

IDENTIFICATION, ISOLATION AND CHARACTERIZATION OF POTENTIAL DEGRADATION PRODUCT IN IDEBENONE DRUG SUBSTANCE

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ABSTRACT

In the stress degradation studies of Idebenone, one major unknown base degradation impurity was identified by LC-MS. This impurity was isolated using preparative high performance liquid chromatography. Based on the spectral data (¹H NMR, ¹³C NMR, DEPT, MS, HR-MS and IR), this degradation impurity is characterized as 2-hydroxy-5-(10-hydroxydecyl)-3-methoxy-6-methylcyclohexa-2,5-diene-1,4-dione. The details of stress studies, identification, isolation, characterization, formation and mechanism of this impurity is discussed and presented in detail.

Keywords: Idebenone, Degradation, Potential degradation product, Identification, Isolation, Characterization.

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INTRODUCTION

Idebenone, 2-(10-hydroxydecyl)-5,6-dimethoxy-3-methylcyclohexa-2,5-diene-1,4-dione belongs to quinone family. It is a powerful antioxidant and an analog of ubiquinone which is most commonly used antioxidant drug¹⁻². Idebenone is used for Alzheimer disease, liver disease, heart diseases and a number of inherited disorders including Leber's disease (an eye condition), mitochondrial encephalomyopathy (nerve and muscle disorders) and Friedreich's ataxia (heart disease and diabetes)³⁻⁸. Unlike vitamin and botanical antioxidants, Idebenone is a respiratory chain drug and is reported to correct the signs of aging and also offers the highest protection against harmful environmental free radical stress. Its Environmental Protection Factor (EPF) is 95, the highest rating of all antioxidants tested⁹. Idebenone enhances long-term potentiation in hippocampal nerve cells, a key part of memory formation and consolidation. Idebenone reaches the tissues more easily than ubiquinone and efficiently protect cells from peroxidative damage due to the modification of the composition and length of its side chain. Idebenone was developed from benzoquinone derivatives in the research laboratories of Takeda Chemical Industries, Ltd., and widely used in Japan to treat mental symptoms associated with old age and cerebrovascular disease¹⁰.

Amit *et.al* and Francis *et.al* developed UV-Spectrophotometric methods for the determination of Idebenone in bulk drug and formulations¹¹⁻¹². Rathi *et.al* developed a validated a stability-indicating HPTLC method for the determination of Idebenone in bulk drug¹³. Several analytical methods have been reported for Idebenone in the bulk drug and formulations using HPLC¹⁴⁻¹⁷. Hu *et.al* developed LC-MS method for the determination of Idebenone in plasma¹⁸. To the best of our knowledge there are no reports on the characterization of degradation products of Idebenone in literature. The present research work describes the identification, isolation and characterization of major unknown degradation product formed during the base degradation studies¹⁹.

EXPERIMENTAL

Chemicals and Reagents

The investigated samples of Idebenone were received from Process Research Department of Custom Pharmaceutical Services of Dr. Reddy's Laboratories Limited, Hyderabad, India. The chemicals used in the present analysis are Trifluoroacetic acid (Across), sodium hydroxide (Rankem), hydrochloric acid

(Rankem), hydrogenperoxide (S.D. Fine Chemicals) and HPLC-grade acetonitrile (Rankem). Water used in the analysis and isolation was purified using Milli-Q plus purification system.

Instrumentation

High-performance liquid chromatography

An Agilent 1100 series HPLC with photodiode array detector with chemstation data handling system was used. The analysis was carried out on Zorbax SB C18 column with 150mm length, 4.6mm internal diameter and 3.5 μ m particle size. Mobile phase A was water and trifluoroacetic acid in the ratio of 100:0.05 (v/v) and mobile phase B was acetonitrile and trifluoroacetic acid in the ratio of 100:0.05 (v/v). UV detection was carried out at 215 nm and flow rate was kept at 1.0 mL/min. Column oven temperature was maintained at 40 °C. The gradient program: time/% of MP-B was 0/40, 25/90, 30/90, 31/40 with post run time 5 min.

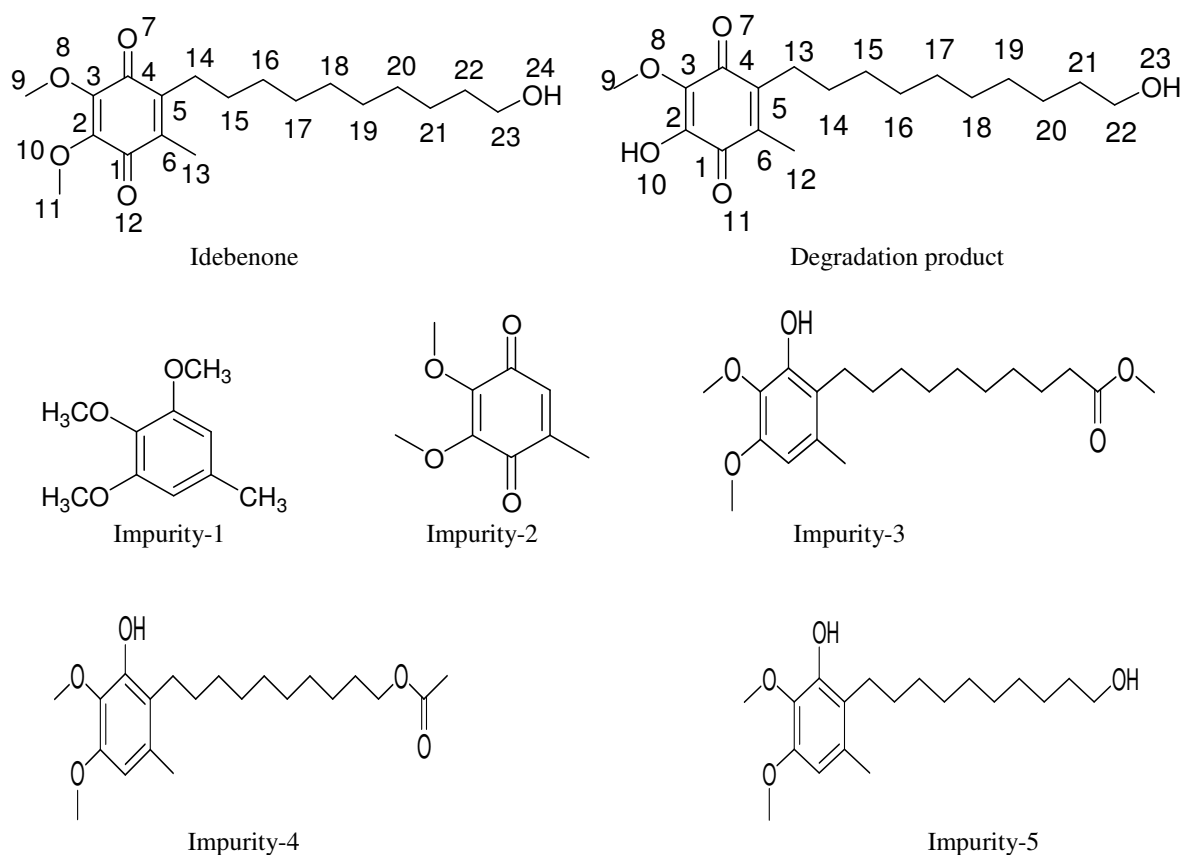


Fig.-1: Chemical structures of Idebenone and impurities

High-performance liquid chromatography (preparative)

An Agilent 1100 series preparative liquid chromatography equipped with a photodiode array detector system was used. Data was processed through chemstation software. Zorbax SB C18 (250mm long \times 9.4mm.i.d.) preparative column packed with 5 μ m particle size was employed for isolation of the impurity. Mobile phase A was water and trifluoroacetic acid in the ratio of 100:0.05 (v/v) and mobile phase B was acetonitrile and trifluoroacetic acid in the ratio of 100:0.05 (v/v). The flow rate was kept at 20 mL/min and the UV detection was carried out at 215 nm. The gradient program employed with T (min)/ % mobile phase-B (v/v) as 0/40, 14/70, 16/90, 19/90, 20/40 with post run time of 5 min.

LC-MS

LC-MS was carried out on the degraded drug substance of Idebenone. The mobile phase used was water : trifluoroacetic acid in the ratio of 100:0.05 (v/v) as a mobile phase -A and acetonitrile: trifluoroacetic acid in the ratio of 100:0.05 (v/v) as a mobile phase - B using the following gradient/elution (T/%B: 0/40, 25/90, 30/90, 31/40) flow rate of 1.0 mL/min and monitored at 215 nm. A Zorbax SB C18 column (150mm×4.6mm i.d., with a particle size of 3.5 μm) was used. The injected volume was 10μL. Nitrogen as nebulizer gas and as collision assisted dissociation gas was used with Capillary voltage 4000V and collision energy 25V. Source gas temperature at 325°C and flow rate at 12 lit/min was used. The data were processed using Mass hunter software.

High resolution mass

Samples were analyzed on the Micromass LCT Premier mass spectrometer equipped with an ESI Lockspray source for accurate mass values. Leucine enkephalin was used as internal reference compound which was introduced via the Lockspray channel using the Waters reagent manager. The mass range was calibrated with the cluster ions of sodium formate using a fifth order polynomial fit. The data were acquired in W mode using the Mass Lynx software.

The mass spectrometer was equipped with a Waters Acquity system. The conditions were used for the analysis are as follows-

Water: trifluoroacetic acid in the ratio of 100:0.05 (v/v) as a mobile phase -A and acetonitrile: trifluoroacetic acid in the ratio of 100:0.05 (v/v) as a mobile phase-B was used. The gradient/elution (T/%B: 0/40, 25/90, 30/90, 31/40), flow rate of 1.0 mL/min and detection at 215 nm was used. A Zorbax SB C18 column (150mm×4.6mm i.d., with a particle size of 3.5μm) was used. Impurity was dissolved in water: acetonitrile (50:50 v/v) diluent at a concentration level of 0.5 mg/ mL. The positive electrospray data were obtained by capillary voltage 2400 V, cone voltage 70V, cone gas 50 L/min, source temperature 125°C, desolvation temperature 250°C and desolvation gas flow 450 L/h.

NMR spectroscopy

The NMR spectra for Idebenone and degradation product were recorded on Varian Mercury plus 400 MHz and Gemini 200 MHz (Gemini-2000) spectrometers (Varian, Germany) in CDCl₃ solvent. The ¹H chemical shift values were reported on the δ scale in ppm, relative to TMS (δ=0.00ppm), while ¹³C chemical shift values were reported relative to CDCl₃ (δ=77.0 ppm) as internal standards. Distortionless enhancement by polarization transfer (DEPT) spectral editing revealed the presence of methyl and methyne groups as positive peaks while the methylenes as negative peaks. The data were processed using Linux software.

Table -1: NMR assignments of Idebenone and Degradation product

Position ¹	Idebenone				Degradation product			
	¹ H	δ (ppm) ² J	¹³ C	DEPT	¹ H	δ (ppm) ² J	¹³ C	DEPT
1	-	-	184.04	-	-	-	184.54	-
2	-	-	142.97	-	-	-	140.55	-
3	-	-	142.97	-	-	-	139.51	-
4	-	-	184.59	-	-	-	185.02	-
5	-	-	114.19	-	-	-	144.88	-
6	-	-	138.57	-	-	-	137.23	-
9	3H	3.99/s	61.06	-CH ₃	3H	4.06/s	60.24	-CH ₃
10	-	-	-	-	1H	6.55/d	-	-
11	3H	3.99/s	61.06	-CH ₃	-	-	-	-

12	-	-	-	-	-	3H	2.03/s	-	12.09	-CH ₃
13	3H	2.01/s	-	11.84	-CH ₃	2H	2.47/t	6.8	32.79	-CH ₂
14	2H	2.45/t	6.8	32.67	-CH ₂	2H	1.31/m	-	29.48	-CH ₂
15	2H	1.34/m	-	29.44	-CH ₂	2H	1.31/m	-	29.35	-CH ₂
16	2H	1.34/m	-	29.30	-CH ₂	2H	1.31/m	-	28.84	-CH ₂
17	2H	1.34/m	-	29.30	-CH ₂	2H	1.31/m	-	25.70	-CH ₂
18	2H	1.34/m	-	25.65	-CH ₂	2H	1.31/m	-	26.18	-CH ₂
19	2H	1.34/m	-	26.31	-CH ₂	2H	1.31/m	-	26.51	-CH ₂
20	2H	1.34/m	-	28.62	-CH ₂	2H	1.31/m	-	28.58	-CH ₂
21	2H	1.34/m	-	29.24	-CH ₂	2H	1.56/m	-	29.69	-CH ₂
22	2H	1.56/m	-	29.71	-CH ₂	2H	3.64/t	6.8	63.05	-CH ₂
23	2H	3.64/t	6.8	62.91	-CH ₂	1H	1.38/s	-	-	-
24	1H	1.48/s	-	-	-	-	-	-	-	-

1: Refer Fig.-1 for numbering

2: This column gives ¹H-¹H coupling constant

3: (s) singlet, (d) doublet, (t) triplet, (m) multiplet

Table -2: FT-IR spectral data of Idebenone and Degradation product

Compound	IR ^a
	Characteristic Absorptions (cm ⁻¹)
Idebenone	3570 (O-H stretching), 2924 (aliphatic C-H stretching), 1654 (C=O stretching), 1460 (C=C stretching), 1273 (C-O stretching).
Degradation product	3566 (O-H stretching), 3382 (O-H stretching), 2924 (aliphatic C-H stretching), 1637 (C=O stretching), 1459 (C=C stretching), 1276 (C-O stretching).

^a KBr

ESI-MS

The Mass spectra were recorded on Agilent 6410 Triple Quadrupole mass spectrometer. The sample was introduced through a union from liquid chromatograph into mass spectrophotometer in positive ionization mode of Electro spray ionisation. Nitrogen gas was used in nebulizer with Capillary voltage 4000V. Source gas temperature at 325°C and flow rate at 12 lit/min was used. The data were processed using Mass hunter software.

FT-IR spectroscopy

FT-IR spectra were recorded as KBr pellet on Perkin-Elmer instrument model-spectrum one.

Preparation of base degradation sample

A solution of Idebenone (50 mg) in 100 mL of 0.025N sodium hydroxide was kept for 24 hr.

RESULTS AND DISCUSSION

Analysis of base degradation sample by analytical HPLC

The base degradation sample was analyzed with analytical high-performance liquid chromatography. The analytical HPLC chromatograms of Idebenone and stressed sample in base degradation are shown in Fig-2 and Fig-4 respectively. The Idebenone eluted about a retention time of 13 min. In the chromatogram of the base stressed sample one prominent unknown degradation impurity was found about 33% by area normalization at relative retention time of 0.73 with respect to Idebenone peak.

Analysis of base degradation sample by LC-MS

To get structural insight, the LC-MS analysis was carried out on the base stressed sample. The mass spectrum thus obtained was shown the protonated molecular ion of degradation impurity at m/z 325,

whereas the Idebenone displayed protonated molecular ion at m/z 339. Thus the degradation impurity has 14 amu less than the molecular ion of Idebenone.

As the molecular ion information is not just enough to arrive at the structure, the drug substance was stressed and subjected to purification by preparative HPLC to isolate more quantity of degradation impurity for spectroscopic studies.

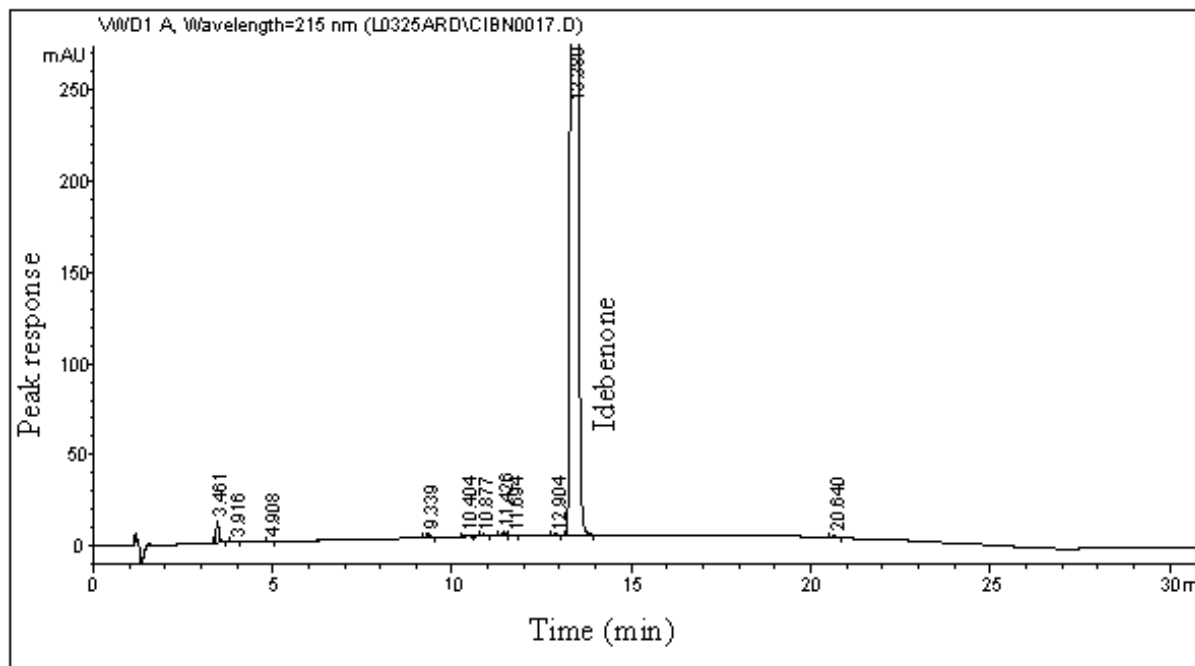


Fig-2 : HPLC Chromatogram of Idebenone

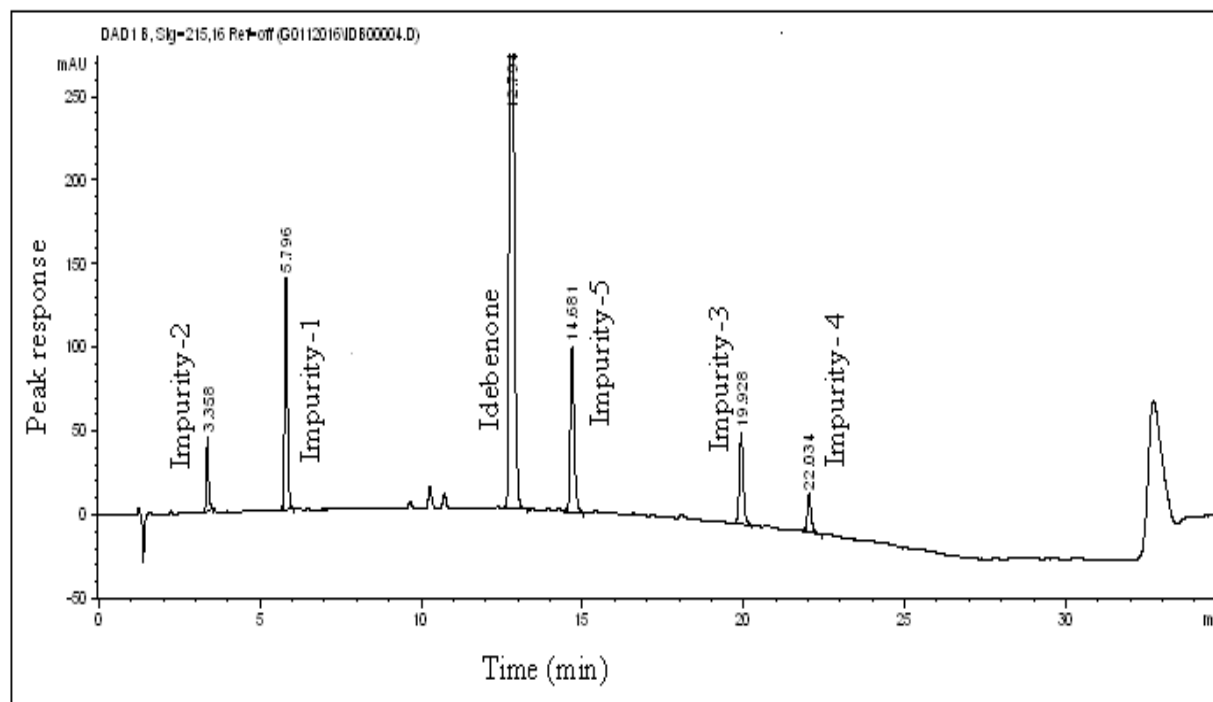


Fig-3 : HPLC Chromatogram of Idebenone with known impurities

Isolation of degradation product by preparative HPLC

Idebenone (1.0g) was treated with sodium hydroxide (0.025N, 25mL) and kept at room temperature for 24 h. Degraded solution was subjected to preparative HPLC. Fractions collected were analyzed by analytical HPLC. Fractions of >95% purity were pooled together and concentrated on rotavapour to remove the solvent mixture. To confirm the retention time of the isolated impurity the isolated fraction was analyzed by analytical HPLC. The impurity obtained as brick red color solid and the chromatographic purity is 98.0% by area percentage. Isolated impurity (Fig-5) was spiked to Idebenone and spiked HPLC chromatogram was shown in (Fig-6).

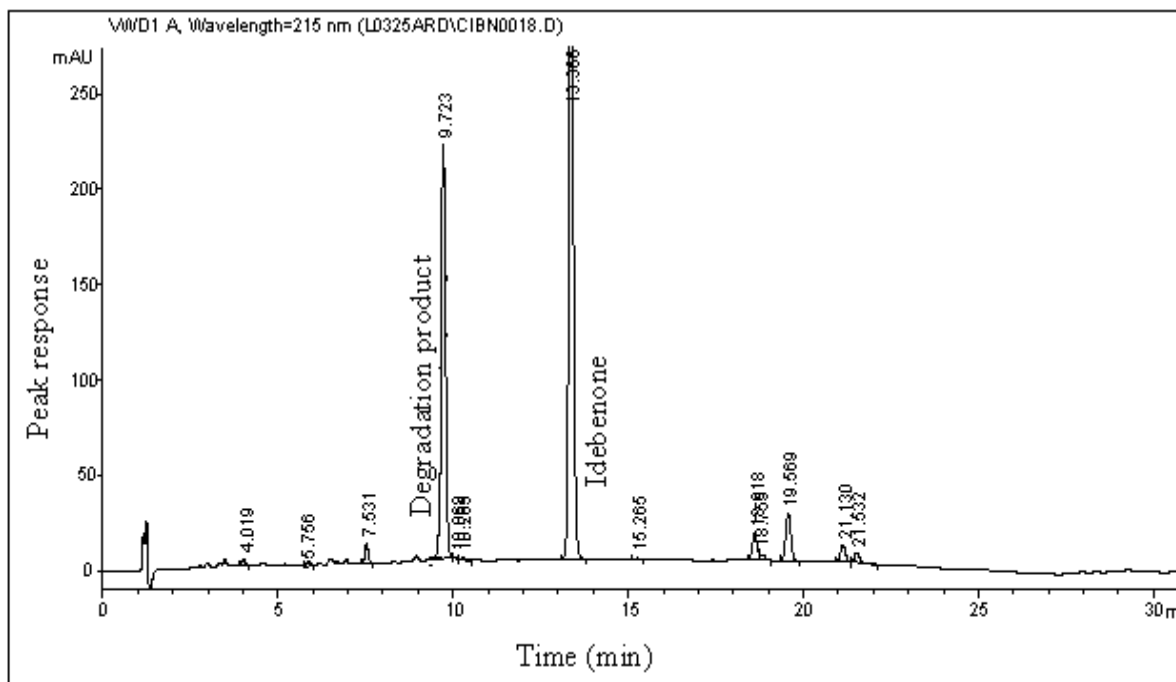


Fig-4 : HPLC Chromatogram of stressed sample by base hydrolysis condition

Structure elucidation of degradation product

The HR MS data of this degradation product showed exact mass of the protonated molecular ion at m/z 325.2003 (Calcd. 325.2015) for $C_{18}H_{29}O_5$ which corresponds to the molecular formula $C_{18}H_{28}O_5$.

The 1H NMR, ^{13}C NMR and DEPT spectral data of degradation product was compared with those of Idebenone in Table-1. The numbering scheme for the NMR assignments is shown in Fig-1. 1H NMR signals for several protons in the structure of the Idebenone and that of the impurity are same or with slight changes. One of the methoxy groups protons of 9th and 11th positions of the Idebenone is missing in the impurity spectrum. This indicates one of the methoxy groups missed in the impurity. One new signal at 6.55 ppm equal to one proton is presented in the impurity spectrum which is not seen in Idebenone spectrum. This new signal may be correspond to hydroxy group proton which might be formed in place of methoxy group. So it can be interpreted that one of the methoxy group replaced by the hydroxy group in base hydrolysis. From ^{13}C NMR data, it is observed that loss of one methoxy group carbon in the impurity. DEPT spectral data also supported to the loss of one methoxy group in the impurity. Thus the degradation impurity structure can be rationalized in terms of loss of one methoxy group and formation of hydroxyl group in the Idebenone molecule.

The electro spray ionization (ESI) mass spectrum of the degradation product (Fig-8) exhibited a molecular ion peak at m/z , 325 amu (MH⁺) in positive ion mode, whereas the Idebenone (Fig-7), displayed protonated molecular ion at m/z 339. Thus the degradation impurity has 14 amu less than the

molecular ion of Idebenone indicating that loss of one carbon atom and two hydrogen atoms in the Idebenone.

IR absorption spectral data of degradation impurity (Table-2) also supporting that formation of -OH functional group. The IR (KBr) spectral data of the degradation impurity was compared with those of Idebenone in Table-2. IR absorption bands for this impurity (cm⁻¹) are 3566 (O-H stretching), 3382 (O-H stretching), 2924 (aliphatic C-H stretching), 1637 (C=O stretching), 1459 (C=C stretching), 1276 (C-O stretching).

From the spectral data, the structure of this degradation impurity is characterized as 2-hydroxy-5-(10-hydroxydecyl)-3-methoxy-6-methylcyclohexa-2,5-diene-1,4-dione with molecular formula C₁₈H₂₈O₅ and molecular weight 324.2.

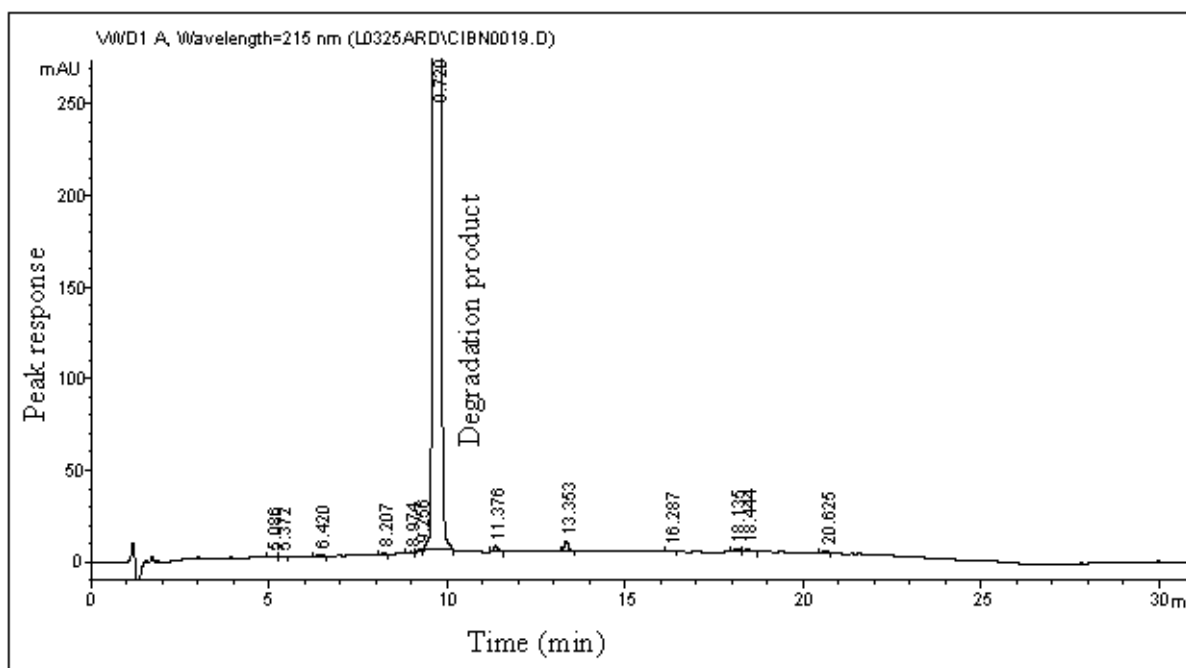


Fig-5 : HPLC Chromatogram of base degradation product

Formation of degradation impurity

The degradation impurity formed in the presence of base stress degradation. The probable degradation pathway is shown in Fig-9. Initially, oxonium ion forms on oxygen of the methoxy group. Then, hydroxide ion attack the same carbon of the methoxy group attached to cyclohexadienedione ring, followed by removal methoxy group happens with simultaneous substitution of hydroxyl group.

CONCLUSION

The major unknown base degradation product was isolated by preparative LC and was characterized by using spectroscopic techniques namely NMR, HR-MS, ESI-MS and IR. This work demonstrated the practical utility of NMR, LC-ESI-MS in the efficient structural elucidation of the novel degradation products of bulk drug substances. Thus in case of impurities where the quantity of the isolated impurity is less, these techniques come to rescue and help in carrying out the structural elucidation efficiently.

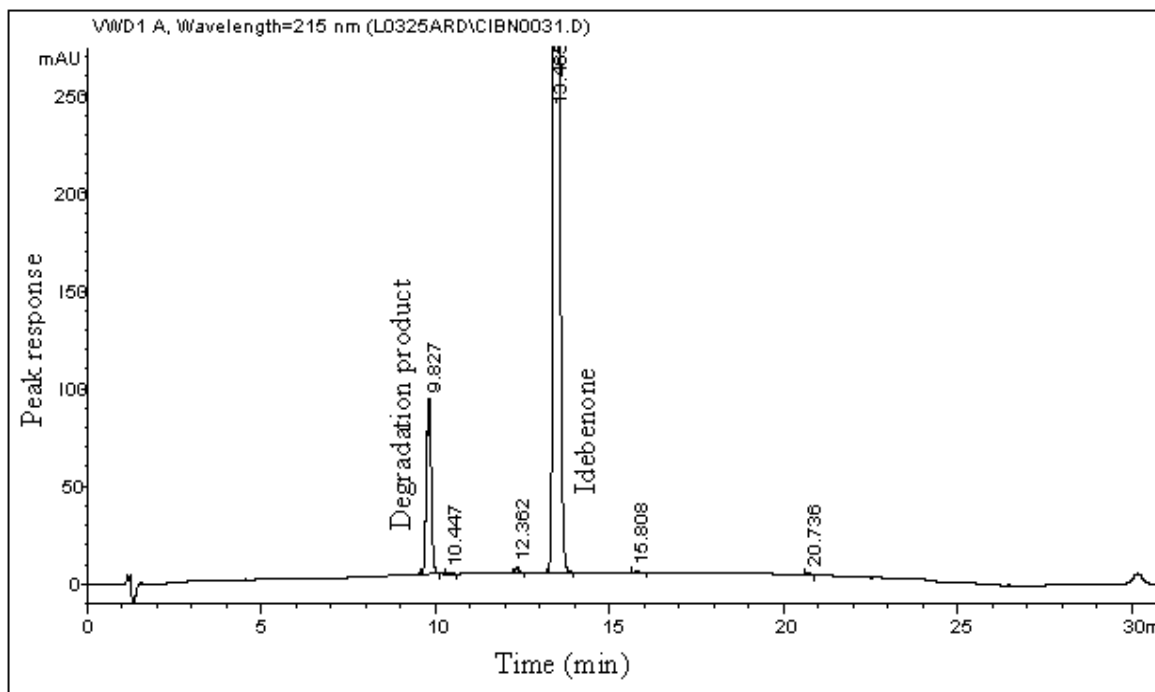


Fig-6: HPLC Chromatogram of Idebenone spiked with degradation product

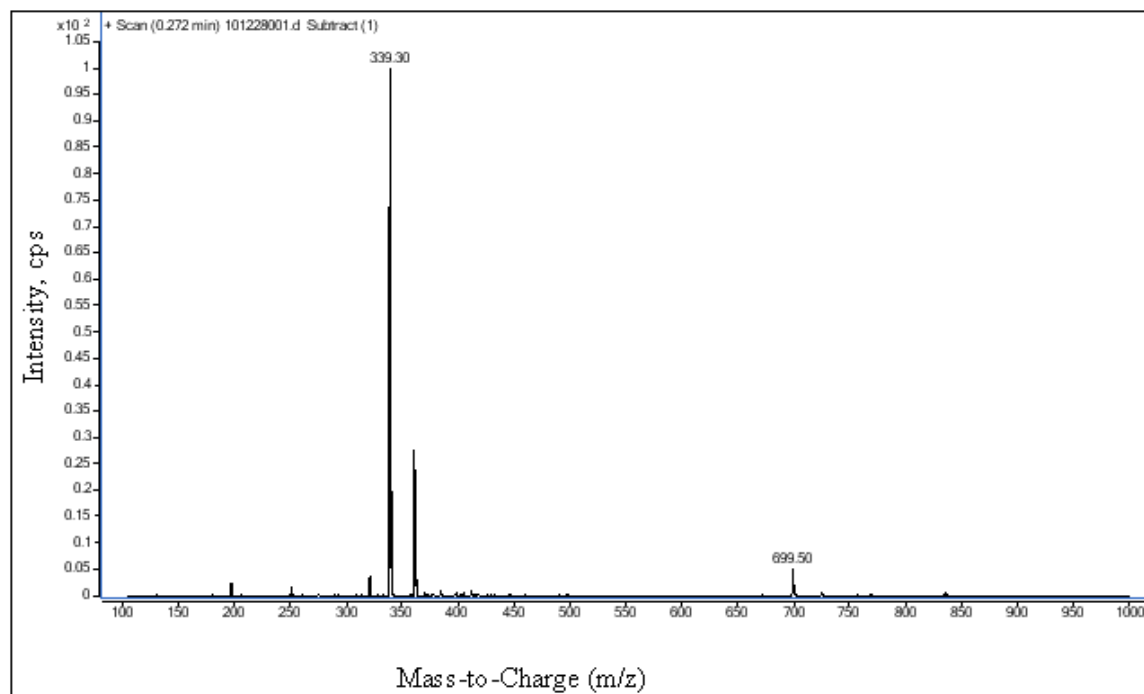


Fig-7: Mass spectrum of Idebenone

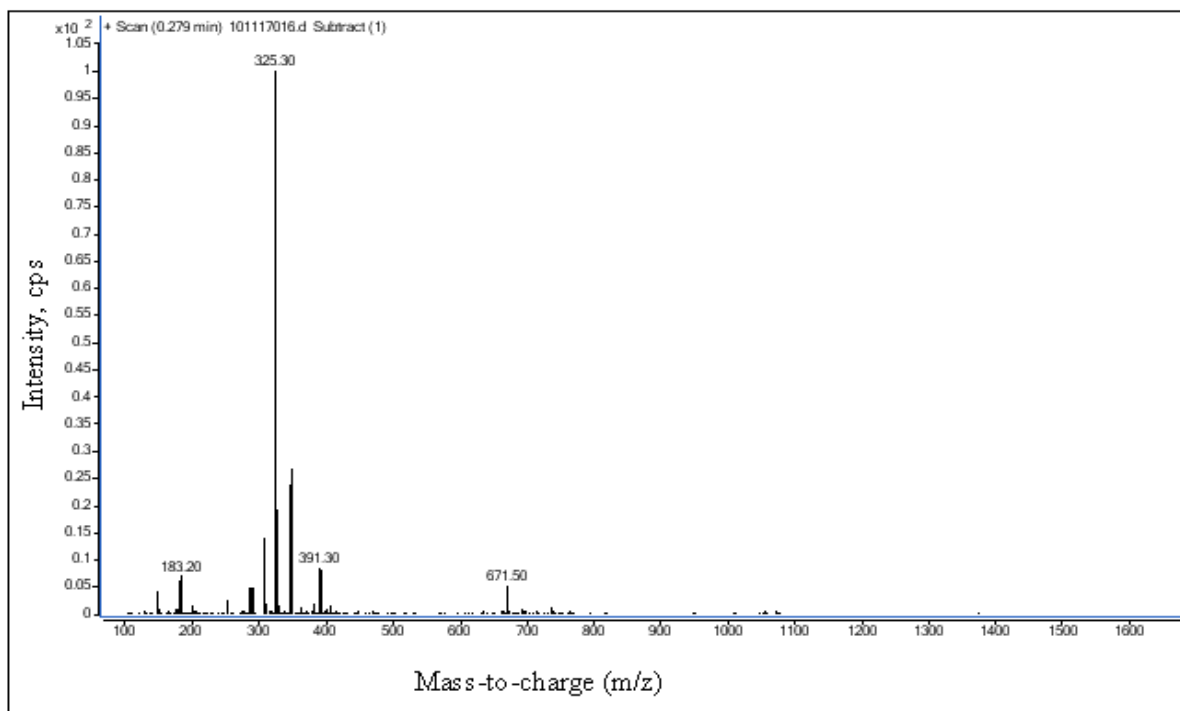


Fig-8: Mass spectrum of degradation product

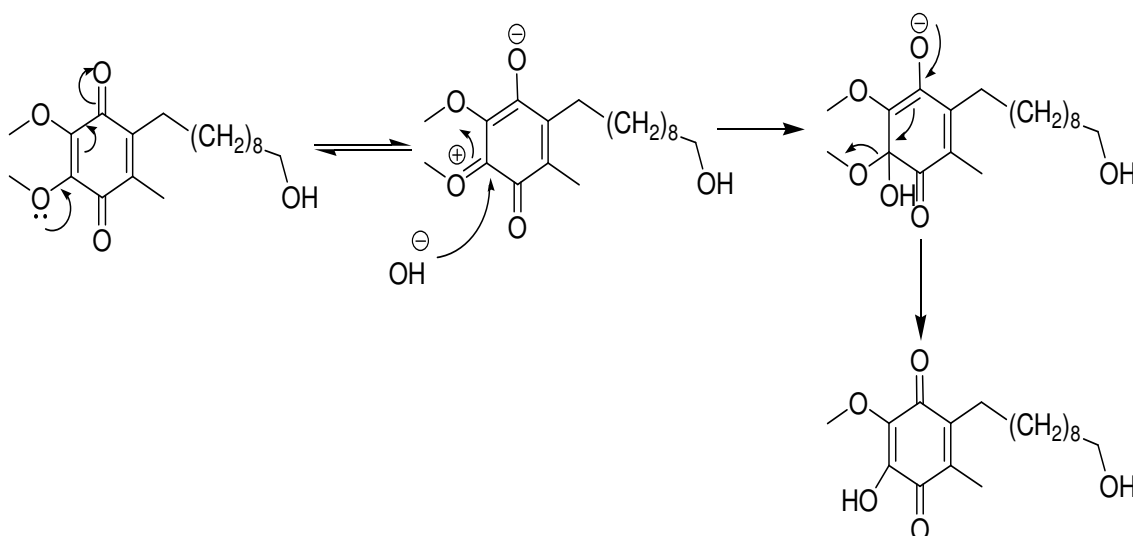


Fig-9: Proposed degradation pathway for the degradation product

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