilization of energy. The broad slope and higher correlation coefficient (>0.95) indicates that BDQ was stable under alkaline condition.

Alkali	Activation	r	Ea(kJ/mol)	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$
0.1N	*ln k* = -424.5x - 1.430	0.99	3.52	2.274	-39.71
1.0N	*ln k* = -337.0x - 1.601	0.99	2.80	2.273	-39.71
2.0N	*ln k* = -336.8x - 1.293	0.96	2.80	2.273	-39.71

Table 4.17 Arrhenius plot and activation parameters for BDQ in alkaline medium

Kinetic parameters and degradation of BDQ

The calculated kinetic parameters are shown in Table 4.18, the effect of factors and degradation of BDQ is already discussed. The massive change in kinetic parameters is observed in 2N NaOH at 80^0C.

		From Graph			From Equation			
Conc. (N NaOH)	**Temp. (^{O}C)**	**Slope**	**k**	**Log k**	**k**	**Log k**	**t_{50} [Min]**	**t_{90} [Min]**
0.1	25	0.00060	0.00138	-2.86	0.00133	-2.87	501.52	75.99
	50	0.00080	0.00184	-2.73	0.00187	-2.72	376.14	56.99
	80	0.00100	0.00230	-2.63	0.00261	-2.58	300.91	45.59
1	25	0.00080	0.00184	-2.73	0.00187	-2.72	376.14	56.99
	50	0.00100	0.00230	-2.63	0.00225	-2.64	300.91	45.59
	80	0.00120	0.00276	-2.55	0.00278	-2.55	250.76	37.99
2	25	0.00160	0.00368	-2.43	0.00368	-2.43	188.07	28.50
	50	0.00210	0.00484	-2.31	0.00486	-2.31	143.29	21.71
	80	0.00240	0.00553	-2.25	0.00552	-2.25	125.38	19.00

Table 4.18 Degradation kinetic parameters for BDQ in alkaline medium

4.6.2.2.Oxidative degradation kinetic

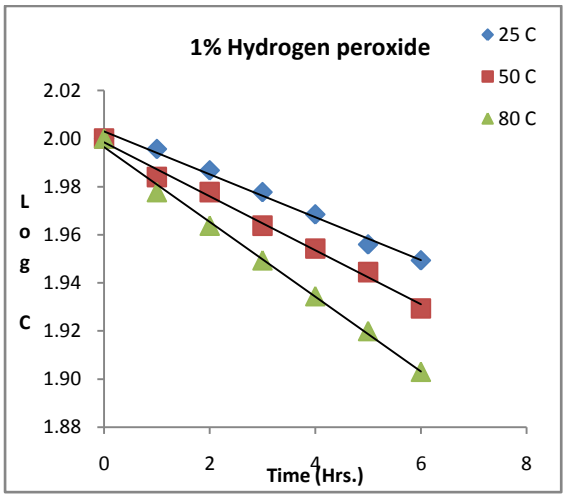
Table 4.6 for stress degradation study of BDQ shows that 63.93% of BDQ was degraded in presence of oxidizing agent (12% hydrogen peroxide), and formed three DPs. To study the degradation kinetics of BDQ three level of hydrogen peroxide concentrations (6% ± 6), temperatures (50^0C ± 25^0) and time intervals (0 to 6 hrs, 7points) were selected. The analysis of %C versus time ,*ln C* versus time and 1/C versus time plot showed that strongest correlation coefficient was achieved in *ln C versus t* plot indicates that oxidation of BDQ followed the first order of reaction. The correlation coefficient and regression equations for order of reactions are shown in Table 4.19.

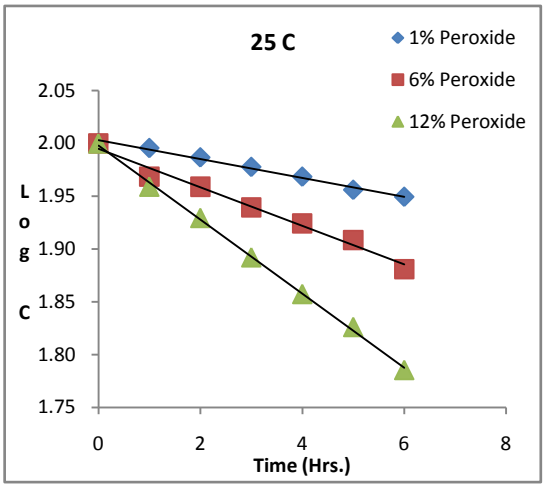
		R^2 Value			Regression equation		
% H_2O_2	T (^{0}C)	Zero order	**First order**	Second order	Zero order	**First order**	Second order
1%	25	0.987	**0.989**	0.988	y = -1.964x + 100.6	y = -0.000x + 2.00	y = 0.0023x + 0.499
	50	0.988	**0.993**	0.992	y = -2.357x + 99.35	y = -0.0002x + 1.99	y = 0.0029x + 0.500
	80	0.988	**0.996**	0.995	y = -3.142x + 98.85	y = -0.0004x + 1.99	y = 0.0041x + 0.500
6%	25	0.982	**0.985**	0.984	y = -3.678x + 98.46	y = -0.0001x + 1.99	y = 0.004x + 0.501
	50	0.975	**0.989**	0.983	y = -4.000x + 97.71	y = -0.0003x + 1.99	y = 0.005x + 0.501
	80	0.984	**0.989**	0.981	y = -4.785x + 97.78	y = -0.0004x + 1.99	y = 0.006x + 0.502
12%	25	0.993	**0.998**	0.997	y = -6.357x + 98.21	y = -0.0001x + 1.99	y = 0.009x + 0.499
	50	0.98	**0.981**	0.969	y = -8.678x + 95.89	y = -0.0003x + 1.99	y = 0.016x + 0.498
	80	0.979	**0.981**	0.944	y = -11.82x + 93.89	y = -0.0005x + 2.00	y = 0.033x + 0.487

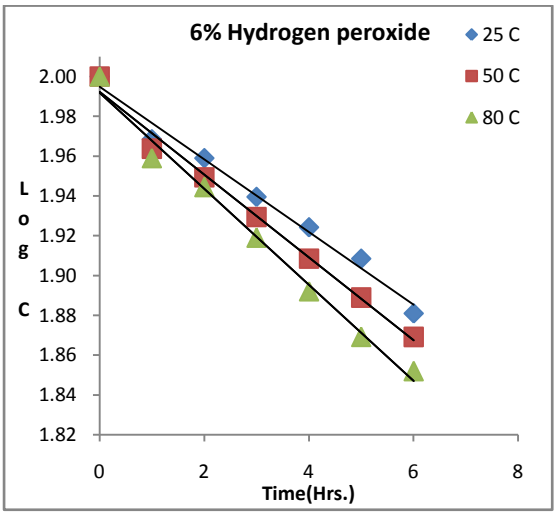
Table 4.19 correlation coefficient and regression equations for oxidative degradation kinetics of BDQ

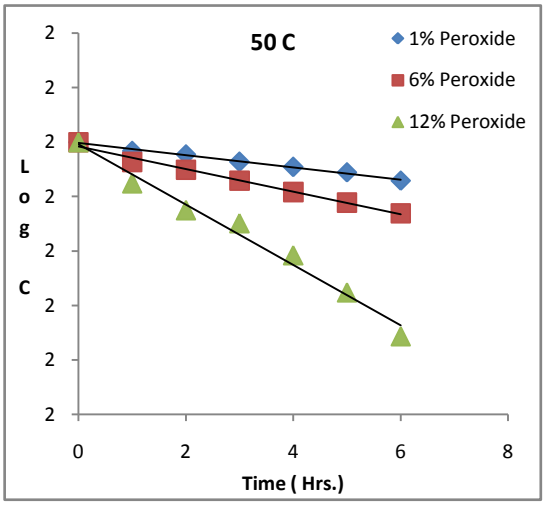
Effect of temperature and stressor concentration on BDQ degradation

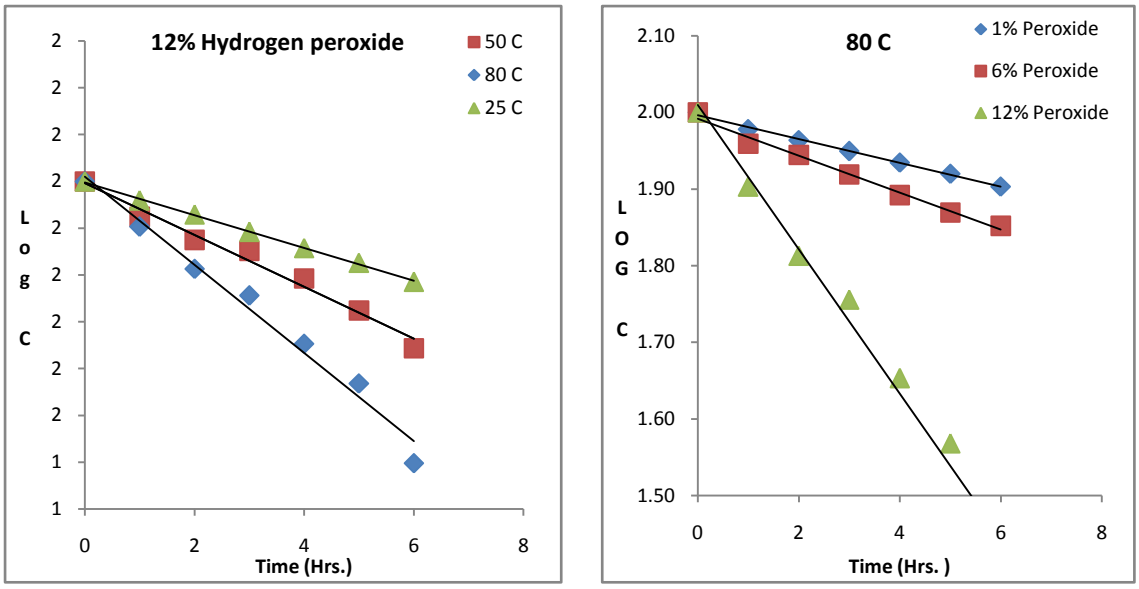
To study effect of temperatures and stressor concentrations on BDQ degradation in oxidative medium the plot of *ln C versus t* was constructed (Sown in Fig. 4.16) at (A) different temperatures and (B) at different stressor concentrations. An ideal degradation plot was observed for BDQ degradation as linear degradation was secured. From plots it can be assumed that both temperatures and stressor concentration played equal role in degradation of BDQ. The highest degradation was achieved in 12% hydrogen peroxide at 80^0C after 6hrs.











A **B**

Fig. 4.16 A) Effect of temperatures and B) effect of stressor concentrations on BDQ degradation

Effect of temperatures and stressor concentrations on kinetic parameters

The kinetic parameters were calculated using equations and evaluated for the impact of factors and degradation of BDQ. Fig. 4.17 shows the relation between kinetic parameters and kinetic factors. It can be determined from plots that oxidation of BDQ was occurred spontaneously in overall used concentrations of hydrogen peroxide except the reaction was slow in 1% hydrogen peroxide at room temperature. Addition of temperatures along with oxidative agent crutched the reaction. Half life and shelf life of reaction decreased with increase in temperatures and concentration of oxide, while degradation rate constant increased with it. The overall activation energy of reaction remained approximately same for all % of hydrogen peroxide, this indicates that the oxidation of bedaquiline can occur at any concentration of hydrogen peroxide but temperature played vital role to speed up the reaction.

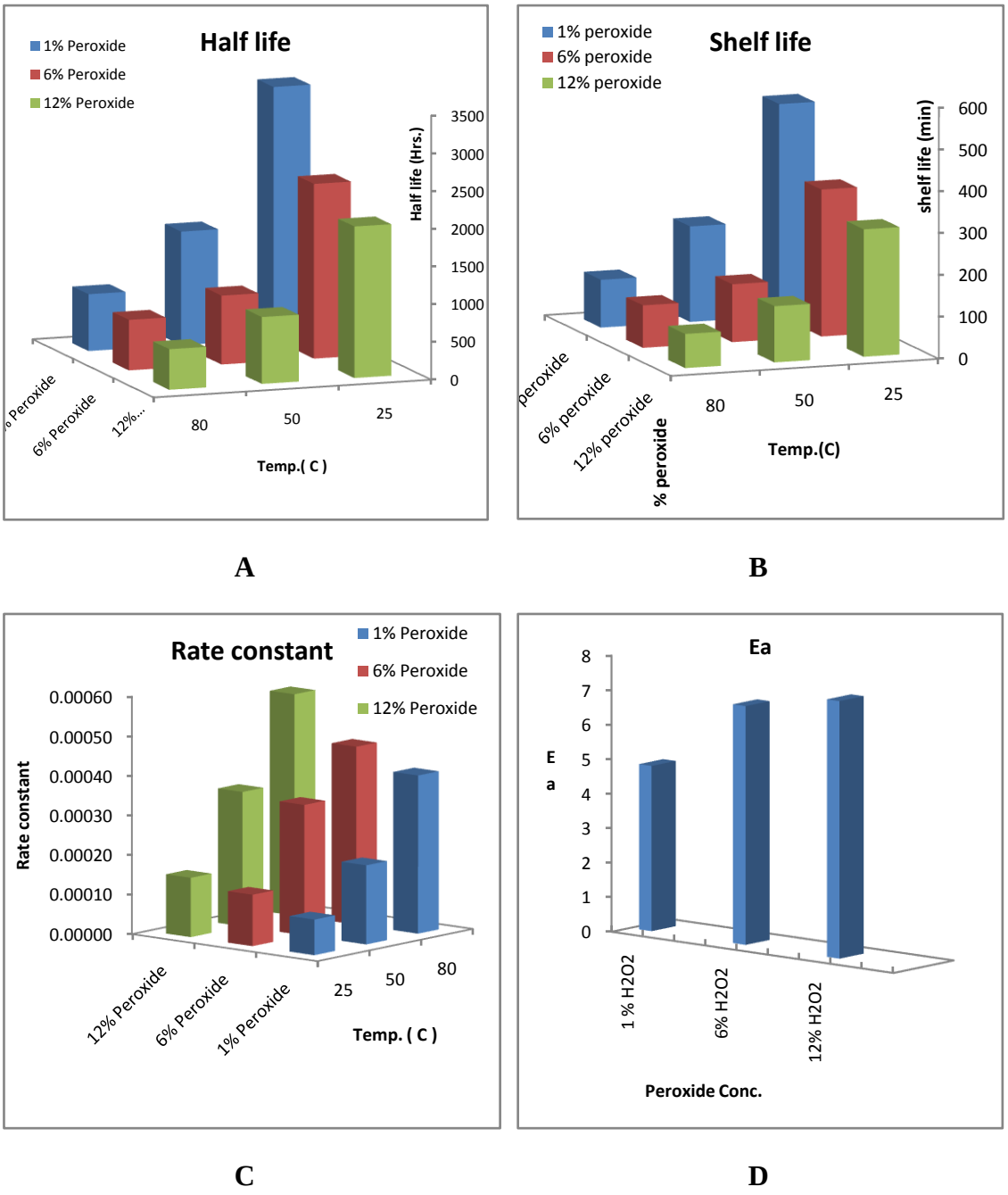


Fig. 4.17 effect of temperature and stressor concentrations on (A) Half life (B) shelf life (C) Rate constant, and (D) Activation energy of reaction

Arrhenius plot and activation parameters for BDQ oxidation

The linear regression line of Arrhenius plot indicates that oxidation of BDQ was linear reaction and activation of energy can be calculated using slope of regression equation of Arrhenius plot(Fig.4.18).The reaction was slow in 1% hydrogen peroxide while it was eventuated almost similar in 6 and 12% peroxide.

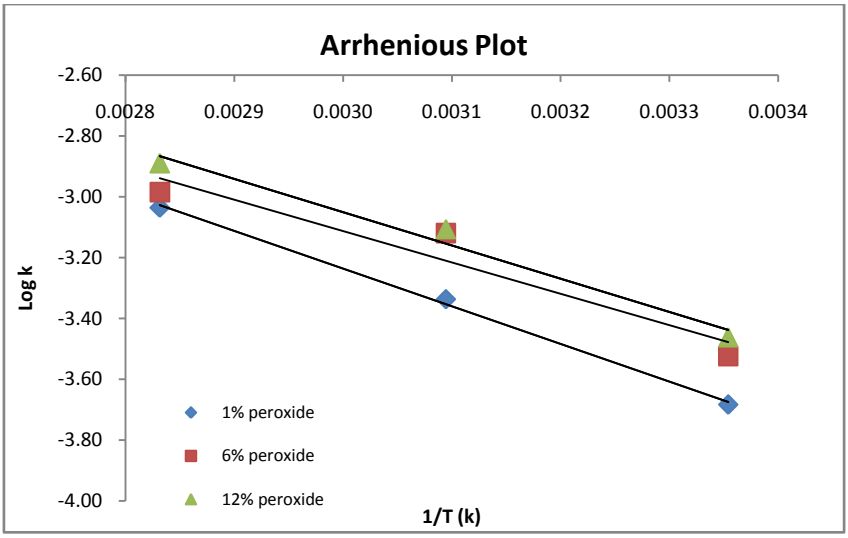


Fig. 4.18 Arrhenius plot for oxidation of BDQ

Activation parameters for BDQ oxidation areshown in table 4.20, to happen the reaction moderately, negative entropy values are insisted. The increase in activation energy and negative enthalpy values turns the arrow towards an exothermic reaction. The oxidation of BDQ released heat in environment.

Oxide	Activation	r	Ea(kJ/mol)	$\Delta H^{\updownarrow}$	$\Delta S^{\updownarrow}$
1%	*ln k*= -576.x + 0.155	0.91	4.78	-4.6	-33.068
6%	*ln k* = -832.x + 0.271	0.92	6.91	-2.47	-33.062
12%	*ln k*= -897.x + 0.330	0.99	7.45	-1.93	-33.061

Table 4.20 Activation parameters for oxidation of BDQ

Degradation kinetic parameters for oxidation of BDQ

Degradation kinetic parameters were calculated using equations and shown in Table 4.21. The effect of temperature and stressor concentrations already discussed. The highest degradation of BDQ was observed in 12% hydrogen peroxide at 80^0C after 6hrs.

		From Graph			From Equation			
Conc. (%)	**Temp. ^{O}C**	**Slope**	**k**	**Log k**	**k**	**Log k**	**t_{50} (Min.)**	**t_{90} (Min.)**
1% H_2O_2	25	0.00009	0.0002	-3.68	0.0020	-2.70	3343.47	506.59
	50	0.00020	0.0005	-3.34	0.0006	-3.26	1504.56	227.96
	80	0.00040	0.0009	-3.04	0.0009	-3.05	752.28	113.98
6% H_2O_2	25	0.00013	0.0003	-3.52	0.0003	-3.52	2314.71	350.71
	50	0.00033	0.0008	-3.12	0.0008	-3.10	911.85	138.16
	80	0.00045	0.0010	-2.98	0.0010	-3.00	668.69	101.32
12% H_2O_2	25	0.00015	0.0003	-3.46	0.0003	-3.52	2006.08	303.95
	50	0.00034	0.0008	-3.11	0.0008	-3.10	885.03	134.10
	80	0.00056	0.0013	-2.89	0.0015	-2.82	537.34	81.42

Table 4.21 Degradation kinetic parameters for oxidation of BDQ

- **Conclusion**

The degradation kinetic study was completed using different temperatures and different stressor concentrations. The *ln C versus t* plot showed strongest correlation coefficients and BDQ followed first order kinetics for acid, alkaline and peroxide induced reactions.BDQ is stable under the thermal conditions for more than 21 days indicates that hydrolytic and oxidative reaction of BDQ was the simultaneous effect of stressor and temperature. Stressor alone can induce degradation of BDQ but temperature pushes the reaction to speed up. The acid and alkali induced reaction were endothermic while oxidative reaction of BDQ was exothermic.

PART-C

4.7 MULTI FACTORIAL DESIGN TOOL FOR DEGRADATION KINETIC STUDY OF BDQ

The degradation kinetic for BDQ is completed using the conventional method of study. The study clearly shows effect of temperature and concentration of stressor on degradation of BDQ and on degradation kinetic parameters. The Design of Experiment (DoE) is novel approach for screening, optimization and predication of response in experiment, degradation kinetic study by conventional method demands time and solvent utilization so it was thought of interest to apply DoE approach for prediction of response. A hypothetical concept was applied to study the degradation kinetics of BDQ using the multifactorial tool for the prediction of kinetic parameters.

4.7.1 EXPERIMENTAL

4.7.1.1 Chemicals and Reagents

The chemicals and reagents utilized in present section were same as described in section 4.5.1.1.

4.7.1.2 Equipment and Chromatographic conditions

Equipment and chromatographic conditions utilized in present section were same as described in section 4.6.1.2. Multi factorial design was performed using software Design Expert ® 12 (state-ease, USA).

4.7.1.3. Analytical sample preparations

As described in section 4.6.1.3.

4.7.1.4. Multi Factorial design generation

The Factorial Experiment was designed using 2 × 3 factorial designs where 2 levels and 3 factors were included in the design. The levels were -1 and +1 indicated for the lower limit and upper limit of design respectively; moreover the central point can be added manually if a researcher wants to study the response of center level of factor.The factors included for the study were stressor concentration, temperature, and time, levels were the upper limit and lower limit of factors; one center point was added in each factor level to ease the analysis of data. Table 4.22 shows the factor and level of multi factorial design.

Factor	Name	Units	Type	Minimum	Maximum	Coded Low	Coded High
A	Acid Concentration	N	Numeric	0.1000	2.00	-1 ↔ 0.10	+1 ↔ 2.00
B	Temperature	C	Numeric	25.00	80.00	-1 ↔ 25.00	+1 ↔ 80.00
C	Time	minutes	Numeric	0.0000	180.00	-1 ↔ 0.00	+1 ↔ 180.00

Table 4.22 Multi factorial design parameters

The responses of factors and levels were selected as kinetic parameters, five responses were selected; R1: % Drug (%), R2: Rate constant (-), R3: Half life (min.), R4: Shelf life (min.), and R5 Activation energy (Kj/mol*K).

4.7.1.5. Analysis of response and generation of data

The responses obtained against the factor and level was performed and data was gathered in Design Expert © 12 software. The analysis of each response was done using the same software. The actual value was gathered by performing the experiment and was compared with the predicted value obtained from the software.

4.7.2. Result and Discussion

Design generation and suitability of design

Design for acid, alkali and oxidative degradation kinetic was generated using 2×3 factorial design; suitability of design was restrained by FDS plot. Standard error mean term was taken in consideration for suitability of design. FDS plot for design is shown in supplementary file. 4.7.2.1. (A)

Significance of factors for design

The relative significance of factors was analyzed by Perturbation plot, the effect of one factor was studied while keeping other factors constant. For acid, alkali and oxidative degradation design, the midpoint was obtained for each factor indicates that factors were significant for study.

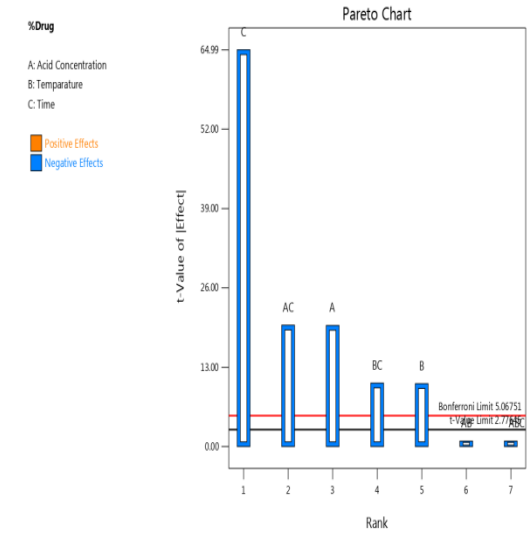
4.7.2.1 Hydrolytic degradation kinetic

Acid degradation kinetic study

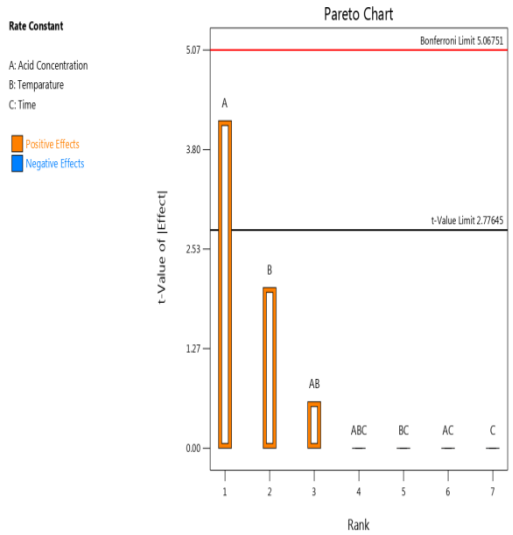
Analysis of response

The run obtained for given level of factor was practically performed and responses were entered into the design. The responses were analyzed against each factors and level. Analysis of response for each factor was confirmed by *half normal plot* and *Pareto Chart*. Both the

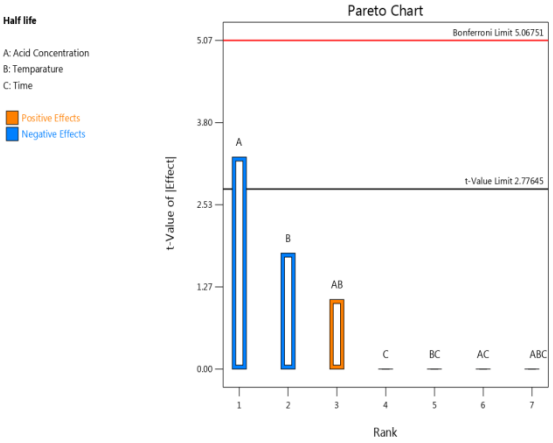
plot can be analyzed for positive or negative effect of factors on response and to identify significant factor for particular response. The analysis of half normal plot and Pareto chart (Fig.4.19) for responses shows following results:



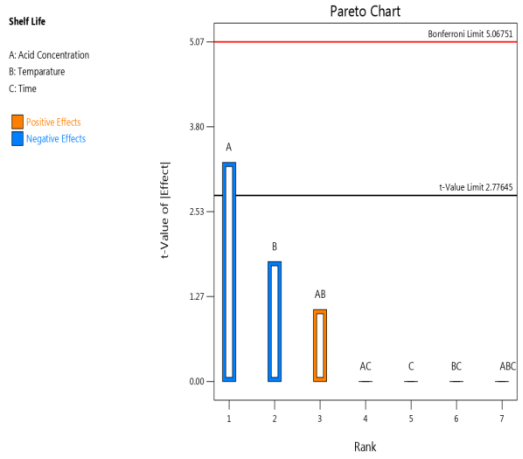
A



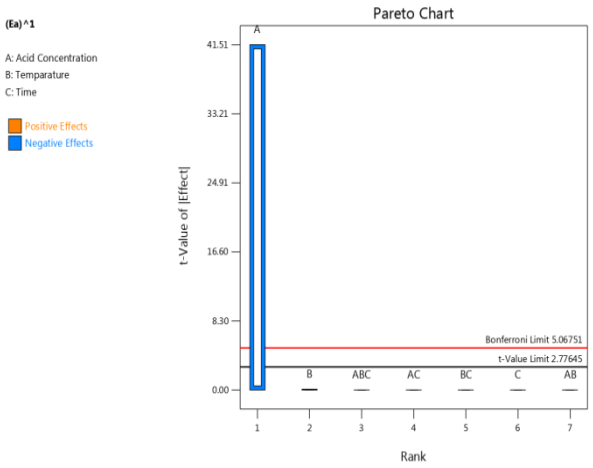
B



C



D



E

Fig. 4.19 Pareto chart for analysis of significance of the factors

R1 (% Drug) – The all three factors and combined effect of factors shows negative effect on %drug of BDQ; Factor C (time) showed the prominent effect while other factors showed moderate to low negative effect.

R2 (Rate constant) – The analysis of Pareto chart for rate constant show that Factor A, acid concentration and Factor B, temperature increased the rate constant while other factors have moderate to low positive effect on R2.

R3 (Half life) – Factor A (Acid concentration) and B (Temperature) has negative effect on half life; increase in factor A and B, decreases the half life on BDQ in acid medium.

R4 (Shelf life) – Increase in factor A (Acid concentration) and B (Temperature) lead to decrease in shelf life of BDQ in acid medium.

R5 (Activation energy) – Acid concentration (factor A) highly affect the energy of activation for reaction.

Analysis of Variance (ANOVA)

ANOVA is a mathematical term used to analyze effect of factor (either positive or negative) on response. For acid degradation study of BDQ mathematical terms are generated for each response;

R1 (%drug): +72.14 – 8.35 A – 4.33 B – 27.38 C -0.3773 AB - 8.38 AC -4.38 BC -0.3750 ABC

R2 (Half life): +0.0048 +0.0018 A +0.0009 B + 0.0000 C + 0.0003 AB + 0.0000 AC + 0.0000BC + 0.000 ABC

R3 (Shelf life): +173.79 -88.78 A -48.50 B +0.0000 C +29.10 AB +0.0000 AC +0.0000 BC +0.0000 ABC

R4 (Rate constant): +26.33-13.45 A -7.35 B +0.0000 C +4.41 AB +0.0000 AC +0.0000 BC +0.0000 ABC

R5 (Activation energy): +3.76-0.4154 A -0.0010 B +0.0000 C +0.0000 AB -0.0000 AC -0.0000 BC +0.0000 ABC

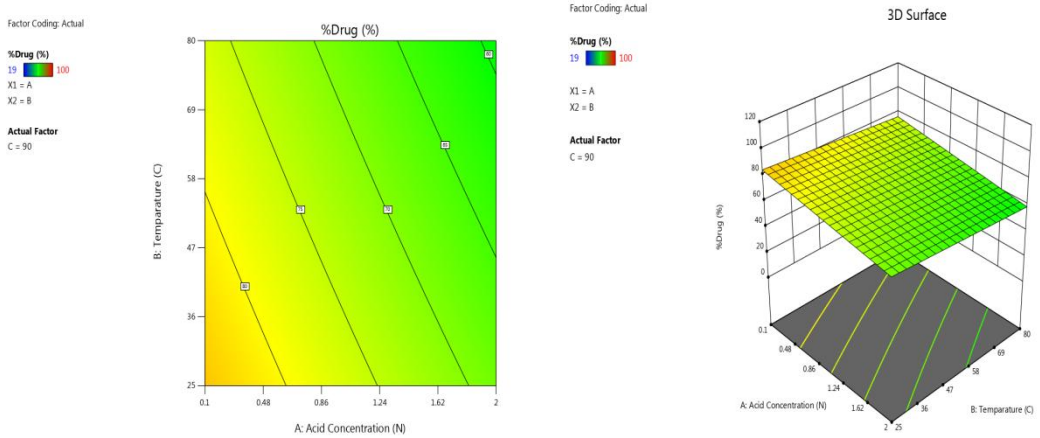
The equation is generated by software, + (positive) sign indicates the supportive effect of factor on response while – (negative) sign indicates the reducing effect of factor. The 00 indicates no effect of factor on response. The factor A (acid concentration) and factor B (temperature) has negative effect on all responses except rate constant. Response R1 (%Drug) is affected by all the factors.

Interaction of factors

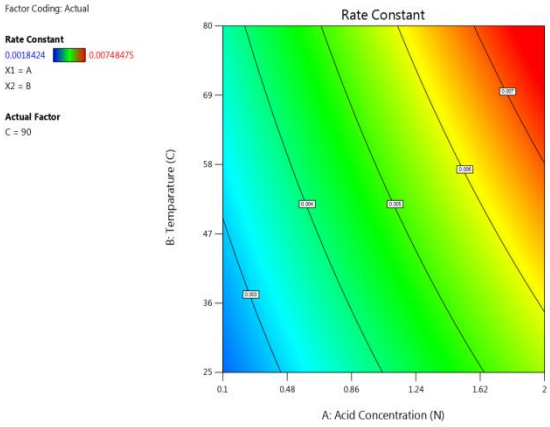
The factors interacting with each other can reduce the factors' effect on responses. Interaction plot showed that if any factor interacted and produced changes in response. To evaluate the interaction between two factors, one factor was kept constant and other factors are studied simultaneously. For BDQ acid degradation kinetic study, factor C (time) was kept constant as factor C had less effect on responses, while factor A and Factor B was studied. Interaction plots are shown in supplement file 4.7.2.1. (D). Factor A and B show no interaction in any responses, the lines are parallel indicates that both factors have their individual effect on response.

Contour Plots and 3-Dimentional study

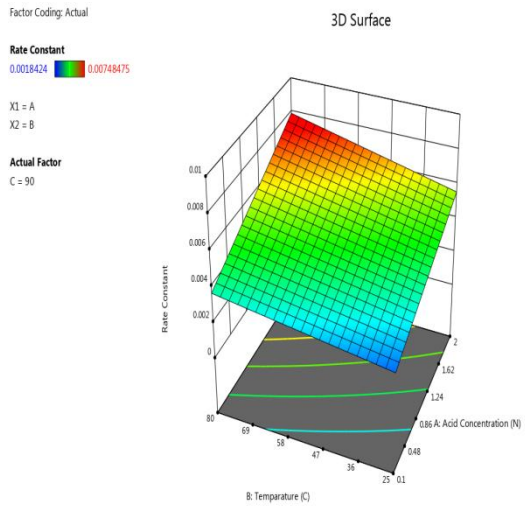
Contour plots are 3D-graphical representation of effects of three factors on response. The plots in 2D and 3D format are shown in Fig. 4.20 for each response. The contour plot is taken by keeping Factor C (time at 90min.) constant so that Factor A and Factor B effect can be analyzed.



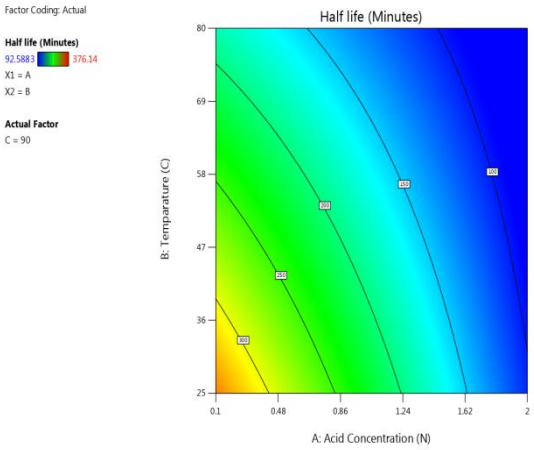
A_1 **A_2**



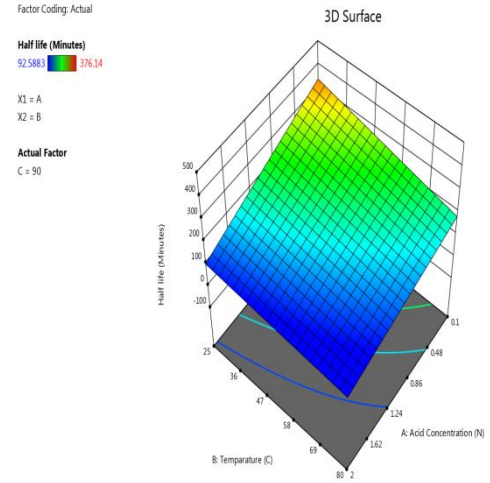
B_1



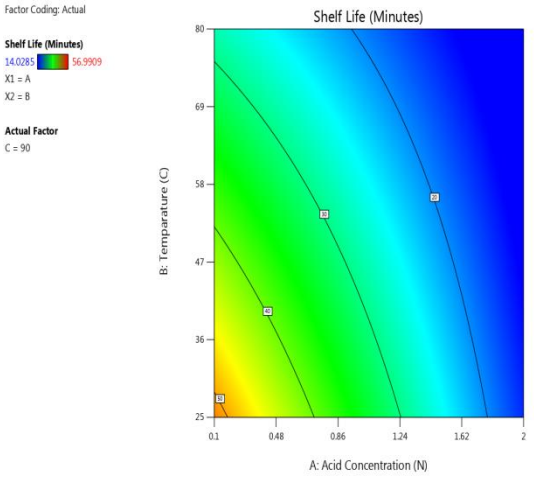
B_2



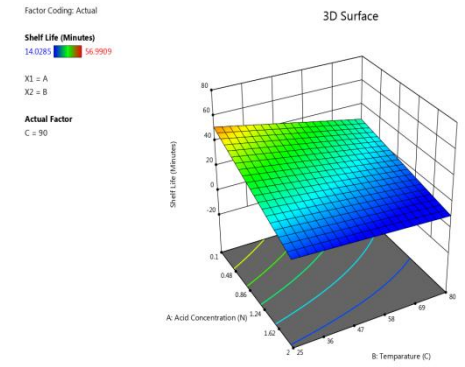
C_1



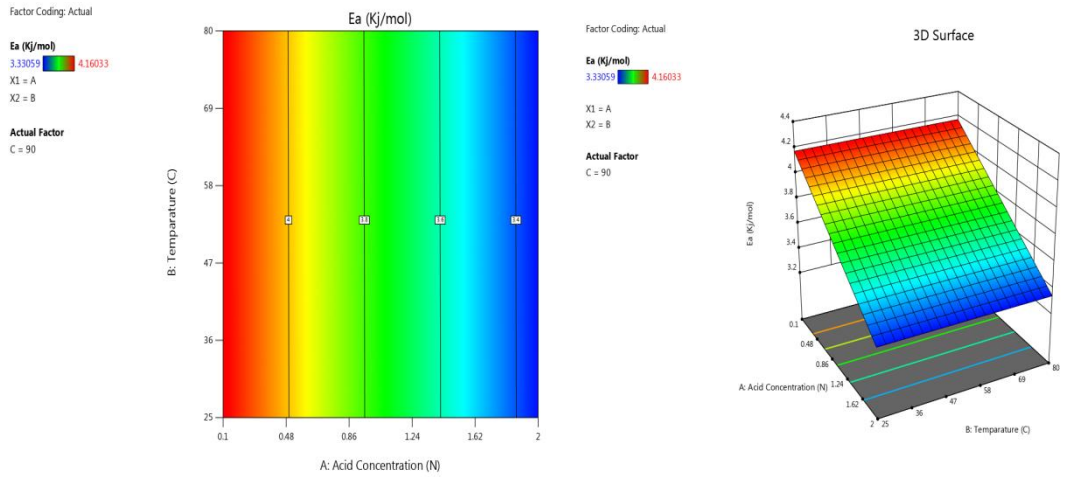
C_2



D_1



D_2



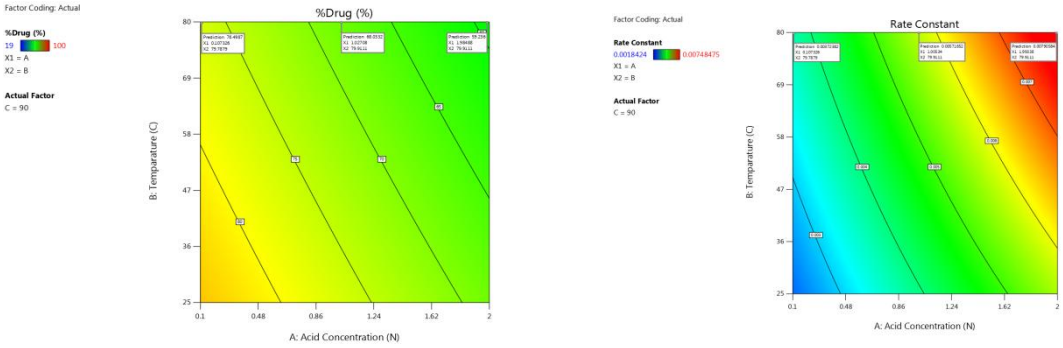
E_1 **E_2**

Fig. 4.20.2D contour for **A_1)** % Drug, **B_1)** Rate Constant, **C_1)** Half life, **D_1)** Shelf life and **E_1)** Activation energy and 3D plot for **A_2)** % Drug, **B_2)** Rate Constant, **C_2)** Half life, **D_2)** Shelf life and **E_2)** Activation energy

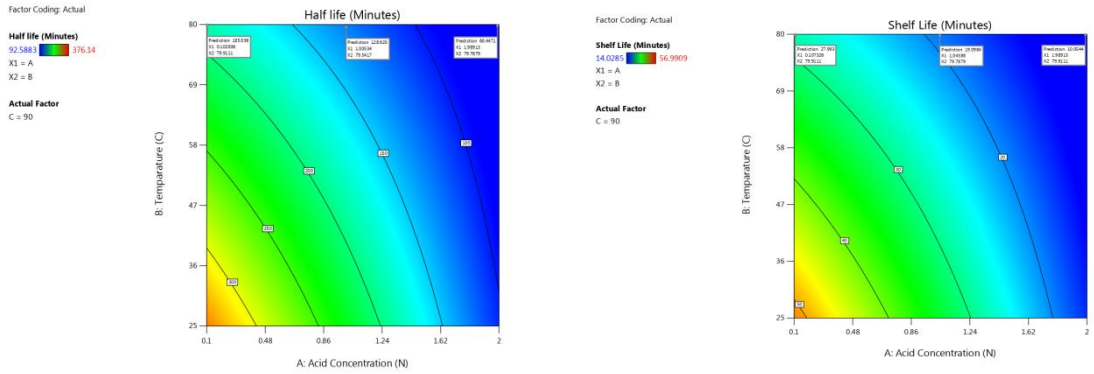
From Fig. 4.23 it can be analyzed that at constant time 90 minutes factor A and factor B has mild to severe effect on responses. Rate constant and energy of activation is strongly driven by both the factors. The effect of factors is similar to the one discussed in section 4.6.1.2.

Prediction of kinetic parameters using Design Expert ^{TM} software

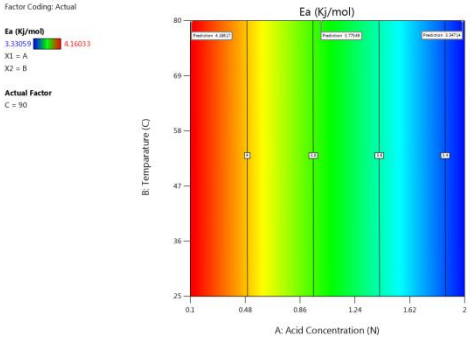
Design expert ^{TM} software was utilized for predication of kinetic parameters using factors and levels 2×3 multi factorial design. The feature of software can predict the response value for the factors at any level of design. For ease of analysis, the prediction point was chosen in a way that includes the point in the conventional study so that a comparison of value can be done easily. The responses were predicted for 0.1N to 2N HCl at 80^0C (constant time 90min.), the values are flagged in Fig. 4.21 for every response.



A **B**



C **D**



E

Fig.4.21 Predication of acid degradation kinetic parameters using Design Expert ™ software

Application of multi factorial tool

For kinetic parameters responses (R1 to R5) values are predicted (flagged in Fig. 4.24) and compared with practical values to evaluate the efficiency of the tool to analyze the factors' effect. The tool featured application is shown in Table 4.23 where kinetic parameters obtained by both methods (Conventional and Multi factorial approach) are compared.

Kinetic parameters	Multi factorial method	Conventional method
	0.1N HCl	
%Drug (%)	76.49	76
Rate constant	0.0037	0.0032
Half life (Min.)	185.03	214.94
Shelf life (Min.)	27.99	32.57
Ea (kj/mol. k)	4.16	4.16
	1N HCl	
%Drug (%)	68.03	67.02
Rate constant	0.0057	0.0064
Half life (Min.)	128.9	107.85
Shelf life (Min.)	19.09	16.34
Ea (kj/mol. k)	3.77	3.80
	2N HCl	
%Drug (%)	59.23	57.9
Rate constant	0.0079	0.0075
Half life (Min.)	66.44	92.59
Shelf life (Min.)	10.05	14.03
Ea (kj/mol. k)	3.34	3.33

Table 4.23 Degradation kinetic parameters by conventional and multi factorial method

The analysis of Table 4.22 shows that %drug, rate constant and activation energy value obtained by both the methods are corresponding to each other while half life and shelf life values are slightly varied in both methods although the variation is in the acceptable range.

Alkaline degradation kinetics

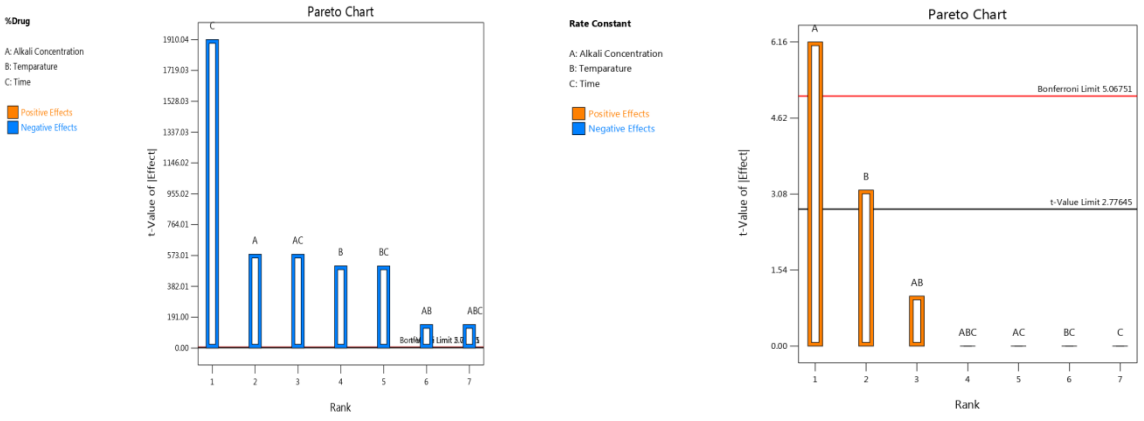
The degradation kinetics of BDQ in alkaline condition is described in brief in section 4.6.2.1. The multi factorial design was created using 2×3 factors and levels. The design criteria are shown in Table 4.24.

Factor	Name	Units	Type	Minimum	Maximum
A	Alkali Concentration	N	Numeric	0.1000	2.00
B	Temperature	C	Numeric	25.00	80.00
C	Time	Minutes	Numeric	0.0000	180.00

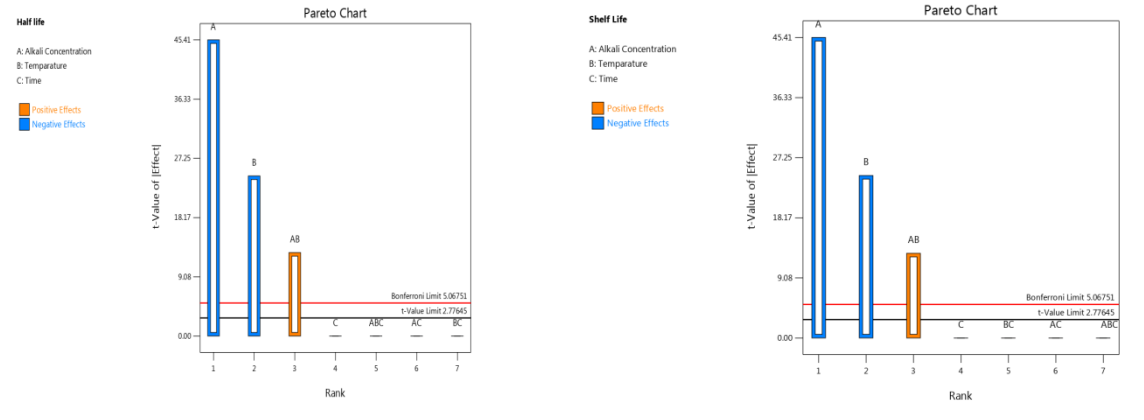
Table 4.24 Multi factorial design criteria for alkali degradation kinetics of BDQ

Analysis of response

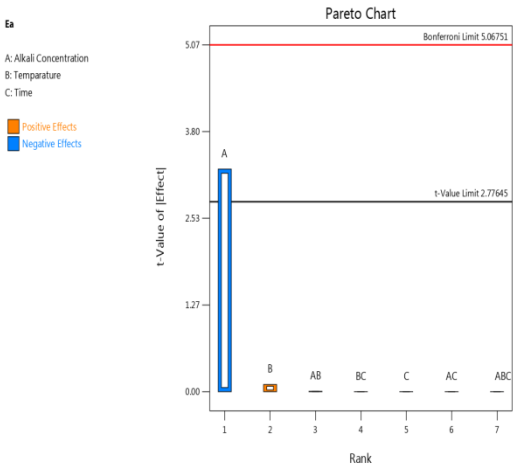
The responses are analyzed against factors and their levels, the run obtained in design were practically imposed to get the data for addition in response block. After completion of collection of data, the responses were analyzed for effect of factors on it. The Pareto chart and half normal plots show the factor effect in the form of chart. The Pareto charts for each response are shown in Fig. 4.22.



A **B**



C **D**



E

Fig.4.22 Pareto chart for responses of alkaline degradation kinetics of BDQ

Analysis of Pareto chart show that %drug (R1) of BDQ is reduced by all the factors while Rate constant (R2) is provoked in high level of factors, half life (R3) and shelf life (R4) is decreasing with high level of factors. The activation energy of reaction is decreasing with increase in acid concentrations.

Analysis of Variance (ANOVA)

The variance analysis is mathematical study of responses; an equation is generated by software which shows positive and negative effect of factors by sign (+) and (-) respectively. The equations are shown below to estimate the factor effect on responses.

R1 (%Drug): +80.24-6.00 A -5.25 B -19.75 C -1.50 AB -6.00 AC -5.25 BC -1.50 ABC

R2 (Rate Constant): +0.0030+0.0014 A +0.0007 B +0.0000 C +0.0002 AB +0.0000 AC +0.0000 BC +0.0000 ABC

R3 (Half life): +282.08-122.41 A -66.11 B -7.599E-06 C +34.49 AB -7.599E-06 AC -7.599E-06 BC -7.599E-06 ABC

R4 (Shelf Life): +42.74-18.55 A -10.02 B +0.0000 C +5.23 AB +0.0000 AC +0.0000 BC +0.0000 ABC

R5 (Activation energy): +3.04-0.3579 A +0.0115 B +0.0000 C -0.0006 AB +0.0000 AC +0.0000 BC +0.0000 ABC

The values shown before the factors are the intensity of the effect of the factor with respect to the numbers; where 0 indicates no or negligible effect.

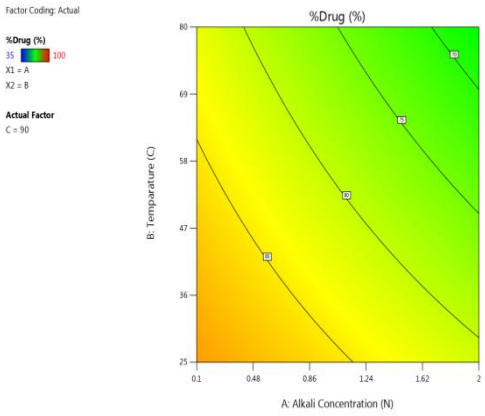
Interaction of Factors

Interacting factors can reduce the effect of factors on responses, so the study of the interaction of factors is important to check whether factors are interacting and diminishing

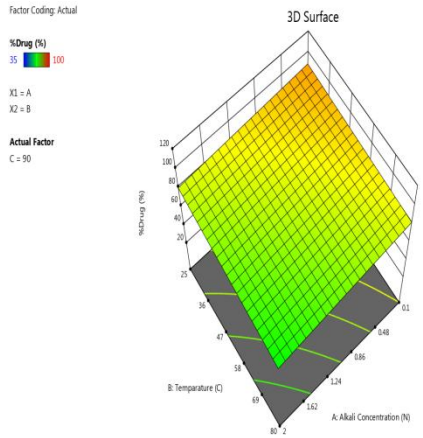
their effect on responses or not. The interaction plot of factors for each response were analyzed and it showed that factor A and B line was parallel (Factor C was constant) in each responses indicates that factors are not interfering with each other to produce effect on response.

Contour plots and 3-Dimentional study

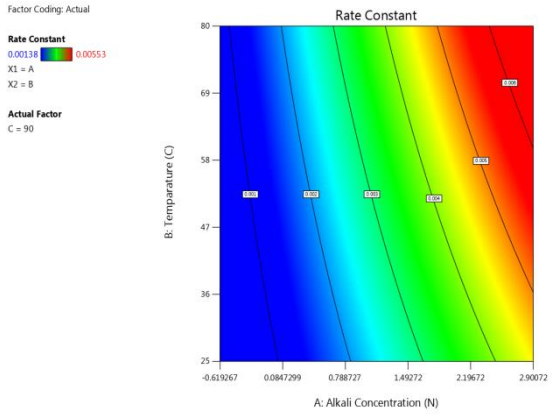
The effect of factors on responses can be generated in 2D and 3-D forms. It can be used to view the pattern, extent, and analysis of the effect on response. The 2-D form shows X and Y axis of plot while 3-D form adds Z axis to generate a chart in 3-dimentional view. These charts can be seen in Fig. 4.23.



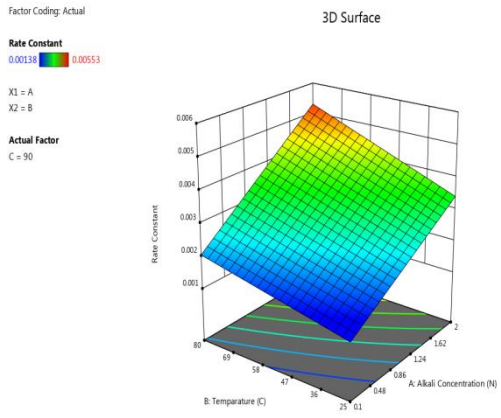
A_1



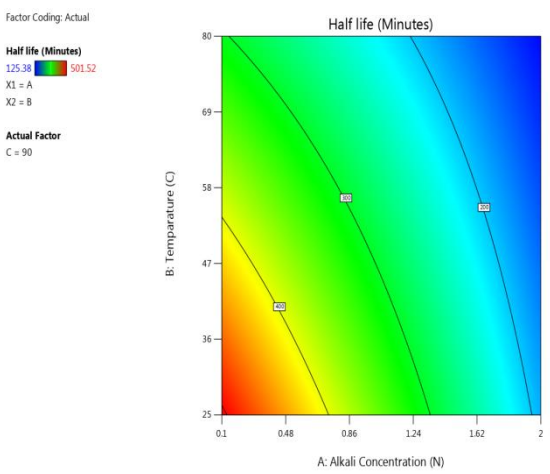
A_2



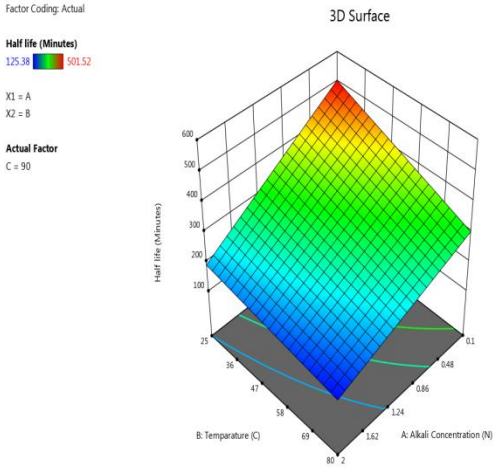
B_1



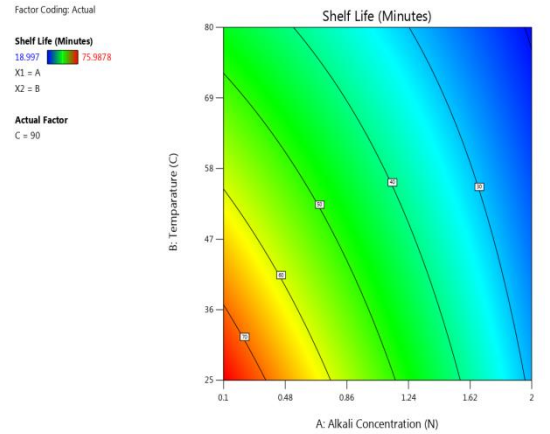
B_2



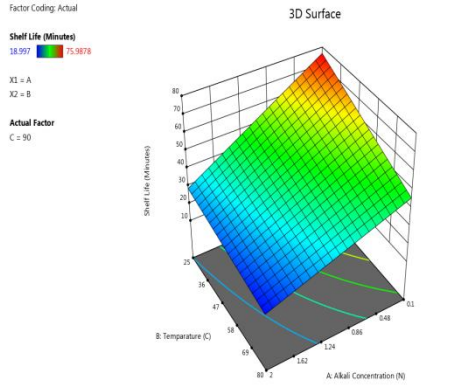
C_1



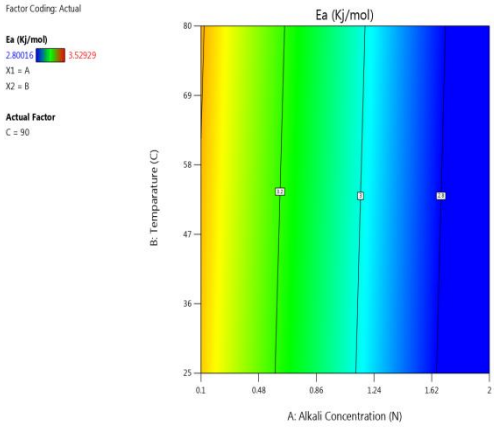
C_2



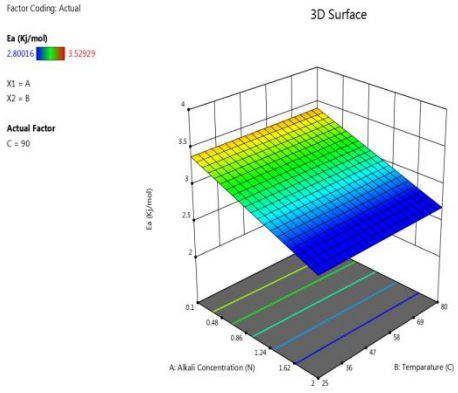
D_1



D_2



E_1



E_2

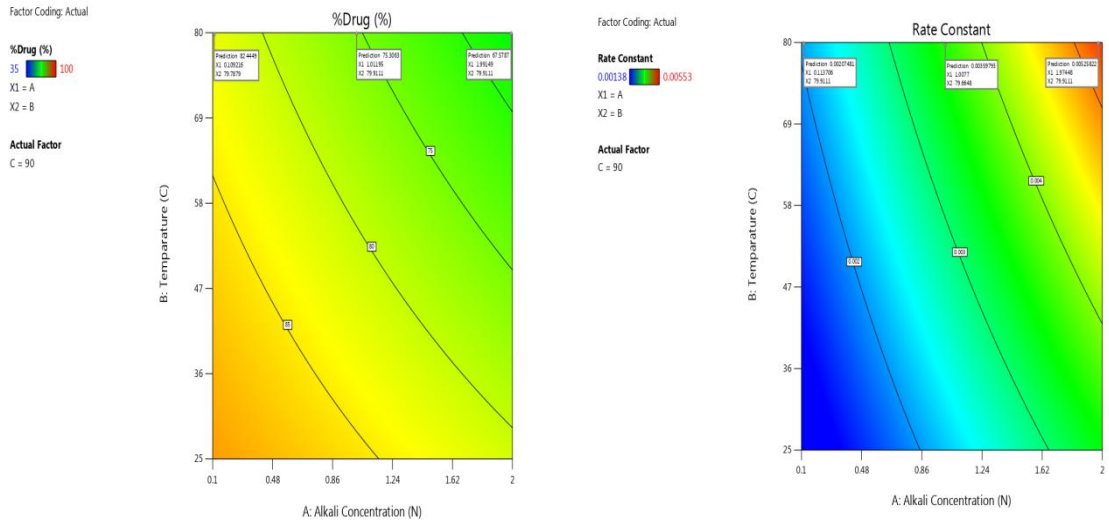
Fig. 4.232D contour plot for **A_1)** % Drug, **B_1)** Rate Constant, **C_1)** Half life, **D_1)** Shelf life and **E_1)** Activation energy and 3D plot for **A_2)** % Drug, **B_2)** Rate Constant, **C_2)** Half life, **D_2)** Shelf life and **E_2)** Activation energy

The color code shows effect intensity on response e.g. red color for highest value of response while blue color for lowest value of response. Blue color is seen at high levels of factors in

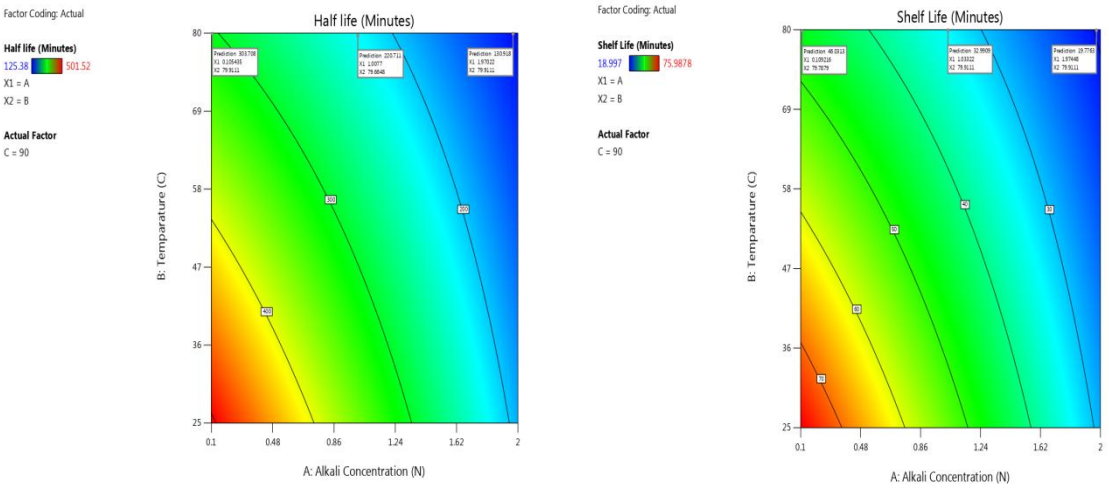
half life, shelf life and activation energy while at low levels of factors in rate constant. The all over effect of factors can be seen with 3-plot including all levels and factors.

Prediction of kinetic parameters using Design Expert [TM] software

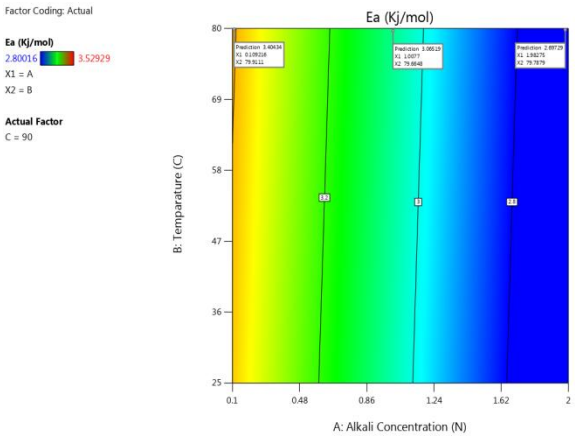
Design Expert [TM] software includes a tool that can predict or estimate the response value for any level of factors included in design. The degradation kinetic parameters (R1 to R5) are predicted for 0.1N to 2.0N NaOH at 80^0C for constant 90minutes. The predicted values are flagged in Fig. 4.24.



A **B**



C **D**



E

Fig.4.24 Predication of alkali degradation kinetic parameters using Design Expert ™ software

Application of multi factorial tool

The multi factorial design analyzed each factor and its impact on responses, from this knowledge software proposed value for responses shown in Fig.4.26. These values are compared (Table 4.25) with practical experiment value to know the application of software. Table shows that for 0.1N NaOH and 2N NaOH parameters obtained by both the methods are corresponding with each other, while for 1N NaOH values are varied but in limited range (<10%). Thus, the software can be used to study degradation kinetic study for drug substance.

Kinetic parameters	Multi factorial design	Conventional method
	0.1N NaOH	
%Drug (%)	82.44	81
Rate constant	0.0020	0.0023
Half life (Min.)	303.70	300.91
Shelf life (Min.)	45.3	45.59
Ea (kj/mol. k)	3.40	3.52
	1N NaOH	
%Drug (%)	75.30	78
Rate constant	0.0035	0.0027
Half life (Min.)	220.7	250.76
Shelf life (Min.)	32.9	37.99
Ea (kj/mol. k)	3.00	2.80
	2N NaOH	
%Drug (%)	67.57	62
Rate constant	0.0052	0.0055
Half life (Min.)	130.9	125.38
Shelf life (Min.)	19.7	19.00
Ea (kj/mol. k)	2.69	2.80

Table 4.25 Degradation kinetic parameters by conventional and multi factorial method

4.7.2.2 Oxidative degradation kinetics

The design was generated inDesign Expert ™ software using 2×3 factorial tool, significance of factors for generated design was checked by FDS plot and perturbation plots.

Analysis of responses

The responses were analyzed against factors effect using half normal plots and Pareto charts. Half normal plots are shown in supplementary file 4.7.2.2 (A) and Pareto charts are shown in Fig. 4.25 for evaluation of factors.

The Pareto chart shows that, for R1 all factors show effect on it, for R2 temperature was the significant factor to degrade BDQ, for R3 and R4 temperature and oxide concentration is the prime factor for degradation of BDQ, for R5 oxide concentration is most affective factor. Primarily the oxide concentration along with temperature boosts the degradation reaction of BDQ.

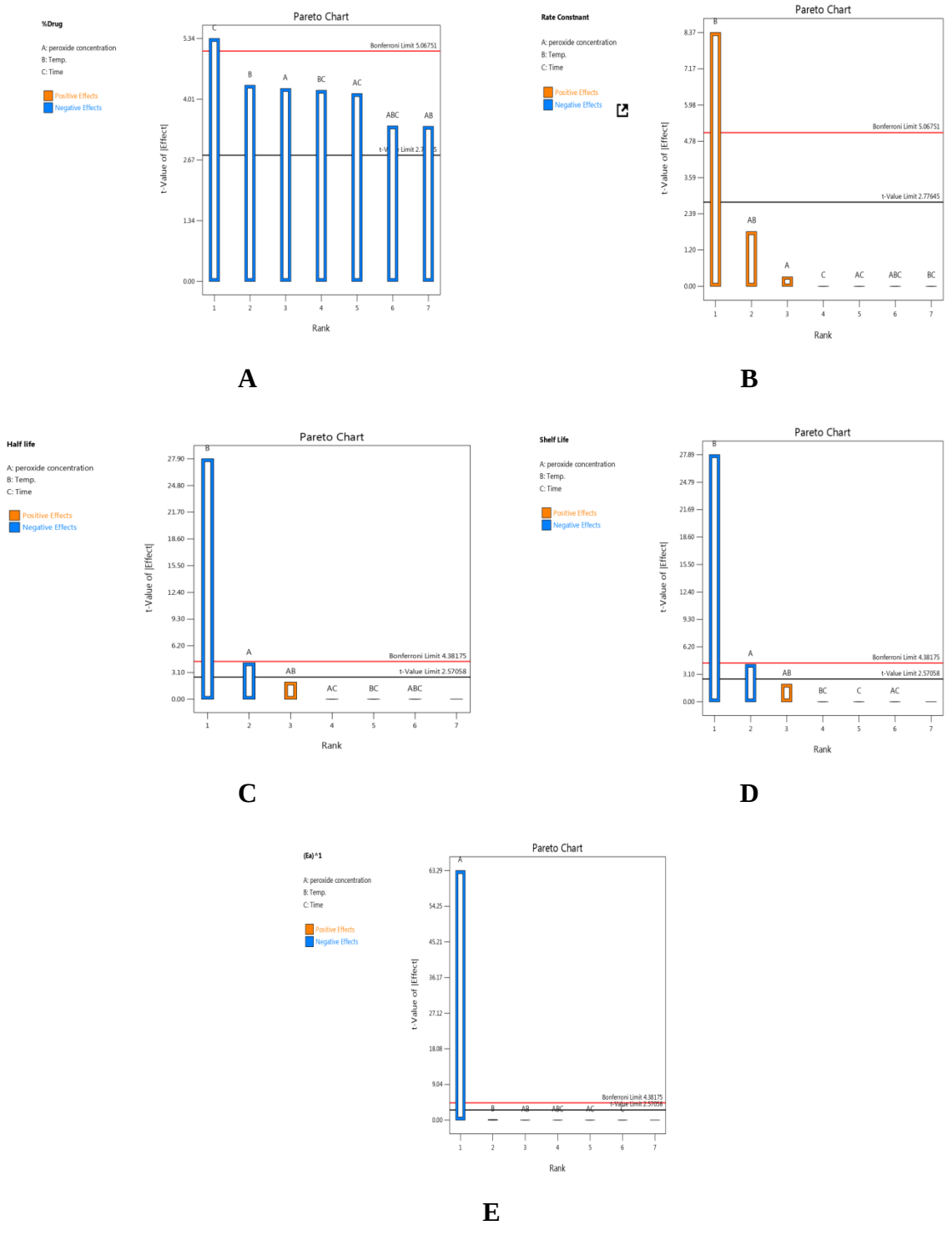


Fig.4.25 Pareto chart for responses of alkaline degradation kinetics of BDQ

Analysis of Variance

The mathematical equation for analysis of variance was generated by software to identify the factors having positive effect and/or negative impact on responses. the equations are shown below;

R1 (%Drug): +98.53-1.49 A-1.51 B -1.88 C-1.20 AB-1.45 AC-1.47 BC-1.20 ABC

R2 (Rate constant): +0.0014 +0.0000A + 0.0010 B +0.0000C +0.0002 AB +0.0000 AC +0.0000 BC+ 0.0000ABC

R3 (Half Life): +618.83 -49.74 A -329.16 B +0.0000 C + 23.41 AB +0.0000 AC +0.0000 BC +0.0000ABC

R4 (shelf life): +93.76 -7.54 A -49.87 B -0.0011 C +3.55 AB -0.0001 AC +0.0011 BC

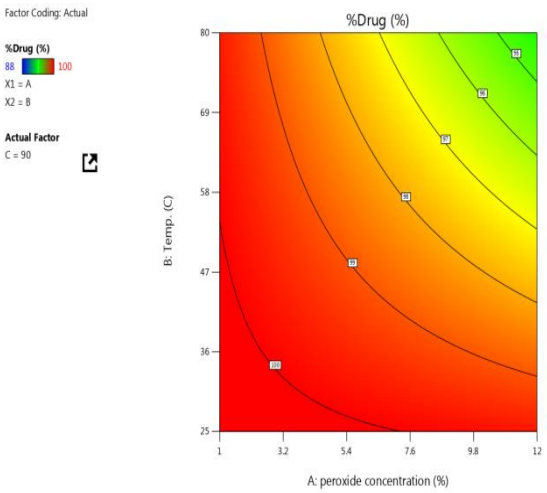
R5 (Activation energy): +8.91-1.42 A -0.0026 B +0.0000C +0.0002 AB +0.0000AC + 0.0000ABC

Interaction of Factors

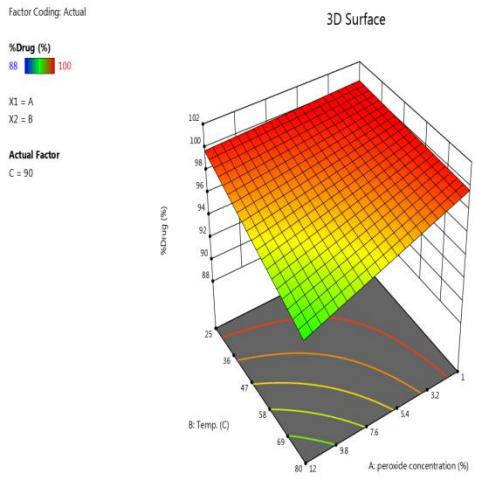
Interaction of factors was determined by interaction plots. The parallel lines in plots show that there is no interaction of factors occurred for any response. Therefore it can be assured that there was not any interference of factor in the impact of the response.

Contour Plots and 3-Dimentional studies

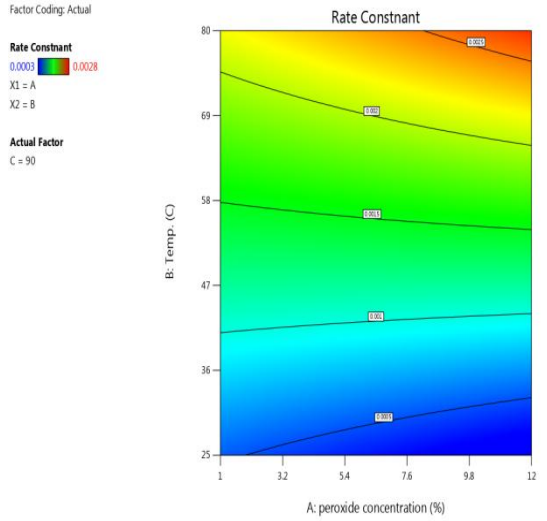
The 2D and 3D plots for oxidative degradation of bedaquiline was generated by software Design Expert $^{TM.}$ The plots can be used to evaluate the impact of factors on responses from low level to the high level; 3D plot can show the impact of two factors simultaneouslyfrom low to the high level. Plots are shown in Fig. 4.26.



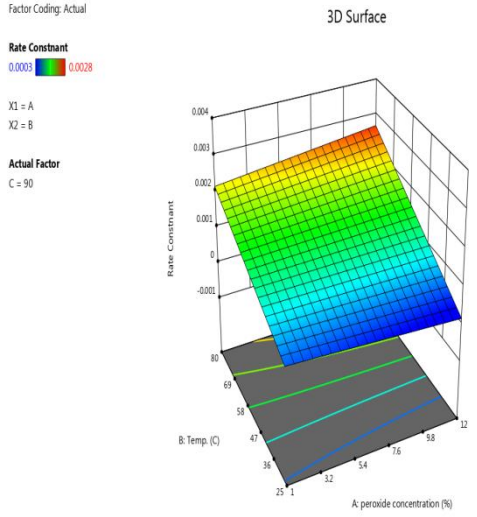
A_1



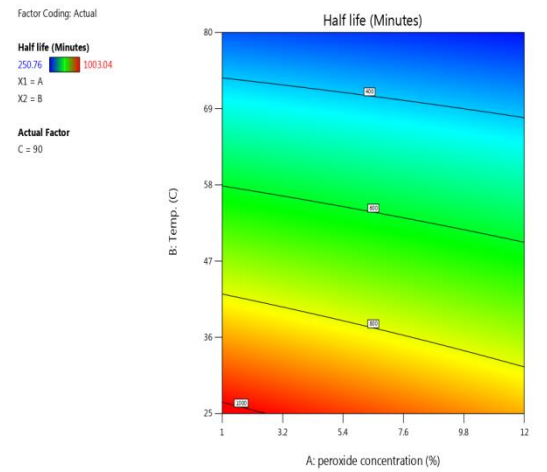
A_2



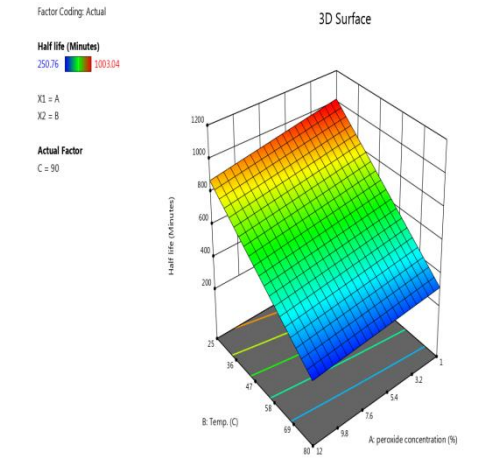
B_1



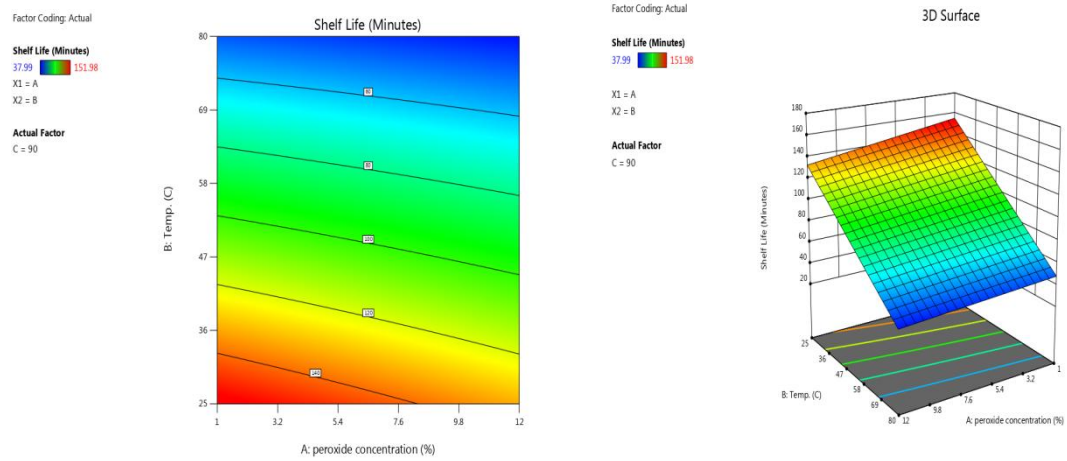
B_2



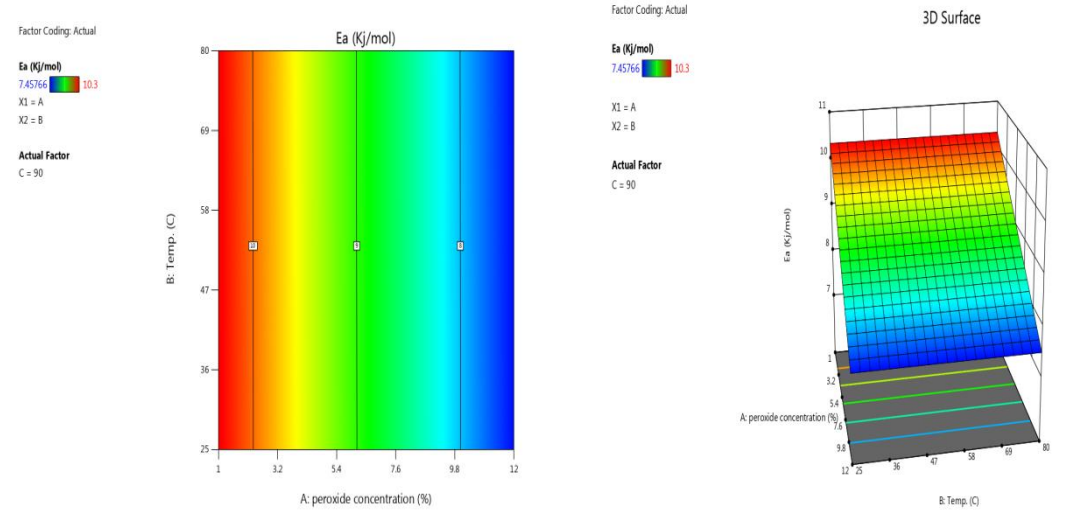
C_1



C_2



D_1 **D_2**



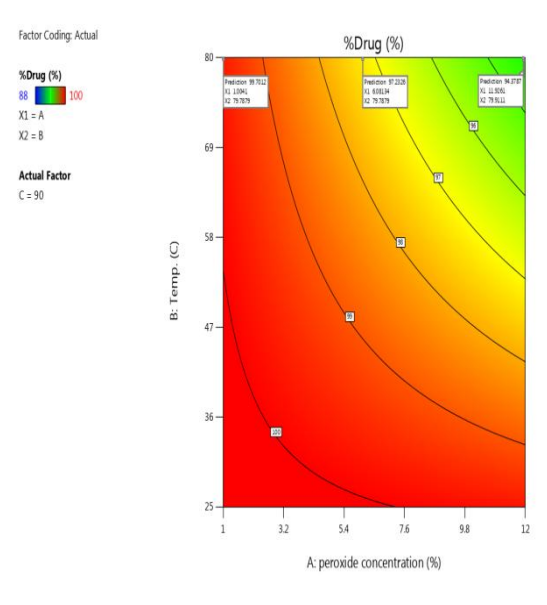
E_1 **E_2**

Fig. 4.262D contour plot for **A_1)** % Drug, **B_1)** Rate Constant, **C_1)** Half life, **D_1)** Shelf life and **E_1)** Activation energy and 3D plot for **A_2)** % Drug, **B_2)** Rate Constant, **C_2)** Half life, **D_2)** Shelf life and **E_2)** Activation energy

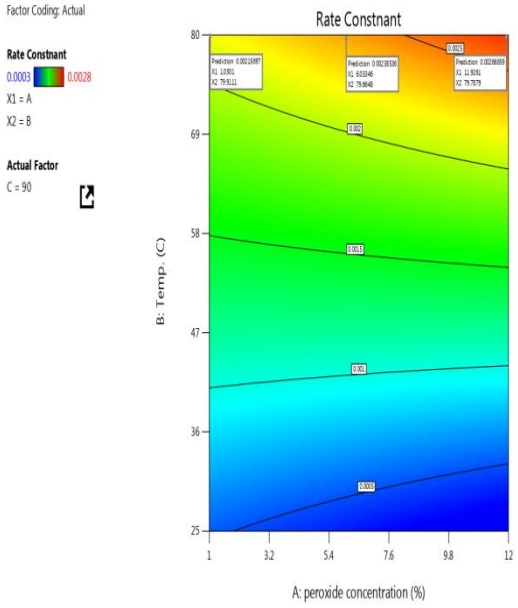
The results of the impact of factors on responses shown in contour plots are matching with the results of the conventional study of degradation kinetics.

Prediction of kinetic parameters using Design Expert ^{TM} software

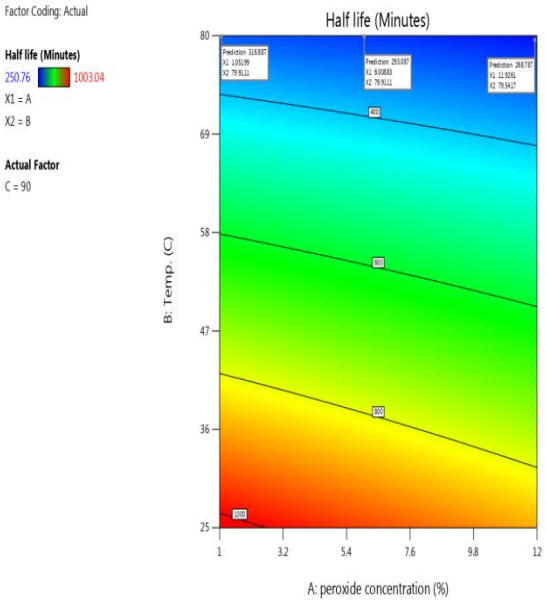
The featured tool of Design Expert ^{TM} software for prediction was used to predict the value of kinetic parameters. The prediction of response value is done for 1% to 12 % hydrogen peroxide at 80^0C for 90minutes. These predicted values are flagged in Fig. 4.27.


Factor Coding: Actual
%Drug (%)
88
100
X1 = A
X2 = B
Actual Factor
C = 90
%Drug (%)
B: Temp. (C)
A: peroxide concentration (%)

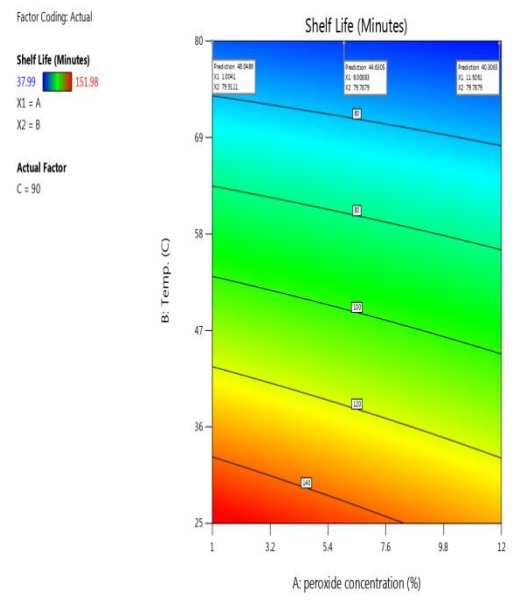
A


Factor Coding: Actual
Rate Constnant
0.0003
0.0028
X1 = A
X2 = B
Actual Factor
C = 90
Rate Constnant
B: Temp. (C)
A: peroxide concentration (%)

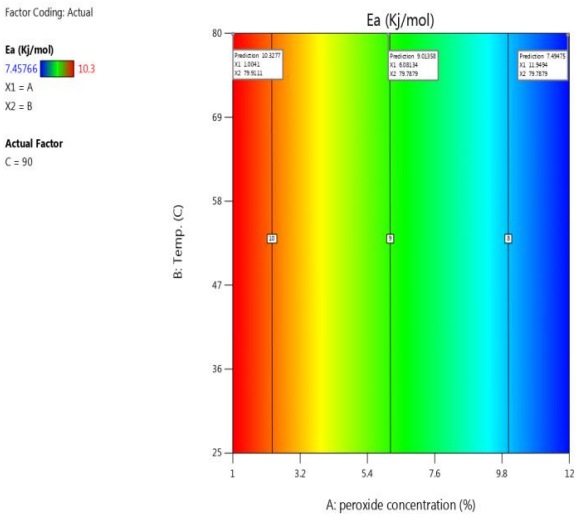
B


Factor Coding: Actual
Half life (Minutes)
250.76
1003.04
X1 = A
X2 = B
Actual Factor
C = 90
Half life (Minutes)
B: Temp. (C)
A: peroxide concentration (%)

C


Factor Coding: Actual
Shelf Life (Minutes)
37.99
151.98
X1 = A
X2 = B
Actual Factor
C = 90
Shelf Life (Minutes)
B: Temp. (C)
A: peroxide concentration (%)

D



E

Fig. 4.27 Predication of oxidative degradation kinetic parameters using Design Expert ™ software

Application of multi factorial tool

The predicted values of kinetic parameters are compared with the observed values in table 4.26. The table shows that values are corresponding to each other by both the methods (deviation <10%) suggests that both the methods can be exhorted for degradation kinetic studies.

Kinetic parameters	Multi factorial method	Conventional method
	1% Peroxide	
%Drug (%)	99.7	99
Rate constant	0.0021	0.0023
Half life (Min.)	316.80	300.91
Shelf life (Min.)	48.0	45.59
Ea (kj/mol. k)	10.3	10.3
	6% Peroxide	
%Drug (%)	97.2	99.3
Rate constant	0.0023	0.0025
Half life (Min.)	398.00	373.56
Shelf life (Min.)	44.9	41.45
Ea (kj/mol. k)	9.02	9.09
	12% Peroxide	
%Drug (%)	94.3	93
Rate constant	0.0026	0.0028
Half life (Min.)	268.70	250.76
Shelf life (Min.)	40.30	37.99
Ea (kj/mol. k)	7.90	7.45

Table 4.26 Degradation kinetic parameters by conventional and multi factorial method

- **Conclusion**

The degradation kinetic study was completed by the conventional method and the data for the conventional method was utilized to apply a multi-factorial approach by Design of Experiment (DoE) in Design Expert ™ software. The hydrolytic and oxidative degradation kinetic studies were performed by this tool. The inferences of results of multi-factorial design are same as the conventional method. There was no interaction between factors indicates that each factor has its specific impact on response. The values predicated for acid, alkaline and oxidative degradation kinetics parameters of BDQ were similar (deviation <10%) to the values obtained by practical suggest that either conventional or multi factorial approach, any of them can be used for degradation kinetics study as both the methods are equally specific for the results.

PART-D

4.8. ISOLATION AND IDENTIFICATION OF MAJOR DPs

4.8.1. Experimental

4.8.1.1 Chemicals and reagents

The chemicals and reagents are same as described in section 4.5.1.1.

UPLC/ESI-MS (Ultra Performance Liquid Chromatography/ Electron Spray Ionization- Mass Spectrometer) grade acetonitrile was purchased from Fisher Scientific chemicals, India. Formic acid was purchased from Merck, USA. Ammonia was purchased from sigma-Aldrich, USA. NMR grade deuterated di Methyl Sulph Oxide (DMSO) was purchased from Sigma-Aldrich (USA).

4.8.1.2 Equipment and chromatographic conditions

The equipment and chromatographic conditions are same as described in section 4.5.1.2 and 3.7.1.2.

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). Mass Lynx software was used for the UPLC-PDA and UPLC/ESI-MS data acquisition and analysis of bedaquiline and its DP.

UPLC/ESI-MS study was completed using mobile phase **a)** 0.1 % Formic acid in Milli Q water (pH= 2.70) and **b):** 0.1%Formic acid in Milli Q water: Acetonitrile (10:90). Gradient elution program was set to T = 0 min (97% A, 3% B) flow : 0.8 mL/min; T = 0.75 min (97% A, 3% B) flow : 0.8 mL/min; gradient to T = 2.7 min (2% A, 98% B) flow : 0.8 mL/min; gradient to T = 3 min (0% A, 100% B) flow : 1mL/min; T = 3.5 min (0% A, 100% B) flow : 1 mL/min; gradient to T= 3.51 min (97% A, 3% B) flow : 0.8 mL/min; end of run at T = 4 min (97% A, 3% B), Flow rate: 0.8 mL/min, analysis time 4 min

Mass probe (Probe temperature 400^0C) was set as source for electro spray ionization in positive mode (temp. 120^0 C) with cone voltage 10 and 30V and capillary voltage 3.25kV. Cone gas flow and desolvation (400^0C) gas flow was 100 and 800L/hr, respectively. Column and auto sampler temperature were set to 35^0 and 5^0C, respectively.

Preparative HPLC: Isolation of major acid stress degradationproduct was completed by preparative HPLC; Shimadzu-LC-20AP, binary, auto sampler and auto fraction collector, high pressure mixing chamber, and UV detector. Manual injector (5ml) was used to load sample in chromatographic system.

Isolation of major acid stress degradation product was completed using stationary phase X-Bridge ($C_{18,}$ 250×19mm, 5) column set to ambient temperature. Mobile phase consisted of **A)** water and **B)**: acetonitrile with flow rate 100ml/min, PDA detection at 227nm. Gradient elution program was set as follows; run time 20minutes; T (min) =**%v/vA): T=** 0.01(50), **T=** 15 (30), **T** = 15.01 (0), **T=** 15 (0), **T=** 18 (50) and stop command after **T=**20 (50).

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

4.8.1.3 Sample preparation

Analytical sample preparation, stress degradation sample and buffer preparations are same as described in section 4.5.1.3.

Enrichment of DPs for isolation

High concentration sample was prepared for isolation; accurately weighed 5gm of BDQ was dissolved separately in 15ml methanol, sonicated for 15minutes with provisional shaking; 50ml final volume was achieved using 1N HCl or 12% hydrogen peroxide, the solution was kept in specified stability condition in dark (acid: 0.5N HCl at 80^0C for 75 minutes and oxidative: 12% H_2O_2 at 80^0C for 5hrs). The 3ml aliquot +1ml acetonitrile + 1ml water was filtered through Whatman filter paper prior to filtering with 0.45µ Pall syringe filter. The sample was injected in described chromatographic condition of preparative HPLC for isolation of degradation impurity with purity <95.0%.

The 5ml sample load was injected in described chromatographic condition of preparative HPLC, The fractions were collected separately and gathered 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 against purity and for other co-eluting substance presence.

Lyophilization process: The concentrated sample was freeze dried to get in solid state after confirmation of purity >98.0 % by HPLC. A degradation product was with purity of > 98.0%. This final product was analyzed for LC/ESI-MS and NMR (^{1}H, ^{13}C and APT).

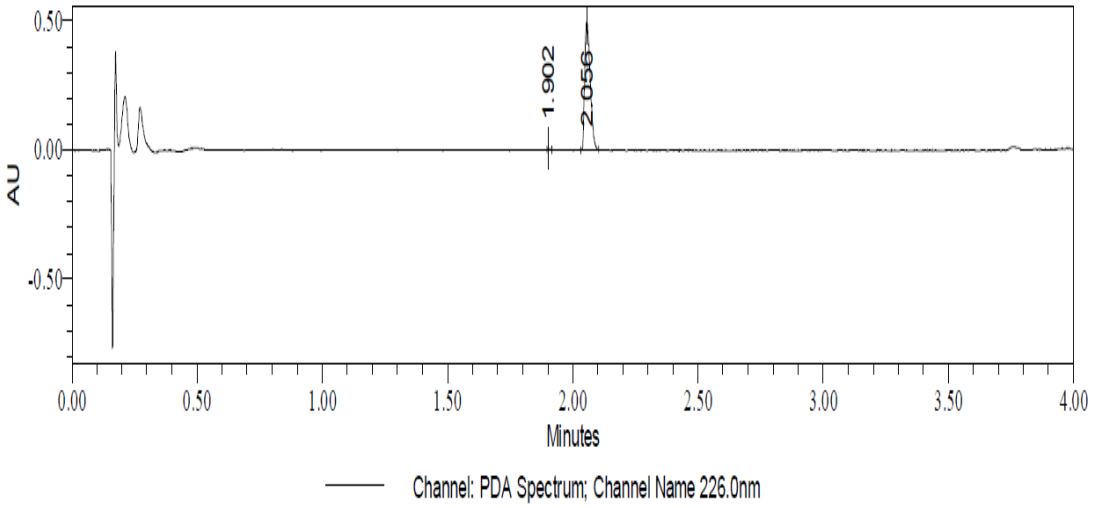
4.8.2. Result and Discussion

4.8.2.1 Spectral analysis of BDQ

BDQ API was analyzed by IR, UV and melting point (DSC), the results are shown in section 4.4. The UPLC/ESI-MS and NMR (^{1}H) is discussed in this section.

UPLC/ESI-MS analysis of BDQ: The equipment details and chromatographic conditions are described in section 4.8.1.2. Reported *m/z* of BDQ is 555.55g/mol [6], it has Br^{-} group in chemical structure which may have different abundance in BDQ, therefore ESI-MS spectrum showed difference of ±2 amu. Here, in this study BDQ showed 555.2 and 557.1 in 50% abundance in ESI-MS spectrum while in UPLC chromatogram BDQ peak was obtained at Rt 2.056min with process related impurity at Rt 1.902min (discussed in section 4.9.2.1 Part E). The UPLC chromatogram and ESI-MS spectrum are shown in Fig. 4.28.

A



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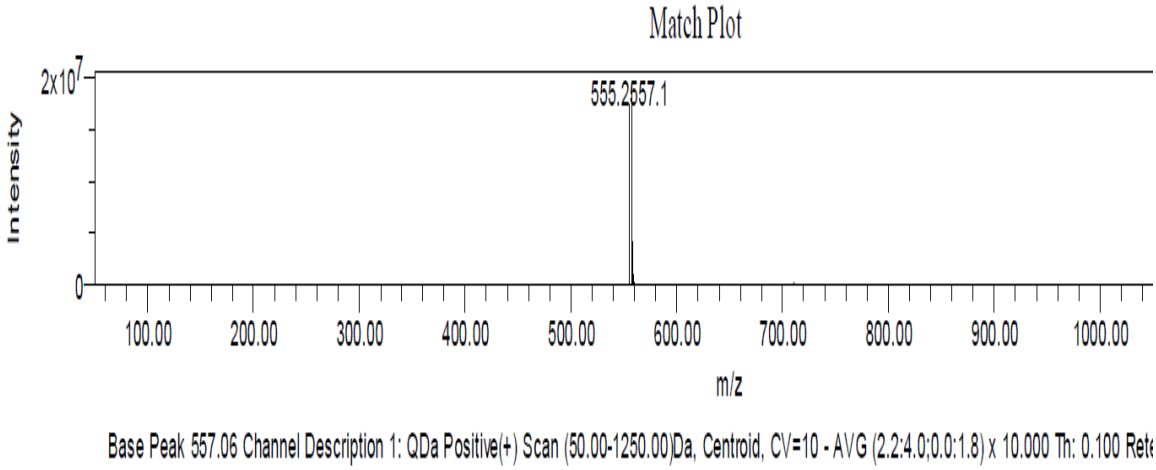


Fig.4.28 A) UPLC chromatogram and B) ESI-MS spectrum of BDQ API

The UPLC chromatogram and ESI-MS spectrum showed that there is no interfering substance in BDQ API other than process related impurity. The process related impurity is reported for the first time in literature for BDQ.

NMR analysis of BDQ: The equipment details are shown in section 4.8.1.2. The proton NMR was performed for BDQ API to analyze the sample and to utilize the obtained data for observing the changes in DPs NMR data.

The NMR spectrum is shown in Fig. 4.29.

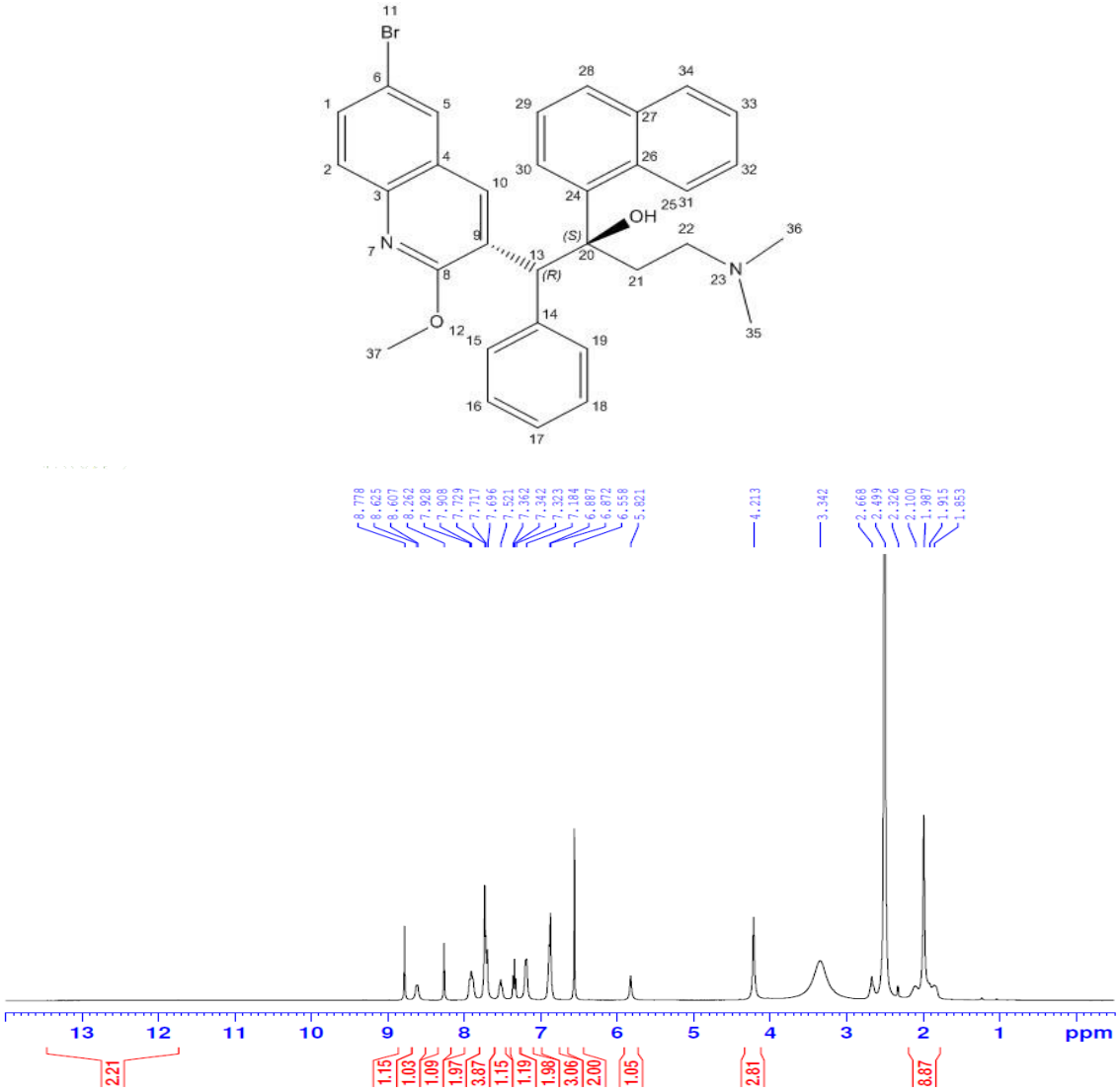


Fig.4.29 Proton NMR spectrum for BDQ API

The proton NMR is analyzed in Table 4.27.

α-position	^{1}H (∂ ppm)
1	7.32
2	7.63
3(-C-)	-
4(-C-)	-
5	7.9
6 (-C-)	-
7(-N-)	-
8(-C-)	-
9(-C-)	-
10	8.7
11(-Br)	-
12(-O)	-
13	5.82
14(-C-)	-
15	7.18-7.30
16	7.18
17	6.87-6.88
18	6.87-6.88
19	7.18-7.30
20(-C-)	-
21	4.21
22	2.19-2.32
23(-N)	-
24(-C-)	-
25	3.3
26(-C-)	-
27(-C-)	-
28	7.18-7.77
29	7.32
30	7.92
31	8.62
32	7.18-7.77

33	7.58
34	7.72
35	2.49-2.66
36	2.49-2.66
37	2.1-2.6

Table 4.27 Proton NMR spectrum analysis for BDQ API

The proton NMR spectrum analysis showed number and possible position of proton in BDQ API;these analyses were utilized for isolated DP's characterization and to elucidate structure for isolated DP of different stress condition.

4.8.2.2 Acid degradation impurity

Acid stress degradation study showed eight degradation products in section 4.5.2.2. The chromatogram for acid degraded sample is shown in Fig. 4.5. The peak purity results are shown in Table 4.7 and Fig 4.7. The major acid degradation product was identified by Rt in acid degradation sample HPLC chromatogram. Peak-3 (in HPLC chromatogram) was identified as major degradation product at Rt 26.4 ±0.05minutes (area: 39.14%). DPs were named as DP-A1 to DP-A8 in increasing order of their molecular weight obtained in UPLC/ESI-MS spectrum, among which DP-A8 was identified as major DP.

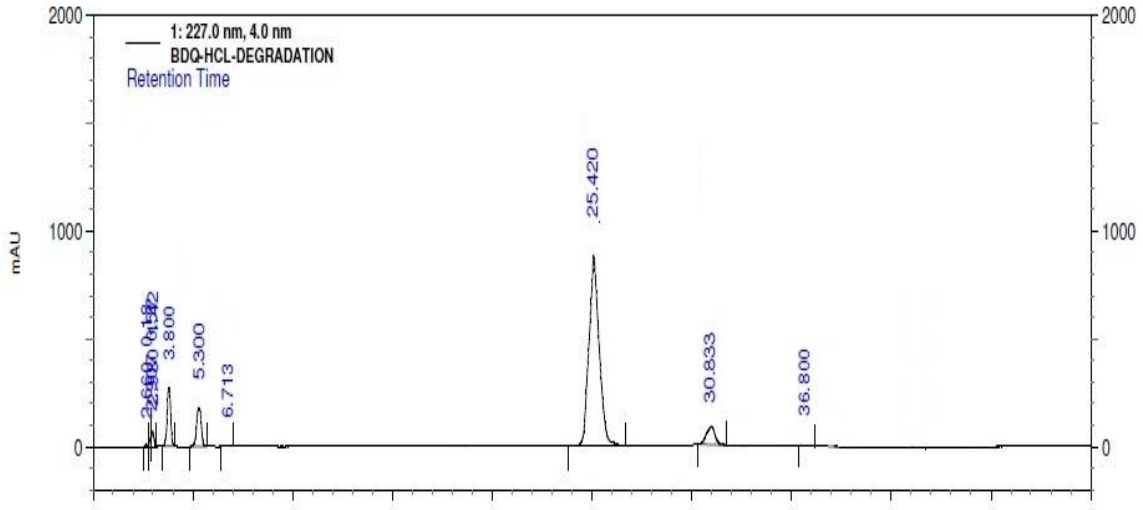
Isolation and purification of DP-A8

The DP-A8was identified as a major DP as it covers highest area in HPLC and UPLC chromatogram with peak purity after BDQ peak area. The degradation impurity was isolated using preparative HPLC, instrumental and chromatographic specifications are described in section 4.8.1.2 and sample preparation is described in section 4.8.1.3.

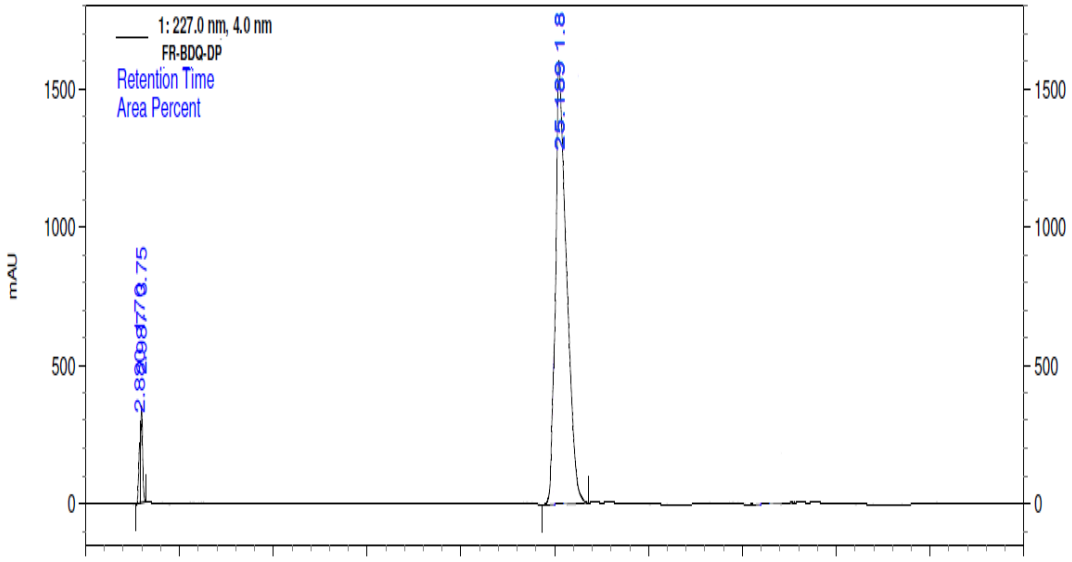
Confirmation and Characterization of DP

The isolated DP-A8 was confirmed as desired DP after performing LC/ESI-MS and HPLC Rt matching test. Structure elucidation and characterization was performed by NMR (Proton, Carbon-13and APT NMR). The isolated crude of DP-A8 and mixture of DPs solutions were injected individually in described chromatographic condition (section 4.5.1.2) for RP-HPLC Rt matching of DP-A8. The chromatograms are shown in Fig. 4.30.

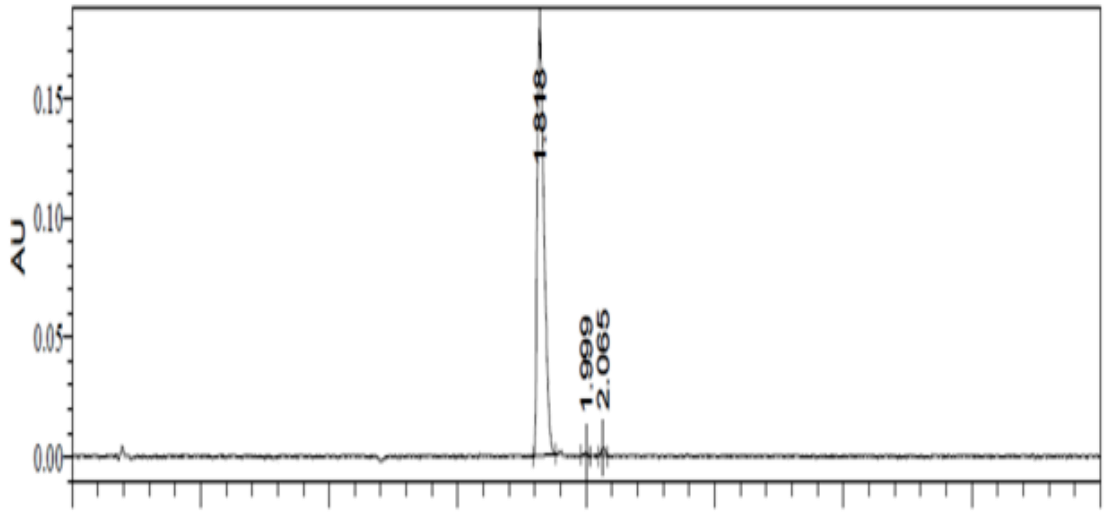
A


1: 227.0 nm, 4.0 nm
BDQ-HCL-DEGRADATION
Retention Time
mAU
2000
1000
0
3.800
5.300
6.713
25.420
30.833
36.800

B


1: 227.0 nm, 4.0 nm
FR-BDQ-DP
Retention Time
Area Percent
mAU
1500
1000
500
0
25.189 1.8
2.889 0.75

C



D

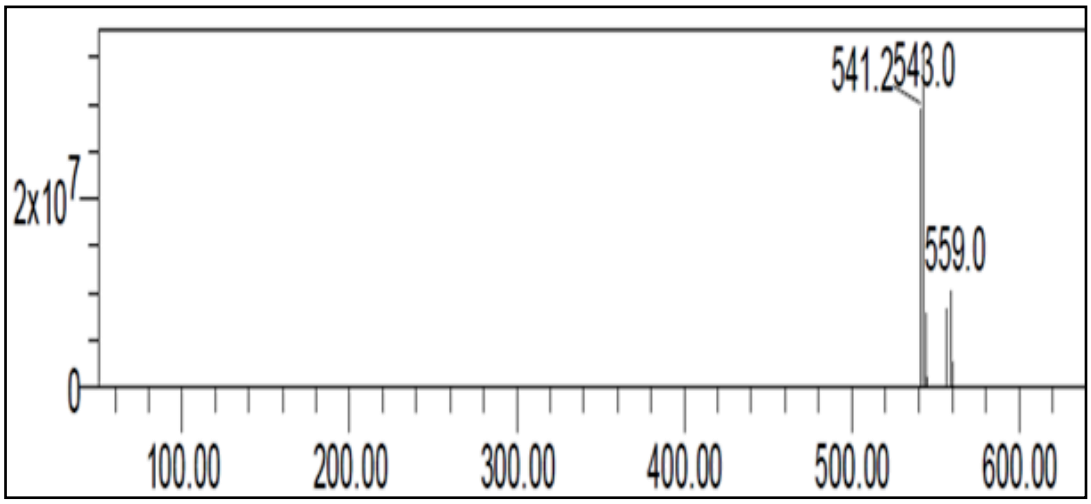


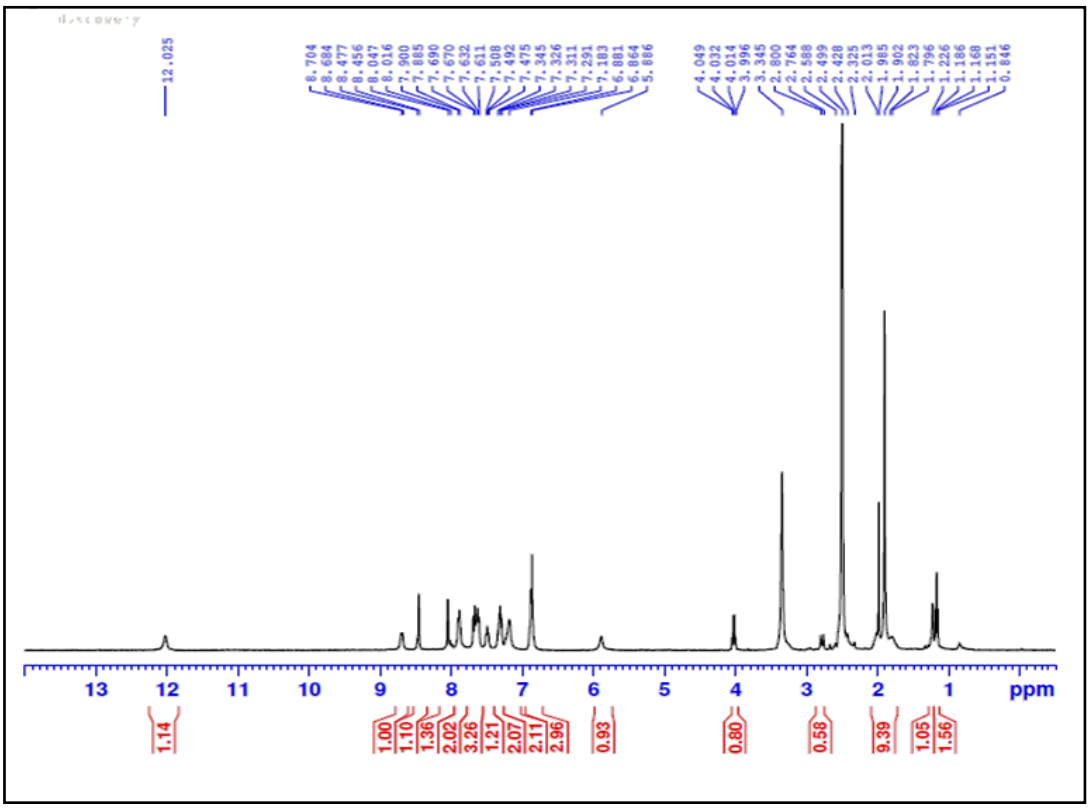
Fig. 4.30 (A) DPs mixture chromatogram of acid degraded sample of BDQ (B) isolated DP-8 chromatogram (C) UPLC chromatogram of isolated DP-8 (D) LC/ESI-MS of isolated DP

The Fig. 4.30 confirms that isolation of desired DP was carried out and DP was pure (>98.0%). The $[M+H^+]$ ion of *m/z* 543.0 matches with the structure elucidated for DP-A8. A water adduct was formed with *m/z* 559 amu with DP-A8ESI- MS spectrum.

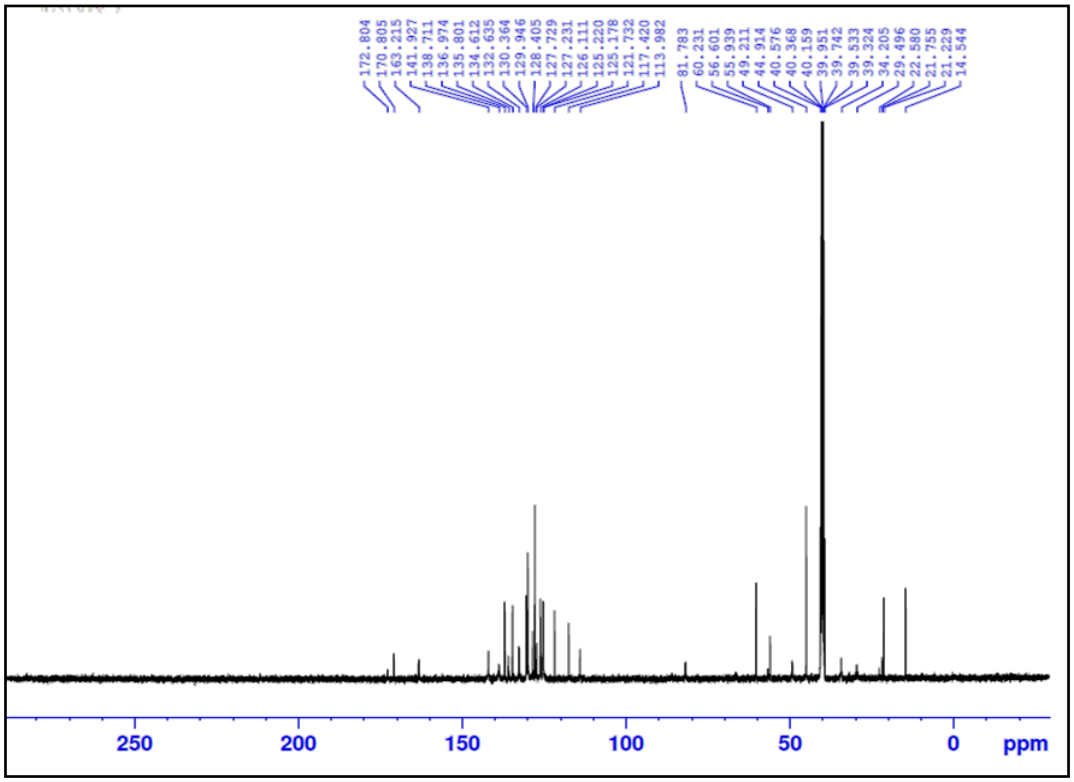
Characterization of DPA8

The NMR spectra are shown in Fig. 4.31. Characterization of DP-A8 was done by Nuclear Magnetic Resonance (NMR) study, three NMR were performed proton NMR to know the hydrogen present in structure, C^{13}NMR and Attached Proton Test (APT) to know the number and valences of carbon atom present in structure. The IR was also performed for DP-A8 to know the groups present in it.

A



B



C

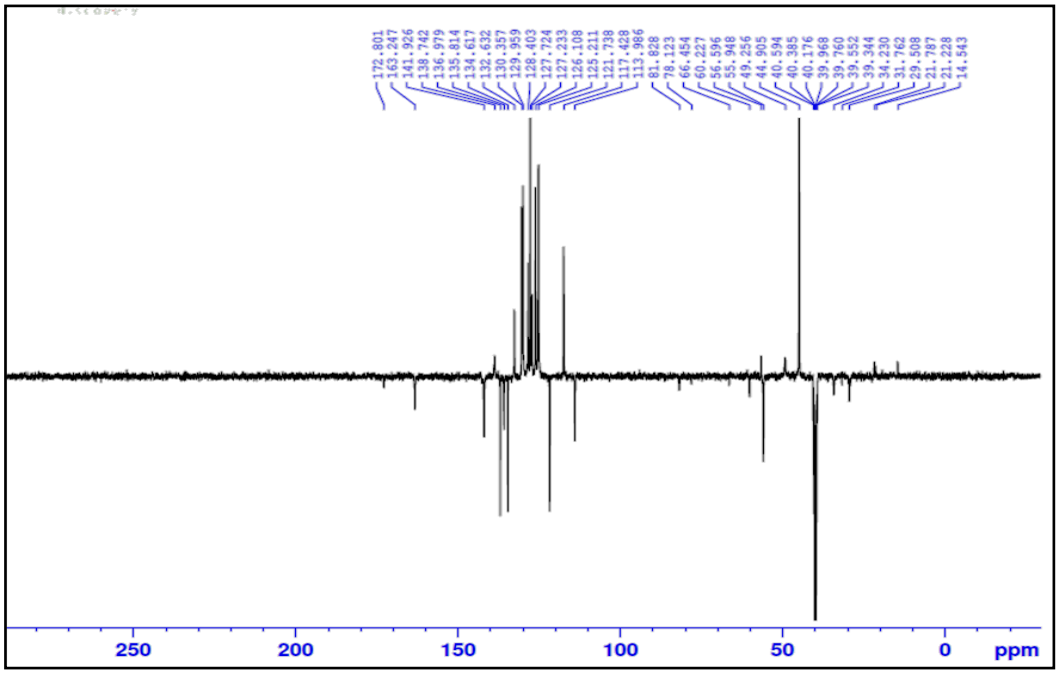


Fig. 4.31 A) Proton NMR B) Carbon ^{13} NMR and C) APT NMR for isolated acid DP-A8

The assignment of NMR spectrum is shown in Table 4.28.ESI mass spectrum of the impurity in positive ion mode displayed a molecular ion peak at *m/z* 541.1 and 543.0 [M^+], which is 14 amu less than that of the bedaquiline protonated molecular ion. Suggested elemental composition of this impurity was $C_{31}H_{30}BrN_2O_2^+$ and molecular weight as 540.14 in ^{1} H NMR

Proton NMR and C^{13} NMR with APT (Attached Proton Test) were performed for DP-8 are shown in Fig.4.31 A), B) and C). The assignment of NMR is shown in Table-4.28. The NMR data suggested removal of methyl group at $O^{12;}$ peak of –OH group was observed in proton NMR at 11.1ppm as the new peak appeared. Aliphatic and aromatic region remains same asthat of obtained in bedaquiline parent NMR spectrum except solvent peaks appeared in DP-A8 NMR spectrum. DMSO peak appeared at 2.5-2.6 PPM. ^{13}Carbon and APT NMR also confirmed removal of methyl group and suggested structure is shown in Fig. 4.32. APT showed –C and -CH_2 in lower field while –CH and –CH_3 group in upper field. The Table 4.28 shows BDQ bulk drug NMR assignment and DP-A8 NMR assignment.

	API	**DP-A8**		
α-position	**^{1}H**	**^{1}H**	**^{13}C**	**APT**
1	7.32	7.32	135.801	*(-CH) uf*
2	7.63	7.632	127.231	*(-CH) uf*
3	-	-	130.634	*(-C) Lf*
4	-	-	113.982	*(-C) Lf*
5	7.9	7.9	136.974	*(-CH) UF*
6	-	-	117.42	*(-C) Lf*
7	-	*8.01	-	*(-NH^+)*
8	-	-	172.804	*(-C) LF*
9	-	-	141.927	*(-C)LF*
10	8.7	8.7	138.74	*(-CH) uf*
11	-	-	-	*-Br*
12	-	*12.02	-	**-OH*
13	5.82	5.8	56.601	*(-CH) UF*
14	-	-	136.97	*(-C) lf*
15	7.18-7.30	7.18-7.30	127.729	*Benzene (-CH) uf*

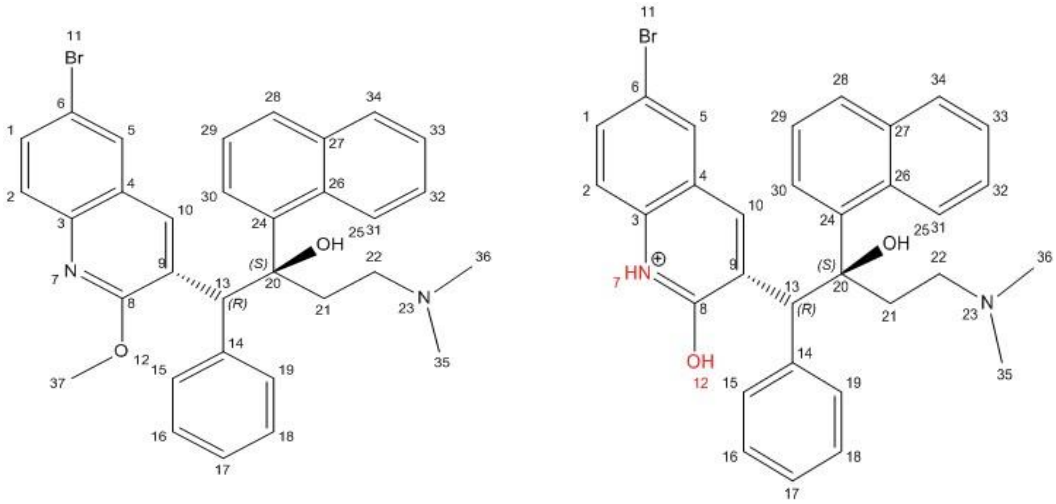
16	7.18	7.18	128.405	*Benzene (-CH) uf*
17	6.87-6.88	6.87-6.88	125.22	*Benzene (-CH) uf*
18	6.87-6.88	6.87-6.88	128.405	*Benzene (-CH) uf*
19	7.18-7.30	6.86	127.729	*Benzene (-CH) uf*
20	-	-	81.783	*aliphatic (-C) lf*
21	4.21	4.04	34.205	*aliphatic (-CH_2)lf*
22	2.19-2.32	2.19-2.32	60.231	*aliphatic (-CH_2) Lf*
23	-	-	-	*-N*
24	-	-	163.215	*1-napthalene (-C) Lf*
25	3.3	3.3	-	*-OH*
26	-	-	135.6	*1-napthalene (-C) Lf*
27	-	-	134.612	*1-napthalene(-C) Lf*
28	7.18-7.77	7.18-7.77	129.946	*1-napthalene(-CH) Uf*
29	7.32	7.32	125.178	*1-napthalene(-CH) Uf*
30	7.92	7.92	126.1	*1-napthalene(-CH) Uf*
31	8.62	8.62	126.1	*1-napthalene(-CH) uf*
32	7.18-7.77	7.18-7.77	126.111	*1-napthalene(-CH) Uf*
33	7.58	7.5	126.111	*1-napthalene(-CH) Uf*
34	7.72	7.47	129.946	*1-napthalene(-CH) Uf*
35	2.49-2.66	2.49-2.66	44.9	*Uf aliphatic CH_3*
36	2.49-2.66	2.49-2.66	44.9	*UF aliphatic CH_3*
37	*2.1-2.6	-	-	-

*indicates changes observed in NMR spectrum

Uf- upper field, Lf – lower field

Table 4.28 NMR assignment for acid DP of BDQ (DP-A8)

Chemical structures of BDQ bulk drug and DP-A8 (elucidated structure) with atom number are shown in Fig 4.32.



A **B**

Fig. 4.32 A) BDQ bulk drug chemical structure B) elucidated structure for DP-A8 of BDQ

IR confirmation of DP-A8

The presence of groups in DP-A8 was confirmed by IR study using instrument described in section 4.5.1.1. The IR spectrum is shown in Fig 4.33 and analysis is shown in Table 4.29.

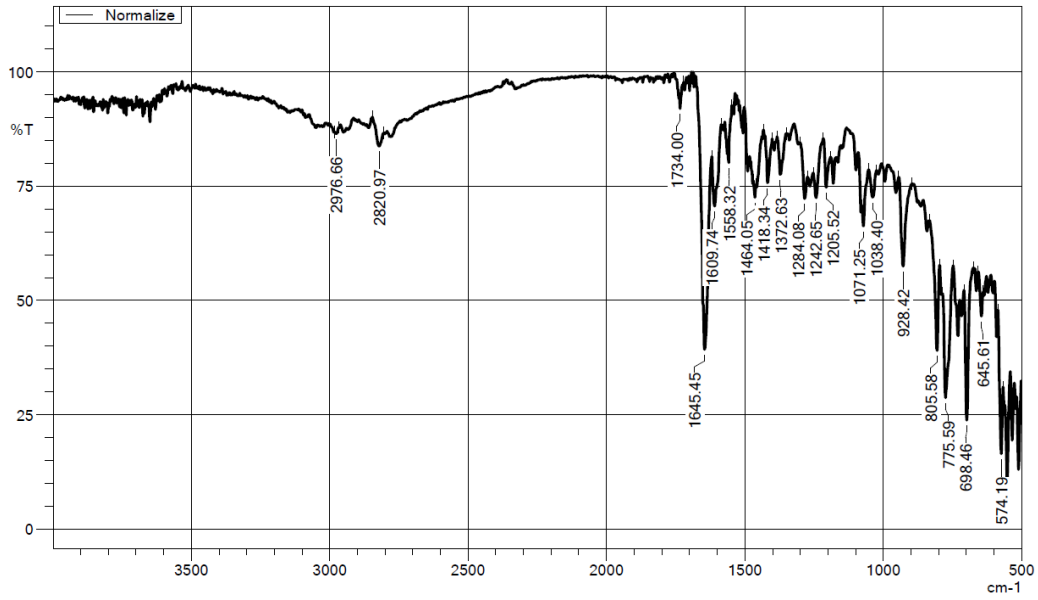


Fig.4.33 IR spectrum of DP-A8

Assigned wave number	Observed Wave number	Observed group
690-515	574.19, 645.61	C-X (bromo compound) stretching
730-665	698.46	C=C bending
755±20	775.59	C-H bending
810±20	805.58	C-H bending
1250-970	1038.40	C-O bending
1300-1000	1071.25	C-O (alcohol)
1020-1220	1205.52	Alkyl amine (C-N)
1342-1266	1284.08	C-N stretching (aromatic amine)
1380-1370	1372.63	C-H bending
1440-1395	1418.34	O-H bending
1600-1400	1464.05,1558.32	Asymmetric C-H bending (-CH_3 and –CH_2),C=C Alkene (aromatic)
1650-1580	1609.74	N-H bending (amine)
1648-1638	1645.45	C=C stretching
3200-2700	2820.97, 2976.66	O-H stretching

Table 4.29 IR spectrum analysis of DP-A8

The analysis of IR spectrum supports the presence of groups in DP-A8 chemical structure shown in Fig 4.32.

4.8.2.3 Oxidative degradation impurity

Oxidative stress degradation study showed three degradation products in section 4.5.2.2. The chromatogram for acid degraded sample is shown in Fig. 4.5. The peak purity results are shown in Table 4.7 and Fig 4.7. For isolation sample was prepared as described in section 4.8.1.3. The major degradation product was identified by area occupied by peak in oxidative degradation sample chromatogram. DP-O3 was identified as major degradation product at Rt 32.4 ±0.05minutes (area: 45.81%) in HPLC chromatogram. In UPLC/ESI-MS spectrum DPs were named as DP-O1 to DP-O3 in increasing order of *m/z* obtained in spectrum, among which DP-O3 was identified as major DP.

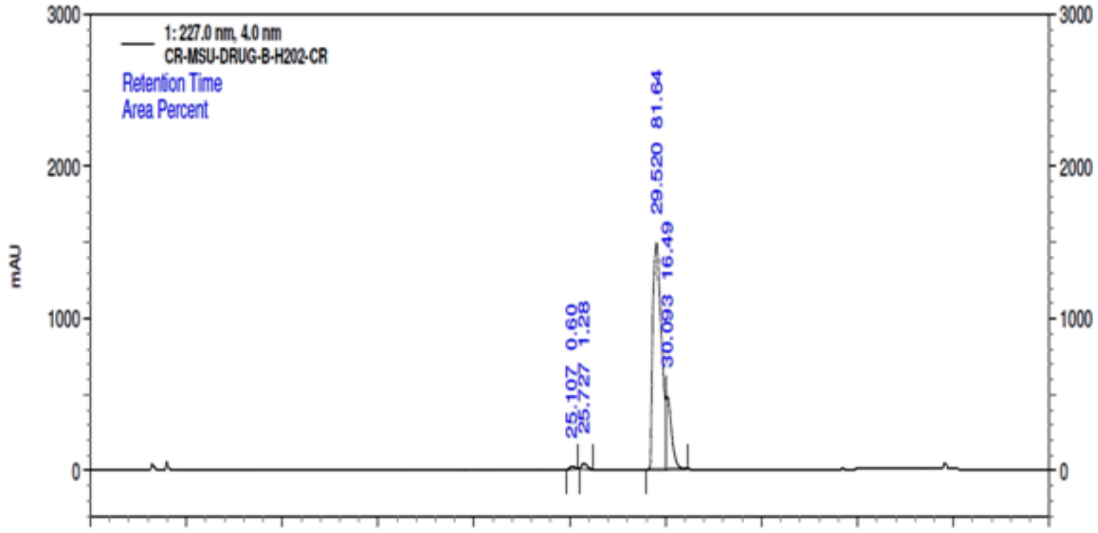
Isolation and purification of DP-O3

For isolation and purification of DP-O3 the same method was used as described in section 4.8.2.1.

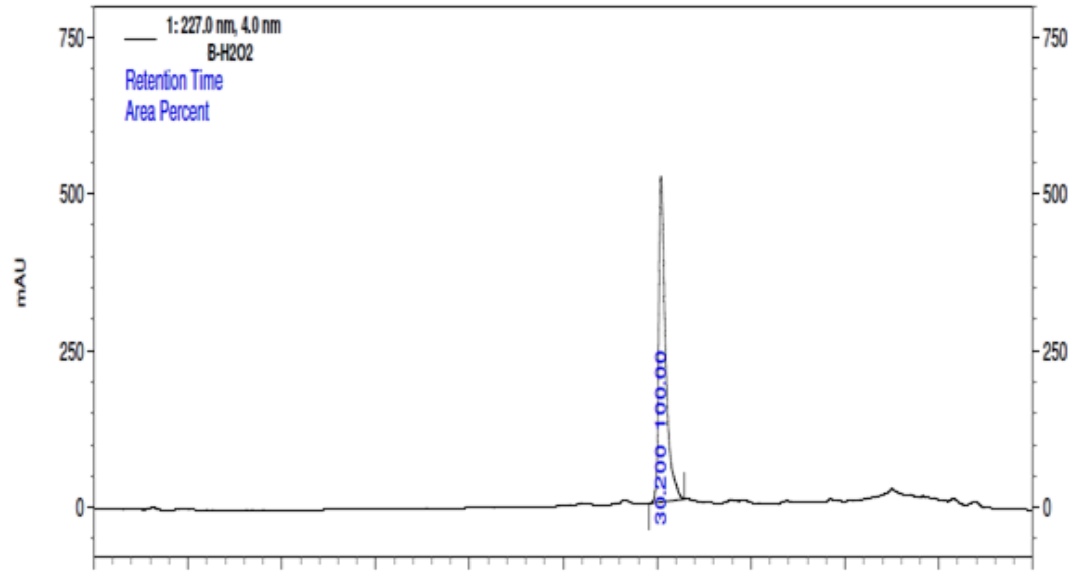
Confirmation of DP-O3

The isolated DP-O3 was matched with Rt in HPLC and LC/ESI-MS data to confirm that desired DP was isolated. The isolated crude and mixture of DPs solutions were injected individually in described chromatographic condition (section 4.5.1.2) for RP-HPLC. The chromatograms are shown in Fig. 4.34.

A



B



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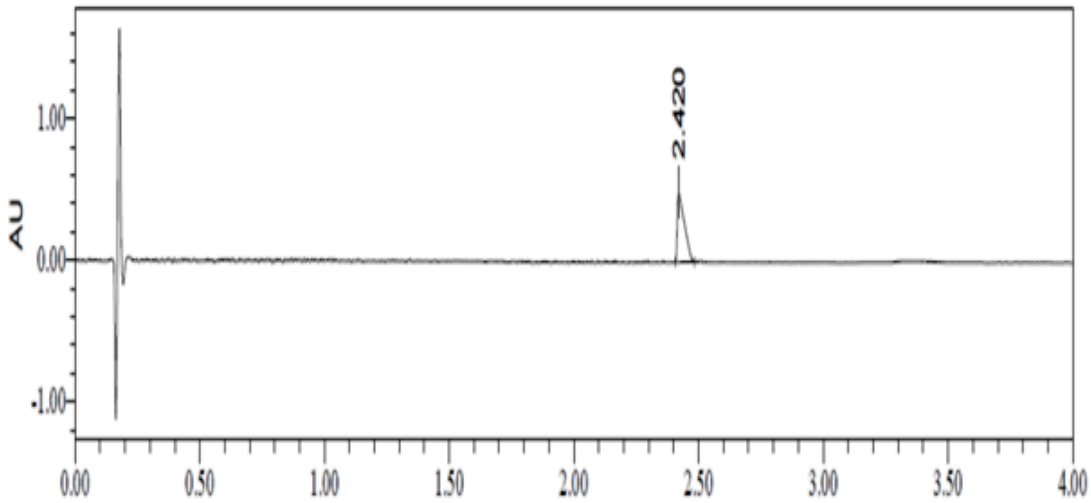


D

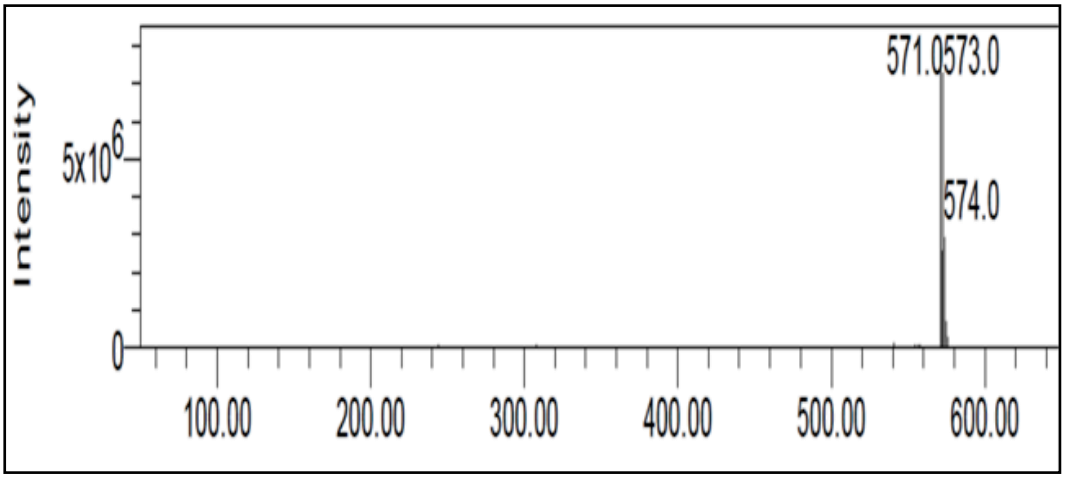


Fig. 4.34 (A) Oxidative DPs mixture chromatogram of BDQ (B) isolated DP-O3chromatogram (C) UPLC chromatogram of isolated DP-O3 (D) LC/ESI-MS of isolated DP-O3

The Fig. 4.34confirms that isolation of desired DP was carried out and DP was pure (>98.0%). The [M+H+] ion of *m/z*572±1.0 (571 and 573 in 50% abundance) matches with the structure elucidated for DP-O3.

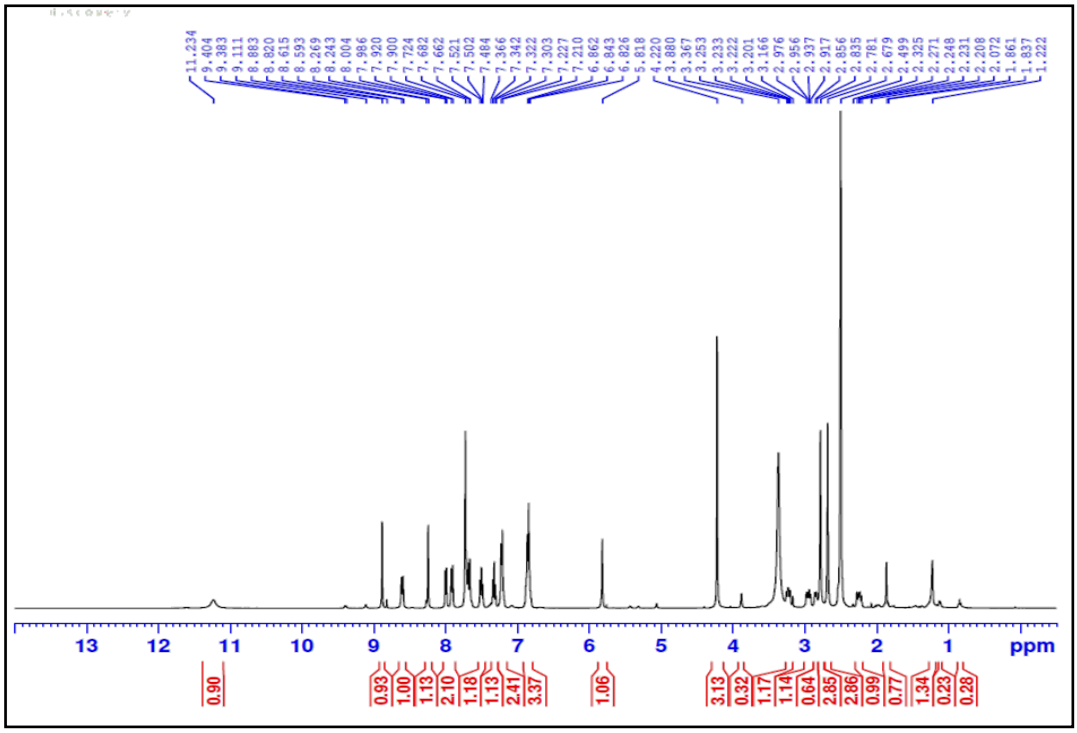
Characterization of DP-O3

The LC/MS data showed $[M+H]^+$ ion at *m/z* of 572±1.0 for DP-O3 which is 16 amu higher than bedaquiline, shows addition of oxide (O^-) in bedaquiline. The presence of oxide and other structural changes were confirmed by combining NMR data; proton, carbon and

attached proton NMR. The assignment of NMR is shown in Table 4.33. Following are the structural changes with NMR conformation;

The changes were observed in aromatic region and aliphatic region of DP-3 ^{1}H NMR (shown in Sheme-2); demethyalation changed the –O-CH_3 group to –OH group, this group was confirmed by chemical shift (ð) 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 of this impurity was $C_{31}H_{29}BrN_2O_4$ and molecular weight as 573.49gm/mol (Fig. 4.35) in ^{1} H NMR Proton NMR and C^{13} NMR with APT (attached proton test) were performed for DP-3 is shown in Fig.4.37. Expected changes in proton NMR of DP-3 were compared with bedaquiline 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 (ð) of 9.404 and 9.383 ppm in ^{1}H NMR. Addition of –OH group to quinoline or naphthalene ring lead to chemical shift around 150-155ppm in C^{13} NMR while absence of this chemical shift confirms –OH group addition at benzene ring.

A



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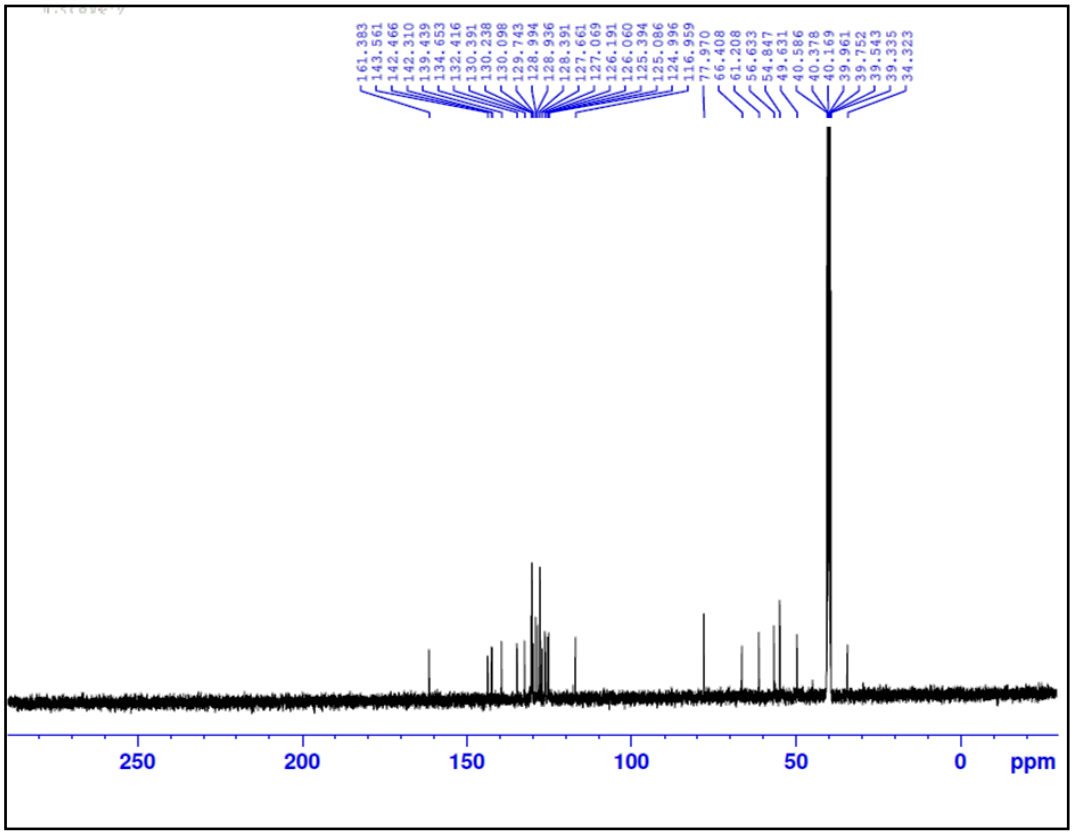

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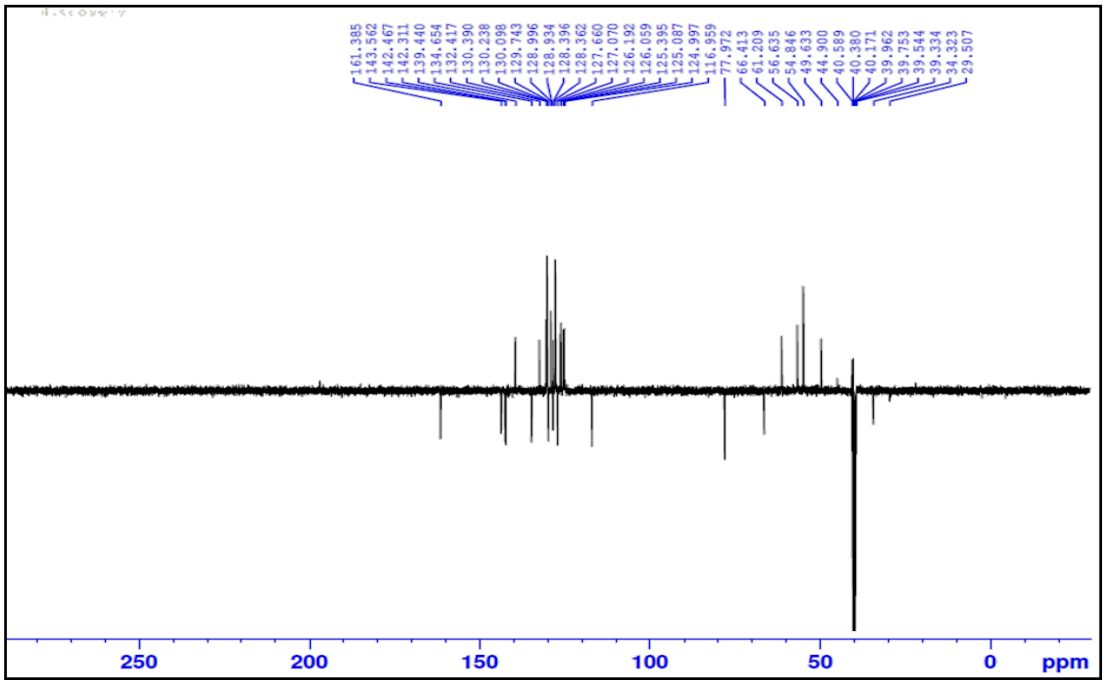


Fig. 4.35 A) Proton NMR B) Carbon ^{13} NMR and C) APT NMR for DP-O3

The BDQ bulk drug NMR assignment is shown in Table 4.30.The assignment of NMR to DP-3 is shown in Table 4.30.

α-position	**API ^{1}H API**	**DP-O3 ^{1}H**	**^{13}C δ (ppm)**	***APT δ (ppm)***
1	7.32	7.32	139.439	*(-CH) uf*
2	7.63	7.66	130.098	*(-CH) uf*
3	-	-	142.51	*(-C-) lf*
4	-	-	126.086	*(-C-) lf*
5	7.9	7.9	129.743	*(-CH) uf*
6	-	-	125.394	*(-C-) lf*
7	-	-	-	*-N*
8	-	-	161.383	*(-C-) lf*
9	-	-	126.191	*(-C-) lf*
10	8.7	8.8	142.466	*(-CH) uf*
11	-	-	-	*Br*
12	-	*11.2	-	*OH*
13	3.3	3.3	39.335	*(-CH) uf*
14	-	-	132.416	*(-C-) lf*
15	7.18-7.30	*7.21-7.36	124.996	*(-CH) uf*
16	7.18	*-	144.461	*(-C-) lf*
17	6.87-6.88	6.86	127.07	*(-CH) uf*
18	6.87-6.88	6.86	125.086	*(-CH) uf*
19	7.18-7.30	*-	143.561	*(-C-) lf*
20	-	-	77.97	*C lf*
21	4.21	4.22	34.323	*lf aliphatic CH_2*
22	2.19-2.32	2.20-2.32	66.4	*lf aliphatic CH_2*
23	-	-	-	*N*
24	-	-	142.31	*(-C-) lf*
25	5.8	5.81	-	*OH*

26	-	-	134.653	*(-C-) lf*
27	-	-	130.238	*(-C-) lf*
28	7.18-7.77	7.4-7.7	127.661	*(-CH) uf*
29	7.32	7.32	128.391	*(-CH) uf*
30	7.92	7.92	127.069	*(-CH) uf*
31	8.62	8.61	128.991	*(-CH) uf*
32	7.18-7.77	7.4-7.7	128.994	*(-CH) uf*
33	7.58	7.52	128.939	*(-CH) uf*
34	7.72	7.72	130.391	*(-CH) uf*
35	2.66	2.67	49.63	*uf aliphatic-CH_3*
36	2.66	2.67	49.63	*uf aliphatic-CH_3*
37	*2.1-2.6	*-	-	-
38	-	*9.11	-	*OH*
39	-	*9.4	-	*OH*

Table 4.30 NMR assignment for DP-3

The suggested chemical structure for DP-3 is shown in Fig 4.36.

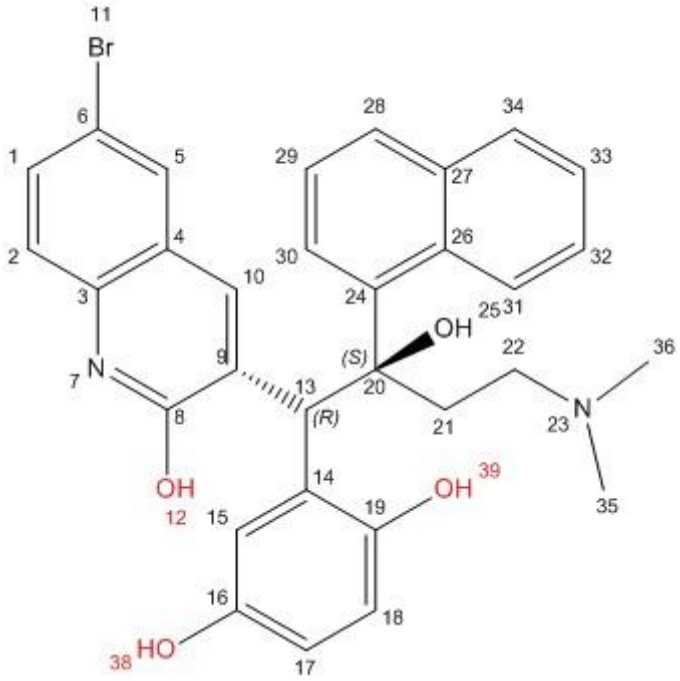


Fig.4.36 Elucidated structure for DP-O3 by ESI-MS and NMR

IR confirmation of DP

The presence of groups in DP-O3 was confirmed by IR study using instrument described in section 4.5.1.1. The IR spectrum is shown in Fig 4.37 and analysis is shown in Table 4.31.

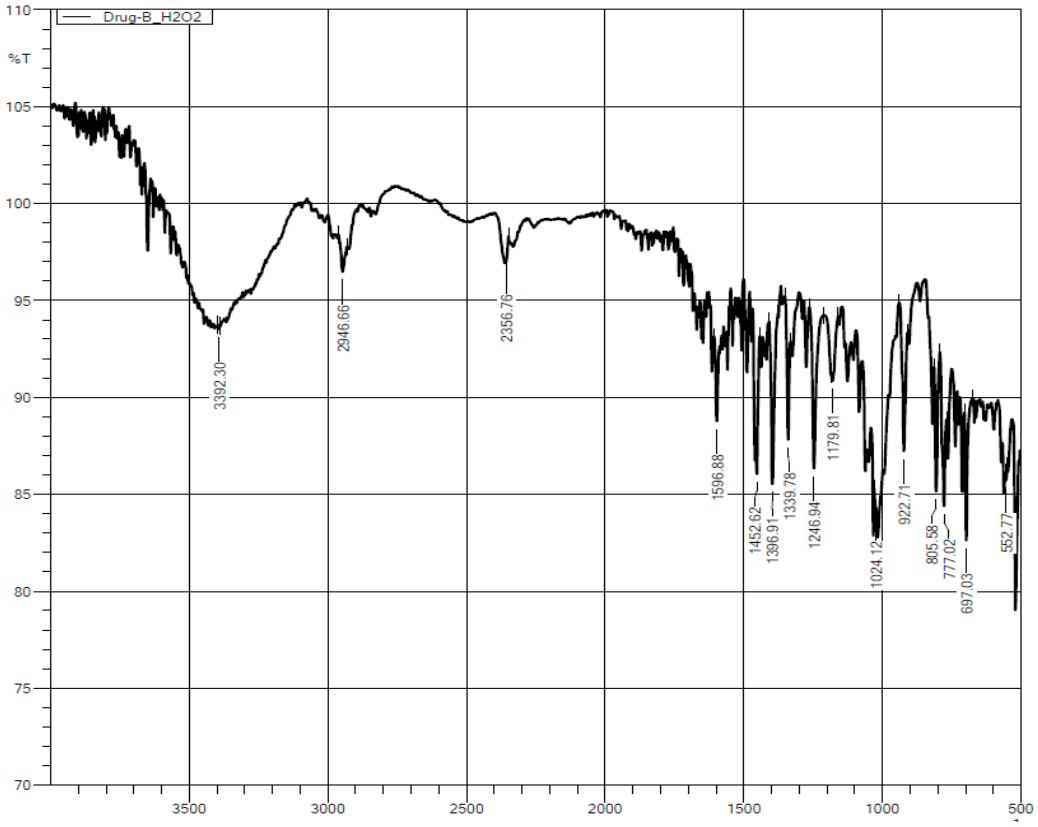


Fig.4.37 IR spectrum of DP-O3

Assigned wave number	Observed Wave number	Observed group
3550-3200	3392.3	O-H stretching
3200-2700	2946.6	O-H stretching
1650-1580	1596.8	N-H stretching
1450	1452.6	C-H bending
1420-1330	1396.9, 1339.7	O-H bending
1250-1020	1246.9, 1024.1	C-N stretching
810±20	805.5	C-H bending
780±20	777.0	C-H bending
730-660	697.3	C=C bending
690-515	552.7	C-Br stretching

Table 4.31 IR spectrum analysis of DP-O3

The analysis of IR spectrum supports the presence of groups in DP-O3 chemical structure shown in Fig 4.36.

PART-E

4.9. IMPURITY PROFILING OF BEDAQUILINE

The process related and degradation impurities are identified in this section. The major degradation impurities were isolated and characterized for structure elucidation using sophisticated instruments.

4.9.1. Experimental

4.9.1.1 Chemicals and reagents

The chemicals and reagents for HPLC are same as described in section 4.8.1.1.
UPLC/ESI-MS grade solvents are described in section 4.8.1.1.).

4.9.1.2 Equipment and chromatographic condition

The equipment and chromatographic conditions are same as described in section 4.5.1.2.

UPLC-MS, Preparative HPLC and NMR (^{1}H NMR, ^{13}C NMR and APT) instrument and conditions are described in section 4.8.1.2.

4.9.1.3. Sample preparations

Analytical sample preparation, stress degradation sample and buffer preparations are same as described in section 4.5.1.3.

Enrichment of DPs for identification

From the stock solution (Section 4.8.1.3)1ml aliquot (neutralized in case of acid sample) was diluted with methanol to produce 10ml (10mg/ml), from this solution 0.5ml aliquot was again diluted with methanol to produce 10ml (500µg/ml). The solution was filtered through 0.45µ Pall syringe filter prior to injection in chromatographic condition described for UPLC/ESI-MS for identification purpose.

4.9.2. Result and Discussion

The process related impurity was noticed in BDQ bulk drug during analysis; it was identified by UPLC/ESI-MS. The DPs formed under acid pH media and in presence of oxidizing agent was identified and structures were proposed for them. The major degradation products from acid and oxidative conditions were isolated and characterized for structure elucidation and confirmation by NMR (^{1}H NMR, ^{13}C NMR and attached proton test (APT).

4.9.2.1 Process related impurity

The process related impurity was observed during primary analysis of RP-HPLC; the process related impurity can be residual solvent, by-product of reaction or product of side reaction.

Analysis of BDQ by LC/ESI-MS and NMR

Bedaquiline contains C-Br^- (C-X halogen) group which has two isotopes in natural abundance which are ^{79} Br and ^{81} Br in 50:50 %extents; the molecule containing Br^- atom might show $[M]^+ \pm 2$ ion in LC/MS analysis with or without same intensity peaks. The LC/MS analysis of bedaquiline showed two *m/z* values 555.0 and 557.0 in positive ion spectrum matched with chemical formula of $C_{32}H_{31}BrN_2O_2$ at retention time 2.05minutes. ESI-MS spectrum for BDQ and process related impurity is shown in Fig. 4.41 (B) and UPLC chromatogram for bedaquiline and process related impurity is shown in Fig.4.41 (A). The result of ESI-MS is analyzed in Table 4.35 with DP quantity in %. The DPs containing Br^- repeated the same condition of $[M^+ \pm 2]$. The proton NMR is shown in Fig.4.29. The analysis of NMR spectrum with assignment of ∂ ppm for BDQ is shown in Table 4.29.

Process related impurity analysis

The RP-HPLC chromatogram showed (Fig. 4.38) process related impurity in bulk drug chromatogram.

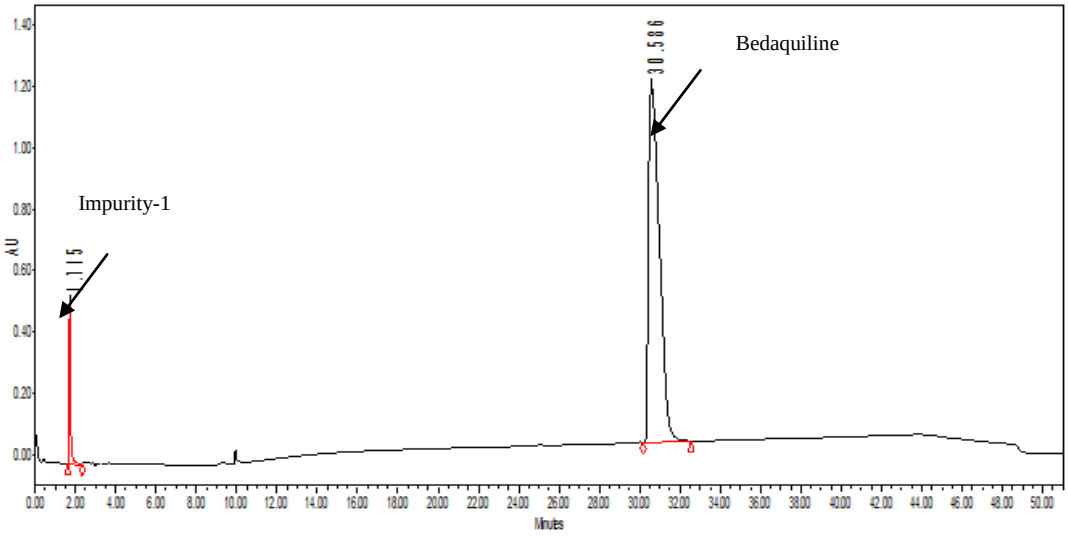


Fig. 4.38 Chromatogram showing BDQ and process related impurity (200 µg/ml)

For ease, the process-related impurity was named Impurity-1; in chromatogram the impurity covered 5.99% area of total area 100%. The Fig. 4.9 also showed the impurity-1 peaks in linearity of BDQ. Table 4.32 showed the chromatographic results for BDQ and impurity-1.

	Name	Retention Time	Purity1 Angle	Purity1 Threshold	Area	% Area
1	Impuirty-1	1.715	5.058	5.198	2805531	5.99
2	BDQ	30.586	0.191	1.012	44040158	94.01

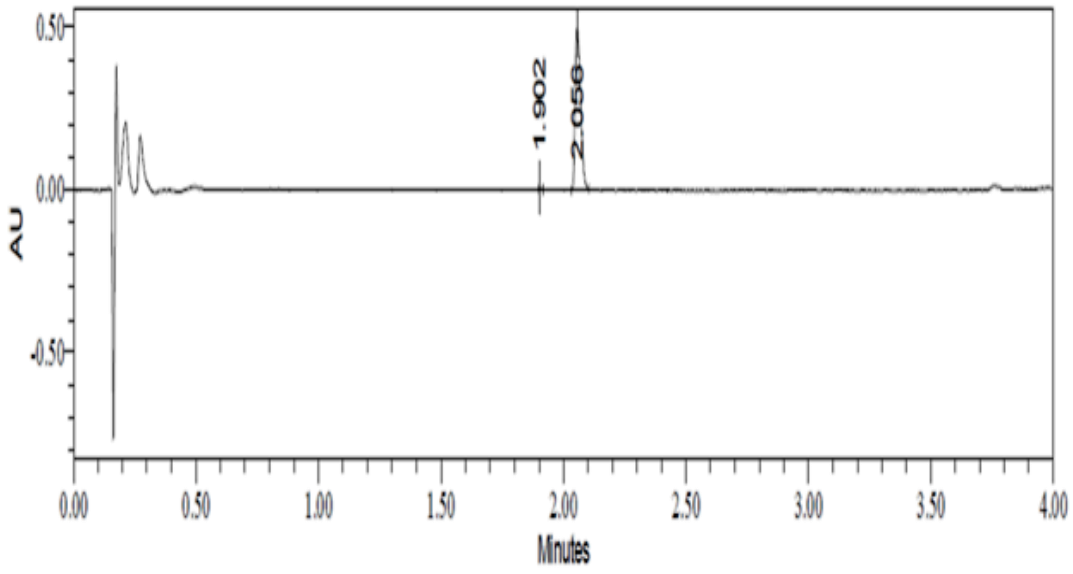
Table 4.32 Chromatographic parameters for BDQ and impurity-1

The total area of chromatogram was 100% among which 5.99% area is covered by impurity-1 led to the identification of it.

LC/ESI-MS identification of impurity-1

The identification of impurity-1 was carried out by [M+H $^{+}$] ion of *m/z*obtained in UPLC/ESI-MS. The UPLC chromatogram and LC/ESI-MS spectrum is shown in Fig.4.39(A) and (B), respectively.

A



B

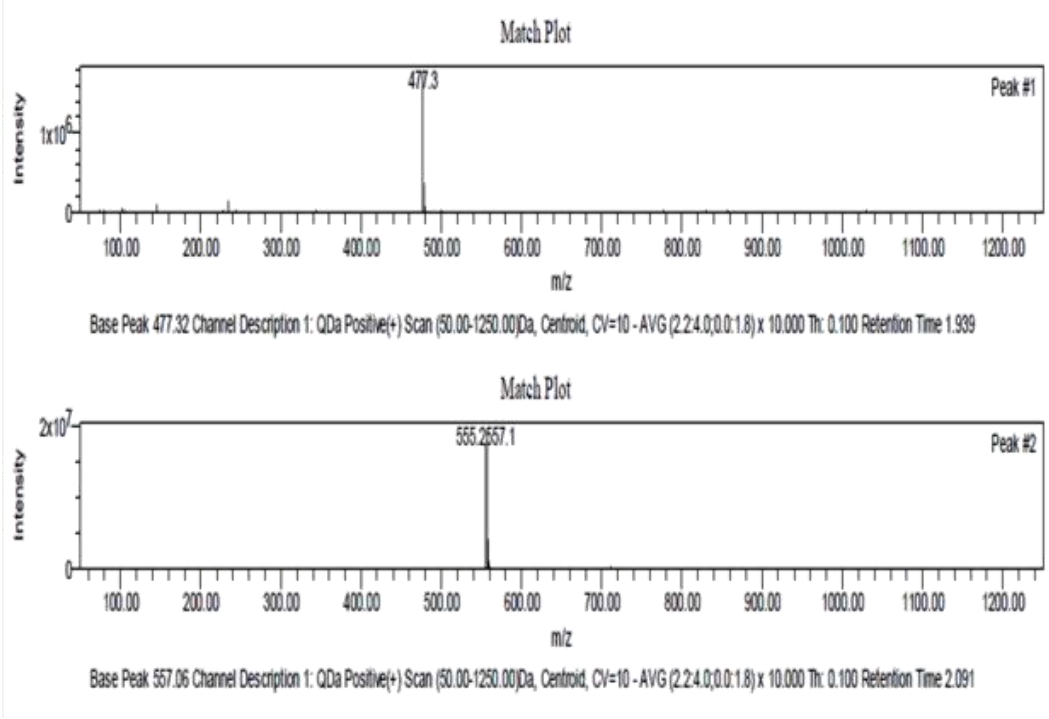
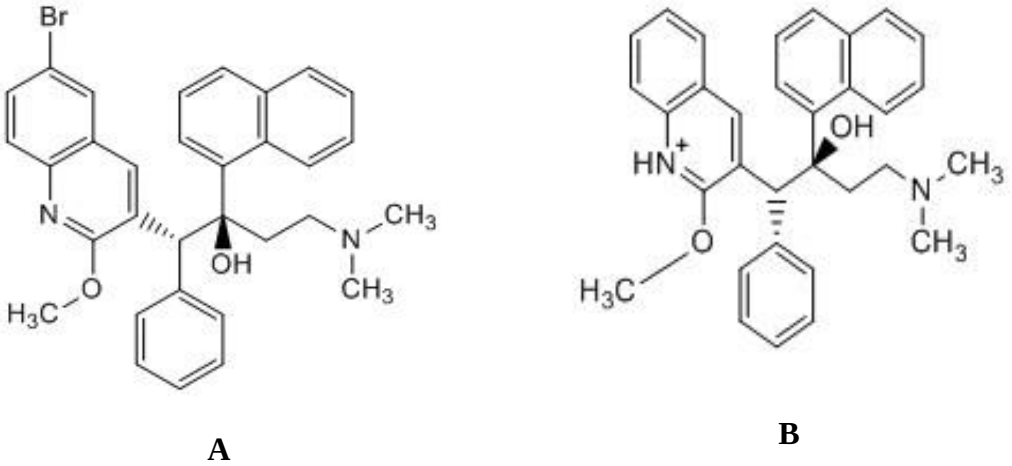


Fig. 4.39 (A) UPLC chromatogram (B) LC/ESI-MS spectrum for Impurity-1 and BDQ

The ESI/MS spectrum showed [M+H$^+$] ion for *m/z* of 477.4 amu, which is 79.8amu less than the bulk drug amu indicates the removal of Br $^-$ ion removal from the chemical structure of BDQ. Suggested chemical structure for impurity-1 is shown in Fig. 4.40.



A **B**

Fig. 4.40 chemical structure for (A) BDQ and (B) Impurity-1

The analysis of structure and LC/ESI-MS spectrum shows that a weak bond between the atom C and Br broken down in BDQ structure to form impurity-1 that matches with chemical formula of $C_{32}H_{33}N_2O_2$. This can be the result of incomplete reaction during BDQ bulk drug

synthesis. The impurity-1 can be chemically named as 3-((1R-2S))-4-(dimethylamino)-2-hydroxy-2-(naphthalen-1-yl)-2methoxyquinolin-1-ium.

4.9.2.2 Acid degradation impurities

The degradation impurities of BDQ in acid pH wereidentified using UPLC/ESI-MS. Major degradation impurity was isolated using preparative HPLC and characterized by NMR (proton, carbon[13] and APT NMR) and IR for structure elucidation and confirmation.

Degradation behavior of BDQ in acid media

The stability study of BDQ by RP-HPLC showed that different degradation impurities formed under acidic pH medium, to identify and characterize it, a concentrated sample of BDQ in acid was formed (section 4.7.1.3) and kept it in prescribed stress condition shown in table 4.6. The acid degraded chromatogram of BDQ is shown in Fig.4.5 with peak purity test in Fig. 4.7 and Table 4.7.

The chromatogram and peak purity plot showed that three peaks are pure degradation impurities while two peaks are co-eluting peaks. Major DP was identified based on criterion of major area occupied by DP in HPLC chromatogram and subjected to isolation and other DPs were identified and structures are proposed for them. The major degradation product was identified at Rt 26.48minutes, the %area covered by each DPs and mass balance(100%) are shown in Table 4.33.

	Name	Retention Time	Area	% Area
1	DP 1-2	2.913	1021331	10.20
2	DP 6-7	3.187	674213	5.99
3	DP 3	13.470	751340	6.06
4	DP 4	25.555	512548	7.15
5	DP 8	26.488	4601660	39.13
6	API	31.830	3991779	25.18
7	DP 5	35.539	841737	6.29

Table 4.33 chromatographic behavior of acid degradation impurity of BDQ

Identification of degradation impurities

The degradation impurities of BDQ were identified using LC/ESI-MS instrument. The instrument and its chromatographic conditions are described in section 4.8.1.2. Samples with high concentration were used to get good signals and better S/N (Signal to Noise) ratio. (Section 4.7.1.3) UPLC/ESI-MS chromatogram is shown in Fig. 4.41for acid DPs mixture

sample. LC/MS spectra showed peak asymmetry and tailing because high sample concentration to detect small fraction of DPs.

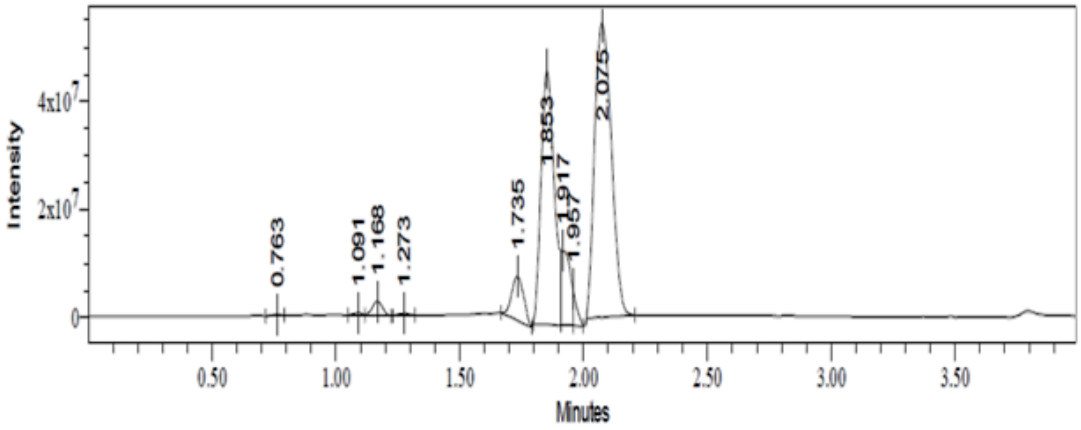
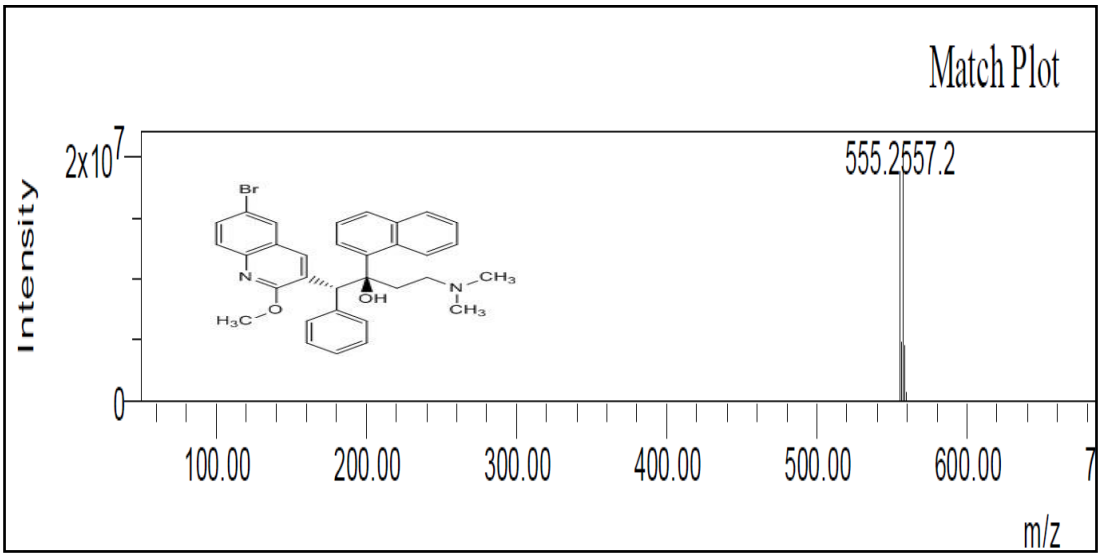


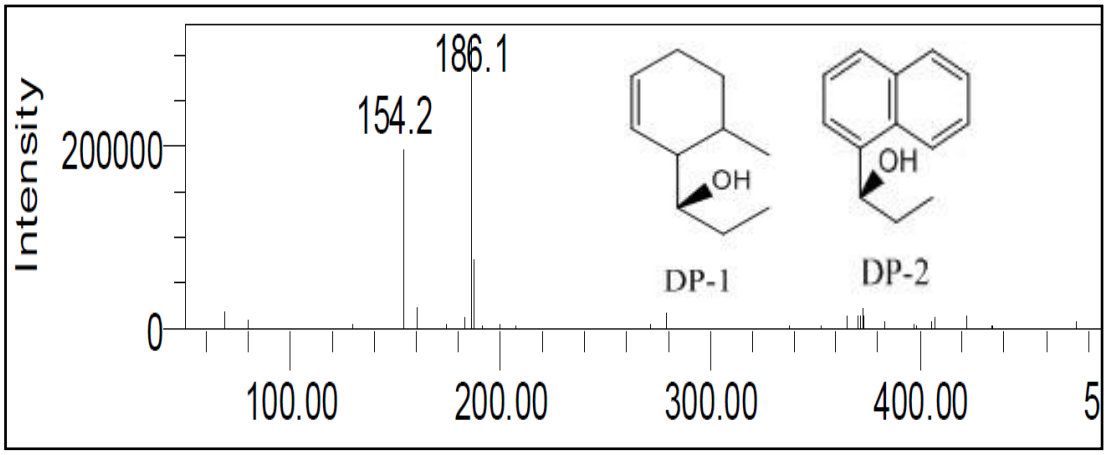
Fig. 4.41 UPLC chromatogram of acid degradation products mixture of BDQ

The chromatogram showed 8 degradation impurities and BDQ peaks, the degradation impurities are named in increasing order of their *m/z*. The LC/MS spectrometry showed four single eluting peaks [M^+] ion without any co-elution of peak while two co-eluting peaks were also observed (Fig.4.42). 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-A8), 1.917 (Co-elution), 1.957 and 2.075 minutes (API), respectively. DPs were named as DP-A1 to DP-A8 in increasing order of their molecular weight, respectively (shown in Fig. 4.40 (B to H)) and data is gathered in Table 4.34. DP-A1 to DP-A8 was identified based on mass spectrometry while DP-A8 was isolated, characterized and structure was elucidated by ^{1}H NMR and ^{13}C NMR and APT.

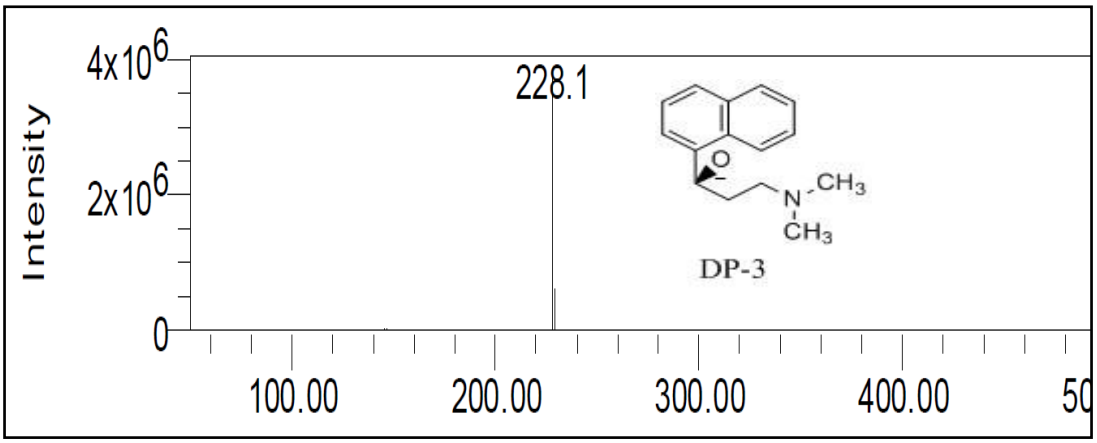
A



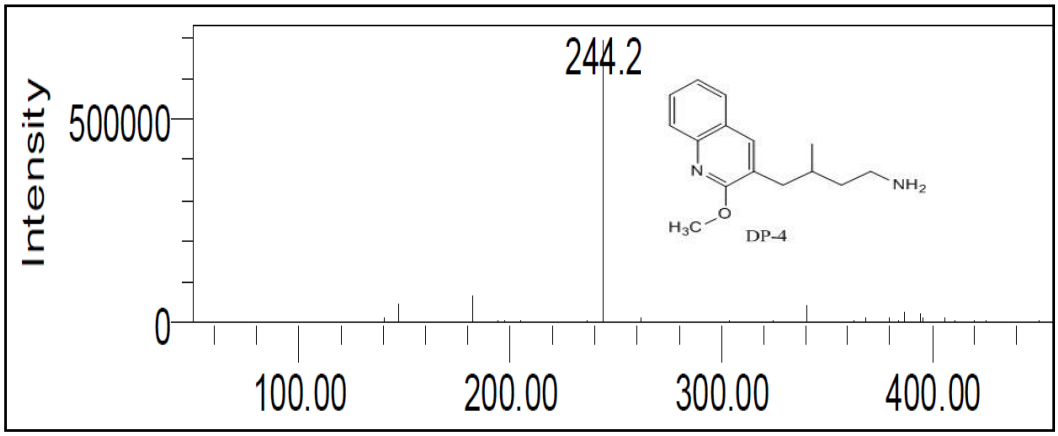
B


186.1
154.2
Intensity
200000
0
100.00
200.00
300.00
400.00
OH
OH
DP-1
DP-2

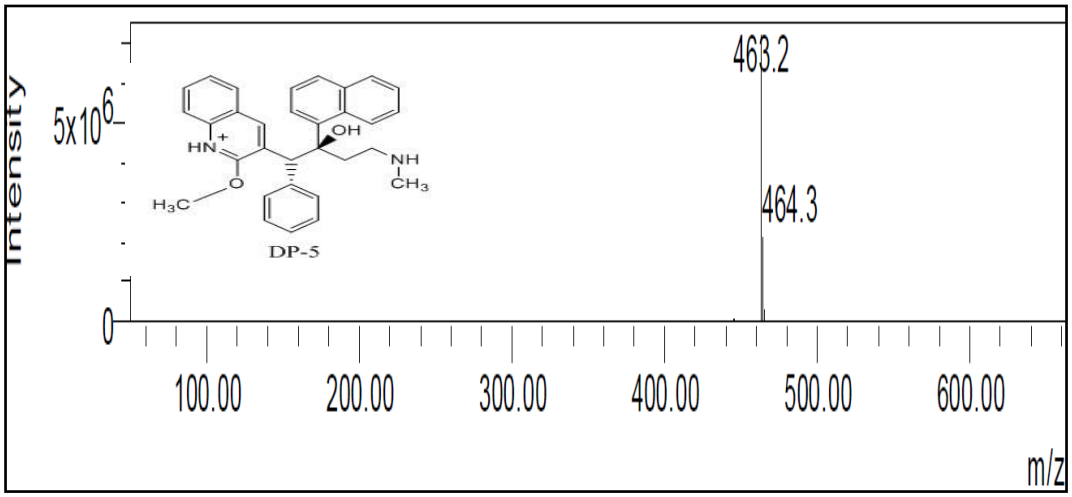
C


228.1
Intensity
4x10^6
2x10^6
0
100.00
200.00
300.00
400.00
O
N
CH3
CH3
DP-3

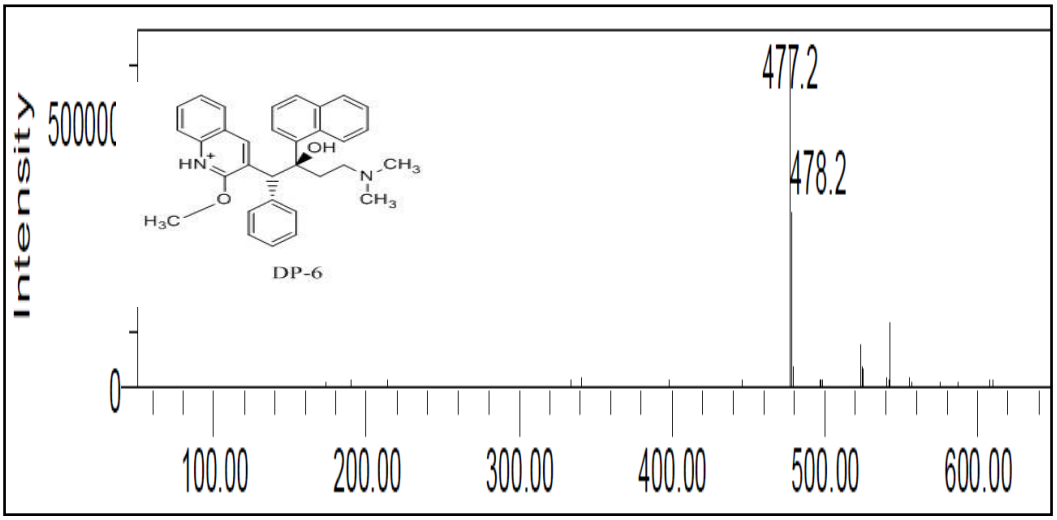
D


244.2
Intensity
500000
0
100.00
200.00
300.00
400.00
N
O
H3C
NH2
DP-4

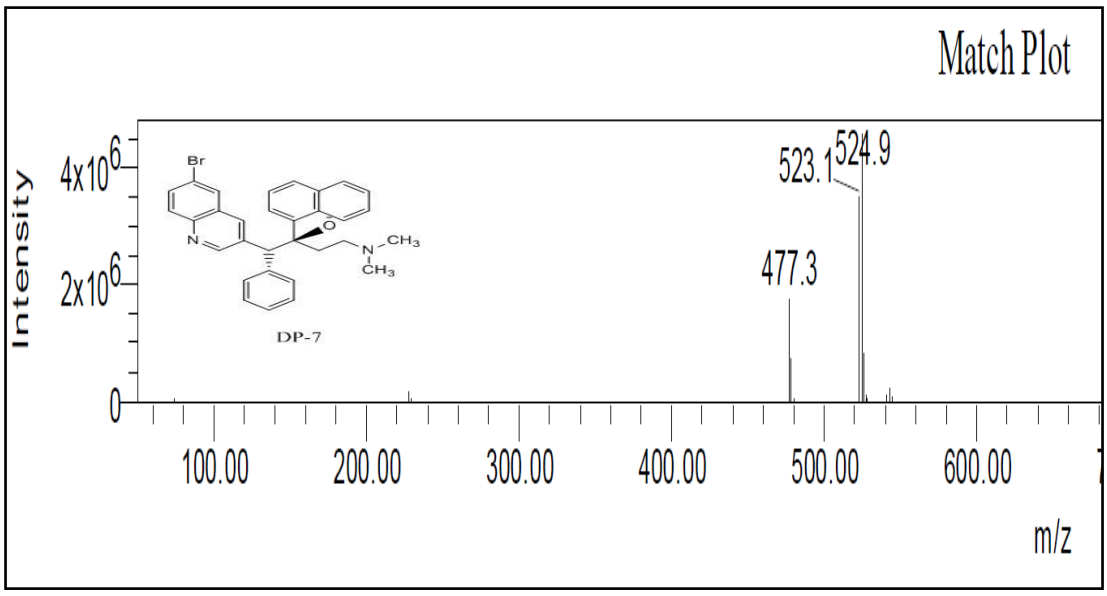
E


Intensity
5x10^6
0
468.2
464.3
DP-5
100.00
200.00
300.00
400.00
500.00
600.00
m/z

F


Intensity
500000
0
477.2
478.2
DP-6
100.00
200.00
300.00
400.00
500.00
600.00

G


Match Plot
Intensity
4x10^6
2x10^6
0
523.1
524.9
477.3
DP-7
100.00
200.00
300.00
400.00
500.00
600.00
m/z

H

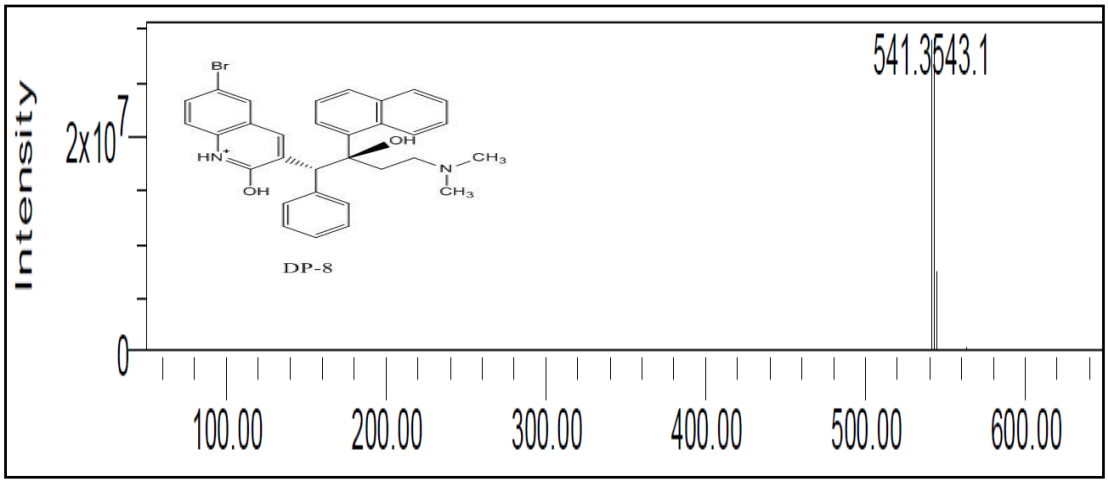


Fig. 4.42 LC/ESI-MS spectra of A) Bedaquiline B) DP-A1 and DP-A2 C) DP-A3 D) DP-A4 E) DP-A5 F) DP-A6 G) DP-A7 and H) DP-A8

The individual DP and its identification with proposed structure are shown below.

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

The LC/ESI-MS spectrum showed two co-eluting peaks at Rt 1.273minutes 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; it was confirmed by absence of isotope peak in LC/ESI-MS with $M^+\pm2$. DP-A1 and DP-A2 can be considered as fraction of bedaquiline formed due to bond breaking at C^{31} and C^{30}. The possible destruction of bedaquiline in DP-A1 and DP-A2 is shown in scheme-1. The DP-A1 and A2 can be chemically named as *(1R)*-1-(6-methylcyclohex-2-en-1-yl) propan-1-yl and *(R)*-1-(napthelen-1-yl)propan-1-ol, respectively.

DP-A3(m/z 228.1)

DP-A3 was eluted at Rt 1.168minutes with $[M]^+$ 228.1, corresponds to molecular formula $C_{15}H_{18}NO^-$. Mechanism for formation of DP-3 is again the result of bond breaking atC^{31} and C^{30} and absence of Br was confirmed by absence of isotope peak in LC/ESI-MS spectrometry. Bond between $C^{31}$and C^{30} was broken by catalyst effect of acid and temperature; resulted in DP-A1, A2 and A3. The DP-A3 can be chemically named as *(R)*-3-(dimethylamino)-1-(napthelen-1-yl) propan-1-olate.

DP-A4 (m/z 244.2)

DP-A4 was corresponding to chemical formula $C_{15}H_{20}N_2O$ with $[M]^+$ 244.2 without isotope peaks. DP-A4 was formed due to removal of benzene ring at $C^{35}$and naphthalene ring at C^{15}.

Br^- and methylene ($-CH_3$) groups were also removed in acid catalyzed reaction. The DP-A4 can be chemically named as 4-(-2methoxyquinolin-3-yl)-3-methylbutan-1-amine.

DP-A5 (m/z 464.2)

DP-A5 was observed at Rt 1.735minutes with $[M]^+$ 463.2 and 464.2. *M/z* value corresponds with chemical formula $C_{31}H_{31}N_2O_2$, structure of DP-A5 showed removal of methylene ($-CH_3$) group at C^2 and Br at C^{21}.The DP-A5 can be chemically named as 3-((*1R, 2S)*-2-hydroxy-4-(methylamino)-2-(naphthalen-1-yl)-1-phenylbutyl)-2-methoxyquinolin-1-ium.

DP-A6 (m/z 477.2)

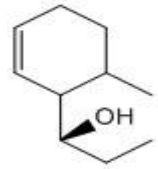
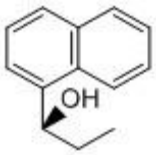
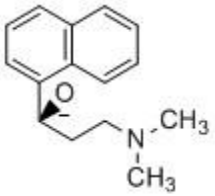
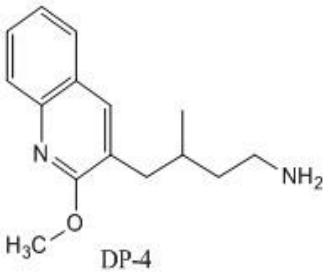
The *m /z* of 477.2 and 478.2 showed H^+ ion interchange in DP-A6 at Rt 1.917 match up with chemical formula $C_{32}H_{33}N_2O_2$. DP-A6 showed removal of Br atom at C^{21} and H^+ ion interchange at N^{27}. The DP-A6 can be chemically named as 3-*((1R, 2S)*-4-(dimethylamino)-2-hydroxy-2-(naphthalen-1-yl)-1-phenylbutyl)-2-methoxyquinolin-1-ium.

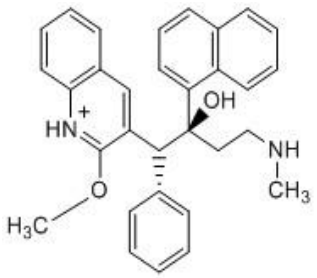
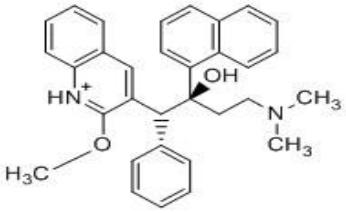
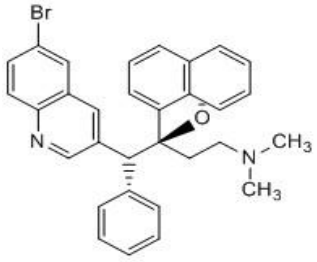
DP-A7 (m/z 524.9)

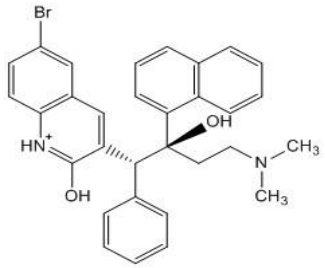
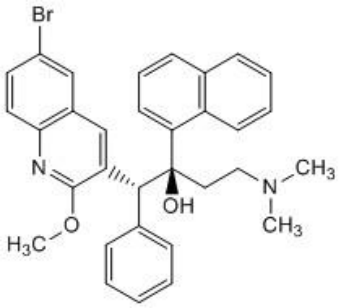
DP-A7 was observed at Rt 1.917 along with DP-A6 with $[M]^+$ 524.9↔ 523.1indicated a lone pair of electron included in structure with high peak intensity of *m/z* of 524.9. Chemical formula for DP-A7 was observed to be $C_{31}H_{28}BrN_2O$. DP-A5, A6, A7 and A8 are formed due to removal of single group from bedaquiline parent molecule. The DP-A7 can be chemically named as *(1R, 2S)*-1-(6-bromoquinolin-3-yl)-4-(dimethylamino)-2-(naphthalen-1-yl-1-phenylbutan-2-olate.

DP-A8 (m/z 541.3)

DP-A1 to A7 was formed in minor quantity while major DP observed was DP-A8. Chemical formula was matched up with $C_{31}H_{30}BrN_2O_2$and $[M]^+$ 541.3↔ 543.11 indicated presence of Br atom at C^{11} as isotope peaks were observed. Bedaquiline API MS spectra showed *m/z* of 555.1 ↔ 557.1; M+2 observed due to presence of Br atom isotopes. The DP-A8 can be chemically named as 6-bromo-3((*1R,2S*)-4-(dimethylamino)-2-hydroxy-2-(napthelen-1-yl)-1-phentlbutyl)-2-hydroxyquinolin-1-ium.

DP No	Chemical structure	*m/z*	Rt	Chemical formula
A1	 DP-1 *(1R)*-1-(6-methylcyclohex-2-en-1-yl) propan-1-yl	154.2(*154.14)	1.27	$C_{10}H_{18}O$
A2	 DP-2 *(R)*-1-(napthelen-1-yl)propan-1-ol	186.13(*186.10)		$C_{13}H_{14}O$
A3	 DP-3 *(R)*-3-(dimethylamino)-1-(napthelen-1-yl) propan-1-olate	228.1(*228.32)	1.16	$C_{15}H_{18}NO^-$
A4	 DP-4 4-(-2methoxyquinolin-3-yl)-3-methylbutan-1-amine	244.2(*244.34)	1.09	$C_{15}H_{20}N_2O$

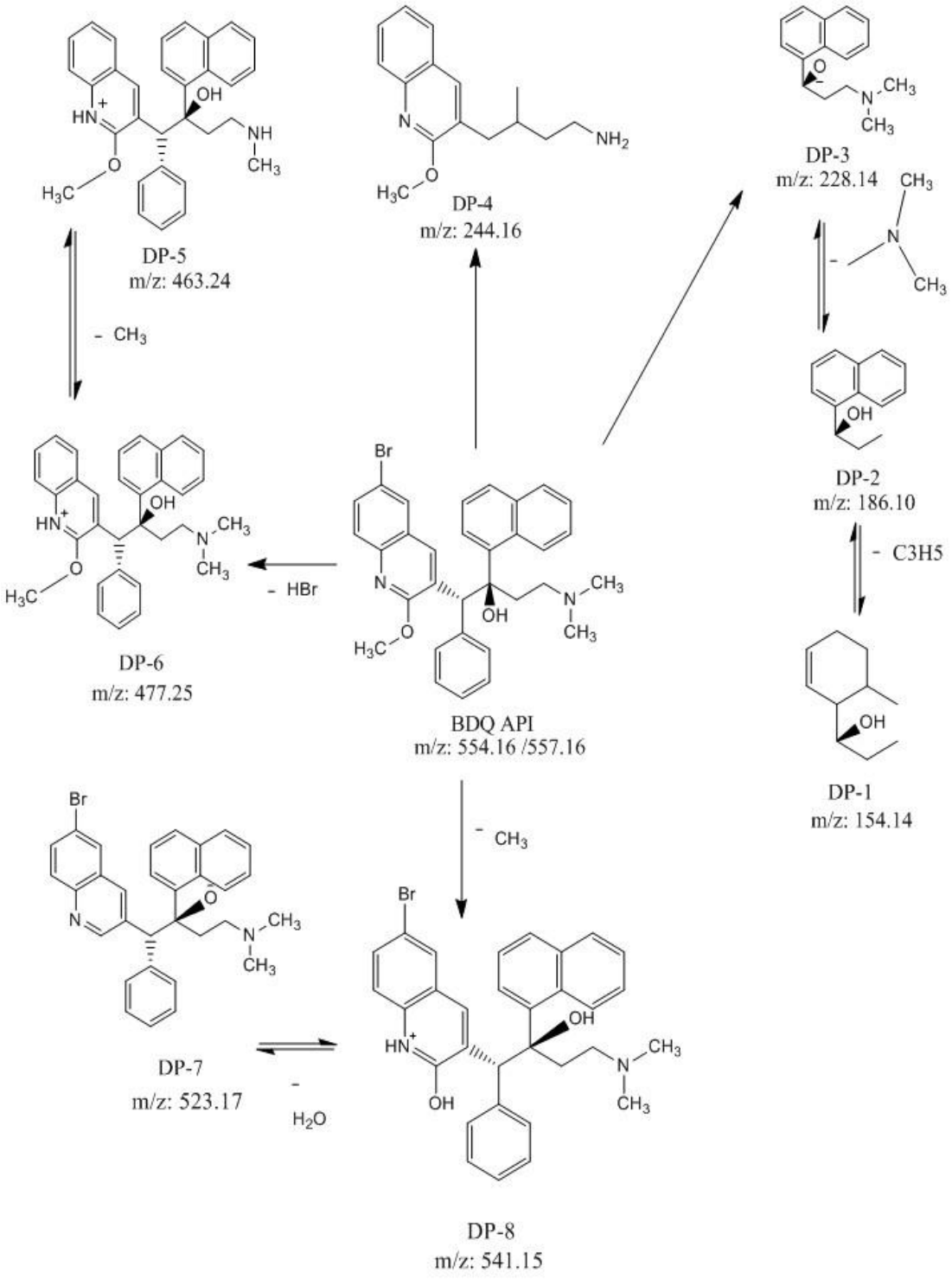
A5	 DP-5 3-((*1R, 2S)*-2-hydroxy-4-(methylamino)-2-(naphthalen-1-yl)-1-phenylbutyl)-2-methoxyquinolin-1-ium	463.2 (*463.60)	1.73	$C_{31}H_{31}N_2O_2^+$
A6	 DP-6 3-*((1R, 2S)*-4-(dimethylamino)-2-hydroxy-2-(naphthalen-1-yl)-1-phenylbutyl)-2-methoxyquinolin-1-ium	477.2 (*477.73)	1.91	$C_{32}H_{33}N_2O_2^+$
A7	 DP-7 *(1R, 2S)*-1-(6-bromoquinolin-3-yl)-4-(dimethylamino)-2-(naphthalen-1-yl-1-phenylbutan-2-olate	524.9(*524.48)		$C_{31}H_{28}BrN_2O^-$

A8	 DP-8 6-bromo-3((*1R,2S*)-4-(dimethylamino)-2-hydroxy-2-(napthelen-1-yl)-1-phentlbutyl)-2-hydroxyquinolin-1-ium	541.3 (*542.50)	1.85	$C_{31}H_{30}BrN_2O_2^+$
API		555.2 (*555.52)	2.07	$C_{32}H_{31}BrN_2O_2$

*****Exact molecular mass

Table 4.34LC/ESI-MS data for bedaquiline and its acid DPs

From above analyzed DP and its *m/z,* the possible degradation pathway and structure for degradation impurities are shown in Scheme 1.



Scheme.1.Proposed mechanism for degradation pathway of BDQ in acid media

Mechanism for formation of DPs

Formation of all the DPs of acid degradation of bedaquiline 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 C^{30} and C^{31}, 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 C^{15} and benzene ring at C^{31} generated DP-A4.

DP-A6 is result of loss of Br^- from the parent molecule, C-Br^- is a weak bond and having partial positive and negative charge seeking for electron accepting nucleolus. Proton (H^+) in acid media provided that electro seeking neuclolus; conjugated with Br^- and left as HBr. Further in DP-A6 demethylation preceded at amine atom N^3 resulted in DP-A5. The DP-A8 was a major degradation product; mechanism for formation of DP-A8can be explained by demethylation by acid. The atom O^- (oxygen)had a lone pair of electron acted as an electrophile and H^+ in acid media served as carrying partial positive charge attracted to oxygen atom resulted in removal of methyl group. In DP-A8 removal of alcohol group at position 19 formed DP-A7, neucleophilic attack of proton in acid media on –OH resulted in H_2O and removal of –OH. DP-A8 was chemically named as 6-bromo-3((*1R,2S*)-4-(dimethylamino)-2-hydroxy-2-(napthelen-1-yl)-1-phentlbutyl)-2-hydroxyquinolin-1-ium.

4.9.2.3 Oxidation of BDQ

The degradation impurities of BDQ in presence of oxidative agent were identified using UPLC/ESI-MS, major degradation impurity was isolated using preparative HPLC and characterized by NMR (proton, carbon ^{13} and APT NMR) for structure elucidation and confirmation.

Degradation behavior of BDQ in oxidative media

The stability study of BDQ by RP-HPLC showed that different degradation impurities formed in oxidative medium, to identify and characterize it, a concentrated sample of BDQ in oxide media was formed (section 4.8.1.3) and kept it in prescribed stress condition shown in table 4.6. The oxide degraded chromatogram of BDQ is shown in Fig.4.5 with peak purity test in Fig. 4.7 and Table 4.7.

The chromatogram and peak purity plot showed that obtained three peaks, are pure degradation impurities. Major DP was identified based on criterion of area occupied by DP in HPLC chromatogram and subjected to isolation and other DPs were identified and structures are proposed for them. The major degradation product was identified at Rt 32.138minutes, the %area covered by each DPs are shown in Table 4.35. For calculation of %DP, chromatogram BDQ bulk drug was considered as standard and 100% API. The calculation was carried out using equation 9.

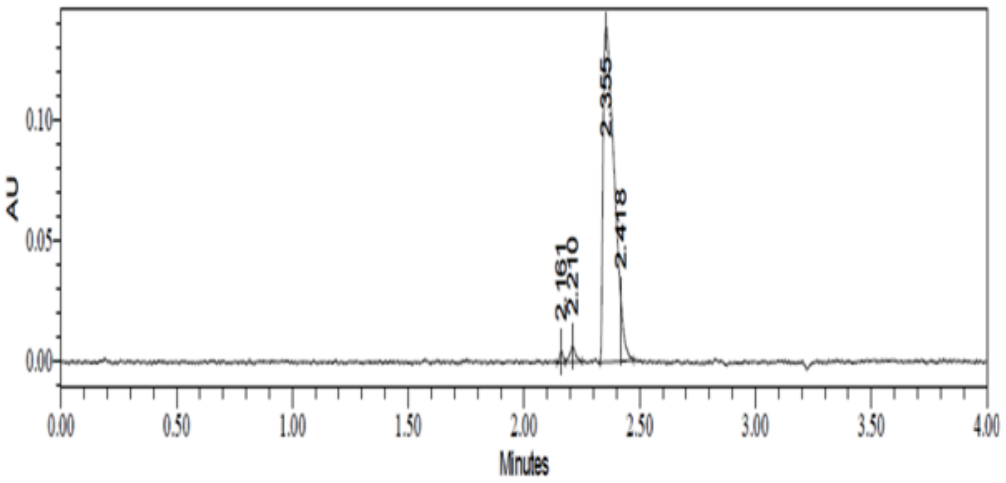
	Name	Retention Time	Area	% DP
2	DP-O2	3.691	971479	8.84
3	DP-O1	27.161	2870615	9.28
4	API	31.358	23270585	36.07
5	DP-O3	32.133	8731296	45.81

Table 4.35 BDQ and its oxidative degradation products in chromatogram

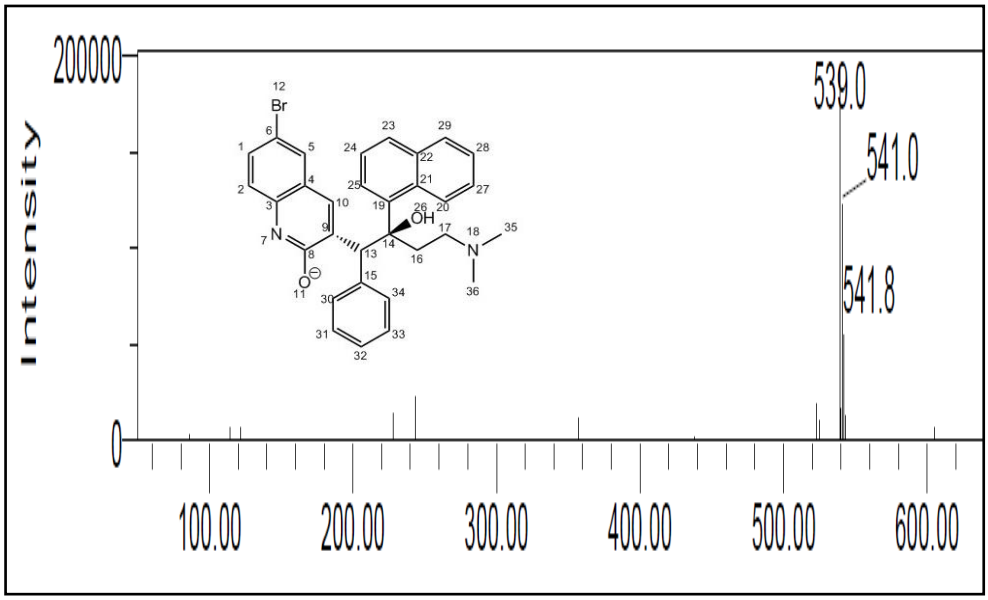
Identification of degradation impurities

The UPLC/MS spectrometric graph of oxidative condition showed two eluting peaks $[M + H]^+$ without any co-elution while DP-O3 and bedaquiline API was co-eluted. Peak elution was obtained at retention time 2.178, 2.228, 2.378-2.434 (Co-elution) minutes for $[M]^+$ ions at *m/z* 543.15, 539.05, 555.0 (API) and 571.0, named as DP-O1, DP-O2 and DP-O3, respectively. (Fig.4.43) The degradation pathway and elucidated structures are shown in scheme-2.

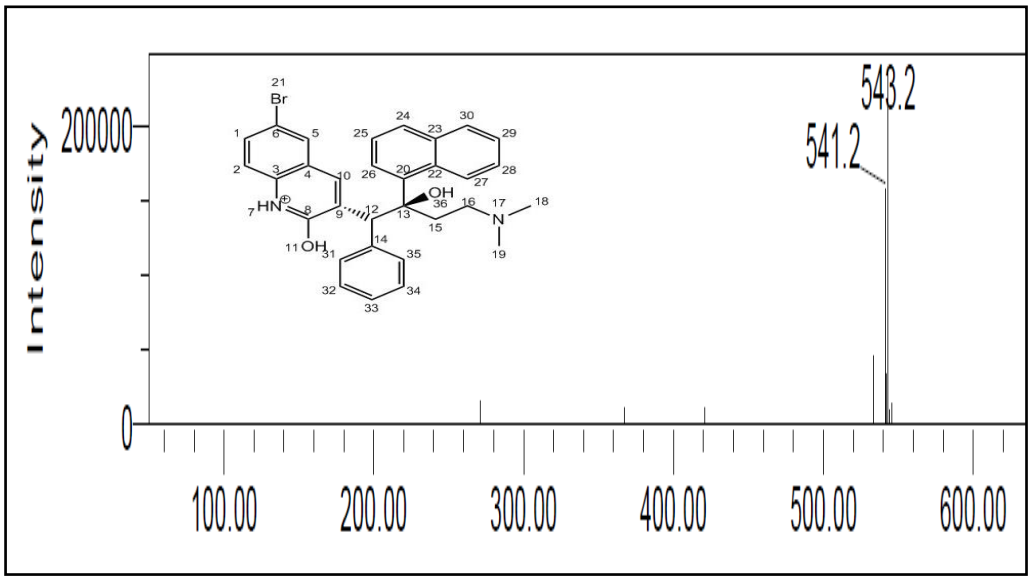
A



B


Intensity
200000
0
539.0
541.0
541.8
100.00
200.00
300.00
400.00
500.00
600.00

C


Intensity
200000
0
543.2
541.2
100.00
200.00
300.00
400.00
500.00
600.00

D

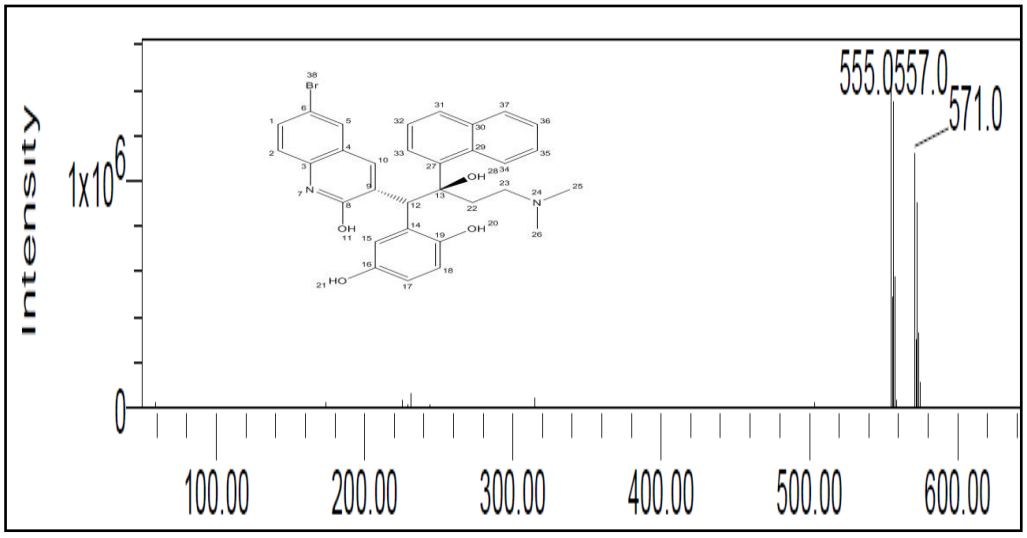


Fig. 4.43 A) UPLC chromatogram for oxide DP mixture ,ESI-MS spectrum for B) DP-O1 C) DP-O2 and D) DP-O3 of BDQ

DP-O1 (*m/z* 539.05)

The DP-O1 was eluted at Rt 2.228minutes in UPLC chromatogram with $[M^+]$ ion at *m/z* 539.05 which is corresponding to chemical formula $C_{31}H_{28}BrN_2O_2$. The intense peak in positive mode showed $[M^+]$ + 2 ion with *m/z* 539.0 and 541.0 indicates presence of Br^- atom. DP-1 could be formed from DP-2 as a parent molecule with H^+ atom loss. The DP-O1 can be chemically named as 6-bromo-3-((*1R, 2S)*-4-(dimethylamino)-2-hydroxy-2-(napthelen-1-yl)-1-phenylbutyl)quinolin-2-olate.

DP-O2(*m/z* 543.15)

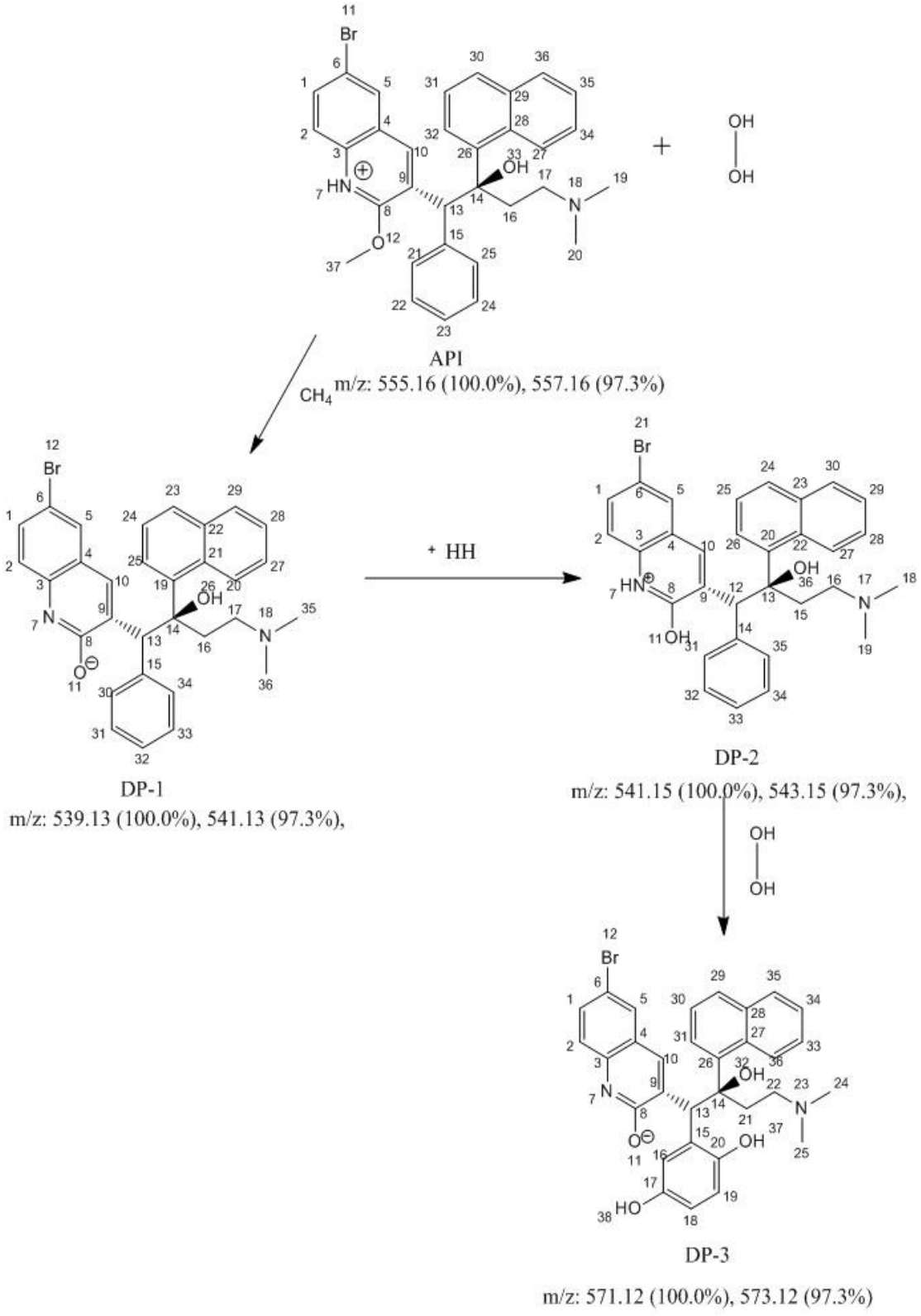
DP-O2 was eluted at Rt 2.178minutes with $[M^+]$ + 2 ion at *m/z* 541.1 and 543.1 indicated presence of Br^- atom and matches with chemical formula $C_{31}H_{30}BrN_2O_2$, the possible reason for formation of DP-2 can be the demethylation of bedaquiline API. The DP-O2 can be chemically named as 6-bromo-3-((*1R, 2S)*-4-(dimethyamino)-2-hydroxy-2-(naphthalen-1-yl)-1-phenylbutyl)-2-hydroxyquinolin-1-ium.

DP-O3 (*m/z* 571.0)

The $[M^+]$ +2 ion was obtained at *m/z* 571.0 which is corresponding to molecular formula of $C_{31}H_{28}$ BrN_2O_4. This indicated the oxidation of bedaquiline at different position. Demethylation of C_{38} left alcohol group at position 11 (confirmed in proton NMR) Addition of oxide at C_{20} and C_{21}positionof benzene ring was confirmed by ^1H NMR. Structure

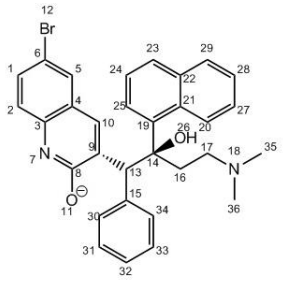
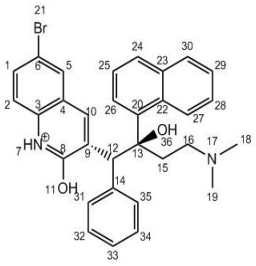
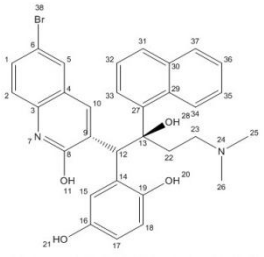
elucidation by NMR is explained briefly in next subsection. The DP-3 can be chemically named as named as 2-((*1R.2S)*-1-(6-bromo-2-hydroxyquinolin-3-yl)-4-(-dimethylmino)-2-hydroxy-2-(napthlen-1-yl) butyl) benzene-1, 4-diol.

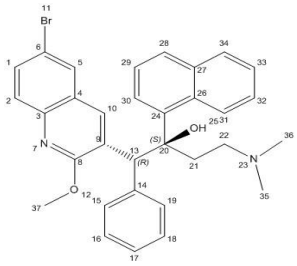
The degradation pathway is shown in Sceme-2.



Scheme.2.The degradation pathway and DPs of BDQ in oxidative medium

The LC/ESI/MS data for DP O1 to O3 and BDQ is shown in Table. 4.36

DP No	Structure	*m/z*	Rt	Chemical formula
DP-1	 6-bromo-3-((*1R, 2S)*-4-(dimethylamino)-2-hydroxy-2-(napthelen-1-yl)-1-phenylbutyl)quinolin-2-olate	539.0 (*540.48)	2.228	$C_{31}H_{28}BrN_2O_2^-$
DP-2	 6-bromo-3-((*1R, 2S)*-4-(dimethyamino)-2-hydroxy-2-(naphthalen-1-yl)-1-phenylbutyl)-2-hydroxyquinolin-1-ium	541.2(*542.50)	2.178	$C_{31}H_{30}BrN_2O_2^-$
DP-3	 2-((*1R.2S)*-1-(6-bromo-2-hydroxyquinolin-3-yl)-4-(-dimethylmino)-2-hydroxy-2-(napthlen-1-yl)	571.0 (*573.49)	2.434	$C_{31}H_{29}BrN_2O_4$

	butyl) benzene-1, 4-diol			
API		555.2(*555.52)	2.434	$C_{32}H_{31}BrN_2O_2$

*****Exact molecular mass

↔ *m/z* [M^++N]

Table 4.36 LC/ESI-MS data for oxidative degradation products of BDQ

Mechanism for formation of DPs

DP-O1 of oxidation DP can be explained by loss of methyl group at α position- 12 and left lone pair of electron on oxygen atom, this elucidation matches with chemical formula $C_{31}H_{28}BrN_2O_2^-$ and molecular weight 540.48 which is relatable with [M^+] ion at *m/z* of 539.13. The formation of DP-O2 can be explained by addition of –H atom at α position 11 on oxygen atom and formation of –OH, and H^+ ion at α position 7 on atom –N. The structure elucidated matches with chemical formula $C_{31}H_{30}BrN_2O_2^+$ and molecular weight 542.20 which is relatable with [M^+] ion at *m/z* 541.15. The DP-3 formation can be explained by mechanism of oxidation, DP-O2 oxidized at α position 17 and 20 by oxidizing agent hydrogen peroxide. The presence of –OH group was confirmed by proton, carbon and APT NMR.

Conclusion

The stability study for BDQ was completed using required conditions and parameters as per ICH Q1 (A) guidelines. It was observed that BDQ was degraded in acidic condition and in oxidative medium; Bedaquiline dosage is ≤2.0 g/day so as per dosage criterion described in ICH guidelines the impurities ≥0.10% should be identified and qualified, shown in Table 4.37 and 4.35, while in other conditions BDQ was found stable or the% quantity of degradation impurity was not sufficient to identify the DP (neutral, alkaline, light, and thermal) (Table 4.6).In acid condition 8 DPs were identified by LC/ESI-MS among which one major DP was isolated by preparative HPLC and characterized by proton and carbon NMR. The structures and degradation pathway for acid and oxidative degradation impurities were evaluated. The major DP of acid was chemically named as 6-bromo-3((*1R,2S*)-4-(dimethylamino)-2-

hydroxy-2-(napthelen-1-yl)-1-phentlbutyl)-2-hydroxyquinolin-1-ium, while in oxidation of BDQ formed major DP-3 can be chemically named as 2-((*1R.2S)*-1-(6-bromo-2-hydroxyquinolin-3-yl)-4-(-dimethylmino)-2-hydroxy-2-(napthlen-1-yl) butyl) benzene-1, 4-diol. For oxidative condition 3 DPs are reported among which one is major DP which is isolated, identified and characterized using sophisticated instruments.

The degradation kinetic studies were performed by conventional method and using multi-factorial tool, the study showed that both the methods are equally effective for evaluation of degradation kinetic parameters. In the case of multi-factorial tool, method would be more effective as it can reduce the utilization of solvent and time used during conventional kinetic study. The study showed that increasing temperature and stressor concentration can effectively reduce the %drug, half life and shelf life of drug in that stressor, while degradation rate increase as rate constant was increasing. The activation of energy depends on exothermic or endothermic reaction.

The developed stability indicating method is precise, specific and reproducible as it can detect the impurity in trace level of 8.22µg/ml. All over the study can be applicable during formulation development of BDQ for suggested storage condition and shelf life of formulation. The process related impurity and major and minor DPs of different condition are reported for the first time in literature for BDQ.

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