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# Determination of DDQ using Ultra Performance liquid Chromatography

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**ABSTRACT: A simple simple, short and accurate UPLC method was developed on Waters Acquity UPLC for the quantification of the reagent, Dihydro dicyano benzoquinone (DDQ) which is employed for the conversion of 6-methylideneandrosta-4-diene-3, 17- dione to the drug substance, Exemestane is presented. The unreacted or the remnant DDQ from the synthesis appears as a carry-over impurity in the final product. The Method was developed on a Zorbax Eclipse Plus C18 column, using a mobile phase consisting of Water and Acetonitrile, each containing 0.02 % Trifluoroacetic acid, and using a linear gradient. The method was validated as per International Conference of Harmonization (ICH) Guidelines in terms of Specificity, Limit of detection (LOD), Limit of Quantitation (LOQ), Linearity, Precision, Accuracy and Solution Stability. The LOD and LOQ values were found to be 0.002 mg/mL and 0.004 mg/mL, respectively for 0.4µL injection volume. The sample concentration were injected was 10 mg/mL. The method is linear within the range of 0.004-0.03 µg/mL for DDQ.**

**Keywords:** DDQ; Exemestane; Ultra Performance Liquid Chromatography (UPLC), 6-methylideneandrosta-4-diene-3, 17- Dione; UPLC Method; Dihydro dicyano benzoquinone;

## INTRODUCTION

In the present work we have developed highly sensitive, rapid and time-efficient UPLC Method for the quantification of the carryover of the impurity, DDQ in the purified drug substance, Exemestane.

Exemestane, a drug used to treat breast cancer belongs to the class of drugs known as aromatase inhibitors. Estrogen is required for the growth of some breast cancers. Breast cancers that have estrogen receptors (ERs) are called ER-positive which might also be referred to as estrogen-responsive, hormonally-responsive or hormone-receptor-positive. Aromatase is the enzyme that synthesizes estrogen. Inhibitors which block the synthesis of estrogen are called Aromatase inhibitors. It lowers the estrogen level, thereby reducing the growth of cancers. (Fig. 1).

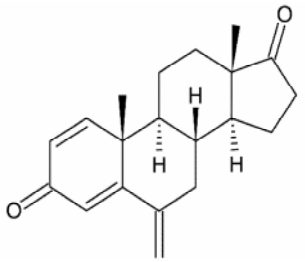


**Fig. 1.** Exemestane

It is an orally administered steroidal aromatase inhibitor used in ER-positive breast cancer in addition to surgery and/or radiation in post-menopausal women. It is also an irreversible, steroidal aromatase inactivator and structurally related to the natural substrate androstenedione. It acts as a false substrate for the aromatase enzyme, and is processed to an intermediate that binds irreversibly to the active site of the enzyme causing its inactivation, an effect also known as "suicide inhibition." Thus, Exemestane prevents the enzymes from converting the androgens into estrogens. Several routes of synthesis of Exemestane had been reported in patent literatures prior to the work undertaken.

One of the common route of synthesis is conversion of the commercially available Androstenedione to 6-methylideneandrosta-4-diene-3, 17- dione, followed by subsequent dehydrogenation of 6-methylideneandrosta-4-diene-3, 17- dione to the final product, Exemestane [1, 2]. DDQ is used in the synthesis of Exemestane. As per ICH guideline Q11 (Control strategy) it is mandatory to check all the reagent(s) and chemical(s) used in the synthesis of the drug substance [3].

There are several methods for the dehydrogenation of 6-methylideneandrosta-4-diene-3, 17- dione to obtain the desired product i.e., Exemestane. The dehydrogenation reaction, with respect to the synthesis of a wide range of dehydrogenating agents that includes Dehydrogenation with Selenium Dioxide, Enzymatic Dehydrogenation and Amino Alkylation and Oxidation of Boldenone. DDQ is a versatile reagent as it has high oxidant ability and relative stability as compared to others. It is a well known fact that Quinones have the property to oxidize compounds. DDQ can be used to make double bonds, aromatic rings and stable aromatic cations. The reaction of DDQ requires a functionality that is capable of stabilizing the formed cation in the transition state and initiate hydrogen transfer is needed, typically an aromatic moiety. Hydrocarbons lacking these functionalities are stable to the action of DDQ. DDQ can also be used to make C-C or C-O couplings [4-6].

DDQ is employed for the introduction of the required 1, 2-double bond to 6-methylideneandrosta-4-diene-3, 17- dione **(Fig. 2).**

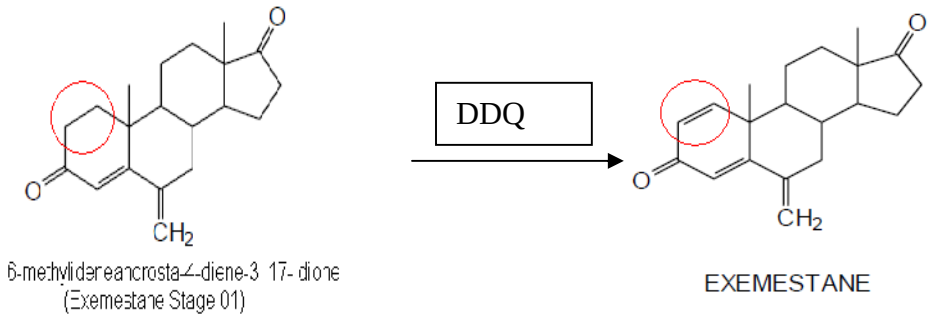


**Fig. 2.** Synthesis of Exemestane.

Several methods are also reported for the analysis of Exemestane by HPLC. However, it was found that were no methods were reported for the quantification of DDQ, a Carry-over impurity in Exemestane. Therefore, a sensitive, rugged and time-efficient method was developed and validated on UPLC (Ultra High Pressure Liquid Chromatography) quantification of process related impurity, DDQ. The chromatographic separation was achieved with Zorbax Eclipse C18, 50 mm x 2.1 mm, 1.8 µm using gradient elution. The developed method was validated for parameters like accuracy, linearity, LOD, LOQ, ruggedness. [7,8]

## EXPERIMENTAL

### Chemicals and Reagents.

DDQ was purchased from Sigma-Aldrich Ltd. The analytical reagents required for the quantification of DDQ by UPLC i.e., Trifluoroacetic Acid and Acetonitrile were purchased from Sigma-Aldrich. HPLC grade water was obtained from Milli-Q water purification system (Millipore, Milford, USA).

### Instrumentation and Software

UPLC analysis was performed using a Waters Acquity system equipped with binary solvent delivery pump and an auto sampler, connected to Waters Empower 2 software.

### Chromatographic conditions

The chromatographic separation was performed using a Zorbax Eclipse C18, 50 mm x 2.1 mm, 1.8 µm at a column temperature of 45°C and liquid flow-rate of 0.5 mL / min using 0.02 % Trifluoroacetic acid in Water (Solvent A) and 0.02% Trifluoroacetic acid in Acetonitrile (Solvent B) as mobile phase with a linear gradient set as: T / %B: 0 / 25, 1.5 / 25, 1.6 / 60, 4.0 / 60, 4.1 / 25 and 5.0 / 25. The chromatogram was monitored at a wavelength of 224 nm. The injection volume was 0.3 µl. Acetonitrile was used as the diluent.

**Preparation of Standard and sample solutions**

Dichloro dicyano benzoquinone (DDQ) Standard was prepared at 20 µg/ml (Limit Level) prepared in the diluent was considered as the Reference Standard solution for the quantification of DDQ in Exemestane. Similarly, sample solution was prepared at a concentration of 20 mg / ml (Fig. 3).

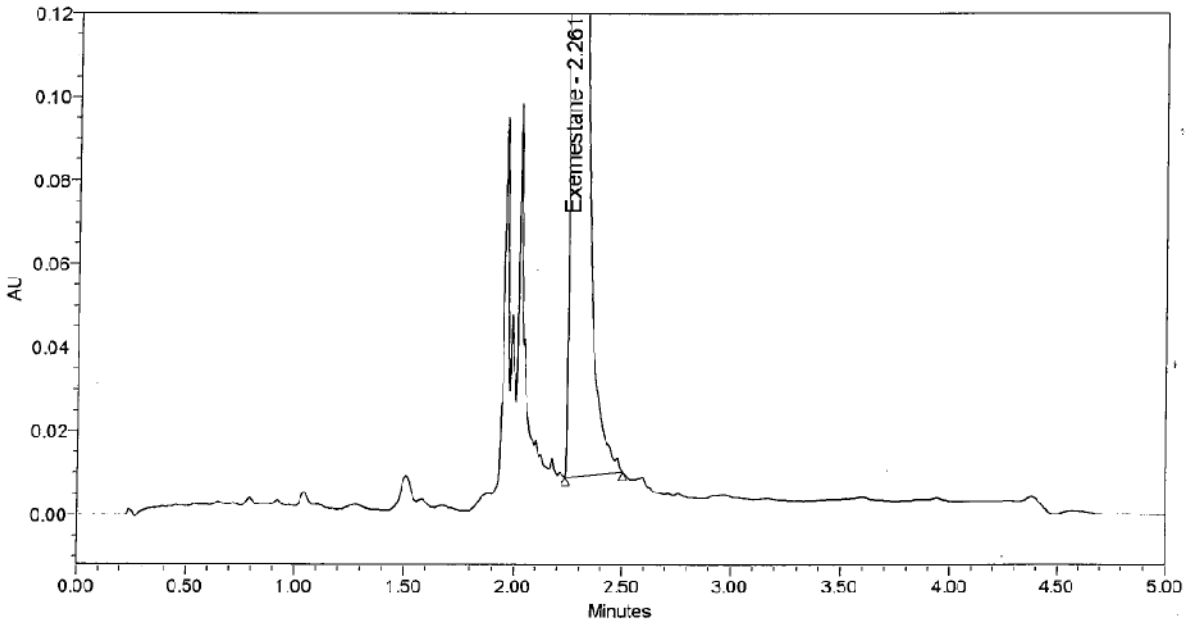


**Fig. 3.**

**Validation of method**

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities and degradation products. In the presented work, since only one impurity was monitored, the impurity standard (DDQ) solution at the Limit Level and the sample solution with the concentration specified above (20 mg / ml) as well as a spiked standard solution with the above mentioned concentrations were prepared and injected.

The LOD and LOQ for DDQ were estimated at a Signal-to-noise ratio of 3:1 and 10:1, respectively by injecting a series of diluted solutions with known concentration. The LOD solution was at 0.004mg/ml, whereas the LOQ solution was 0.008mg/ml.

Linearity test solutions for DDQ were prepared at four concentration levels from 0.008mg/ml, to 0.030mg/ml. The peak area versus concentration data was performed by least-squares linear regression analysis. Linearity test solutions were prepared by diluting the impurity stock solution. The calibration curve was drawn by plotting the peak areas of impurities versus its corresponding concentration. The % R.S.D. value of the slope and *Y*-intercept of the calibration curve was calculated. The Method precision was evaluated by carrying out six independent injections of test sample of Exemestane for the DDQ content and calculated the % R.S.D of the content. The precision of the related substance was checked by injecting six individual preparations of DDQ (20.0 mg/ml) Exemestane spiked with 0.10 % of DDQ with respect to Exemestane concentration.

The accuracy of method was carried out by injecting known concentration of DDQ to the Exemestane. The accuracy was calculated in terms of recovery (%). The study was carried out in duplicate at covering from 0.008mg/mL to 0.030µg/mL (0.008, 0.010, 0.020 and 0.030 mg/mL) in diluent. The recovery of DDQ was calculated.

## RESULTS AND DISCUSSION

**Optimization of Chromatographic Condition**

Standard stock solution and standard solution prepared in the mobile phase was used for method development. A number of column chemistries supplied by different manufacturers were employed along with various suitable and appropriate mobile phase compositions to obtain good peak shape for the peak of DDQ. Poor peak shape and resolution was observed with Acquity BEH C8 and Acquity BEH Phenyl column.

Another trial with a mixture of Mobile phase i.e., Water and Acetonitrile: Methanol (80:20) as the organic modifier using Inertsil C18 column and Fortis Biphenyl column, where DDQ eluted closely to Exemestane. Good peak shape and resolution was achieved using a mobile phase composition of Water and Acetonitrile each containing 0.02% TFA and employing a linear gradient (Fig. 4).

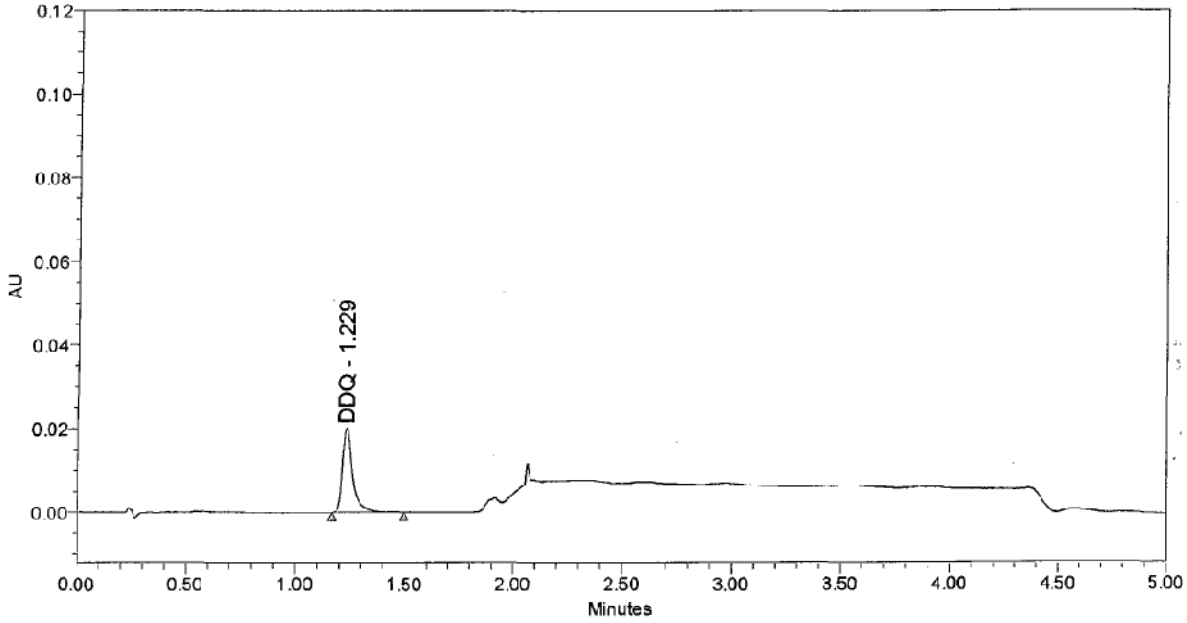


**Fig. 4.** Standard chromatogram of DDQ.

**Validation Results of the method**

The above method on UPLC was finalized after evaluation of the validation parameters of Specificity, LOD, LOQ, Linearity, Accuracy, Precision and Stability. The Specificity of the method was determined by injecting DDQ and Exemestane and subsequently determining the peak purity of the peak of DDQ.

The LOD and LOQ concentrations were estimated at 0.004 mg / mL and 0.008 mg / mL respectively for DDQ. The % RSD for Area of the peak of DDQ for the LOQ Level solution was less than 0.6. Therefore, this method had adequate sensitivity for the detection and estimation of DDQ in Exemestane. (Fig. 5, 6).

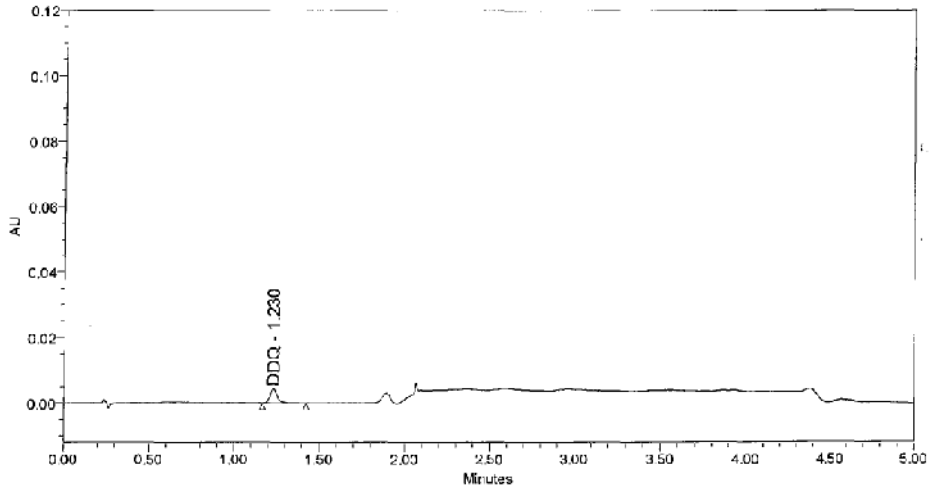


**Fig 5:** Limit of Quantitation.

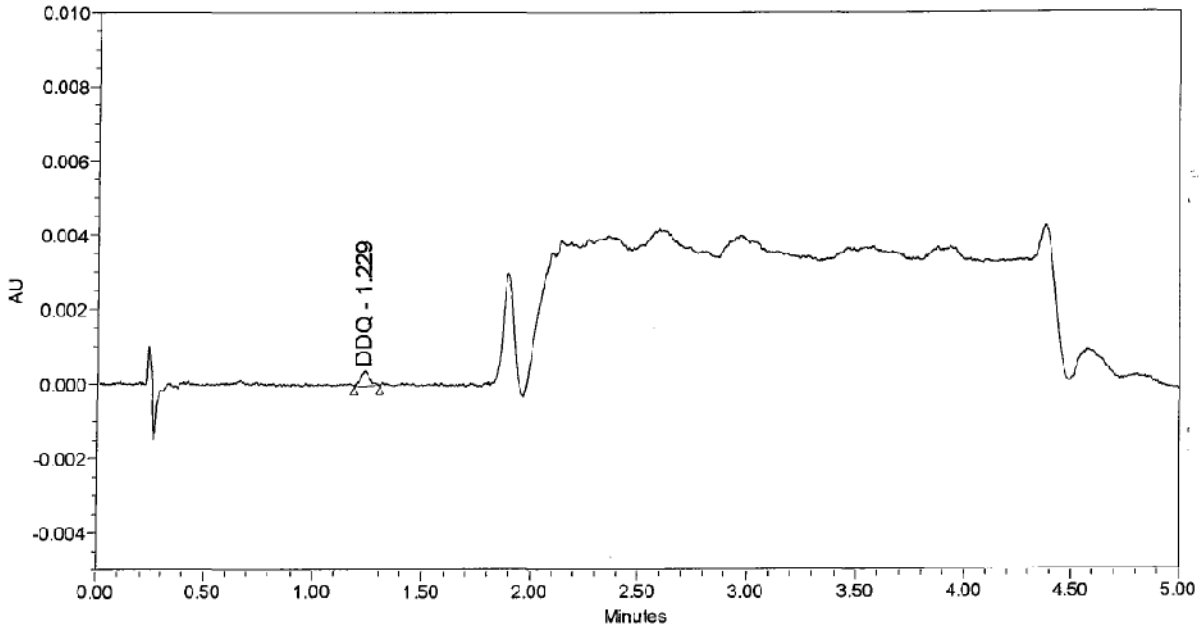


**Fig. 6.** Limit of Detection.

Linearity of DDQ was evaluated over four levels of DDQ solutions ranging from 0.008 mg/mL to 0.030 mg/mL, with the linear regression equation y = mx + c, where x is the concentration in mg/mL, and y is the corresponding peak area of DDQ. We observed linear results with respect to concentration of DDQ. The correlation coefficient value is more than 0.999. The linearity graph was shown in Fig. 7.

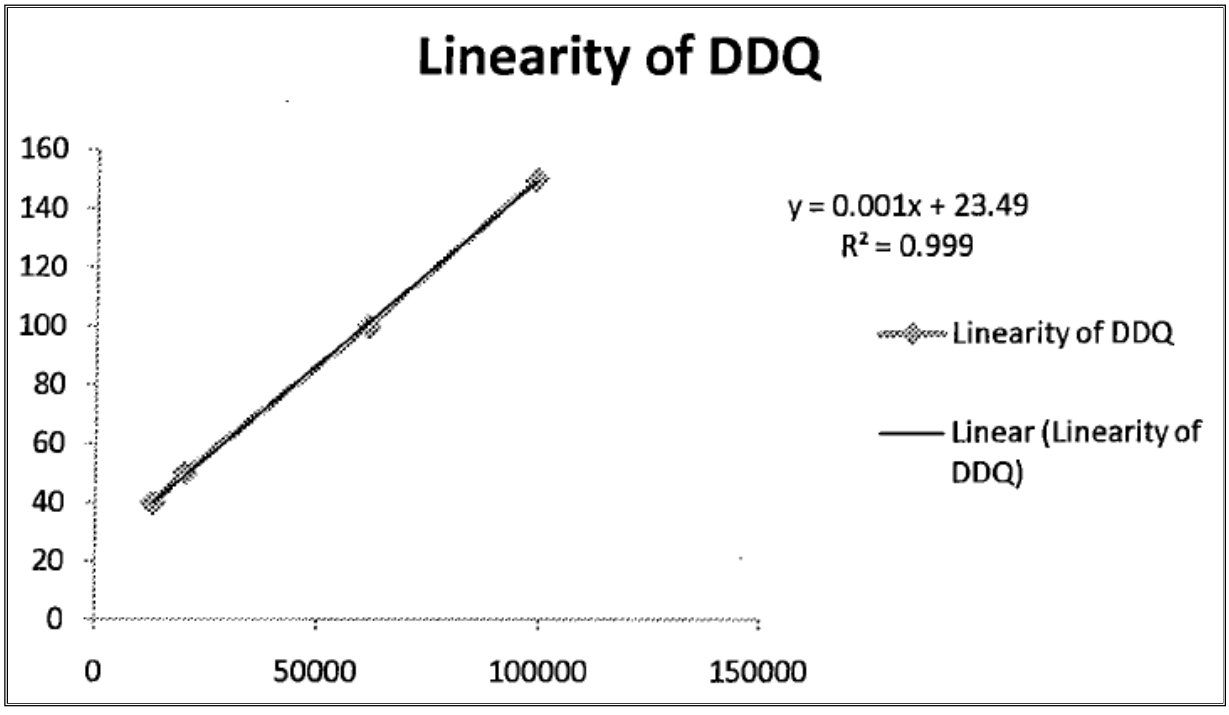


**Fig. 7.** Linearity of DDQ.

The Standard Addition and Recovery experiments were conducted for DDQ in Exemestane samples in duplicate at 0.008mg/mL to 0.030mg/mL (0.008, 0.010, 0.020, and 0.030 mg/mL). The accuracy was performed in terms of Recovery (%). The Recovery was calculated by concentration at each level in each preparation. The recovery was found to be not less than 90.0% and not more than 110.0% Fig (Table 1). (Fig 8).

**Table 1. Accuracy Level of DDQ.**

| Levels | Test 1 | Test 2 | Test 3 |
|---|---|---|---|
| Level 1(0.008mg/ml) | 97.33 | 95.72 | 94.60 |
| Level 2 (0.020mg/ml) | 92.52 | 92.90 | 93.06 |
| Level 3 (0.030mg/ml) | 104.21 | 103.71 | 103.23 |

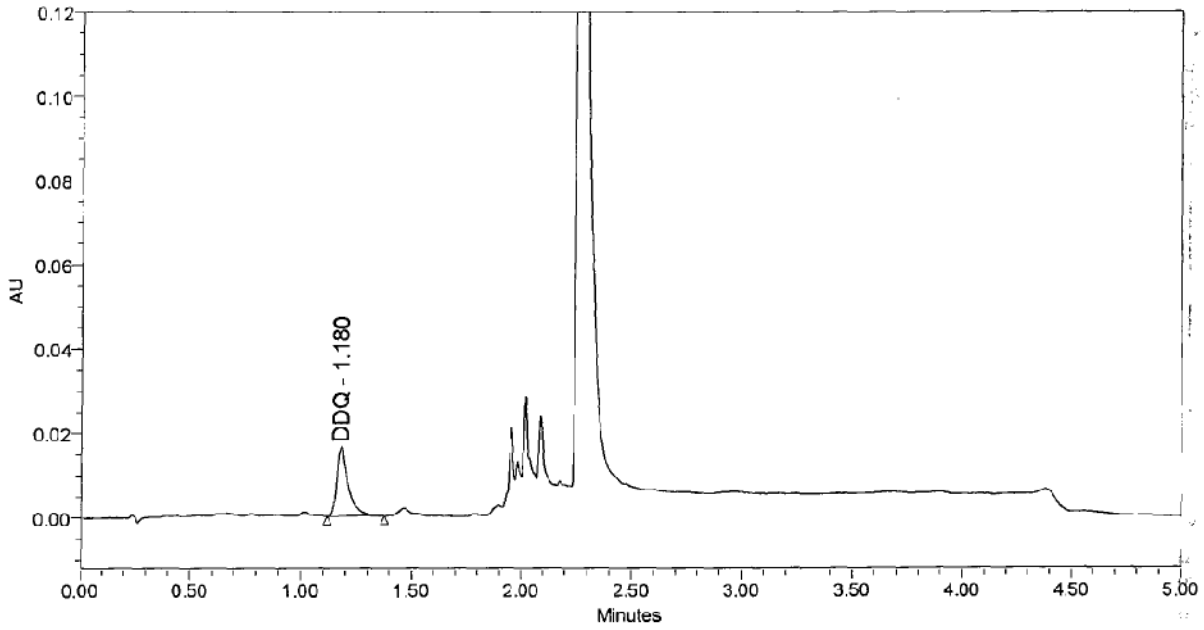


**Fig. 8.**

The Repeatability criterion was expressed as relative standard deviation (RSD). For this study, solution of Exemestane (20 mg/mL) spiked with DDQ (0.1%, 0.020 mg/mL) was analyzed in six replicate injections to establish repeatability. RSD values were better than 1.0% for the retention times of DDQ. All these values indicated that the method was precise.

The stability of the solution and the mobile phase used in the method was tested over a long period of time. However, no significant change in DDQ content was observed in Exemestane sample during the solution stability experiments, and the RSD values were less than 2.0% for peak area of DDQ. Hence, it could be concluded that the DDQ sample solution and the mobile phase were stable for at least 24 hours.

## CONCLUSION

A simple, specific, linear, accurate and precise Reverse phase UPLC method was successfully developed for the quantification of the carry-over impurity – DDQ in Exemestane. Zorbax Eclipsed UPLC column was found to be suitable for the DDQ content in Exemestane. This validated method can be employed for the analysis of DDQ content in Exemestane. The method is also stable and therefore, can be used to monitor the carry-over of DDQ impurity in the final product, and thereby control it in the drug substance.

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