

Forced Degradation Studies and Impurity Characterisation of Thymoquinone in Drug and Formulation

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ABSTRACT

Background: The present research focuses on the development of robust, precise, and stability-indicating analytical methods for Thymoquinone. The study aims to establish validated analytical procedures, conduct systematic forced degradation studies, and characterize impurities formed under stress conditions in both bulk drug substances and pharmaceutical formulations.

Materials and Methods: Thymoquinone is a bioactive quinone compound of natural origin, and it is light, heat, and oxidative sensitive. Therefore, there was great care in handling, storage and preparation of the thymoquinone standard solutions to prevent its careless degradation.

Results: Linearity: A linear regression analysis of the calibration data demonstrated an excellent linear relationship, providing a correlation coefficient (R-squared) value of 0.9991. Precision: The precision was observed well within the generally recognized acceptable threshold of less than 2%. Repeatability at the lowest concentration level (5 µg/mL) showed a percent Relative Standard Deviation (RSD) of 0.90%. Furthermore, intraday precision RSD values ranged from 0.24% to 0.84%, while interday precision RSD values ranged from 0.12% to 0.79%. Accuracy: Accuracy recovery studies were performed, and the mean percent recoveries were found to be 99.31%±0.45 at the 50% level, 99.78%±0.38 at the 100% level, and 99.76%±0.45 at the 150% level. Additional testing at 80%, 100%, and 120% target concentrations yielded mean recoveries of 98.26%, 99.23%, and 98.10%, respectively. The forced degradation experiments indicated that thymoquinone is highly susceptible to thermal and photolytic stresses with a lesser susceptibility at acidic and basic hydrolytic stresses. This disparity in stability is typical of the chemical constitution of thymoquinone and is consistent with already established degradation behaviour of other structurally analogous quinone like substances. **Conclusion:** Thymoquinone exhibited a moderate acidic and basic hydrolytic degradation, compared to having a high degree of thermal and photolytic stress degradation. The retarded hydrolytic degradation suggests that the quinone ring system is comparatively inactive to catalytic reactions by protonic (or hydroxide) ions at the assayed conditions. The formation of different impurity peaks was also the effect of the photolytic stress, particularly of the thymoquinone, the structure of which is photoreactive quinone.

Keywords: Force Degradation, ICH Guideline, Impurity, Method Developments, Stability.

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INTRODUCTION

Chemical stability of pharmaceutical molecules is a matter of great concern as it affects the safety and efficacy of the drug. The FDA and ICH Guidance state the requirements of stability testing data to understand how the quality of a drug substances and drug products changes with time under the influences of various environmental factors. The behaviour of thymoquinone also demonstrated erratic degradation behaviour in various strains with which the drug was subjected to and is the result of

the chemical form of the drug being a quinone-based molecule (Kelly *et al.*, 2017). This high level of hydrolytic resistance implies that pH caused hydrolysis of the quinone structure of thymoquinone is relatively resistant to the pH conditions in the examined system. These observations reveal that thymoquinone is particularly sensitive to high temperatures and light that could lead to structure rearrangement, and photochemical reactions. This very distinction proves the stability-signalling nature of the developed approach to an analytical method and its versatility to stability testing and profiling of impurities. These degradation products were detected under some severe stress factors which included oxidative, thermal, photolytic stress, and the force of quinone structure of thymoquinone to redox, photochemical reaction. The structural clarification of the degradation products of thymoquinone was explained through the mass spectrometry and nuclear magnetic resonance spectroscopy. The behaviour of



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thymoquinone in stability as it was perceived in the current study is noteworthy to the comprehension of the intrinsic chemical characteristics of the bioactive agent basing on the thymoquinone and the implication of the identical on the pharmaceutical development and quality control (Gaudin *et al.*, 2018). This is highly concerned with the processes of drug processing with high temperatures in drying, granulation and storage used by pharmaceutical companies. Hydrolytic degradation is less prone to lack of hyper-labile functional groups (e.g. esters or amides), but more prone to oxidative, thermal and photochemical reaction due to the presence of a conjugated quinone system. The experimental findings agree with such a structural basis of degradation and give the mechanistic concept of the instability of thymoquinone. Formulation Thermal and photolytic Thus, there are implications of some importance to thymoquinone, whose thermal and photolytic affinity is high. The formulation strategies should aim at minimizing the exposure of heat and light during the manufacturing and storage process. This may include low temperature methods of processing, stabilizing excipients, and omission of manufacturing processes that subject the drug to protracted heat (Paloukopolou *et al.*, 2023). In addition, stability of the product depends on the choice of the appropriate packaging materials. The acquired knowledge in this research provides a plausible scientific foundation to the deliverance of quality, safety and efficacy to thymoquinone based drug prescription lifecycle. the forced degradation experiments that are conducted in this research shows that thymoquinone is very sensitive to thermal and photolytic-stress and relatively stable in acidic and basic conditions (Sarma *et al.*, 2025). This stability behaviour is not only in line with the chemical behaviour of quinone compounds, but also possesses significant implication on formulation development, choice of packaging, prescription of storage as well as regulation.

MATERIALS AND METHODS

Materials

- Instruments Specification.
- Instrumentation: HPLC LC-2010 CHT Liquid Chromatography.
- Detector: UV detector LC-2010 CHT.
- Column: Phenomenex-luna C18 (250 x 4.6 mm, 5 µm).
- Chromatographic software: Lab Solution.

Reagents and Materials Used

- Acetonitrile HPLC Merck, Rankem.
- Water HPLC Merck, Rankem.
- Ortho-phosphoric acid AR Merck, Rankem.

- Methanol HPLC Merck, Rankem.

Mobile Phase

A mixture of Methanol and Buffer in the ratio of 55:45 (v/v) with pH of mixture adjusted to 3.65 using ortho-phosphoric acid was prepared and the mixture was filtered through 0.45 µm membrane filters and it was degassed.

Preparation Of Standard Solution

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 100 µg/mL of Thymoquinone.

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 min and final volume was made to the mark with mobile phase. The solution was filtered through 0.45 µm membrane filter. The sample solution of 20 mcg/mL was assayed as per proposed method and % assay was calculated.

Chromatographic Conditions For RP-HPLC Method

- Column: Enable C18 (250 x 4.6 mm, 5µm).
- Mobile Phase: Buffer: Methanol (45: 55) (pH 3.65 using o-phosphoric acid).
- Detection wavelength: 290 nm.
- Flow rate: 1.0 mL/min.
- Injection Volume: 20µL.
- Run Time: 10 min.

Preparation Of Calibration Curve

Take 0.5, 1, 1.5, 2, 2.5, 3 mL of aliquots to the 10 mL volumetric flasks from standard stock solution and make up to mark with mobile phase to get final concentration. Plot the graph for area vs. concentration to get calibration curve.

METHODOLOGY OF FORCED DEGRADATION STUDY OF THYMOQUINONE

Acid Degradation

To 2 mL stock solution Thymoquinone, 2 mL of 5 N hydrochloric acid was added and refluxed for 8 hr 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 hr 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 (H₂O₂) 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°C for 8 hr 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 Photolytic chamber for exposure of 1.2 Mill. Lux light and samples were subjected to analysis (20 µg/mL) after dilution with mobile phase. (20 µg/mL).

RESULTS AND DISCUSSION

Force Degradation of Thymoquinone

The summary of findings in Table 1 shows that thymoquinone was rather stable on both acidic and basic hydrolytic conditions with the maximum degradation of 1.53% and 0.78%, respectively. This high level of hydrolytic resistance implies that pH caused hydrolysis of the quinone structure of thymoquinone is relatively resistant to the pH conditions in the examined system. On the contrary, thymoquinone was vulnerable to oxidative stresses

whereby a proportion of 5.25% of the molecule was influenced when thymoquinone made contact with the hydrogen peroxide. This fact is correlated with oxidative reactivity of quinone moieties which are apt to be redoxed. The greatest degradation was observed on thermal stress and photolytic stress and the respective degradation condition was 14.68% and 12.11%, respectively (Taleuzzaman *et al.*, 2017). These observations reveal that thymoquinone is particularly sensitive to high temperatures and light that could lead to structure rearrangement, and photochemical reactions. The overlay chromatogram of Figure 4 shows the chromatographic profiles of the stressed thymoquinone samples and it can be observed that there is the formation of other peaks relative to the degradation products. It is important to note that the peaks of degradation were well distinct relative to the parent drug peak, and co-elution and interference were not evident. This very distinction proves the stability-signalling nature of the developed approach to an analytical method and its versatility to stability testing and profiling of impurities.

The choice of stability of the drug is explained by silencing degradations of dislocation of the parent drug as shown in Figure 1-3. (Overlay chromatogram of stressed samples) on the stigma proves that the stability properties were fulfilled.

Impurity Characterization of Thymoquinone

Clear studies of degradation thymoquinone led to the emergence of many degradation products that were identified by simply replacing the names with D-1, D-2, D-3, and D-4, in order to be easily identified and discussed. The structural clarification of the degradation products of thymoquinone was explained through the mass spectrometry and nuclear magnetic resonance spectroscopy. Results of the mass spectrometry provided molecular ion peaks of the modified molecular weight, which displayed the chemical alteration of the parent molecule (Kumar *et al.*, 2011). D-1 and D-2 products of degradation were primarily the products of the oxidative stress, so one can suppose that it is the interactions with the oxidative reactions that may lead to the change or breakdown of the quinone moiety. Degradation products D-3 and D-4 were largely formed under thermal stress and photolytic stress that is to say that heat and light can result in a rearrangement of the structure or photochemical reaction within the quinone system (Figures 4-7).

Table 1: Forced Degradation Results for Thymoquinone.

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

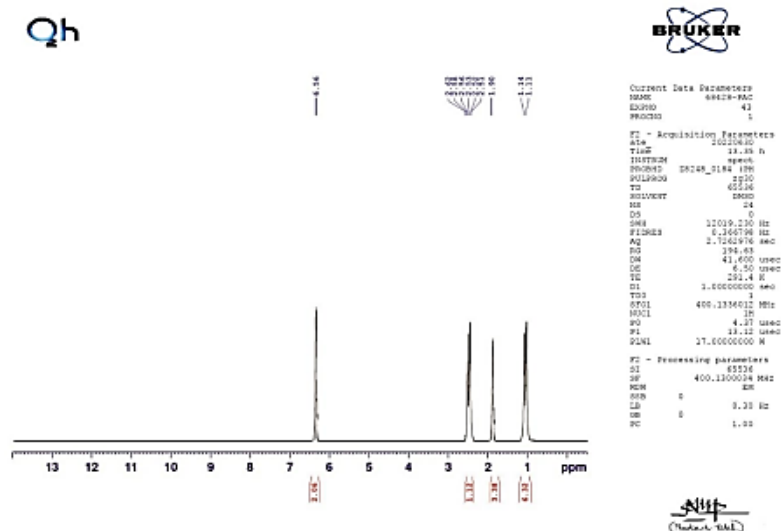


Figure 1: NMR Data.

Synthesis and Characterization

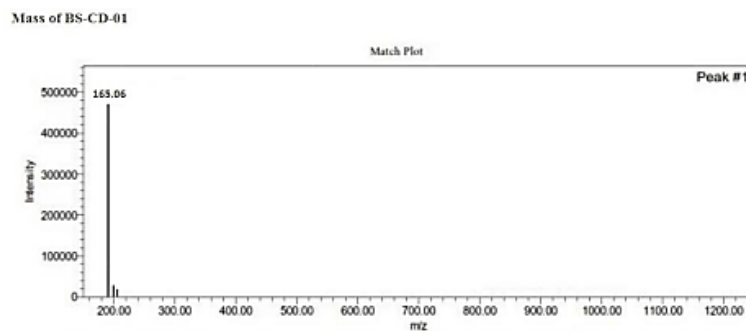


Figure 2: Synthesis and Characterization.

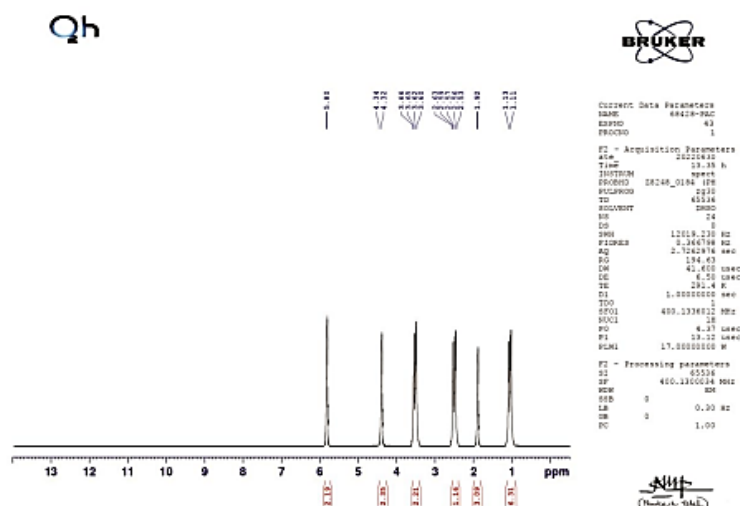
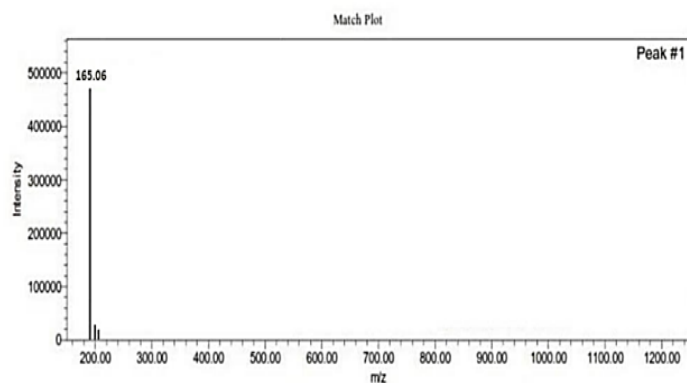


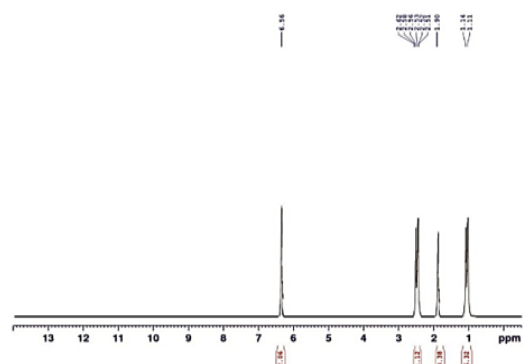
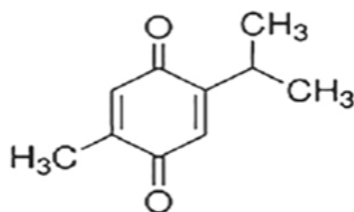
Figure 3: Predicted Structure (D-1) NMR Structure.

Thymoquinone (Mass: 165)

Mass of BS-CD-01



Qh



NMR Interpretation:

a) 1.11-1.14(d,6H)

b) 1.90(s,3H)

c) 2.51-2.62(H,1H)

d) 6.56(s,2ArH)

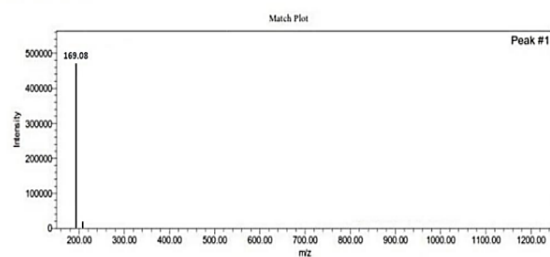
Figure 4: Thymoquinone Impurity NMR Interpretation.

Thymoquinone Impurity D1

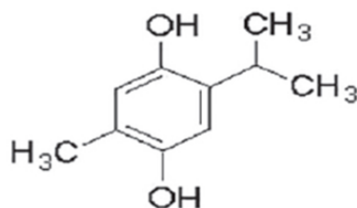
Mass Spectra

Synthesis and Characterization

Mass of BS-CD-02



Predicted Structure (D-1)



NMR Interpretation:

a) 1.11-1.13(d,6H)

b) 1.92(s,3H)

c) 2.53-2.63(H,1H)

d) 4.32-4.34(d,2H)

e) 5.80(s,2ArH)

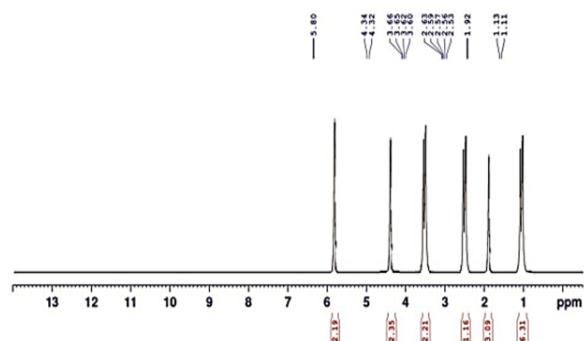


Figure 5: Thymoquinone Impurity D1 NMR Interpretation.

Thymoquinone Impurity D2

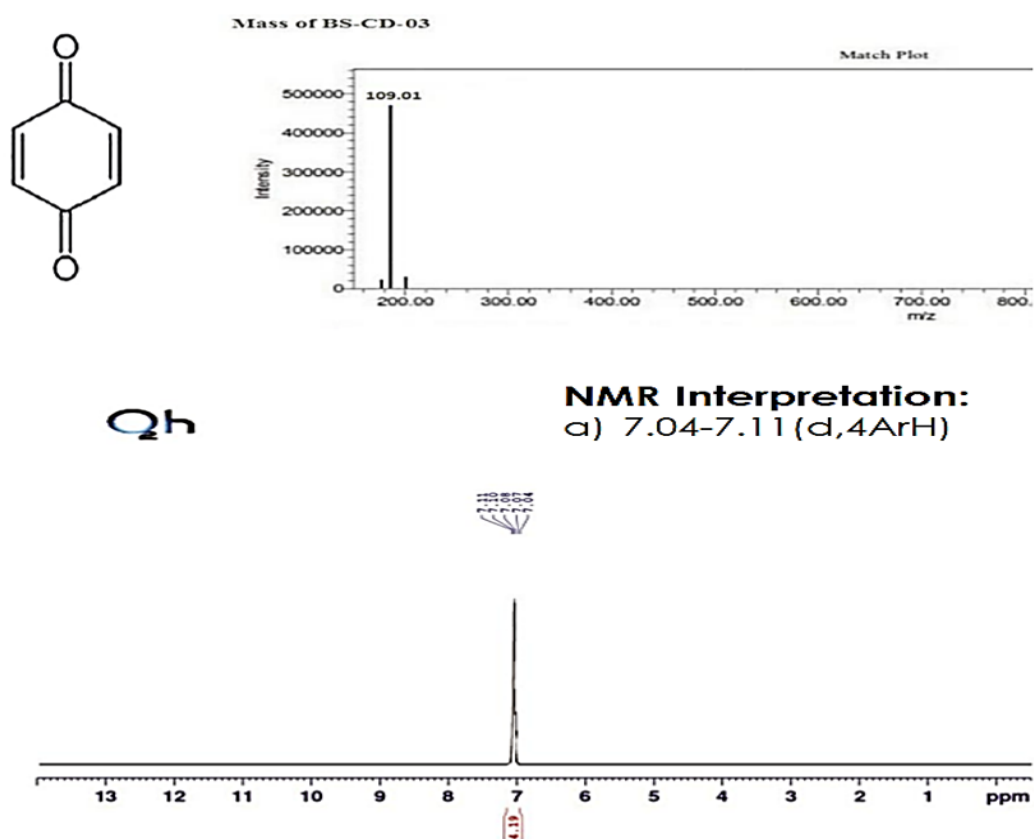


Figure 6: Thymoquinone Impurity D2 NMR Interpretation.

Thymoquinone Impurity D3

Predicted Structure (D-3)

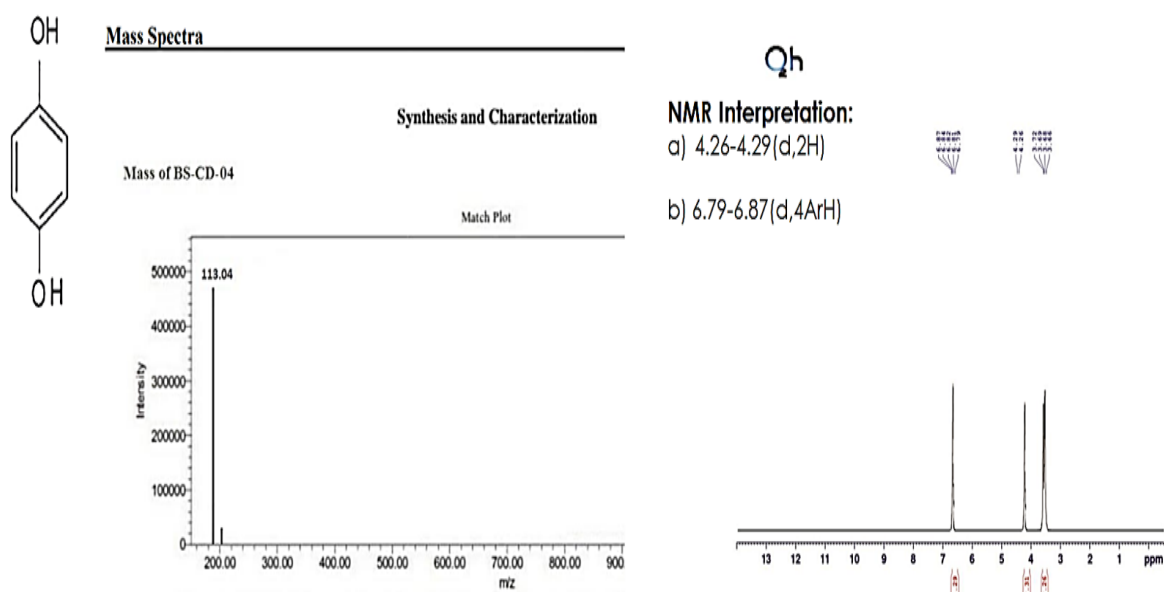


Figure 7: Thymoquinone Impurity D3 NMR Interpretation.

CONCLUSION

The determination of intrinsic stability profile of thymoquinone was vital after the forced degradation experiments. The compound was highly vulnerable to thermal and photolytic stresses and relatively low vulnerability to acidic and basic hydrolytic stress. This is congruent with the fact that quinones is a chemical compound, which is known to undergo photochemical reactions and thermal rearrangements. The findings suggest that the storage conditions and the packaging plans of the formulations which include thymoquinone must be considered particularly (Kazi *et al.*, 2020). Protecting the product factors of light and high temperatures is one of the requisite conditions that will ensure the stability of chemicals and quality of products in the shelves. The trends of degradation experienced during stressful services would be of great assistance in formulation development as it can be applied to choose the relevant excipients, packaging materials, and storage rule to minimize the possibility of degradation.

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ABBREVIATIONS

HPLC: High-Performance Liquid Chromatography; **RSD:** Relative Standard Deviation; **NMR:** Nuclear Magnetic Resonance; **ICH:** International Council for Harmonisation; **FDA:** Food and Drug Administration; **API:** Active Pharmaceutical Ingredient; **RP-HPLC:** Reversed-Phase High-Performance Liquid Chromatography; **UV:** Ultraviolet; **AR:** Analytical Reagent.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTIONS

Mayur Raval: Investigation, Methodology, Data curation, Writing - original draft preparation. **Dr. Ishan Panchal:** Conceptualization, Supervision, Validation, Writing - review and editing.

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