Stability-Indicating Forced Degradation Study of Thymoquinone Using RP-HPLC

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ABSTRACT

Thymoquinone (TQ), a bioactive compound derived from Nigella sativa, exhibits a range of therapeutic properties, but its chemical instability under various environmental conditions presents a challenge to its pharmaceutical formulation. This study aimed to evaluate the forced degradation behavior of Thymoquinone under different stress conditions, including hydrolytic, oxidative, thermal, and photolytic degradation. A stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated in accordance with ICH guidelines, assessing parameters such as specificity, linearity, precision, accuracy, and robustness. The method demonstrated excellent resolution and sensitivity, enabling the separation and quantification of Thymoquinone and its degradation products. Forced degradation studies revealed that Thymoquinone is most susceptible to thermal and oxidative degradation, with thermal conditions causing a significant degradation of 14.68% and oxidative stress resulting in a 5.25% degradation. Photolytic degradation led to a 12.11% reduction in Thymoquinone content, while acid and base hydrolysis caused minimal degradation (1.53% and 0.78%, respectively). The findings underscore the importance of optimizing storage, packaging, and formulation strategies to maintain Thymoquinone's stability. The validated RP-HPLC method can be employed for routine quality control and stability testing of Thymoquinone-containing pharmaceutical products, ensuring regulatory compliance and product efficacy over its shelf life.

Introduction

Pharmaceutical stability is a critical factor in drug development, as the chemical integrity of an active pharmaceutical ingredient (API) directly influences the efficacy, safety, and shelf life of the drug product. Thymoquinone (TQ), a key bioactive component isolated from the seeds of *Nigella sativa*, has demonstrated a wide range of therapeutic properties, including anti-inflammatory, antioxidant, antimicrobial, and anticancer activities. (1-2) Despite its promising

therapeutic potential, the inherent chemical instability of thymoquinone in various environments poses a significant challenge to its formulation and storage. (3)

Given the susceptibility of thymoquinone to environmental stressors, understanding its degradation profile under forced conditions is essential for the development of stable pharmaceutical formulations. The objective of a forced degradation study is to identify the degradation pathways and potential degradation products of an API when subjected to conditions beyond normal storage. These studies simulate extreme environments such as hydrolytic (acidic and basic), oxidative, photolytic, and thermal conditions, offering valuable insights into the compound's stability profile. (3, 4) Additionally, forced degradation helps distinguish between degradation-related impurities and the active pharmaceutical component, a critical step for ensuring product safety and quality. (5)

Forced degradation studies are conducted in line with the International Council for Harmonisation (ICH) Q1A(R2) guidelines to define the stability profile of pharmaceutical products. These studies also aid in the development of a stability-indicating method, which is an analytical method that can effectively differentiate between the API and its degradation products. A well-designed stability-indicating method is integral to ensuring the reliability and accuracy of routine quality control processes, supporting formulation development, and meeting regulatory requirements. (6)

Reverse-phase high-performance liquid chromatography (RP-HPLC) is widely regarded as the analytical method of choice for stability-indicating studies due to its high resolution, precision, and reproducibility. RP-HPLC has the capacity to separate the API from its degradation products with excellent sensitivity, enabling comprehensive profiling of degradation pathways. The versatility of RP-HPLC stems from its ability to handle a variety of compounds with differing polarities, making it especially suited for the forced degradation of structurally diverse compounds like thymoquinone. The method's ability to monitor the degradation process in real-time provides invaluable information that can be used to optimize formulation strategies and assess product stability over time. (7-8)

Thymoquinone's quinone structure, characterized by its reactive nature, makes it particularly prone to degradation under oxidative and hydrolytic conditions. Previous studies have indicated that thymoquinone is unstable under light, temperature, and oxidative stress, leading to the generation of various degradation products that could compromise its therapeutic efficacy. (9-10) Thus, the development of a validated stability-indicating RP-HPLC method is crucial for evaluating the degradation behaviour of thymoquinone under different stress conditions.

This study focuses on evaluating the forced degradation behaviour of thymoquinone by subjecting it to a series of stress conditions, including Hydrolytic, oxidative, photolytic and thermal conditions.

Each stress condition will be evaluated using a stability-indicating RP-HPLC method that will be developed and validated according to ICH guidelines for linearity, precision, accuracy, and robustness. This method will ensure the reliable detection and quantification of thymoquinone and its degradation products, providing a thorough understanding of its stability under varying conditions. (11)

The results of this forced degradation study will aid in the optimization of pharmaceutical formulations containing thymoquinone, ensuring they meet the necessary stability criteria throughout the product's shelf life. (12) Moreover, by identifying the conditions under which thymoquinone is most susceptible to degradation, the study will provide key insights into the storage, packaging, and formulation considerations required to preserve its therapeutic properties. The validated RP-HPLC method developed during this study will also serve as a powerful tool for routine quality control, helping to ensure that thymoquinone-containing products remain consistent in their quality, safety, and efficacy.

Materials and Methods

Materials

Thymoquinone and its formulation were generously provided as gift samples by Intas Pharmaceuticals, Ahmedabad, India. High-performance liquid chromatography (HPLC)-grade solvents, including methanol, Potassium dihydrogen Phosphate, O-Phosphoric acid and HPLC-grade water were sourced from Ricca Chemical Company. HPLC-grade water was used for the preparation of all solutions to ensure accuracy and consistency.

Preparation of Standard stock Solutions

Accurately weighed quantity of Thymoquinone 10 mg was transferred into 100 ml volumetric flask and final volume of solution was made 100 ml with mobile phase to get stock solution containing 100µg/ml of Thymoquinone in 100 ml volumetric flask. The standard solutions are prepared in the range of 5-25 µg/ml.

Preparation of Sample solution

Powder equivalent to 10 mg of Thymoquinone was weighed and transferred in a 100 ml volumetric flask and sufficient mobile phase was added to dissolve the drug. It was sonicated for 20 minutes and final volume was made to the mark with mobile phase. The solution was filtered through 0.45 µ Membrane filter. 10 ml of aliquot from above stock solution was further diluted to 100 ml with mobile phase to get the final concentration of 10 mcg/ml. The sample solution was assayed as per proposed method and % assay was calculated.

Chromatographic Procedure

The chromatographic separation was performed using a Enable C18 column (250 mm × 4.6 mm, 5 µm particle size). The mobile phase consisted of buffer and methanol in a ratio of 45:55 (v/v), adjusted to pH 3.65 using o-phosphoric acid. The detection wavelength was set at 290 nm, with a flow rate of 1 ml/min. The injection volume was 20 µL, and the total run time for the method was 10 minutes. The column temperature was maintained at 30°C, with methanol used as the diluent.

Method Validation

Method validation is a fundamental aspect of analytical chemistry, designed to establish the reliability, accuracy, and reproducibility of a method for analyzing specific compounds. It involves a detailed assessment of critical parameters, including specificity, linearity, precision, accuracy, detection and quantitation limits, robustness, and system suitability. These evaluations ensure that the method is appropriate for its intended purpose and can consistently produce dependable results under prescribed conditions. Proper validation is especially important in regulated environments, as it provides documented evidence of the method's performance and ensures the credibility of the generated data. In this study, the validation process adhered to the ICH Q2 (R1) guidelines, confirming that the method meets stringent criteria for the accurate and reliable quantification of the target analytes in pharmaceutical formulations.

Specificity

The specificity of the analytical method was assessed by analyzing a blank solution (diluent only), a placebo (sample matrix without the analyte), and a standard solution of the analyte (Figure 1). The chromatograms of the blank and placebo samples were reviewed to ensure no peaks were present at the analyte's retention time, confirming the absence of interference from the diluent or matrix components. Forced degradation studies were conducted under various stress conditions, including acid and base hydrolysis, oxidation, thermal degradation, and photolysis. The resulting degraded samples were analyzed to check for potential co-elution of degradation products with the analyte peak. The method's specificity was validated by demonstrating that the analyte peak was well-separated from other peaks, with no significant interference detected in the chromatograms. Additionally, no interference peaks were observed

in the blank or placebo samples, and blank degradation samples showed no additional peaks at the analyte's retention time, aside from the degradation peaks.

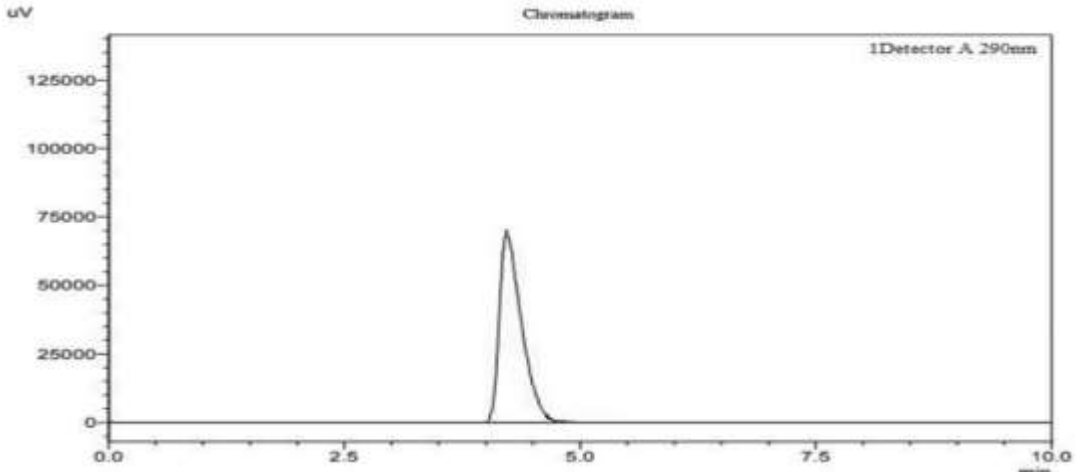

Figure 1: Chromatogram of Standard solution of Thymoquinone

Linearity

To construct the calibration curve, aliquots of 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mL of the standard stock solution were transferred into separate 10 mL volumetric flasks. The flasks were then filled to volume with the mobile phase, resulting in final concentrations ranging from 5 to 30 µg/mL. The chromatographic method was used to measure the area under the curve for each concentration. A calibration curve was plotted with the area against the concentration, and the linearity of the response was evaluated by analyzing the curve for Thymoquinone. The correlation coefficient (R^2) and the regression equation were calculated to confirm the linear relationship between the area and the concentration of Thymoquinone.

Table 1: Linearity Data of Thymoquinone (n=6)

Concentration (µg/ml)	Avg. area (µV*sec.) ± SD	% RSD
5	2359098 ± 21217.74	0.90
10	4468256 ± 19213.7	0.43
15	6158495 ± 56766.4	0.92
20	8125238 ± 100502	1.24
25	10270248 ± 57328.9	0.56
30	12258199 ± 150791.9	1.23

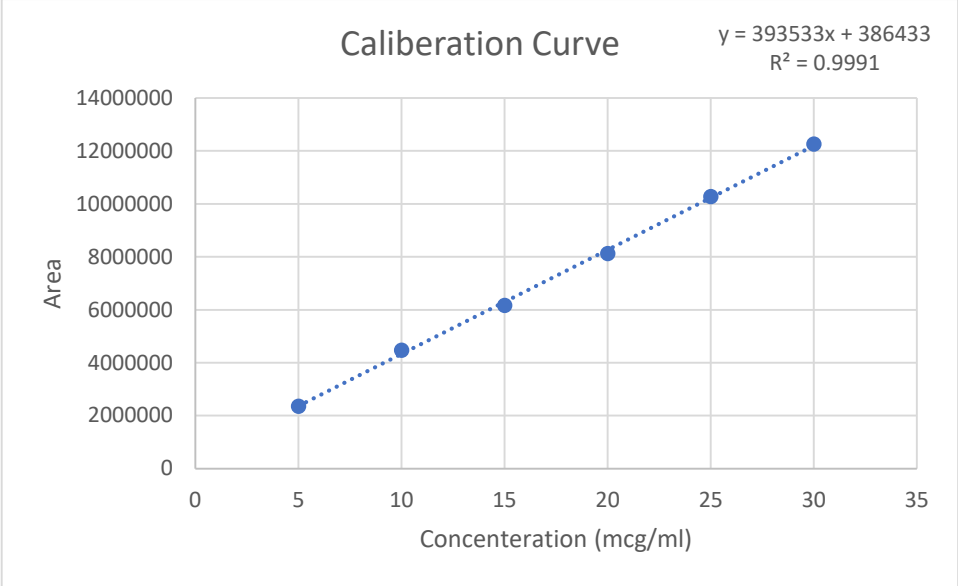

Figure 2: Calibration Curve of Endoxifen

The linearity of the analytical method was evaluated over a concentration range of 5 to 30 µg/mL for Thymoquinone. The average peak areas, along with the standard deviation (SD) and percent relative standard deviation (%RSD), demonstrated consistent and reproducible results. At 5 µg/mL, the average area was 2,359,098 ± 21,217.74 with a %RSD of 0.90%. For 10 µg/mL, the average area was 4,468,256 ± 19,213.70 (%RSD 0.43%), while at 15 µg/mL, it was 6,158,495 ± 56,766.40 (%RSD 0.92%). Similarly, at 20 µg/mL, the average area was 8,125,238 ± 100,502 (%RSD 1.24%), at 25 µg/mL, 10,270,248 ± 57,328.90 (%RSD 0.56%), and at 30 µg/mL, 12,258,199 ± 150,791.90 (%RSD 1.23%). These results confirm the method's linearity and precision across the tested concentration range.

Precision

Repeatability

Six replicate solutions of 20 µg/ml for Thymoquinone were prepared and analyzed using the optimized chromatographic conditions. The areas of the solutions were determinedand % RSD was calculated.

Table 2: Repeatability study of Thymoquinone

Sr. no.	Conc (µg/ml)	Area (µV*sec.)	Average (µV*sec.) ± SD	% RSD
1	20	8027834	8057112 ± 53486.9	0.66
2	20	8122637		
3	20	8022384		
4	20	8013485		
5	20	8128948		

Intra-day and Inter-Day Precision

To assess intra-day and inter-day precision, three concentrations of Thymoquinone (15, 20, and 25 µg/mL) were prepared by diluting the standard stock solution with the mobile phase. For intra-day precision, each concentration was analyzed in triplicate within a single day under identical experimental conditions, and the average peak areas, standard deviations (SD), and percentage relative standard deviations (%RSD) were calculated. For inter-day precision, the same concentrations were analyzed in triplicate across three separate days, maintaining consistent experimental conditions. The average areas, along with SD and %RSD, were determined for each concentration over the three days. This approach ensured the method's

reproducibility and reliability were evaluated both within a single day and over multiple days. As shown in Table 3, the %RSD values for all concentrations were below 2%, confirming that the method meets the precision criteria.

Table 3: Intra-day and Inter-day Precision study of Thymoquinone (n=3)

Sr. no.	Conc.(µg/ml)	Intra-day precision (n=3)	
		Mean area (µV*sec.) ± S.D	% RSD
1	15	6238441 ± 14731.8	0.24
2	20	7979348 ± 66848.7	0.84
3	25	10270085 ± 4466.4	0.43
Inter-Day Precision			
1	15	6240408 ± 7380.24	0.12
2	20	8056568 ± 63350.8	0.79
3	25	10262923 ± 16791.91	0.16

Accuracy

The accuracy of the method was assessed through a recovery study using a marketed formulation, performed at three levels of standard addition (50%, 100%, and 150% of the label claim). Twenty tablets were accurately weighed, and a quantity of powder equivalent to 10 mg of Thymoquinone was transferred to a 100 mL volumetric flask. The powder was dissolved in the mobile phase, and the solution was sonicated for 20 minutes to ensure complete dissolution before adjusting the volume to the mark with the mobile phase. For the recovery study, fixed aliquots of the prepared sample solution were transferred to 10 mL volumetric flasks, to which Thymoquinone standard solution was added to achieve concentrations corresponding to 50%, 100%, and 150% of the label claim. The volumes were then adjusted to the mark with the mobile phase. Each concentration level was prepared in triplicate (n=3) and analyzed under the optimized chromatographic conditions. The percentage recovery of Thymoquinone was calculated by comparing the recovered amount from the spiked samples to the added amount, confirming the accuracy of the method.

Table 4: Accuracy study data of Thymoquinone (n=3)

Level (%)	**Test amount (µg/ml)**	**Spiked std. amount (µg/ml)**	**Total amount (µg/ml)**	**Avg. Amount recovered (µg/ml)**	**% Mean recovery ± SD. (n=3)**
50	10	5	15	14.90	99.31 ± 0.45
100	10	10	20	19.96	99.78 ± 0.38
150	10	15	25	24.94	99.76 ± 0.45

LOD and LOQ

Calibration curve was repeated for three times and the standard deviation (SD) of the intercepts wascalculated. Then LOD and LOQ were measured as follows.

LOD=3.3 * SD/Mean slope of
calibration curveLOQ=10 *
SD/Mean slope of calibration curve

SD = Standard deviation of y intercepts of calibration curves

LOD and LOQ were shown in Table no: 5

Table 5: LOD and LOQ value for Thymoquinone

Sr. No.	Parameter	Mean slope	SD	Result (µg/ml)
1	LOD	366336	1692. 59	0.15
2	LOQ			0.46

Assay

The assay was carried out on three batches of Thymoquinone. A quantity of powder equivalent to 10 mg of Thymoquinone was accurately weighed and transferred to a 100 mL volumetric flask. Sufficient mobile phase was added to dissolve the drug, and the solution was sonicated for 20 minutes to ensure complete dissolution. The volume was then adjusted to the mark with the mobile phase, and the solution was filtered using a 0.45 µm membrane filter. A 10 mL aliquot of this stock solution was further diluted to 100 mL with the mobile phase, resulting in a final sample solution of 10 µg/mL. This solution was analysed using the proposed method, and the percentage assay was calculated for each batch.

Table 6: Assay result of Thymoquinone

Sr. No	Amount taken (µg/ml)	Amt. found (µg/ml)	% Assay	%RSD
1	20	19.83	99.18	0.63
2	20	19.66	98.29	
3	20	19.60	98.00	

Forced Degradation Study

All the procedures were carried out in triplicates.

Acid Degradation:

To 2 ml stock solution Thymoquinone, 2 ml of 5 N hydrochloric acid was added and refluxed for 8 hours at 60°C. The acidic degradation was performed under dark condition to exclude the possible photolytic degradation. The degradation sample was cooled at room temperature and neutralized the sample with same strength of NaOH. Suitable aliquots of resultant degradation samples were pipette out and made up the volume with the mobile phase.

Base Degradation

To 2 ml stock solution of Thymoquinone, 2 mL of 5 N sodium hydroxide was added and refluxed for 8 hours at 60°C. The basic degradation was performed under dark condition to exclude the possible photolytic degradation. The degradation sample was cooled at room temperature and neutralized the sample with same strength of HCl. Suitable aliquots of resultant degradation samples were pipette out and made up the volume with the mobile phase.

Oxidative Degradation

To 2 ml stock solution of Thymoquinone, 2 ml of 3% hydrogen peroxide (H_2O_2) was added separately. The solutions were refluxed for 30 min at 60°C The oxidative degradation performed under dark condition to exclude the possible photolytic degradation. The degradation sample was cooled at room temperature. Suitable aliquots of resultant degradation samples were pipette out and made up the volume with the mobile phase.

Thermal Degradation

For dry heat degradation the sample were placed in the oven at 80^{O}c for 2 hours under the dark condition and then cooled at room temperature. Degradation sample were subjected to analysis after its dilution (20 µg/ml) with the mobile phase.

Photolytic Degradation:

For Photolytic degradation samples were kept in UV chamber for 8 hours and samples were subjected to analysis (20 µg/ml) after dilution with mobile phase. (20 µg/ml)

Results:

Parameter	Standard Area	Area After Degradation of Thymoquinone (API)	% Degradation of Thymoquinone (API)
Acid Degradation	8125238	8000963	1.53
Base Degradation		8062259	0.78
Peroxide Degradation		7698663	5.25
Thermal Degradation		6932453	14.68
Photolytic degradation		7141271	12.11

Conclusion

The forced degradation study conducted on Thymoquinone under various stress conditions has provided significant insights into its stability profile and degradation behavior. The results revealed that Thymoquinone is most susceptible to thermal and oxidative degradation, with significant degradation observed under thermal conditions (14.68%) and oxidative stress (5.25%). Photolytic degradation also led to a considerable decrease in Thymoquinone content (12.11%), while acid and base hydrolysis caused relatively minor degradation (1.53% and 0.78%, respectively).

The developed and validated reverse-phase high-performance liquid chromatography (RP-HPLC) method proved to be robust and effective in monitoring Thymoquinone degradation under these conditions. The method demonstrated high specificity, linearity, and precision, and was able to accurately quantify the degradation products, ensuring its reliability for stability-indicating studies. The findings from this study are crucial for the formulation and storage of Thymoquinone containing pharmaceutical products. The observed degradation patterns highlight the need for careful consideration of storage conditions, packaging, and formulation strategies to preserve the drug's therapeutic efficacy. The validated RP-HPLC method can be utilized for routine quality control and stability testing, ensuring consistent product quality and regulatory compliance.

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