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Development and validation of stability indicating method for the quantification of Idebenone and its impurities

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ABSTRACT

A reverse phase liquid chromatographic (RP-LC) method was developed for the quantification of Idebenone and its related impurities in drug substance. The method was optimized in a Zorbax SB C₁₈ stationary phase for the separation of known impurities with excellent detection limit in (i.e. less than 75 ng mL⁻¹) the drug substance. A linear gradient elution was involved with the water-acetonitrile-trifluoroacetic acid as mobile phase. The flow rate, wavelength was set as 1.0 mL min⁻¹ and 215 nm respectively. The stability indicating capability of the developed method was demonstrated using the stressed sample at hydrolysis, oxidative, thermal and photolytic conditions. The developed RP-LC method was validated in compliance with ICH requirements. The developed method can be used for the determination of synthetic and degradation impurities in the regular quality control analysis for the drug substance.

Key words: Reverse phase liquid chromatography, Idebenone, Stability indicating method Validation, Forced degradation (Stress studies), Drug substance.

INTRODUCTION

Idebenone [6 - (10 - hydroxydecyl) - 2, 3 - dimethoxy - 5 - methyl - 1, 4 - benzoquinone] is a powerful antioxidant which is the analog of ubiquinone, a well known and the most commonly

used antioxidant drug [1]. Due to the modification of the composition and length of its side chain, Idebenone reaches the tissues more easily than ubiquinone and efficiently protect cells from peroxidative damage. Idebenone is being used for the treatment and also in the clinical phases for several neurological disorders: Friedreich ataxia, mitochondrial encephalo myopathies, duchenne muscular dystrophy, leber's hereditary optic neuropathy and senile dementia [2- 8]

Idebenone is available as Cerestabon in 30, 45, 50, 90, 100 and 180 mg tablets and also as Cantena in 150 mg tablets. Few analytical methods were reported for the quantification and characterisation of Idebenone and its metabolites in the biological samples and formulation. The reported analytical method employs the quantification by GC, colorimetric, UV-spectrophotometric, capillary electrophoresis, HPLC with electro chemical detection and mass detection [9-15]. Mary Francis *et al.*[16] used a HPLC method specifically for the determination assay of Idebenone with UV detection for the drug product using internal standard. The method was not targeted for separation of the impurities and the detection of impurities at low level. The method was claimed that it was stability indicating assay method; however it was not describing the separation of the degradation products in the stressed conditions which are forming during the hydrolysis and oxidation degradation. Thus with the available information, no method was reported for the determination of Idebenone, its potential impurities and its degradants with the very low detection limit in the bulk drug using HPLC in the quality control laboratory. The objective of this research work is to develop a simple LC method for the related substance and assay determination of Idebenone with the limit of quantitation below 500 ng mL⁻¹ and proving the stability-indicating capability of the developed method. And the method should be also compatible with mass detector. The forced degradation was performed as per ICH recommended conditions, i.e acid, base and water hydrolysis, oxidative, thermal and photolytic stressed conditions to prove the stability-indicating ability of the method. The method was validated as per ICH requirements [17]

EXPERIMENTAL SECTION

Chemicals and Reagents

Idebenone and its impurities were synthesized and purified using column liquid chromatography by process research department of Custom Pharmaceutical Services, Dr. Reddy's laboratories limited, Hyderabad, India. HPLC-grade of acetonitrile and AR grade of sodium hydroxide, hydrochloric acid and hydrogen peroxide were purchased from Rankem (Mumbai, India) and used for the studies. Across organics (Geel, Belgium) of trifluoroacetic acid was used. Millipore Milli Q plus (Bangalore, India) purification system was used to prepare high pure water.

Instrumentation

The method development attempts, forced degradation studies and the method validation were performed in Agilent 1100 series LC systems with a diode array and variable wave length detectors (Agilent Technologies, Waldbronn, Germany). The data were collected and processed using chemstation software. The photolytic degradation was carried out using Binder KBS240 photolytic chamber (New York, USA). The peak homogeneity was established in Agilent LCMS 6410 QqQ instrument (California, USA).

Chromatographic conditions

The chromatographic separation was optimized in the Zorbax SB C₁₈ column with the dimension of 150 mm x 4.6 mm and 3.5 μm as particle size. The gradient elution involves 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 mobile phase B. The HPLC gradient program was set as: time/% mobile phase B: 0/40, 25/90, 30/90 and 31/40 with gradient delay of 5 min. The flow rate of the mobile phase and the column temperature was set as 1.0 mL min⁻¹ and 40 °C. The detection wave length was optimized at 215 nm. The column loading was finalized as 5 μg of Idebenone in 10 μL injection volume. A mixture of water and acetonitrile in the same ratio was used as diluent.

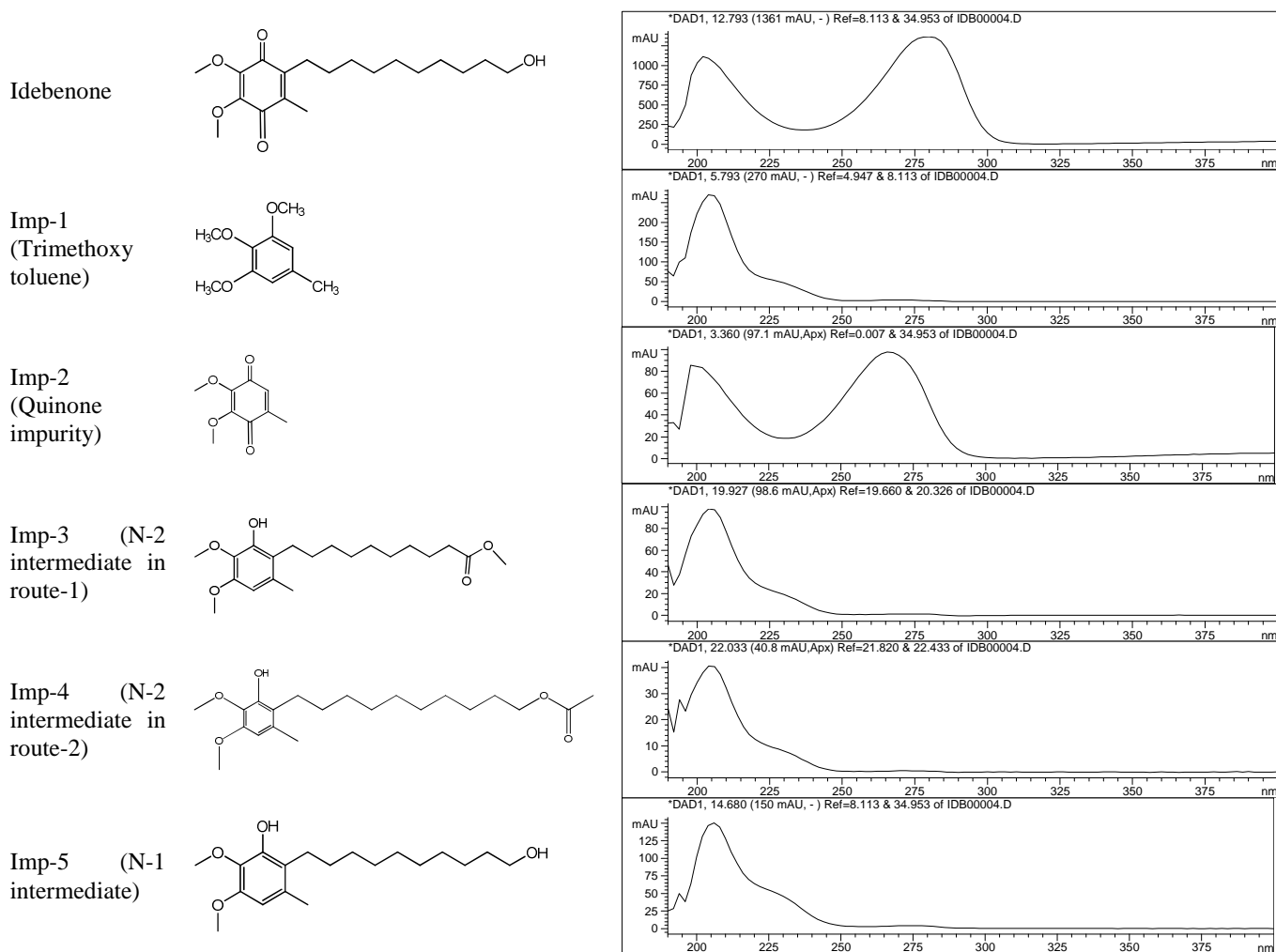
Sample preparation

Idebenone solution was prepared at target analyte concentration (TAC) which is 500 $\mu\text{g mL}^{-1}$ in the diluent for related substances and assay determination. A stock solution with the blend of impurity-1 (starting material), impurity-2 (process related impurity), Impurity-3, Impurity-4 (intermediates of Idebenone in two different synthetic routes) and impurity-5 (precursor intermediate) was also prepared in diluent for the preparation of system suitability solution with 0.15 % w/w of each impurity at TAC of Idebenone.

Method development and optimization

The core objective of the chromatographic method is the detection and separation of all the known and degraded impurities of Idebenone with better baseline. Impurity-5 is the potential impurity in the process developed in Dr. Reddy's Laboratories for Idebenone. The final stage involves oxidation of methoxy group to quinone. Due to the chromophore change during this oxidation, the λ_{max} is changing from 215 nm to 280 nm. At 280 nm there was no response for the precursor and the related impurities. At 215 nm all the intermediates, related impurities are having good response and also Idebenone is showing reasonable response. Crude samples of Idebenone was quantified for the assay and the related substances against Idebenone standard and found the mass balance is close to 100% which is supporting the selection of UV as 215 nm. Though the relative response factor (RRF) of the known impurities are more than unit, majority of the unknown impurities which are present in the sample are the related impurities of Idebenone, so that the mass balance is satisfactory.

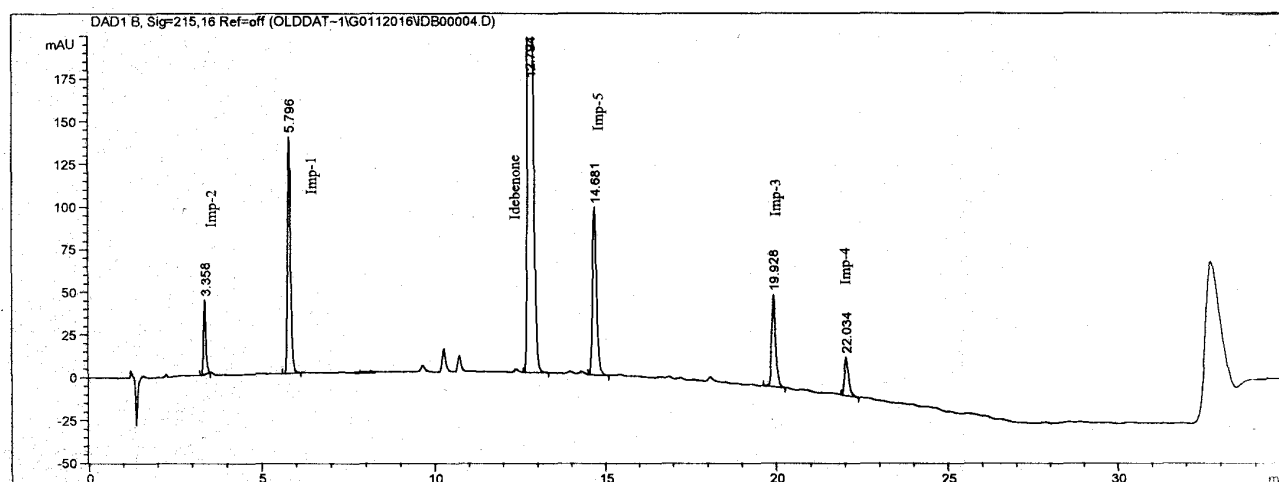
Initial attempts for the method development were made in ammonium acetate as a mobile phase in ODS column in order to get the method is compatible to mass detector. Though the separation of the impurities is good enough, the baseline was observed to be very poor at 215 nm in the gradient elution. With water / acetonitrile / trifluoroacetic acid as mobile phase the base line was observed to be satisfactory at 215 nm. Slight change in the wavelength, results either positive or negative drift in the baseline. Different stationary phases with different selectivity were used for the determination of Idebenone and its impurities as the initial attempts. However good peak shape with less peak width and the resolution of all the related impurities were achieved satisfactorily in Zorbax SB C₁₈ column with the dimension of 150 mm x 4.6 mm and 3.5 μm as particle size, by using solutions A and B as mobile phase. Though the selected stationary phase is not end-capped, there was no interaction between the analyte and the stationary phase as the Idebenone is almost a neutral molecule.

Fig. 1. Chemical structure of Idebenone and its impurities and UV spectra

The selected stationary phase is very stable at highly acidic pH which is suitable for the mobile phase with trifluoroacetic acid. The solution A contains a mixture of water: trifluoroacetic acid [100:0.05 (v/v)] and solution B contains a mixture of acetonitrile: trifluoroacetic acid [100:0.05 (v/v)]. The flow rate of the mobile phase was 1.0 mL min⁻¹. The gradient program was optimized to get the required retention of Idebenone and the separation of impurities. The gradient program was set as: time/% solution B: 0/40, 25/90, 30/90 and 31/40 with gradient delay of 5 min. The column temperature was set as 40 °C in order to reduce the back pressure of the column with the optimized gradient program and to get better peak shape of Idebenone and its related impurities. In the optimized conditions it was observed that the Idebenone, Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5 were well separated with a resolution of greater than 2. The system suitability results were within the acceptance criteria and the developed LC method was found to be specific for Idebenone, its known impurities and its degradation impurities.

Table 1. System suitability test results

| Compound (<i>n</i> = 3) | Capacity factor (<i>k'</i>) | Resolution (<i>R_s</i>) | USP Tailing factor (<i>T</i>) | No. of Theoretical plates (<i>N</i> -Tangent method) |
|-----------------------------|----------------------------------|-------------------------------------|------------------------------------|----------------------------------------------------------|
| Imp-2 | 1.8 | - | 1.4 | 12307 |
| Imp-1 | 3.8 | 18.1 | 1.3 | 22026 |
| Idebenone | 8.9 | 40.0 | 1.3 | 61297 |
| Imp-5 | 11.0 | 9.4 | 1.2 | 77647 |
| Imp-3 | 15.4 | 25.5 | 1.2 | 138727 |
| Imp-4 | 17.1 | 10.2 | 1.2 | 171945 |

Fig. 2. Typical system suitability test chromatogram

Specificity

Specificity is the ability of the method to measure the analyte in the presence of its potential impurities; it might be process related or the degradation impurities. The specificity of the developed LC method for Idebenone was established in the presence of its known impurities in the process developed in Dr Reddy's laboratories, namely Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5 and its degradation product. Forced degradation studies were performed on Idebenone to grant the signal of the stability-indicating property and specificity of the developed method. The stress conditions engaged for degradation studies as per the ICH preferred conditions includes photolytic, thermal, oxidation and hydrolysis at acid, base and water. The photolytic stressed studies were performed for 11 days as per ICH Q1B [18]. The thermal stress was done at 50 °C for 10 days. The acid stress was performed at 0.5 N HCl at the concentrated sample solution at ambient temperature (25±2 °C) for 10 days and further dilution was done to analyte concentration for the quantification of Idebenone and its degradants. The base stress was performed at 0.025N NaOH for 24 hours at ambient temperature. Water hydrolysis was performed for 10 days at 50 °C. The oxidation stress was done using 3% hydrogen peroxide for 7 days at ambient temperature [19, 20]. All the stressed samples were quantified for Idebenone and the impurities. Peak purity of stressed samples of Idebenone and the spiked solutions of Imp-1, Imp-2, Imp-3, Imp-4 and Imp-5 were checked by Agilent HPLC instrument with diode array detector (DAD). Also the peak homogeneity was checked using LCMS for all the stressed samples.

Method validation**Precision**

Precision is the closeness of agreement between a series of measurements obtained from multiple sampling of same sample under the prescribed conditions. Six individual preparation of Idebenone solution was made with each of the known impurities at 0.15 % *w/w* level to TAC. Quantification of individual impurities and Idebenone was done for each of the preparations and the % RSD of the content of the impurities and the assay were determined. Method precision experimentation was repeated with different lot of column and different instrument in the same laboratory to evaluate the intermediate precision.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ of an individual analytical procedure are the lowest amount of analyte in a sample which can be detected and can be quantitatively determined with suitable precision and accuracy respectively. The LOD and LOQ for each of the components were established by attaining signal-to-noise ratio of around 3:1 and 10:1, respectively from a series of dilute solutions with known concentration [17]. Six individual solutions of known impurities and Idebenone were also prepared at LOQ level and recorded the area of impurities and Idebenone. The LOQ precision was established by calculating the %RSD of each of the area of the known impurities and Idebenone. Accuracy of impurities at this level was established by spiking the known LOQ quantities of impurities in test sample and calculating the recovery of impurities.

Linearity

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the amount of analyte in the sample. The linearity of method was established at two different levels separately which are impurity and assay levels. The solution of Idebenone and its known impurities were prepared at five different concentrations from 0.05 % to 0.30 % *w/w* of analyte concentration for the linearity at impurity level. For the assay level, five different solid weighing of Idebenone from 80 % to 120 % *w/w* with respect to analyte concentration were prepared and injected. Using method of least-squares analysis, the regression line was plotted with area versus concentration. The value of the slope and *Y*-intercept of the plot were calculated. The relative response factor (RRF) of impurities was determined using the slopes of each component at impurity level.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the values arrived from the method and conventional true value or an accepted reference value. Accuracy of impurities at each level was established by standard addition of the known quantities of impurities in test sample and calculating the recovery. The study was carried out in triplicate at 0.075, 0.15 and 0.225% *w/w* of the TAC. The recovery of impurities was calculated by calculating the amount of the impurities spiked and the amount of the impurity calculated from the spiked sample after RRF correction. The accuracy of the assay was evaluated in triplicate at three concentration levels from 80 to 120 % of analyte concentration by assaying the reference standard against 100 % of the same. The percentages of recoveries at each level were calculated against the Idebenone standard, considered 100% as the true value which was prepared by several re-crystallisation to get pure sample.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. Deliberate small variations were made from original chromatographic conditions to record the resolution between Idebenone and the impurities to determine the robustness of the developed method. Eight different conditions were studied by a combination of three different small variations which are flow rate, gradient program and the column temperature from optimized condition.

Solution stability and mobile phase stability

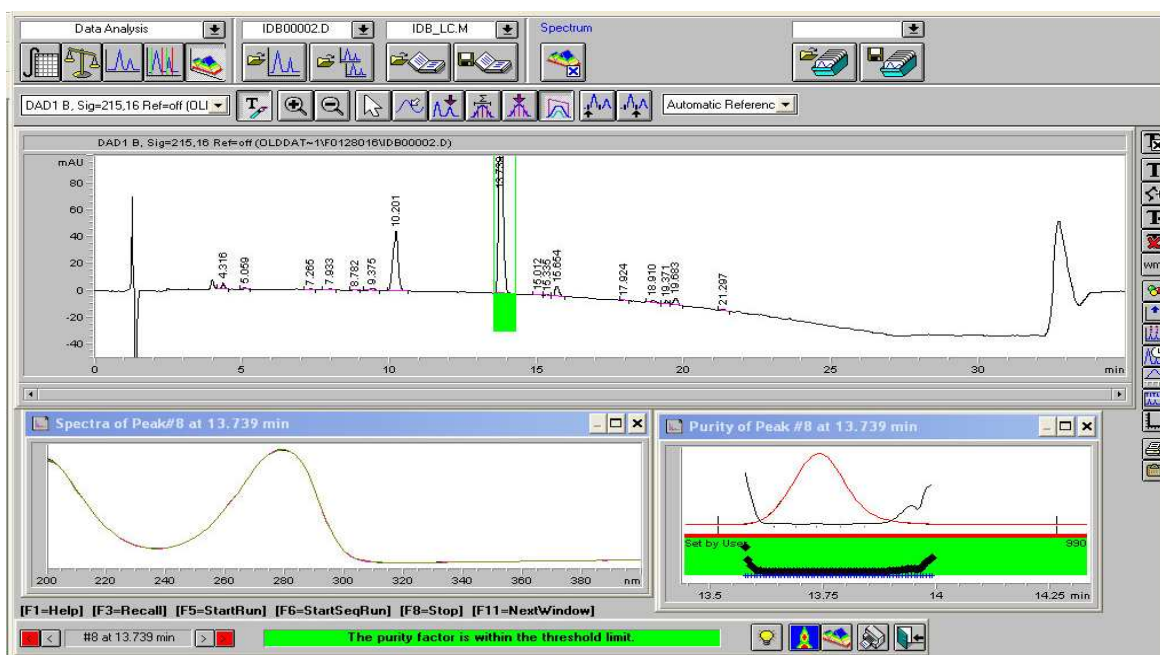
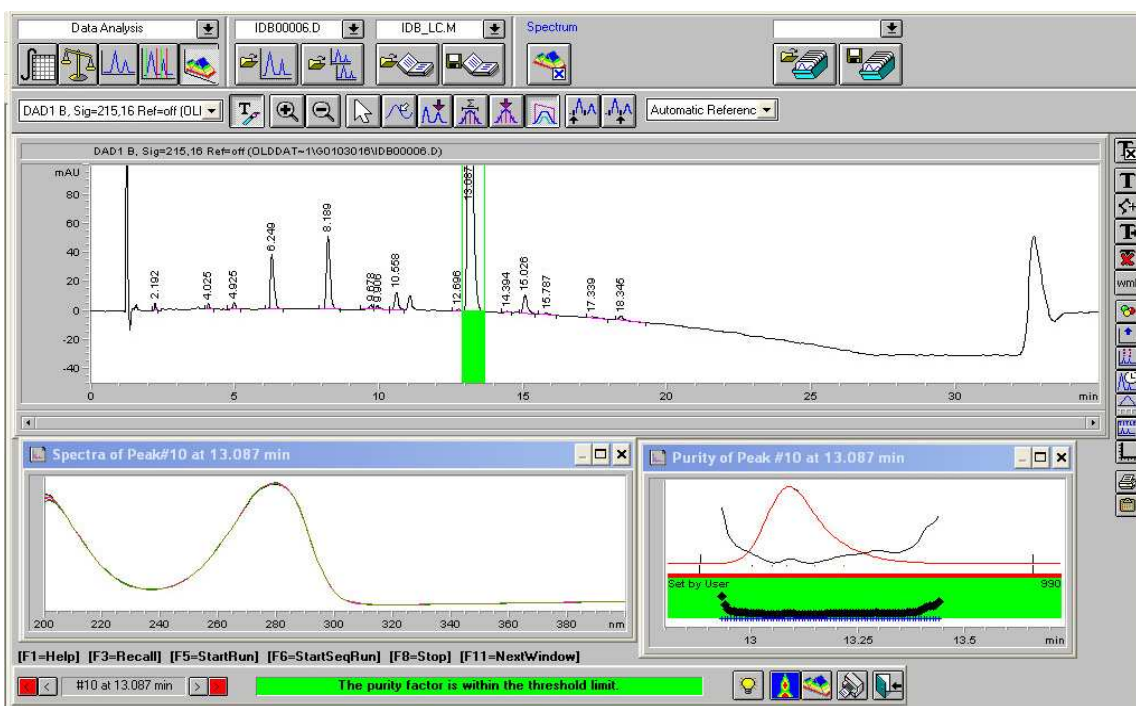
Solution stability and mobile phase stability provides an indication of its reliability during normal usage during the storage of the solutions used in the method. The solution stability of Idebenone was established for 48 hours at room temperature. The reference standard of Idebenone and the sample spiked with impurities at specification level were injected for every 6 hours. The content of impurities and Idebenone were quantified at each interval up to the study period. The mobile phase stability was also established by quantifying the freshly prepared sample solutions against freshly prepared reference standard solutions for every 6 h. During the study period the prepared mobile phase was sustained. The recovery of the assay of Idebenone and the content of each impurity was calculated against the initial value for the study period.

RESULTS AND DISCUSSION**Forced degradation studies**

Degradation was not observed in stressed conditions when the analyte was subjected to photolytic, thermal, acid and water hydrolysis. The degradation of drug substance was observed only under oxidative conditions and base hydrolysis (Figs.3, 4). The Idebenone drug substance under oxidative conditions leads to the formation of two significant unknown degradation peaks at the RRT 0.48 and 0.63. Idebenone is very sensitive to base and there was a significant unknown degradation peak observed at the RRT of 0.74. The peak purity factor over the threshold obtained in all stressed samples for the analyte peak demonstrates the specificity. Also the peak homogeneity study by LC-MS confirmed that the Idebenone peak is homogeneous and pure in all the stress samples (Figs 5, 6).

Table 2. Summary of forced degradation results

| Stress condition | Duration | Assay of Idebenone HCl after forced degradation | Content of major degradant | Remarks |
|-----------------------|----------|-------------------------------------------------|----------------------------|----------------------------------------|
| Acid hydrolysis | 10 Days | 99.2 | - | No degradation products formed |
| Base hydrolysis | 24 hours | 78.2 | 19.4 | Significant degradation product formed |
| Oxidation | 7 days | 89.3 | 4.3 and 6.2 | Significant degradation product formed |
| Water hydrolysis | 10 days | 99.4 | - | No degradation products formed |
| Thermal (50 °C) | 10 days | 99.0 | - | No degradation products formed |
| Photolytic as per ICH | 11 days | 99.1 | - | No degradation products formed |

Fig. 3. Chromatogram in base degradation**Fig-4 Chromatogram in oxidative degradation**

The assay of Idebenone is unaffected in the presence of Imp-1, Imp-2, Imp-3, Imp-4, Imp-5 and its degradation products confirm the specificity and stability indicating ability of the developed method. The RRF of the degradants were considered to be one while quantifying of the

impurities and the mass balance was close to 100 %. The summary of the forced degradation was captured in table 3. During the peak homogeneity study, it was observed that the molecular weight of the impurities is less than the active component which proves the impurities are the degradation products of Idebenone. Further studies can be done on characterization of degradation impurities to understand the possible impurities during the stability and the metabolites of the Idebenone apart from the known.

Fig.5. Peak homogeneity in base degradation by LCMS

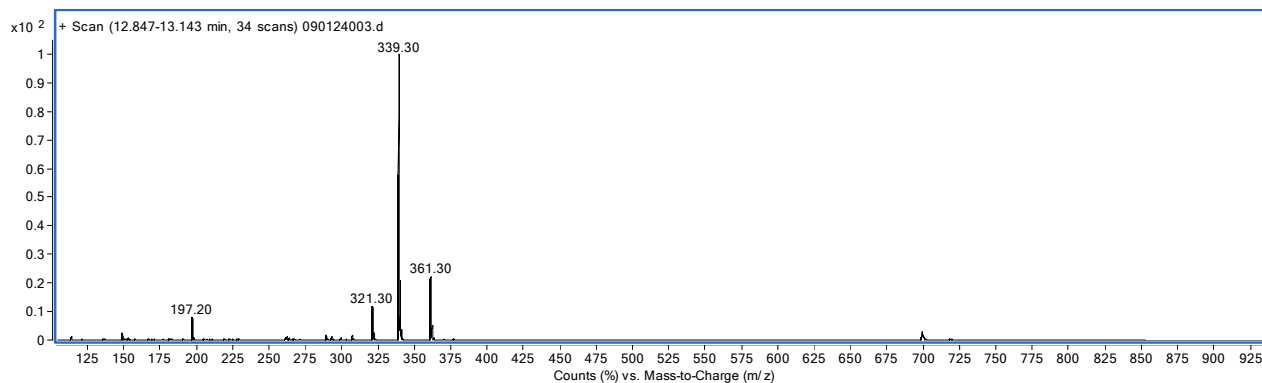
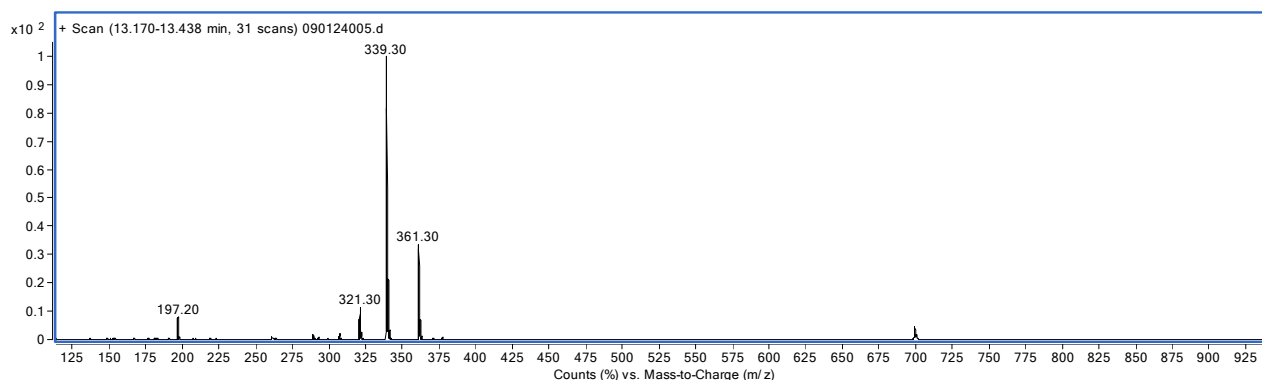


Fig.6. Peak homogeneity in oxidative degradation by LCMS



Precision

All the individual values of impurity content and the assay in the precision and intermediate precision studies are falling well in the range of confidence interval of average confirming the excellent precision of the method. The recommended precision values in terms of percentage of RSD should be not more than 15 for the related substance [21] and not more than 2.0 for the assay. However the percentage RSD of the content of impurities and the assay of Idebenone in the precision study including intermediate precision were well within 4.7% and 0.91 % respectively

Limit of detection and limit of quantification

The limit of detection of Idebenone was 0.02% w/w (of TAC) for 10 μ L injection volumes. The limit of quantification of Idebenone is at the reporting limit i.e 0.05%. As the dosage of the Idebenone is less than 2 g per day, the limit of quantification at this reporting level is good enough to quantify the unknown impurities and the same concentration is meeting the criteria of

all the other validation parameters. Due to that the response of the all the known impurities are quite good at 215 nm, the concentration at LOD and the LOQ of the impurities were very much encouraging (Table 3). This reveals the capability of the method for the quantification of the process related impurities at lower level not only during the analysis of the sample at quality control but also to control them during the optimization of the process.

Table 3. Limit of detection and limit of quantitation results

| Compound | Limit of detection | | Limit of quantitation | |
|-----------|-----------------------------------------|------------------------|-----------------------------------------|------------------------|
| | % with respect to analyte concentration | In ng mL ⁻¹ | % with respect to analyte concentration | In ng mL ⁻¹ |
| Idebenone | 0.02 | 176 | 0.05 | 496 |
| Imp-1 | 0.004 | 42 | 0.01 | 119 |
| Imp-2 | 0.007 | 71 | 0.02 | 223 |
| Imp-3 | 0.006 | 64 | 0.02 | 207 |
| Imp-4 | 0.007 | 74 | 0.02 | 242 |
| Imp-5 | 0.007 | 68 | 0.02 | 202 |

Linearity

Excellent correlation was achieved for the regression line of Idebenone and its related impurities at reporting limit to 200% of the specification level. The correlation coefficient obtained for all the plots were greater than 0.997. The RRF of each impurity was more than 1.5 for all the impurities and it is close to 3 for the Impurity-5 at the optimized condition.

Table 4: Results of accuracy for related substances

| Compound | Level (%) | Spiked quantity n= 3 (ng mL ⁻¹) | Recovered quantity n =3 (ng mL ⁻¹) | Recovery in % | | |
|----------|-----------|------------------------------------------------|------------------------------------------------------|---------------|-------|-------------|
| | | | | Individual | Mean | CI for Mean |
| Imp-1 | 0.075 | 744.6 | 727.0 | 97.6 | 97.8 | ± 2.4 |
| | 0.150 | 1489.2 | 1443.1 | 96.9 | | |
| | 0.225 | 2233.8 | 2207.8 | 98.8 | | |
| Imp-2 | 0.075 | 735.6 | 747.2 | 101.6 | 102.7 | ± 5.0 |
| | 0.150 | 1471.2 | 1545 | 105.0 | | |
| | 0.225 | 2206.8 | 2239.6 | 101.5 | | |
| Imp-3 | 0.075 | 748.2 | 744.3 | 99.5 | 102.8 | ± 7.1 |
| | 0.150 | 1496.4 | 1563.6 | 104.5 | | |
| | 0.225 | 2244.6 | 2343.6 | 104.4 | | |
| Imp-4 | 0.075 | 738.8 | 708.4 | 95.9 | 97.5 | ± 4.1 |
| | 0.150 | 1477.6 | 1465.6 | 99.2 | | |
| | 0.225 | 2216.4 | 2157.8 | 97.4 | | |
| Imp-5 | 0.075 | 743.8 | 754.2 | 101.4 | 101.6 | ± 1.6 |
| | 0.150 | 1487.6 | 1522.4 | 102.3 | | |
| | 0.225 | 2231.4 | 2255.8 | 101.1 | | |

The Y-intercept of the each plots are below 3.0 % of the response at 0.15 % w/w level of corresponding impurity. This sounds good to get the nearest value of RRF since the plots are going almost through the origin. Linear calibration plot for assay was obtained over the calibration ranges tested, i.e. 400 to 600 $\mu\text{g mL}^{-1}$. An excellent correlation existed between the peak area and concentration of the Idebenone by getting correlation coefficient greater than 0.999. The Y- intercept for the assay concentration is also supportive that the plot is going almost through the origin.

Accuracy

Recovery of each impurity is falling in the range of 94 to 103%. Individual assay value at each level in triplicate is close to 100%. All the individual recovery value of the assay and impurities were fallen well with in the confidence interval of mean values. Good recovery values reflecting the exact values of RRF of impurities as well as the capability of accuracy of the method.

Table 5: Results of accuracy for assay

| Compound | Level (%) | Assay by HPLC (<i>n</i> =3) | True assay value | Recovery in % |
|-----------|-----------|---------------------------------|------------------|---------------|
| Idebenone | 80 | 99.3 | 100 | 99.3 |
| | 100 | 99.5 | 100 | 99.5 |
| | 120 | 99.8 | 100 | 99.8 |

Robustness

The resolution between all the known components, baseline and the tailing factor of the Idebenone in all the eight deliberate varied chromatographic conditions were found to be satisfactory. It was observed that a minor variation in the resolution and tailing factor in all the robustness conditions which is illustrating the method is robust enough.

Solution stability and mobile phase stability

The RSD of assay of Idebenone during solution stability and mobile phase stability experiments were within 1.0 %. No significant changes were experienced in the content of any of the impurities during solution stability and mobile phase stability experiments. The percentage recovery of the assay at each time point against the initial value is between 99.5 and 100.8. The percentage recovery of the content of each impurity against the initial value is between 95.7 and 104.5. The solution stability and mobile phase stability experiment data confirms that sample solutions and mobile phase used were stable up to 48 h. It is an advantage to have lengthy sequence which reduces the number of standard preparation in the quality control during regular analysis

CONCLUSION

The developed stability-indicating analytical method for related substance and assay determination of Idebenone and its impurities is precise, accurate, linear and specific. The validation carried out for the method in accordance with the ICH requirements are satisfactory. The developed method can be used conveniently for the routine analysis of production samples and also to check the stability of bulk samples of Idebenone during its storage. The same method can also be attempted for the drug products for getting the information of impurities and degradation products at lower level.

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