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DoE ASSISTED UV-VISIBLE SPECTROPHOTOMETRIC METHOD DEVELOPMENT FOR CRITICAL FACTOR ESTIMATION INVOLVED IN NORFLOXACIN DEGRADATION

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ABSTRACT: Degradation studies are essential to determine the inference of degradation routes and stability of pharmaceuticals at different stress conditions. Norfloxacin is an extensively used antibiotics; hence, it is also commonly found in wastewater effluent. For proper risk assessment of norfloxacin and for better understanding of the factors involved in its environmental fate, different factors were studied which were involved in the degradation of norfloxacin. Effect on degradation was studied for three factors, *i.e.,* time (24 h, 48 h, and 72 h), temperature (30°, 45°, and 70°), and concentration of oxidant (1% H_2O_2, 3% H_2O_2, and 5% H_2O_2) at three different levels using Design of experiment (DoE) approach. Absorbance was selected as a response that would signify the extent of degradation of the drug. It was observed that all three factors were involved in degradation but when the factors are studied one at a time, the effect of temperature and concentration of oxidant was more significant and time has a very small effect on the degradation of norfloxacin. When multiple factors were studied at one time, the interaction between the factors is observed, which are represented by 2D plots and 3D contour plots.

INTRODUCTION: Norfloxacin is one of the commonly used first-generation fluoroquinolone based antibacterial drug. It is being used for the treatment of urinary tract infections for past many years. Chemically, it is 1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7(1-piperazinyl)-3-quinoline carboxylic acid [1-4]. The Norfloxacin acts by inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are essential for bacterial DNA replication, transcription, repair, and recombination [5].

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Norfloxacin acts differently from penicillins, cephalosporins, aminoglycosides, macrolides, and tetracyclines; therefore, the microorganisms which are resistant to these classes of drugs may be susceptible to and other fluoroquinolones [6]. Due to the common usage of Norfloxacin, it has been very commonly found in wastewater effluent. This can lead to a potential risk to the ecosystem and human health due to increased bacterial drug resistance [7].

In a study, 20 degradation products were found for ciprofloxacin and norfloxacin in ozonated waste-water [8]. Several analytical methods for norfloxacin have been described in scientific literature, such as UV spectrophotometry, liquid chromatography, high-performance thin-layer chromatography, *etc*., amongst others [9-12].

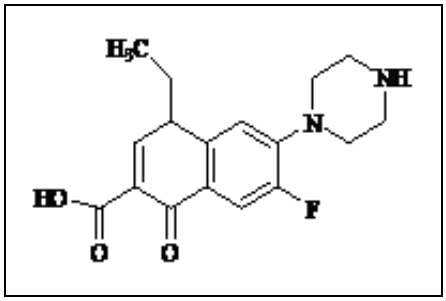

FIG. 1: NORFLOXACIN

Forced Degradation Studies: Forced degradation studies or stress studies are conducted on the sample using different conditions such as acid, alkaline, oxidative, thermal, and photolytic degradations. The sample is exposed to these conditions for different time intervals followed by instrumental analysis, usually by HPLC or LC-MS [13-15].

Regulatory Guidelines: There are various International guidelines that recommend forced degradation studies [16-18]. The most commonly followed guidelines are of ICH:

ICH Q1A: Stability Testing of New Drug Substances and Products,

ICH Q1B: Photostability Testing of New Drug Substances and Products,

ICH Q2B: Validation of Analytical Procedures: Methodology.

The objective of forced degradation is to (i) Development of degradation pathways of drug substances and drug products. (ii) Recognizing the chemical properties of drug molecules. (iii) Elucidation of the structure of degradation products. (iv) Resolve stability-related problems. (v) Establishment of the intrinsic stability of a drug substance in the formulation. (vi) Identification of the degradation mechanisms of the drug substance and drug product. (vii) Distinguish between degradation products related to drug products from those that are generated from non-drug products in a formulation. (viii) Generation of stability-indicating nature of a developed method. (ix) Production of more stable formulations and determination of expiry date of a particular formulation. (x) Generation of degradation profile same as that observed in a formal stability study under ICH conditions [19-22].

Degradation can be induced by the Several Conditions:

Acidic and Basic Conditions: The stability of the drug substance depends upon the class and concentrations of acid or base taken. Hydrochloric acid or sulphuric acids (0.1-1 M) used for acid hydrolysis, whereas sodium hydroxide or potassium hydroxides (0.1-1M) are used for base hydrolysis [23, 24]. For poorly soluble compounds, co-solvents are used [24].

Thermal Condition: According to ICH Q1A accelerated testing conditions, thermal degradation (*e.g.,* dry heat and wet heat) should be carried out under more strenuous conditions. For dry and wet heat, samples of solid-state drug substances and drug products should be exposed. For dry heat, liquid drug products should be exposed. At higher temperatures, studies performed at a shorter period. Thermal degradation of a substance can be studied by Arrhenius equation [26].

Photolytic Condition: The drug substance didn't affect by light exposure but affected by photo stability. For primary degradants of drug substance photostability performed by exposure to UV or fluorescent conditions. Various ICH guidelines are recommended for photo stability testing [27].

Humidity: The potential degradants in the finished product and active pharmaceutical ingredient is humidity. For the establishment of forced degradation samples 90% humidity for one week is recommended [28].

Oxidation Condition: Hydrogen peroxide is commonly used for oxidation. Metal ions, oxygen, MnO_2, ozone, and radical initiators: azobi-isobutyro-nitrile (AIBN) also used for oxidation degradation. The structure of the drug helps in selecting the concentration and condition of oxidizing agents. In the oxidative degradation of drug substance, an electron transfer mechanism is most common [25]. There are various oxidative degradation products reported in literature. The most common degradation pathway for degradation is (i) oxidation of the piperazinyl substituent, (ii) monohydroxylation, and (iii) formation of dimeric products. **Fig. 2** shows some of the common degradation products of Norfloxacin formed by oxidation of piperazinyl substituent [29].

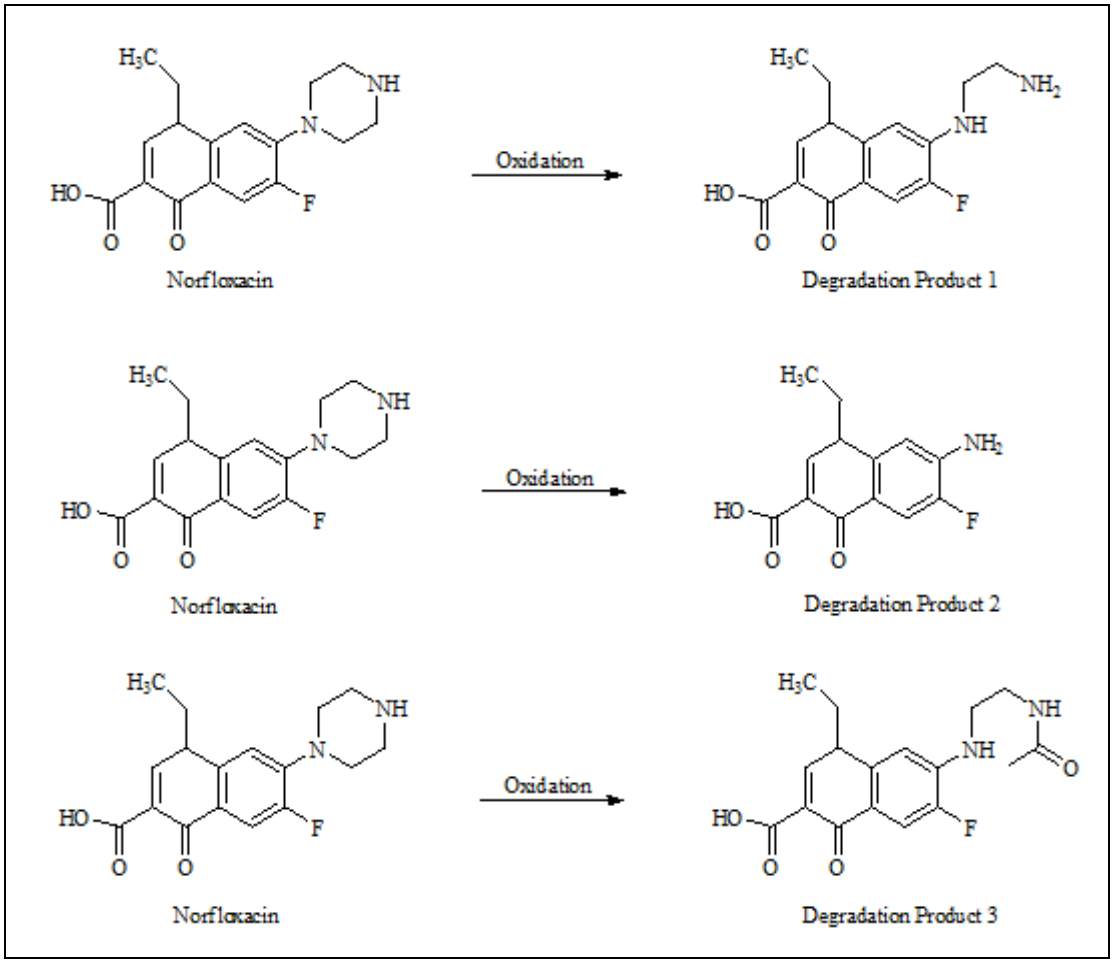

FIG. 2: DEGRADATION PRODUCTS OF NORFLOXACIN FORMED BY OXIDATION OF PIPERAZINYL SUBTITUENT USING LACCASE MEDIATOR 2,2-AZINO-BIS-(3-ETHYLBENZTHIAZOLINE-6-SULFONIC ACID) DIAMMONIUM SALT

MATERIALS AND METHODS:

Chemicals and Reagents: Analytically pure Norfloxacin was obtained as a gift sample from Nakoda Chemicals Hyderabad, India. All chemicals and reagents used were of analytical grade. Hydrogen peroxide was obtained from Central Drug House (P) Ltd. Sodium hydroxide was obtained from Central Drug House (P) Ltd.

Instrumentation: UV Visible Spectrophotometer (Shimadzu 1800), Hot Plate (HICON), Water Bath (HICON), Micropipette (CWS Series), Weighing Balance (Scale-Tec), Distilled water (In-house distillery).

Sample Preparation:

Forced Degradation in Oxidation Condition: A solution of 3% H_2O_2 was prepared by adding 10ml H_2O_2 in 100ml distilled water. 5mg Norfloxacin was added in a volumetric flask, and volume was made up to 100ml with H_2O_2, and it was labeled as stock solution (50ppm). 10ml was drawn from the stock solution in a volumetric flask, and the volume was makeup to 100ml with 3% H_2O_2 (5ppm). A volumetric flask containing 5ppm norfloxacin was kept at 80°C for 96 h in a water bath. At an interval of 24 h (24, 48, 72, 96 h) sample was drawn, and a spectrum was taken.

Central Composite Design (CCD) part of Response Surface Design was used to find the impact of temperature, oxidizing agent, and time on the degradation of Norfloxacin under oxidative conditions. The factors which were selected for the study were the concentration of the oxidizing agent, time, and temperature, as shown in **Table 1**. Each factor was studied at three different levels

1. **Concentration of oxidizing agent:** Levels- 1%, 3% and 5%.
2. **Time:** Levels- 24-h, 48-h and 72-h.
3. **Temperature:** Levels- 30°, 45° , and 70°.

TABLE 1: DESIGN WITH FACTORS AS CONCENTRATION OF OXIDANT, TIME AND TEMPERATURE

Run	Concentration of Oxidant	Time	Temp.
1	0	1	0
2	1	-1	-1
3	-1	1	1
4	0	0	1
5	0	-1	0
6	0	0	0
7	-1	-1	1
8	-1	0	0
9	0	0	-1
10	0	0	0
11	1	1	1
12	-1	-1	-1
13	0	0	0
14	0	0	0
15	1	0	0
16	0	0	0
17	0	0	0
18	1	1	-1
19	1	-1	1
20	-1	1	-1

TABLE 2: CODING THE FACTORS AFFECTING OXIDATION DEGRADATION

Factors	Codes		
	-1	0	1
Concentration of oxidant (%)	1	3	5
Time (hours)	24	48	72
Temperature (°C)	30	45	70

TABLE 3: ABSORBANCE OF NORFLOXACIN AT DIFFERENT CONCENTRATION

S. no	Concentration (ppm)	Absorbance
1	0	0
2	2.5 ppm	0.462
3	5 ppm	0.813
4	7.5 ppm	1.146
5	10 ppm	1.546

RESULTS: The samples were taken according to table 1 and were analyzed using spectrum mode of UV spectrometer at wavelength 272.4 nm. Absorbance was taken as the response, and the data was analyzed using Design expert 11. The responses obtained are shown in **Table 4**.

Spectra of different samples are shown from **Fig. 3-11**. A calibration plot was made using different concentrations of norfloxacin (2.5ppm, 5ppm, 7.5ppm, and 10ppm) to check linearity. With an increase in time (24 h to 72 h), a decrease in absorbance is observed, which means increased degradation though the effect is low as compared to that of temperature and concentration of oxidant. **Fig. 6** shows the graph for this trend. When two factors (a) concentration of oxidant and (b) time (temperature is kept constant as 45 °C) are studied simultaneously, it is observed that degradation is minimum at low concentration and at low time point. The degradation increases with increased concentration of oxidant and for a longer period of time. **Fig. 7** shows the graph for this trend. When two factors (a) concentration of oxidant and (b) temperature (time is kept constant as 48 h) are studied simultaneously, it is observed that degradation is minimum at low concentration and at low time point. The degradation increases with increased concentration of oxidant and for a longer period of time. **Fig. 8** shows the graph for this trend. When two factors (a) time and (b) temperature (concentration of oxidant is kept constant as 3%) are studied simultaneously, it is observed that degradation is minimum at low concentration and at low time point. The degradation increases with increased concentration of oxidant and for a longer period of time. **Fig. 9** shows the graph for this trend.

Contour plots **Fig. 10, Fig. 11,** and **Fig. 12** shows the interaction between the factors. Absorbance is less when all three factors are at their lowest levels. When the concentration of H_2O_2 has increased to 3% the absorbance decreases (time and temperature at their lowest level), but in **Fig. 12** the absorbance increases to some extent when all the three factors are at their maximum levels.

TABLE 4: ABSORBANCE (RESPONSE) FOR DIFFERENT LEVELS OF SELECTED FACTORS CONCENTRATION OF OXIDANT, TIME, AND TEMPERATURE

Run	Factor 1 (Conc. of Oxidan)	Factor 2 (Time)	Factor 3 (Temp.)	Response (Abs)
1	0	1	0	0.629
2	1	-1	-1	0.501
3	-1	1	1	0.560
4	0	0	1	0.568
5	0	-1	0	0.548
6	0	0	0	0.502
7	-1	-1	1	0.541
8	-1	0	0	0.567
9	0	0	-1	0.533
10	0	0	0	0.524
11	1	1	1	0.495
12	-1	-1	-1	0.987
13	0	0	0	0.509
14	0	0	0	0.511
15	1	0	0	0.599
16	0	0	0	0.516
17	0	0	0	0.512
18	1	1	-1	0.642
19	1	-1	1	0.595
20	-1	1	-1	0.585

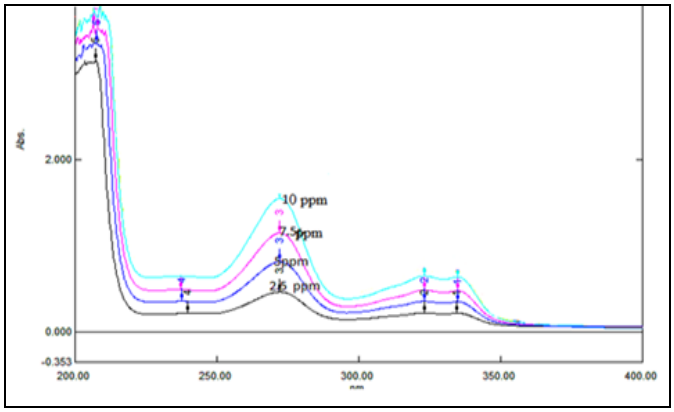

FIG. 3A: OVERLAY SPECTRUM OF NORFLOXACIN WITH 2.5ppm, 5ppm, 7.5ppm AND 10ppm

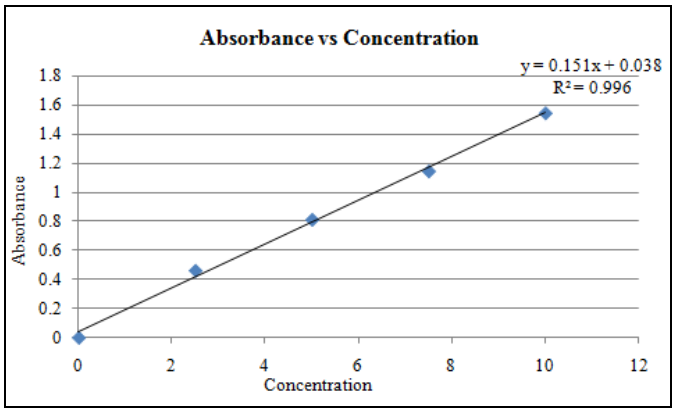

FIG. 3B: CALIBRATION PLOT OF NORFLOXACIN WITH 2.5ppm, 5ppm, 7.5ppm AND 10ppm

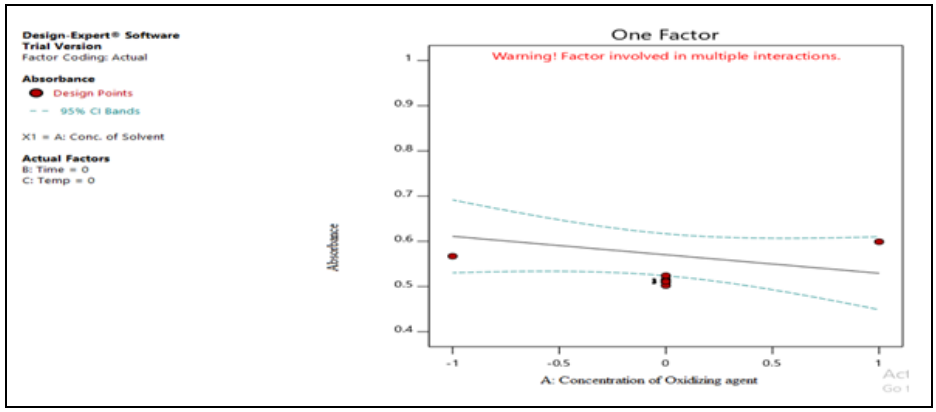

FIG. 4: PLOT SHOWING EFFECT OF SINGLE FACTOR *i.e.*, CONCENTRATION OF OXIDIZING AGENT

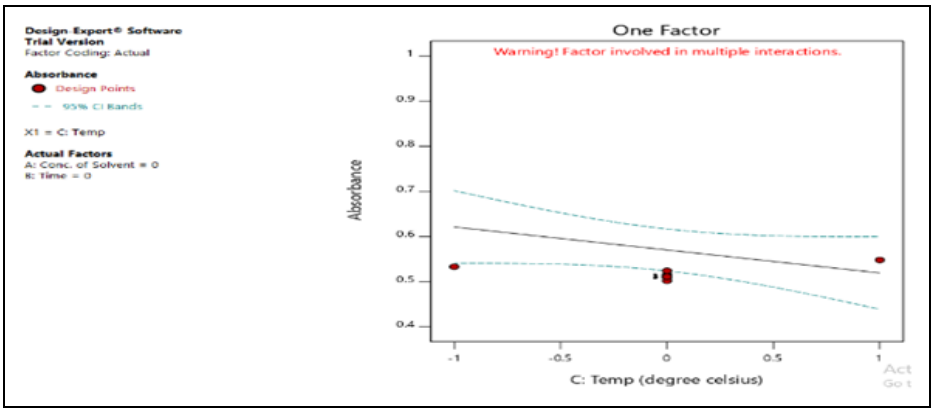

FIG. 5: PLOT SHOWING EFFECT OF SINGLE FACTOR *i.e.*, TEMPERATURE

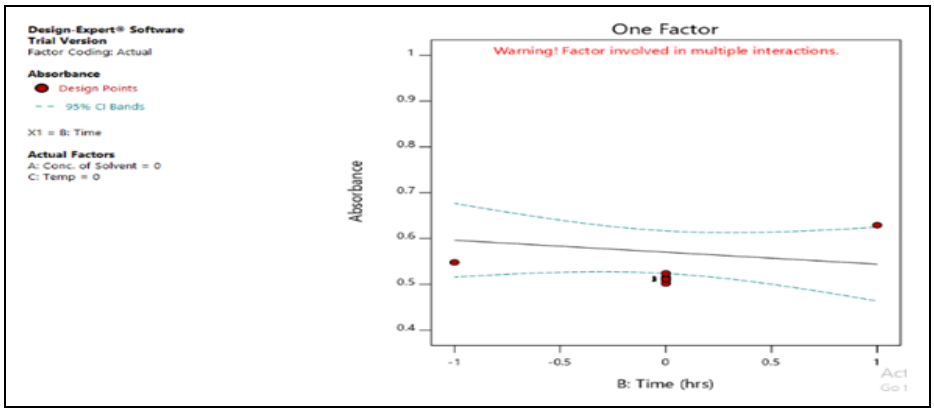

FIG. 6: PLOT SHOWING EFFECT OF SINGLE FACTOR *i.e.*, TIME

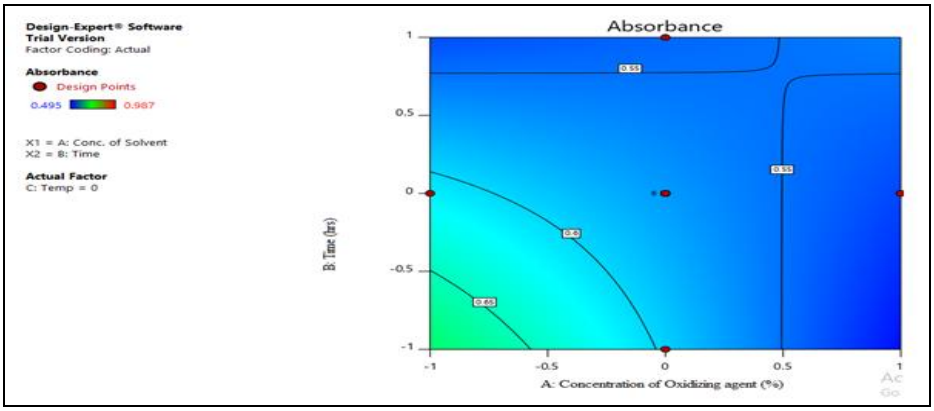

FIG. 7: PLOT SHOWING SIMULTANEOUS EFFECT OF TWO FACTORS *i.e.*, CONCENTRATION OF OXIDANT AND TIME

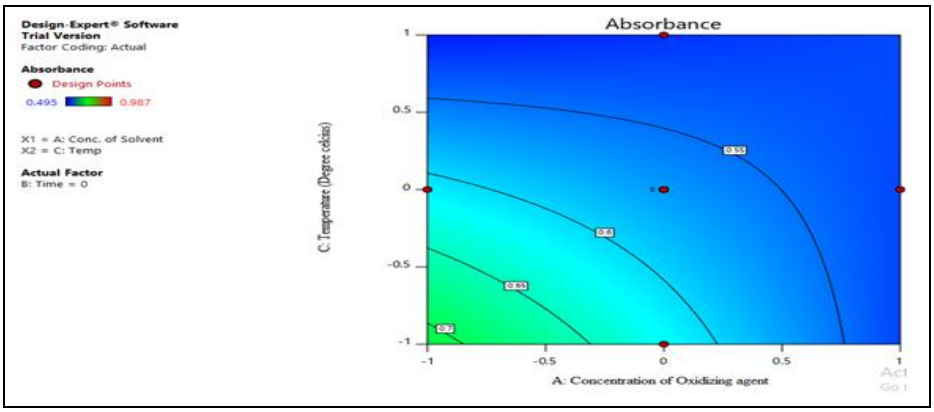

FIG. 8: PLOT SHOWING SIMULTANEOUS EFFECT OF TWO FACTORS *i.e.*, CONCENTRATION OF OXIDANT AND TEMPERATURE

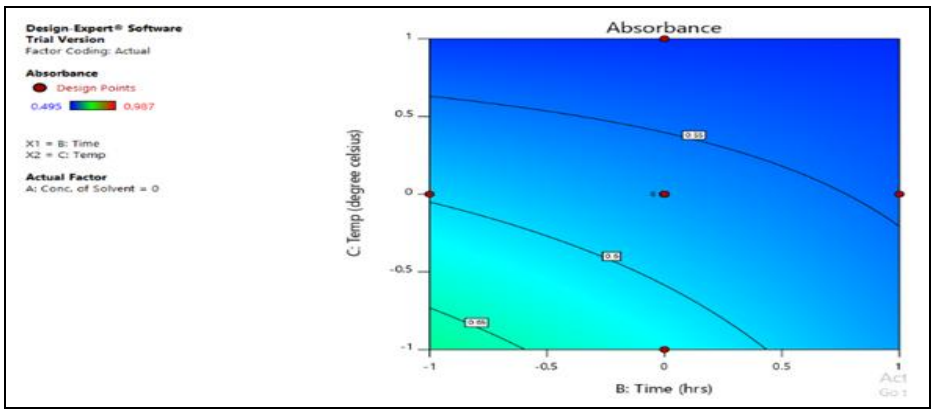

FIG. 9: PLOT SHOWING SIMULTANEOUS EFFECT OF TWO FACTORS *i.e.*, TIME AND TEMPERATURE

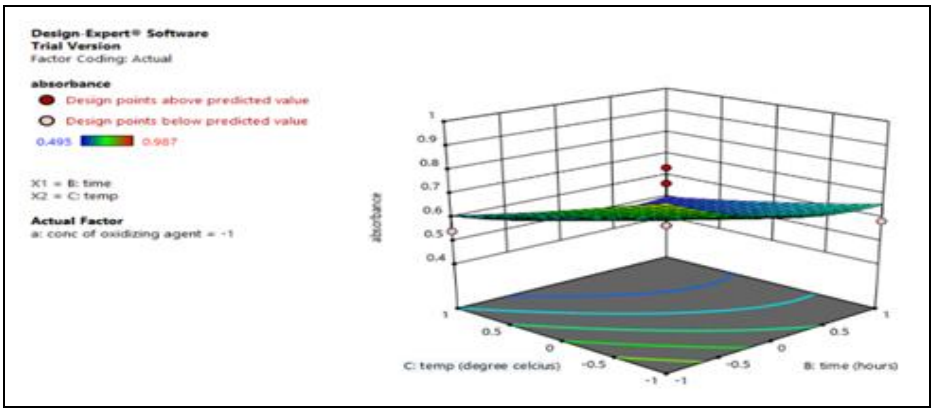

FIG. 10: CONTOUR PLOT BETWEEN TEMPERATURE AND TIME WHEN H_2O_2 IS 1%

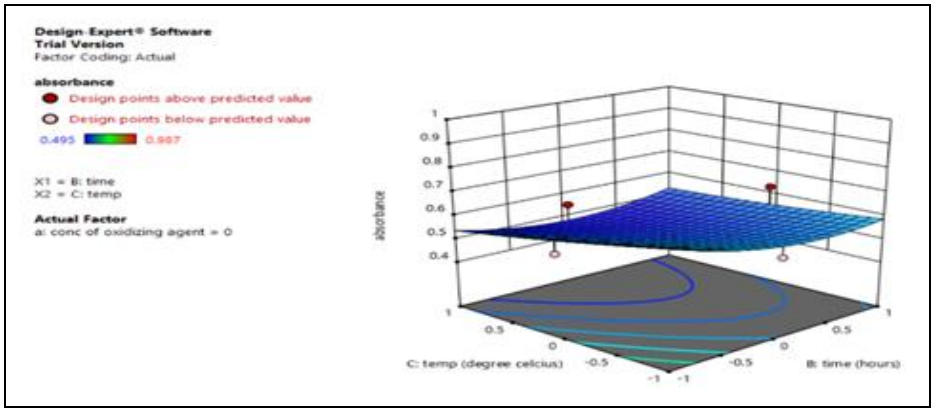

FIG. 11: CONTOUR PLOT BETWEEN TEMPERATURE AND TIME WHEN H_2O_2 IS 3%

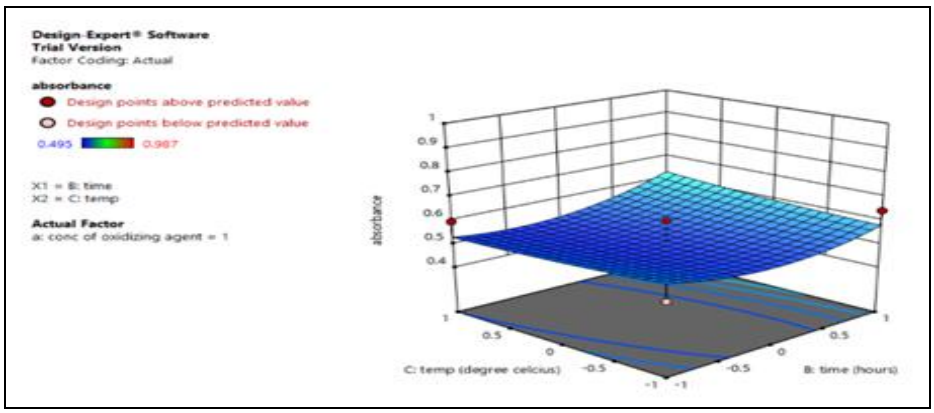

FIG. 12: CONTOUR PLOT BETWEEN TEMPERATURE AND TIME WHEN H_2O_2 IS 5%

CONCLUSION: Study of factors affecting norfloxacin's degradation gives a better picture of the environmental fate of norfloxacin, which is often found as a wastewater effluent in a significant amount. All three factors, the concentration of oxidant, temperature, and time were involved in degradation. The single-factor response shows degradation is mostly affected by the temperature and concentration of oxidant. When multiple factors are studied at one time, it is observed that all factors have a significant effect. Degradation is less at a low concentration of oxidant, temperature, and time but it increases when any of the factors is increased. Contour plots showed an interaction between factors and degradation significantly increases with increase in the concentration of oxidant even when the temperature and time are at low levels. It is also observed that there is some increase in absorbance when all three factors are at their maximum levels.

This is an interesting observation, and the increase in response may be due to the formation of a degradation product with higher absorbance than the parent compound. Though, to validate it, sophisticated instruments LC-MS are required. To recapitulate, the study will be helpful to control or manipulate the degradation of Norfloxacin to reduce its risk. It will give a better understanding of the factors involved in the rate of degradation of Norfloxacin.

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CONFLICTS OF INTEREST: Nil

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