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# VALIDATED STABILITY-INDICATING RP-UHPLC METHOD FOR THE ESTIMATION OF SOFALCONE IN DRUGS, RECONCILING MASS BALANCE IN FORCE DEGRADATION STUDIES AND LC-MS IDENTIFICATION OF ITS DEGRADATION PRODUCTS

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**Keywords:**

Sofalcone, RP-UHPLC, Validation, Stability, LC-MS/MS

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**ABSTRACT:** A highly sensitive, simple, accurate and precise Ultra high-performance liquid chromatographic (UHPLC) method was developed and validated for the quantitative determination of sofalcone in the presence of its degradation products. The analysis was performed on Agilent symmetry analytical column Eclipse plus C18 (100mm × 2.1 mm, 1.8µm) ultra-performance liquid chromatography-Diode Array Detectors (UHPLCDAD), while the detection was performed on 350 nm using Diode Array Detectors. Water (0.1% Formic Acid) and Ammonium Acetate in Methanol were the mobile phases, run at a flow rate of 0.3 ml/min for gradient elution. The mean values of recovery were found to be 100.02% and 100.55%. The linear regression data for the calibration plot showed a good relationship with high correlation coefficients. The performance of the method was validated for precision, accuracy and robustness. Sofalcone was subjected to the ICH-prescribed acidic, alkaline, oxidative, photolytic, and thermal stress conditions, and it undergoes degradation with well-resolved degradation products. These degradation products were further analyzed by mass spectrometry to elucidate the structure of degradation products and proposed the prediction of the degradation pathway and Mass Balance.

**INTRODUCTION:** Sofalconeis a potent Gastro Protective Agent. It is shown to inhibit gastric mucosal injuries and prevent gastric ulceration & formation of acute mucosal lesions. This compound belongs to the class of organic compounds known as linear 1,3-diarylpropanoids. These are organic compounds with a structure based on a C6-C3-C6 skeleton, where the two benzene rings are not linked together [1]. Literature shows Bioanalytical method has been developed for the estimation of Sofalcone. Still, to the best of our knowledge, there is no published chromatographic method for in bulk as well as in the dosage form.

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The present work aimed to develop a sensitive and specific UHPLC method for the quantification of Sofalcone in the presence of its degradation products with its structure elucidation. Mass balance correlates the measured loss of a parent drug to the measured increase in degradation products.

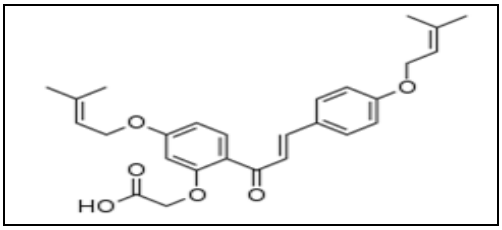



**FIG. 1: CHEMICAL STRUCTURE OF (A) SOFALCONE**

It is a good quality control check on analytical methods to show that all degradation products are adequately detected and do not interfere with the quantitation of the parent drug.

The method was fully validated and tested for stability studies per the International Conference on Harmonization (ICH) guidelines **Fig. 1.**

**MATERIALS AND METHODS:** All the chemicals used were of AR grade. The solvents used are of HPLC grade and purchased from MERCK. The water was distilled and deionized using the Millipore (ELIX) system.

**Chromatographic Conditions:** C18 (100 mm × 2.1 mm, 1.8 µm particles) (Agilent) was used as the stationary phase. The mobile phase comprises two different components Water (0.1% Formic Acid) and Ammonium Acetate in Methanol, as gradient elution mode. The flow rate was optimized at 0.3ml/minute, and the injection volume was kept 2.0 µl with a run time of 20 minutes. The chromatograms were recorded at 350nm, and the column temperature was maintained at 25°C throughout the study period. Different samples prepared and the mobile phase, were filtered using 0.22µm filter and degassed by ultrasonication (LMUC 6) before use.

**Preparation of Standard Stock Solution:** A stock solution of Sofalcone of concentration 1000µg/ml was prepared by dissolving 100 mg of Sofalcone in 5ml Tetrahydrofuran and making volume up to 100ml by using Diluent *i.e.* Water and Acetonitrile (20: 80) in a volumetric flask. Thereafter solutions of different concentration (50- 150µg/ml) were prepared by diluting the stock solution.

**Method Validation:** The method was validated and carried out as per the guidance of ICH Q2 (R1) [2].

**Linearity & Range:** Stock solution of concentration 1000µg/ml was diluted to get the different concentrations 50 µg/ml, 75 µg/ml, 100µg/ml, 125 µg/ml, 150 µg/ml and 2µl of each concentration was then injected using autosampler unit and the chromatograms were recorded. A graph of Area under curve vs. concentration was then plotted to get the Calibration curve. The equation, slope, intercept, and regression coefficient ($R^2$) were determined in **Fig. 2** and **3 Table 1.**

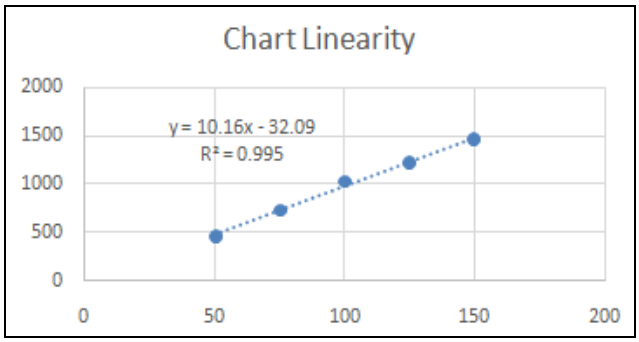



**FIG. 2: STANDARD PLOT OF SOFALCONE**

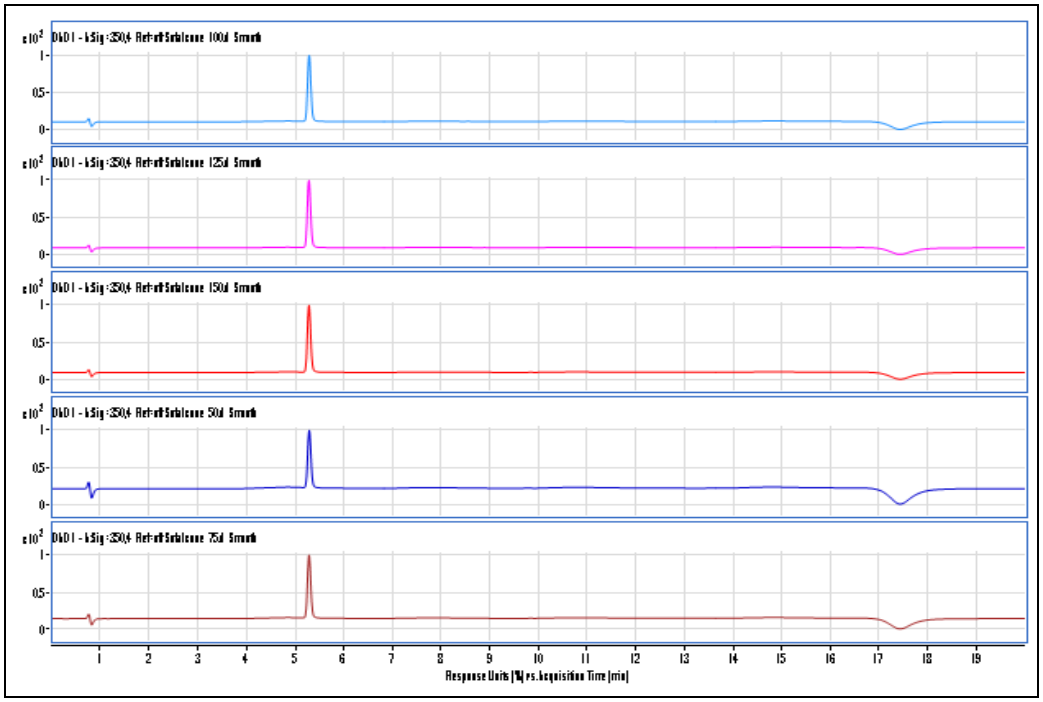


**FIG. 3: OVERLAY CHROMATOGRAM OF SOFALCONE [(A) 50µg/mL (B) 75 µg/mL (C) 100 µg/mL (D) 125µg/mL (E) 150µg/mL]**

**Precision and Accuracy:** The method's precision was verified by repeatability and intermediate precision studies at 100µg/ml concentrations. Six replicate measurements were carried out per day for the repeatability study. The intermediate precision of the method was evaluated by repeating studies three times within the day for intra-day and between the days for inter-day precision **Tables 2** and **3.**

**Specificity:** Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. Typically, these might include impurities, degradants, *etc*. Major degradation impurity peaks are separated from the analyte peak. The purity was found above 990. Hence, the method can be termed as specific **Fig. 4**.

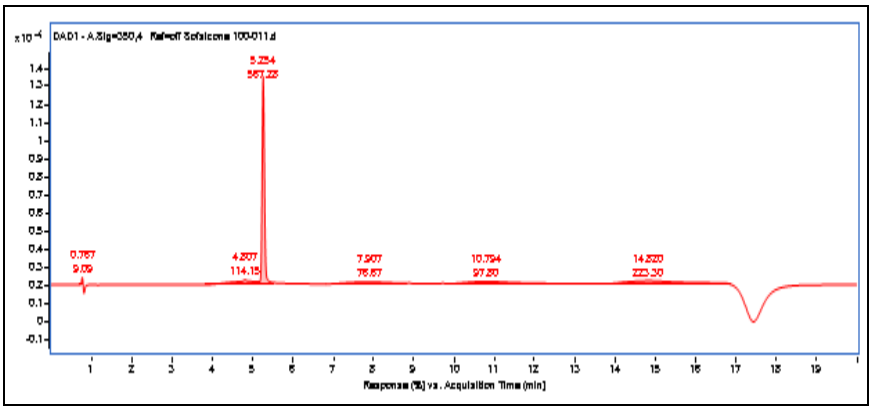


**FIG. 4: CHROMATOGRAM OF MARKETED FORMULATION WITH OPTIMIZED CHROMATOGRAPHIC CONDITIONS**

**Limit of Detection (LOD) and Limit of Quantification (LOQ):** As per ICH guidelines following equations were used to determine the LOD & LOQ

$$LOD = 3.3 \times a/ S$$

$$LOQ = 10 \times a/ S$$

Where a is the standard deviation of y-intercepts & S is the calibration curve slope regression line.

**Robustness:** Deliberate changes were made in the optimized working parameters of the developed method. Changes were made in temperature conditions, wavelength & instrument. Retention time, plate count and peak asymmetry parameters were observed.

**Force Degradation of Sofalcone:**

**Acidic and Alkaline Degradation:** Capsule powder equivalent to 100 mg of Sofalcone (2 Capsule) was transferred in 100 ml volumetric flask. To it 1 ml of 1N HCl was added and kept for 1 hour at room temperature. After that, neutralization was done by using 1ml of 1N NaOH. makeup volume with diluent, sonicate it for 10 min, then centrifuge it for 25min at 5000 rpm and then filter by using a syringe filter and further dilute by taking 1ml from the above solution and up to 10ml with diluent. (Same procedure was carried out for condition of 1ml of 1 N HCL for 3hours room temperature.

**Oxidative Degradation:** Capsule powder equivalent to 100 mg of Sofalcone (2 Capsule) was transferred in 100 ml volumetric flask. 1 ml of 3 % $H_2O_2$ was added and kept for 3 hours at room temperature. Make up volume with diluent, sonicate it for 10 min then centrifuge it for 25min at 5000 rpm and then filter by using syringe filter and further diluted with taking 1ml from the above solution and up to 10ml with diluent.

**Thermal Degradation:** 100 mg of drug was stored at 80°C for 3 hour in oven separately. The drug was then properly diluted to reach a final concentration of 100 µg/ml. The chromatograms were run by injecting the sample in the column.

**Photolytic Degradation:** 100 mg of drug was dissolved in 10 ml of Millipore water. The solutions were kept in the UV light for 3 h. The drug was then properly diluted to reach a final concentration of 100µg/ml & analyzed by the optimized HPLC method.

**Analysis of Marketed Formulation:** A marketed formulation of Sofalcone (Sofalco) was procured from a local pharmacy. The tablet was crushed & it was diluted to get a solution equivalent to 100µg/ml of Sofalcone and was analyzed using the optimized chromatographic conditions. The chromatogram was also observed for the unwanted peaks due to excipients present in the formulation at the optimized RT, to confirm the specificity of the method

## RESULTS AND DISCUSSION:

**Linearity and Range:** The proposed method was found linear in the range of 50µg/ml-150µg/ml with a regression coefficient of 0.9954 and the regression line had a slope of 10.163 and y-intercept 32.09 (equation y = 10.163x –32.09) **Table 1 Fig. 1.**

**TABLE 1: LINEARITY & RANGE PARAMETERS**

| Sr. no. | Parameter | Result |
|---|---|---|
| 1 | Linearity (range) (µg/ml) | 50-150 |
| 2 | Retention time (min) | 5.3 |
| 3 | Regression coefficient ($r^2$) | 0.9954 |
| 4 | Slope | 10.163 |
| 5 | Intercept | 32.09 |
| 6 | Equation | y =10.163x -32.09 |

**Precision:** In precision studies, the area values were obtained for both intraday & interday precision. The % RSD of three concentrations 100 µg/ml for intraday were 0.98, 0.69 & 0.60, while it was 0.46, 0.81 & 1.09 for interday studies.

All three concentrations were found with no significant change as the values of % RSD was within the limit (<2%) **Table 2.**

**TABLE 2: INTRADAY & INTERDAY PRECISION PARAMETERS**

| Inter-day (n=3) | | | Intra-day (n=3) | | |
|---|---|---|---|---|---|
| Conc. (µg/ml) | Mean ± SD | %RSD | Conc. (µg/ml) | Mean ± SD | %RSD |
| 100 | 100.92 ± 1.21 | 1.20 | 100 | 100.00 ± 1.03 | 1.03 |
| 100 | 100.28 ± 1.01 | 1.00 | 100 | 101.20 ± 0.81 | 0.80 |
| 100 | 100.67 ± 0.93 | 0.92 | 100 | 100.53± 1.39 | 1.38 |

**Accuracy:** In this study 50%, 100% & 150% of 100 µg/ml solution was added to the 100 µg/ml& analyzed by the proposed method & the % RSD for these three added concentrations, found were 0.0527%, 0.0153% and 0.0903% respectively. The method was found accurate as good recoveries (100.02- 100.55%) were obtained for various added concentrations **Table 3.**

**TABLE 3: ACCURACY PARAMETER OF VALIDATION FOR THREE DIFFERENT CONCENTRATIONS**

| Sr. no. | (%) Spiked | Conc. from formulation | Standard Conc. Added | Conc. Recovered | % Recovery | % RSD |
|---|---|---|---|---|---|---|
| 1 | 50% | 100 | 50 | 50.15 | 100.30 | 0.0527 |
| 2 | 50% | 100 | 50 | 50.20 | 100.40 | |
| 3 | 50% | 100 | 50 | 50.19 | 100.38 | |
| 4 | 100 % | 100 | 100 | 100.02 | 100.02 | 0.0153 |
| 5 | 100 % | 100 | 100 | 100.05 | 100.05 | |
| 6 | 100 % | 100 | 100 | 100.03 | 100.03 | |
| 7 | 150 % | 100 | 150 | 150.58 | 100.38 | 0.0903 |
| 8 | 100 % | 100 | 150 | 150.79 | 100.52 | |
| 9 | 100 % | 100 | 150 | 150.82 | 100.55 | |

**Limit of Detection (LOD) and Limit of Quantification (LOQ):** LOD and LOQ were found to be 10.419 µg/ml and 31.575 µg/ml, respectively, for sofalcone, which means the method can detect and quantify small amount of drug.

**Robustness:** Robustness was examined by evaluating the influence of small variations in the experimental parameters on the proposed method's analytical performance and indicates method reliability. For each small variation, an amount of 2 µl standard solution was applied on TLC plate. Method parameters were changed in wavelength (± 2 nm), Temperature (±5° C), and Flow rate (± 0.05) from their normal values. %RSD values of peak areas (n = 6) were considered for the assessment, and lower values indicated the method's robustness.

**Analysis of Marketed Formulation:** The solution of the marketed formulation (concentration 100µg/ml) was injected at the optimized chromatographic conditions, and 100.91 % recovery was obtained **Table 4 Fig. 4.**

**TABLE 4: ANALYSIS OF MARKETED FORMULATION**

| Formulation | Labeled amount (mg) | Amount found (mg) | %Label claim ± SD Assay (n=3) | %RSD |
|---|---|---|---|---|
| Sofalco | 100 | 100.25<br>100.75<br>101.75 | 100.91 ± 0.763 | 0.756 |

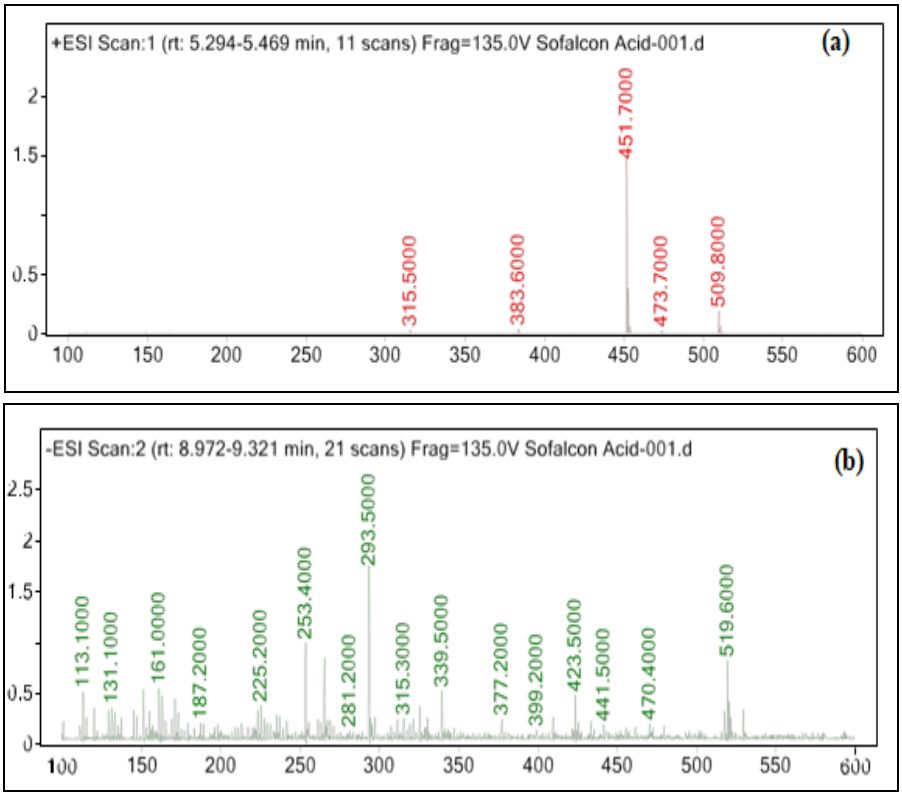
**Reconciling Mass Balance in Force Degradation Studies:** Mass balance correlates the measured loss of a parent drug to the measured increase in degradation products. It is a good quality control check on analytical methods to show that all degradation products are adequately detected and do not interfere with the quantitation of the parent drug (i.e., stability-indicating methods). Regulatory agencies use the mass balance to assess the appropriateness of the analytical method as a stability-indicating method and determine whether all degradants have been accounted.

**Stability-indicating Property:** The stability-indicating properties were tested by applying various stress conditions such as acidic, alkaline, oxidative, thermal and photolytic degradation on standard sofalcone samples.

The degradation studies were optimized to achieve the degradation different reagents, concentrations and time intervals were set to allow appropriate degradation of sofalcone at room temperature. For acidic, the sofalcone undergoes 24.33% degradation by 1 N HCl and 3.27% degradation by 1 N HCl within 3 hours. The acidic degradation product was formed at RRT value of 0.58 and well resolved from the standard sofalcone peak at RT value of 0.58. It indicates that the sofalcone drug is more prone to degrade and sensitive towards acidic conditions. For alkaline, it undergoes 3.27% degradation by 1 N NaOH within 3 h. The alkaline degradation product was formed at RRT value of 0.52.

For oxidative, sofalcone experiences 8.57% degradation by 3% $H_2O_2$ with Two degradation product at RRT value of 4.78 and 1.39 within 3 h. The degradation products were observed and well resolved from the standard peak.

For Photolytic, sofalcone experiences 32.48 % degradation by 3% $H_2O_2$ with one degradation product at RRT value of 1.39 within 24 h. The sofalcone drug was stable over thermal conditions because no degradation was observed at 80°C for 30 minutes **Table 5 Fig. 5(A), (B), (C), (D), (E), (F).**



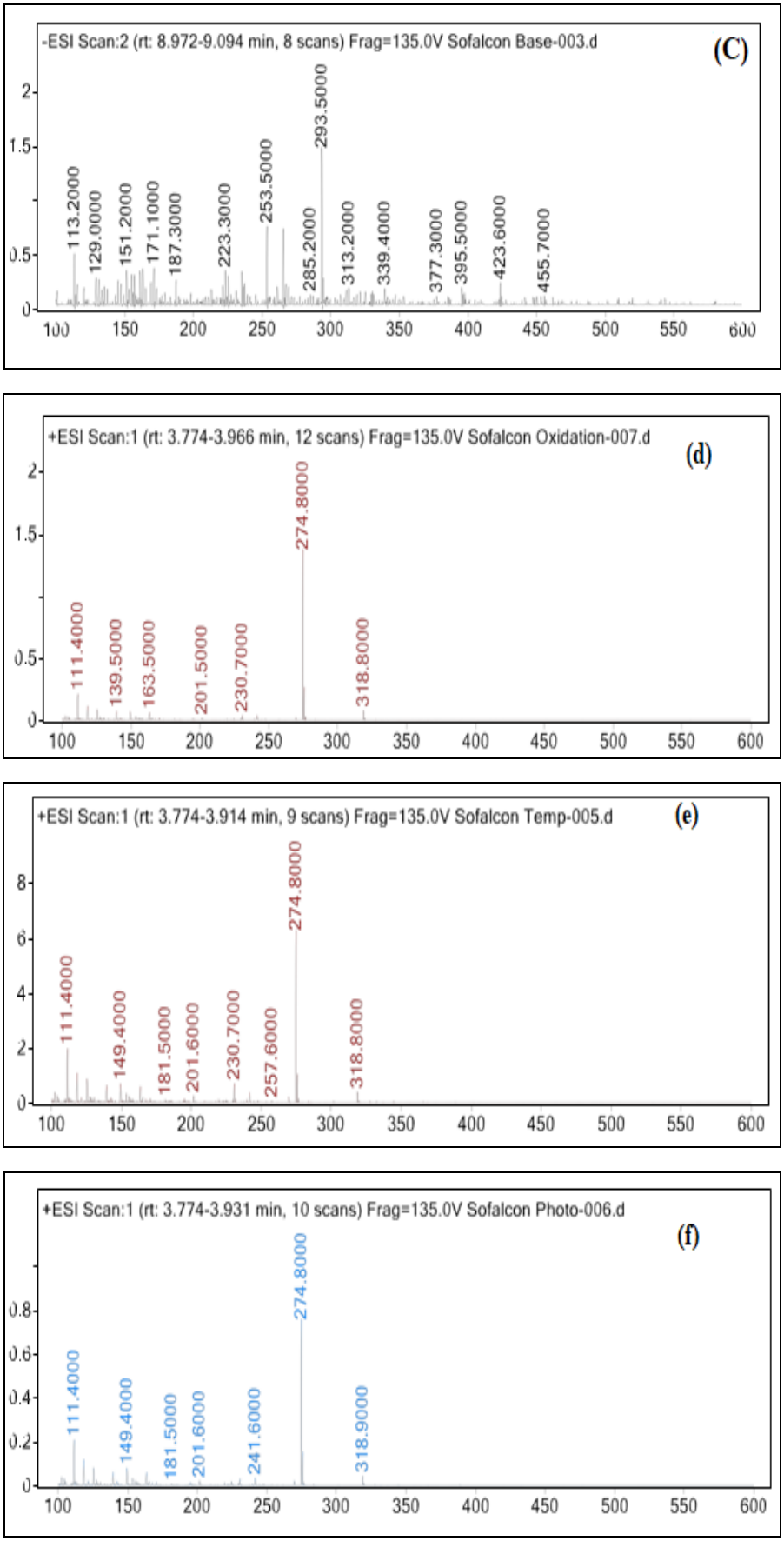



**FIG. 5: MASS SPECTRA OF (A) STANDARD SOFALCONE AND DEGRADATION PRODUCT OF (B) ACIDIC (1 N HCL), (C) BASIC (1 N NAOH), (D) OXIDATIVE (30% $H_2O_2$), (E) THERMAL (80°C), AND (F) PHOTO (UV LIGHT)**

**TABLE 5: ANALYSIS OF RECONCILING MASS BALANCE IN FORCE DEGRADATION STUDIES**

| Conditions | Unspecified impurity at RRT 0.58 | Unspecified impurity at RRT 4.7 | Unspecified impurity at RRT 1.39 | %Total impurities | Assay (%) | Mass Balance (% Total impurities + % Assay) (%) | Mass Balance wrt to as such sample |
|---|---|---|---|---|---|---|---|
| As such (Unstressed Sample) | ND | ND | ND | 0 | 102.5 | 102.5 | NA |
| Acid degradation (1N HCl, RT, 3 Hours) | 8.9 | ND | ND | 24.33 | 77.67 | 102.0 | 99.5 |
| Base degradation (1N NaOH, RT, 3 Hours) | 2.14 | ND | ND | 3.27 | 95.71 | 98.98 | 96.6 |
| Oxidation degradation (5ml 30 % $H_2O_2$, RT , 1 Hours) | ND | 1.55 | 5.21 | 8.57 | 92.47 | 101.04 | 98.6 |
| Thermal degradation (80°C, 3 hours) | ND | ND | 2.12 | 3.49 | 97.01 | 100.5 | 98 |
| PhotoDegradation (UV light for 3hours) | ND | ND | 19.73 | 32.48 | 66.92 | 99.4 | 97 |

**Analysis of Degradation Products:** To identify the degradation products by LC-MS/MS, the standard and processed test solutions were direct-injected to MS in full scan mode (Q1 with positive and negative ionization modes) with water (0.1% FA)–Ammonium acetate in methanol (30:70, v/v) as the mobile carrier phase at 0.3 ml flow rate. The applied collision energy was 25 eV, and the total run time was 20 min. The ESI positive ionization mode showed the mass spectra of major peaks, and the obtained values of mass-to-charge ratio (m/z) were used to identify degradation products.

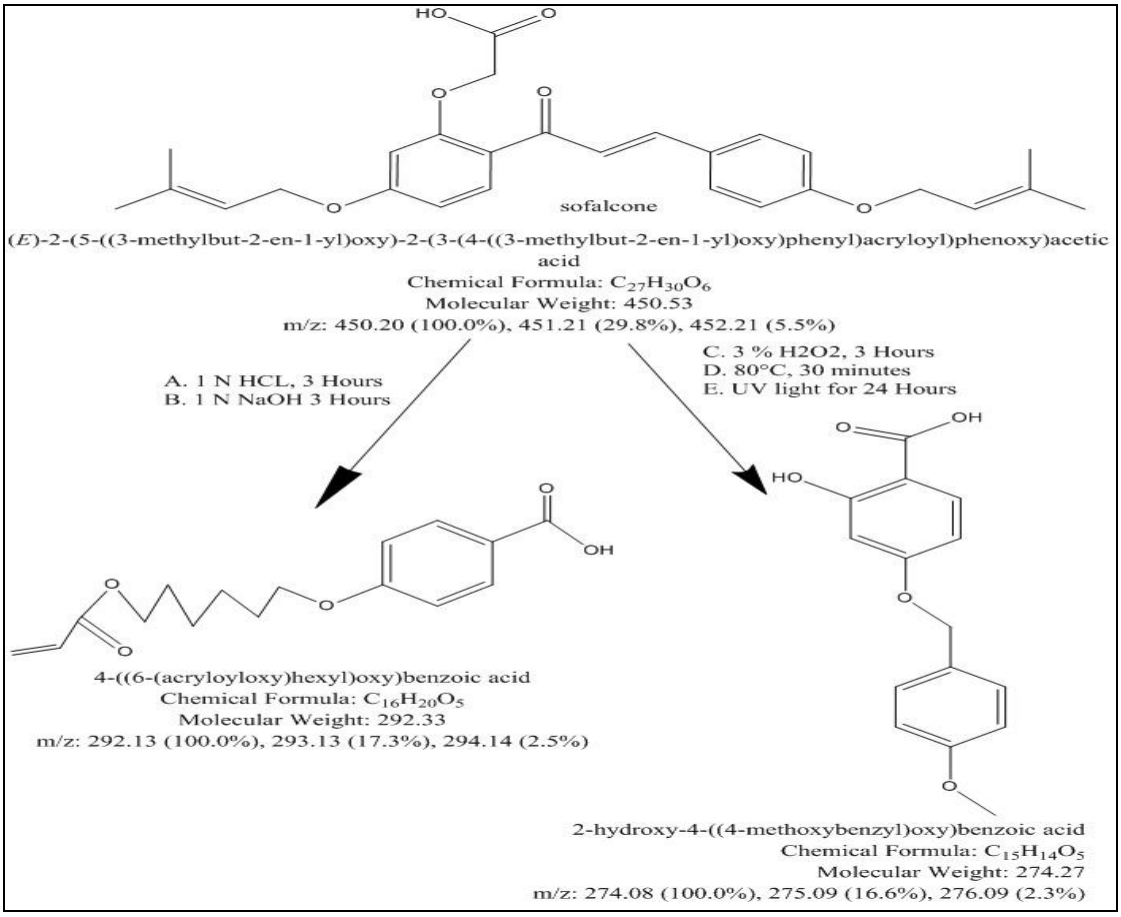



**FIG. 6: THE PROPOSED DEGRADATION PATHWAY OF SOFALCONE IN DIFFERENT STRESS CONDITIONS**

The mass spectra of standard and degradation products are shown in **Fig. 5** to **6**. The standard mass spectra of sofalcone have observed values of m/z 293.50, 201, 327, 543, 274.8 and 465 as major fragments, out of which m/z 451.7 is the molecular ion peak of sofalcone. The mass spectra of degradation products of acidic, alkaline and oxidation samples showed common m/z at 293.50 apart from the major observed values of m/z in standard sofalcone mass spectra. Likewise, the mass spectra of another oxidative & Photolytic degradation product showed 293.50 and 274.8 m/z values. From there, it could be proposed that the values m/z 293.50 (impurity 1) and m/z 274.8 (impurity 2) are matched with the corresponding molecular formula $[C_{16}H_{20}O_5]$ + (calc. 292.33) and the molecular formula $[C_{15}H_{14}O_5]$ + (calc. 274.27) after the elimination of Benzoic acid $[M\text{-}C_7H_6O_2]-$ from the moiety of {(acryloyl) phenoxy}, respectively.

The small and constant difference in the observed and calculated values indicates that the efficient protonation of sofalcone in positive mode could be expected due to the mobile phase used for LC-MS/MS. Therefore, the m/z 293.50 was a major degraded product in acidic, alkaline and oxidative conditions, and m/z 274.8 was a second degradation product in oxidative and photolytic conditions. The proposed structure and molecular formulas of all degraded products are shown in **Fig. 6** and the outcome of this study was a counterpart in terms of degradation seen under acid hydrolysis, Photolytic and oxidative degradation conditions and stable over thermal and Base. The first UHPLC MS/MS method for identifying and quantitatively determining sofalcone and its degradation products has been developed.

**CONCLUSION:** The first UHPLC method for the identification and quantitative determination of Sofalcone and its degradation products has been developed. The chromatographic development was done by using simple mobile phase Water (0.1% Formic Acid) and Ammonium Acetate in Methanol were the mobile phase, run at a flow rate of 0.3 ml/min for gradient elution. The mean values of recovery were found to be 100.02% and 100.55%, which shows the higher and reproducible sensitivity of the method. The developed method meets the acceptance criteria for the validation parameter per the ICH guidelines. The method does not allow any interference of matrices. Higher recoveries and lower RSD (<2%) of peak areas demonstrated that the method is specific, accurate, precise, and robust for the quantitative determination of sofalcone. The result of forced degradation reveals that the method is selective and stability-indicating. The drug was highly susceptible to acidic, alkaline, and oxidative degradation. The drug was found stable from thermal and photolytic effects.

This study was also extended to identify the degradation product using LC-MS/MS analysis and the proposed degradation pathway. The proposed method is simple, precise, specific, accurate, less time-consuming, and cost-effective, and it can separate the drug from its degradation products. The developed method can be used in quality control laboratories for drug stability studies.

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**CONFLICTS OF INTEREST:** Nil

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