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ICH GUIDANCE IN PRACTICE: DEVELOPMENT OF A VALIDATED STABILITY-INDICATING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY METHOD FOR FEBUXOSTAT AND DEGRADATION KINETIC STUDY IN ACID HYDROLYTIC CONDITION

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Keywords:
Febuxostat, Stability-indicating assay method, HPLC, UV, LC-MS, Degradation Kinetic

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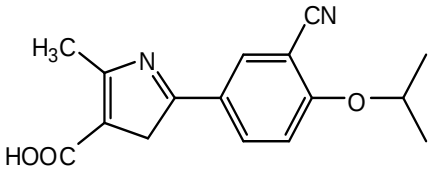
ABSTRACT

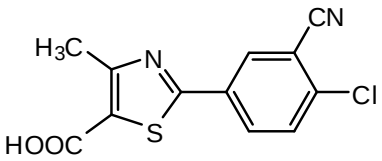
The degradation behavior of Febuxostat was investigated under different stress degradation (hydrolytic, oxidative, photolytic and thermal) conditions recommended by International Conference on Harmonization (ICH) using HPLC and LCMS. Febuxostat was found to degrade significantly in acidic and alkaline conditions as well as in neutral hydrolysis. The drug was stable to dry heat, photolytic degradation and under oxidative condition. Resolution of drug and the degradation products formed under different stress studies were successfully achieved on a C-18 column utilizing Methanol- water (with 0.02%v/v TFA) in the ratio of 95:5 and at the detection wavelength of 315 nm. The method was validated with respect to linearity, precision, accuracy, selectivity and specificity. The degradation kinetic of Febuxostat in acidic condition at different temperature was studied. The reaction order for Febuxostat in aqueous solvent system followed pseudo first order degradation kinetic. The catalytic rate constant and half-life at particular condition were determined. The Arrhenius plot showed the temperature dependence of Febuxostat.

INTRODUCTION: The stability-indicating assay is a method that is employed for the analysis of stability samples in Pharmaceutical industry. The revised parent drug stability test guideline Q1A(R2) issued by International Conference on Harmonization (ICH) requires that stress testing on the drug substance should be carried out to establish its inherent stability characteristics and for supporting the suitability of the proposed analytical procedures [1].

Febuxostat, antigout agent is chemically 2- [3- cyano- 4- (2- methlypropoxy) phenyl]- 4- methlythiazole- 5 - carboxylic acid. It is a non purine selective inhibitor of xanthine oxidase [2] (**Fig. 1**).



A



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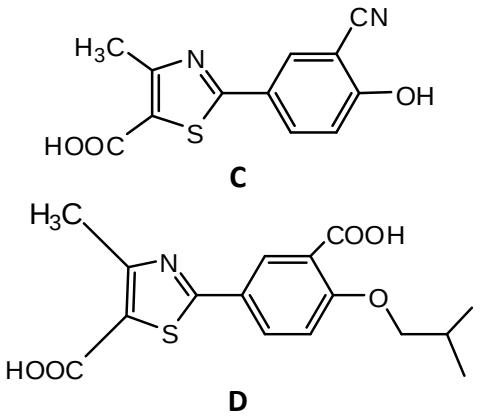


FIGURE 1: STRUCTURE OF FEBUXOSTAT(A) AND DEGRADATION PRODUCTS [DP-1(B), DP-2(C), DP-3(D)].

It inhibits both oxidized and reduced forms of xanthine oxidase [3, 4] and has very less effects on other enzymes of purine and pyrimidine metablism [4, 5]. It is not official in any pharmacopoeia. Based on the literature survey, it shows that very few analytical methods have been reported for the estimation of Febuxostat which includes UV (Drug determination [7] and dissolution [6]), HPLC [8], GC [9] and LC/MS/MS (Impurity profiling) [10].

There is no information in literature on the stability behavior of this drug under any stress conditions. The aims of the present study accordingly were to establish inherent stability of Febuxostat through stress studies under a variety of ICH recommended test conditions[11] and to develop a validated stability-indicating assay.

EXPERIMENTAL:

Materials: Febuxostat bulk drug was obtained from Cadila Healthcare Ltd., Ahmedabad and used without further purification. Tablets (Urifix 40mg) were obtained from the market (manufactured by Precise Biopharma Ltd.) Sodium hydroxide, Hydrochloric acid (were from Astron, Ahmedabad, India) and Hydrogen peroxide (were from Chemdyes corporation, Rajkot, India). HPLC grade methanol, acetonitrile and water were purchased from Rankem, Delhi, India). LCMS grade water and Methanol were purchased form Sigma-Aldrich.

Instrumentation: Stress studies (Hydrolysis) were performed using Heating mentle (EIE instrument Pvt. Ltd. Ahmedabad) with reflux condenser. Dry air oven (SENTWIN, India) was used to study the effect of dry heat.

Photolytic experiments were performed in a photostability chamber equipped with light source (UV Lamp). The study was performed by keeping the samples at a distance of 9 inch from the light bank.

The HPLC system consisted of YL9110 Quaternary solvent delivery Pump, an YL9160 photodiode array (PDA) detector, a Rhenodyne autoinjector, and a degasser module (Lifecare equipment Pvt. Ltd., Mumbai). Data acquisition and processing was performed by use of YL-Clarity software. LC-MS analysis were performed on Agilent Technology 6520, Accurate Mass Q- TOF LC/MS consist of C-18 (4.6 * 50 mm) (3.5 µm) colum and detection was done at ESI positive mode. Degradation kinetic study was performed by both Shimadzu- UV spectrophotometer and HPLC.

Force Degradation studies: Stock solution of Drug in methanol at a concentration of 4 $mgml^{-1}$ was used in all degradation studies. The pH of solutions was measured before and after the reaction and no change was observed. Conditions employed for performing stress studies were as follows.

- **Hydrolytic studies:** Acid decomposition studies were performed by refluxing the solution of drug in 2N HCl at 80 °C for 6 h. The studies in alkaline conditions were done in 0.1N NaOH and the solution was heated at 80 °C for 1 h. For study in neutral conditions, stock solution of drug was refluxed at 80 °C for 18 hr. In acidic and basic hydrolytic study, the solutions were neutralized with proper strength and quantity of base and acid respectively and finally the solution were diluted with methanol to get 20µg/ml.

- **Oxidative studies:** Initial studies were performed in 3% H_2O_2 at room temperature. Subsequently, the stock solution of drug was exposed to 30% H_2O_2 at room temperature for a period of 48 hr and finally the solution was diluted with methanol to get 20µg/ml.

- **Photolytic studies:** Solid drug was spread in 1 mm thickness uniform layer on a Petri dish and exposed in UV stability chamber for some days. From the above sample, at specified time interval, 10mg of drug was accurately weighed in 10ml volumetric flask and made up to mark with

Methanol to get 1000 µg/ml of solution. From the above solution, 0.2 ml of solution was pipette out in 10ml volumetric flask and made up to mark with Methanol to get 20 µg/ml of solution.

- **Thermal studies:** Susceptibility of the drug to dry heat was studied by exposing the solid drug to 80^{o}C for 10 weeks. From the above sample, at specified time interval, 10mg of drug was accurately weighed in 10ml volumetric flask and made up to mark with Methanol to get 1000 µg/ml of solution. From the above solution, 0.2 ml of solution was pipette out in 10ml volumetric flask and made up to mark with Methanol to get 20 µg/ml of solution.

Separation studies on Stressed Samples: In all HPLC runs, the mobile phase was filtered through 0.45µm nylon membrane and degassed before use. The injection volume was 20µl and the mobile phase flow rate was 1 mlmin^{-1}. The analytical wavelength was 315 nm. Preliminary HPLC analyses were performed using a C-18 column and mobile phase composed of different ratios of Methanol, Acetonitrile and water. As satisfactory resolution of the drug and the degradation Products was achieved by using Methanol- water (with 0.02%v/v TFA) in the ratio of 95:5. Separation studies were carried out on samples of different stress conditions (after appropriate dilution) withdrawn at different time periods individually.

LC-MS studies were also performed using the preliminary HPLC method. Satisfactory resolution was obtained with same mobile phase. Retention times of peaks were different as the chromatographic parameters were changed but the sequences of separation remain same.

Degradation kinetic study in acid hydrolytic condition

- **For studying the kinetic order of the reaction:** Into a 250 ml measuring flask, 1 ml of stock solution of Febuxostat (4mg/ml) in methanol was added and diluted up to 20 ml with 2N HCl. These solutions were refluxed in a thermostatically controlled water bath at 80 ºC for 10 hr. 1 ml sample solutions were taken at 1 hr intervals, placed into 10ml measuring flasks, neutralized with 1mL, 2N NaOH and the volume was completed with Methanol. The solutions were injected in the liquid chromatograph using the specific chromatographic conditions. The same solutions were detected by UV. The concentration of Febuxostat was calculated from the regression equation. The log % undegraded concentration against time was plotted.

- **For studying the effect of HCl concentration on the reaction rate:** Into a series of 250 ml measuring flasks, 1 ml of stock solution of Febuxostat (4mg/ml) in methanol was added and diluted up to 20 ml with 2N HCl and 5N HCl respectively. These solutions were refluxed in a thermostatically controlled water bath at 80^{o}C for 10 hr. 1 ml sample solutions were taken at 1 hr intervals, placed into 10ml measuring flasks, neutralized with 1ml, 2N and 5N NaOH respectively and the volume was completed with Methanol. The solutions were injected in the liquid chromatograph using the specific chromatographic conditions. The same solutions were detected by UV. The concentration of Febuxostat was calculated from the regression equation. The log % undegraded concentration against time was plotted for different molarities of HCl and the rates constant and $t_{1/2}$ were calculated.

- **For studying the effect of the temperature on the reaction rate:** Into a series of 250 ml measuring flasks, 1 ml of stock solution of Febuxostat (4mg/ml) in methanol was added and diluted up to 20 ml with 2N HCl and 5N HCl respectively. These solutions were refluxed in a thermostatically controlled water bath at 20, 40, 60 and 80 ºC for 10 hr. 1 ml sample solutions were taken at 1 hr intervals, placed into 10 ml measuring flasks, neutralized with 1ml, 2N and 5N NaOH respectively and the volume was completed with Methanol.

  The solutions were injected in the liquid chromatograph using the specific chromatographic conditions. The same solutions were detected by UV. The concentration of Febuxostat was calculated from the regression equation. The log % undegraded concentration against time was plotted. Also the Arrhenius plot for the effect of temperature on the rate of hydrolysis was constructed.

Validation of the method [12]:

- **Linearity and range**: Linearity of the method was studied by injecting five concentrations of the drug prepared in the mobile phase in the range of 5-25 μgml^{-1} in triplicate into the HPLC system keeping the injection volume constant.

- **Precision:** Precision of the method was verified by repeatability and inter and intraday precision studies. Repeatability studies were performed by analyses of any one concentration of the drug in hexaplicate on the same day. Intraday precision of the method was checked by repeating the studies on same day within different time period and interday precision on three different days.

- **Accuracy:** Accuracy was evaluated by fortifying a mixture of standard drug solutions with four known concentrations of the drug. The recovery of added drug was determined.

- **Specificity:** Specificity of the method towards the drug was studied by determination of purity for drug peak in a mixture of stressed samples using PDA detector.

  The study of resolution factor of the drug peak from the nearest resolving degradation product peak was also done.

- **Robustness:** Change following parameters, one by one and observe their effect on system suitability test and assay. Change flow rate by 10%. (i.e. 1.1 ml/minute and 0.9 ml/minute) and Change the minor components in the mobile phase. If the minor change does not produce the variability in assay, the method is Robust.

RESULTS AND DISCUSSION:

- **Degradation behaviour of Febuxostat:** Stress testing of Febuxostat under different conditions using Methanol:water (with 0.02% v/v TFA) (95:5) as solvent system showed the following degradation behaviour.

Hydrolytic studies:

- **Acidic condition:** It was observed that around 25% of the drug degraded on heating it in 2N HCl for 6 hr at 80°C, 2 separate peaks at 2.6 and 6.7 min were observed (**Fig. 2**).

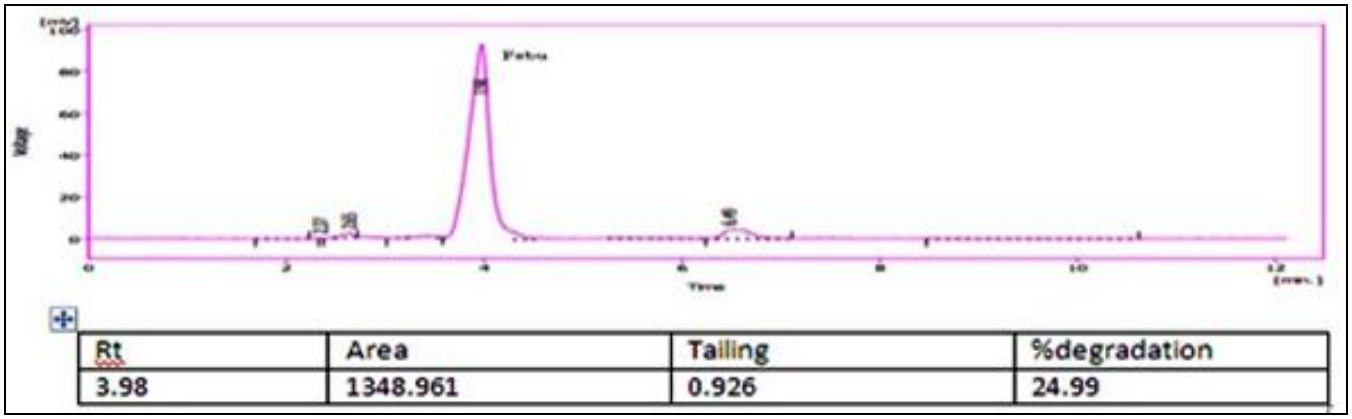
- **Alkaline condition:** It was observed that around 37% of the drug degraded on heating it in 0.1N NaOH for 1 hr at 80°C and 1 separate peak at 3.12 min was observed (**Figure 3**).

- **Neutral condition:** In neutral condition, 23% degradation of the drug was seen after heating the drug for 18 hr at 80 ◦C and 1 separate peak at 3.12 min was observed (**Figure 4**).

- **Oxidative studies:** The drug was found to be stable in 30% H_2O_2 for 18 hr at room temperature (**Figure 5**).

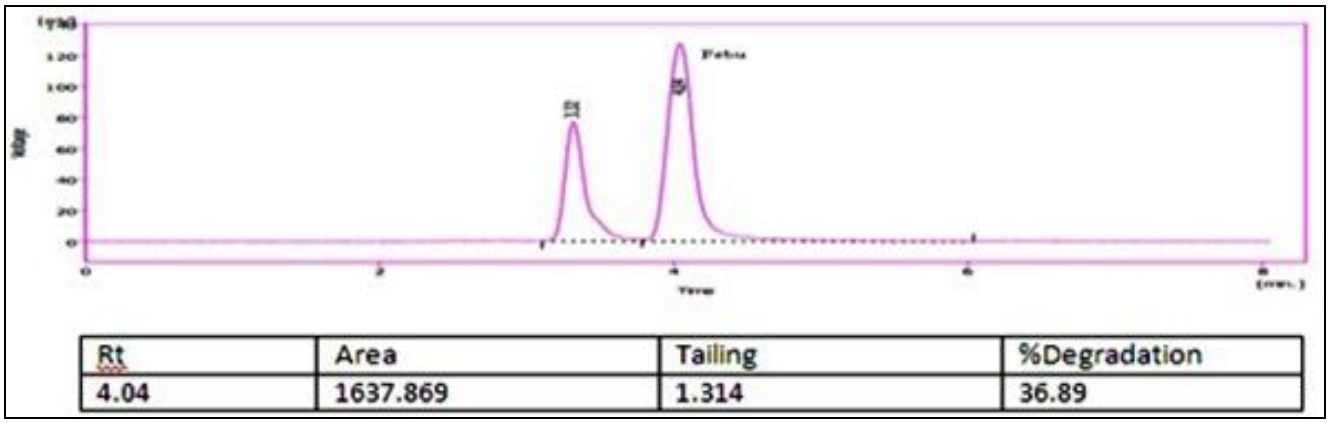
- **Thermal stress:** The drug was found to be stable in 80°C for 8 weeks (**Figure 6**).

- **Photolytic studies:** The drug was found to be stable in UV light for 7 days (**Figure 7**).



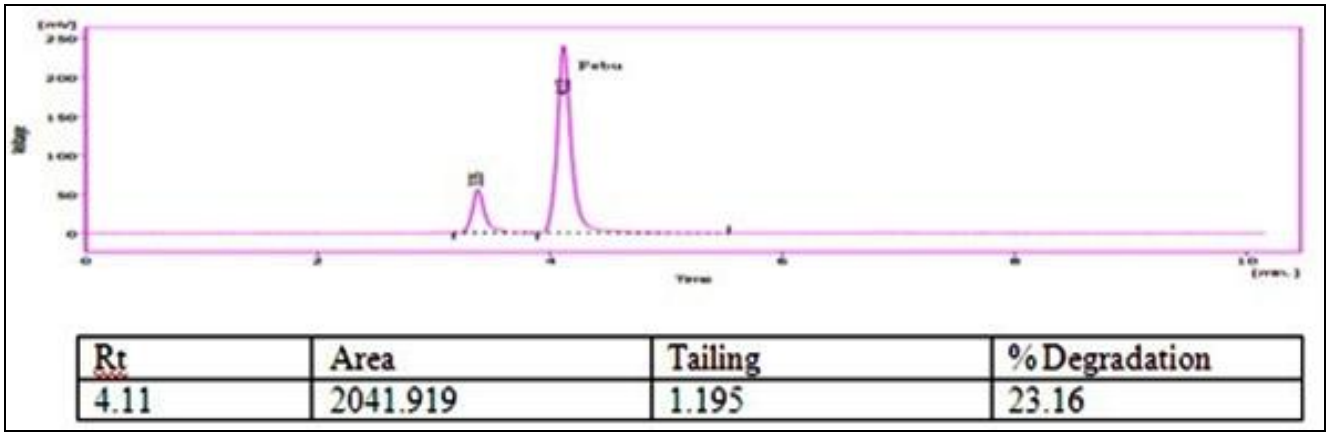
Rt	Area	Tailing	%degradation
3.98	1348.961	0.926	24.99

FIGURE 2: SHOWS DEGRADATION OF FEBUXOSTAT IN 2N HCl AFTER 6 HR



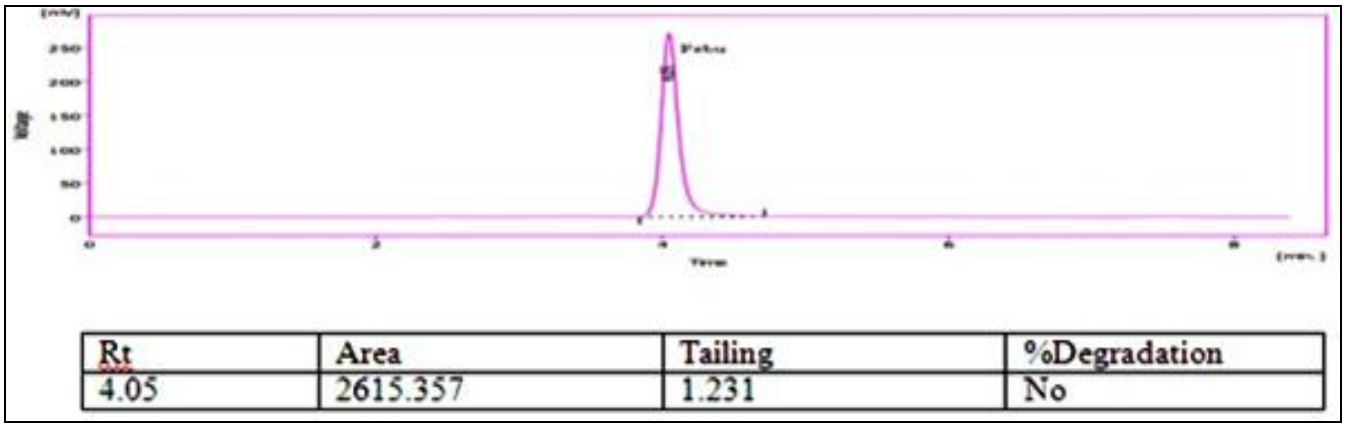
Rt	Area	Tailing	%Degradation
4.04	1637.869	1.314	36.89

FIGURE 3: SHOWS DEGRADATION OF FEBUXOSTAT IN 0.1 N NaOH AFTER 1 HR



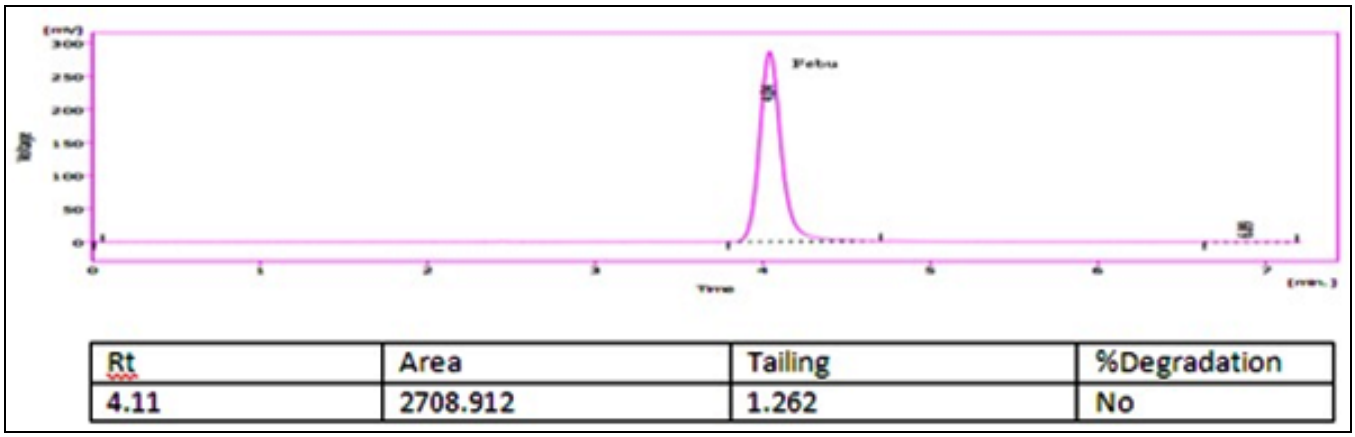
Rt	Area	Tailing	%Degradation
4.11	2041.919	1.195	23.16

FIGURE 4: SHOWS DEGRADATION OF FEBUXOSTAT IN NEUTRAL HYDROLYSIS AFTER 18 HR



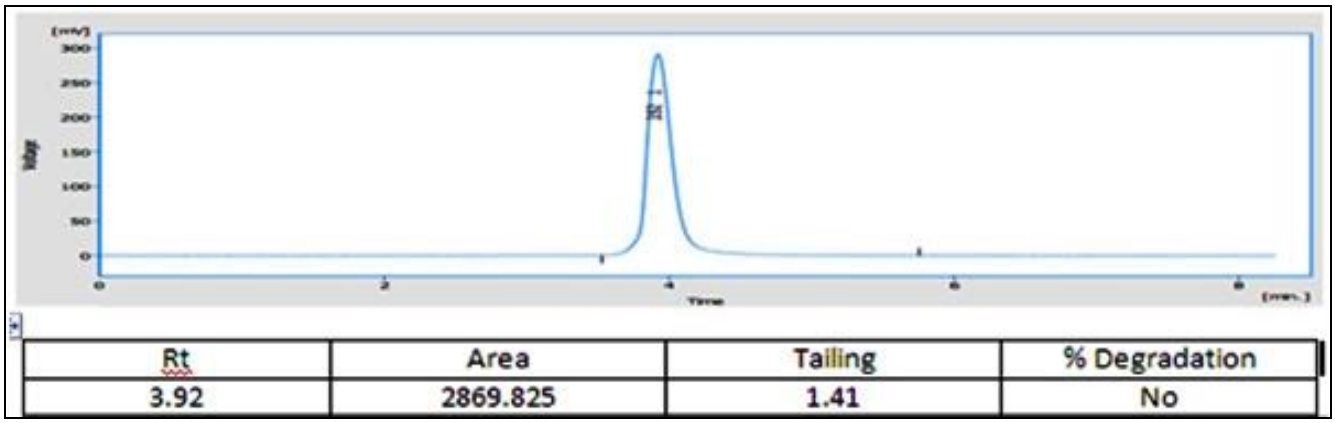
Rt	Area	Tailing	%Degradation
4.05	2615.357	1.231	No

FIGURE 5: SHOWS DEGRADATION OF FEBUXOSTAT IN 30%H_2O_2 AFTER 18 HR



Rt	Area	Tailing	%Degradation
4.11	2708.912	1.262	No

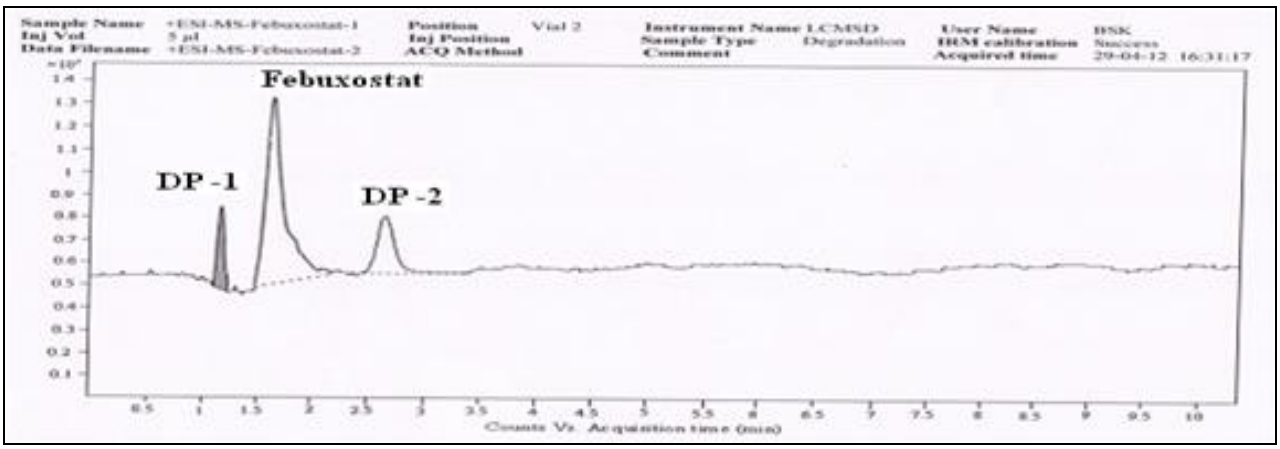
FIGURE 6: SHOWS DEGRADATION OF FEBUXOSTAT IN DRY HEAT OVEN(80°C) AFTER 8 WEEK



Rt	Area	Tailing	% Degradation
3.92	2869.825	1.41	No

FIGURE 7: SHOWS DEGRADATION OF FEBUXOSTAT IN UV LAMP AFTER 7 DAYS.

Characterization of degradation products The LC-MS analyses of degraded samples (In acid hydrolysis and Neutral hydrolysis) were done and the results are shown in **Fig. 8 and 9**.



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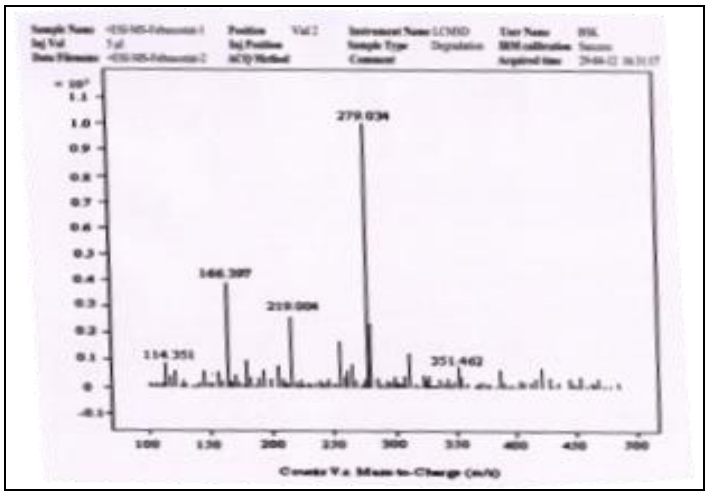

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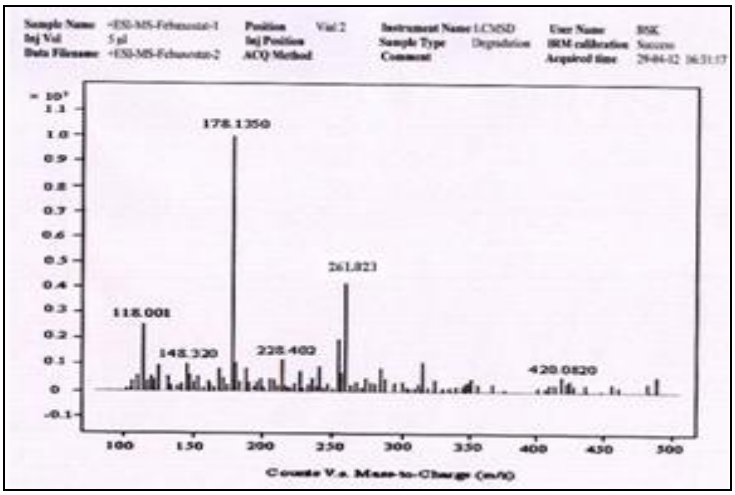
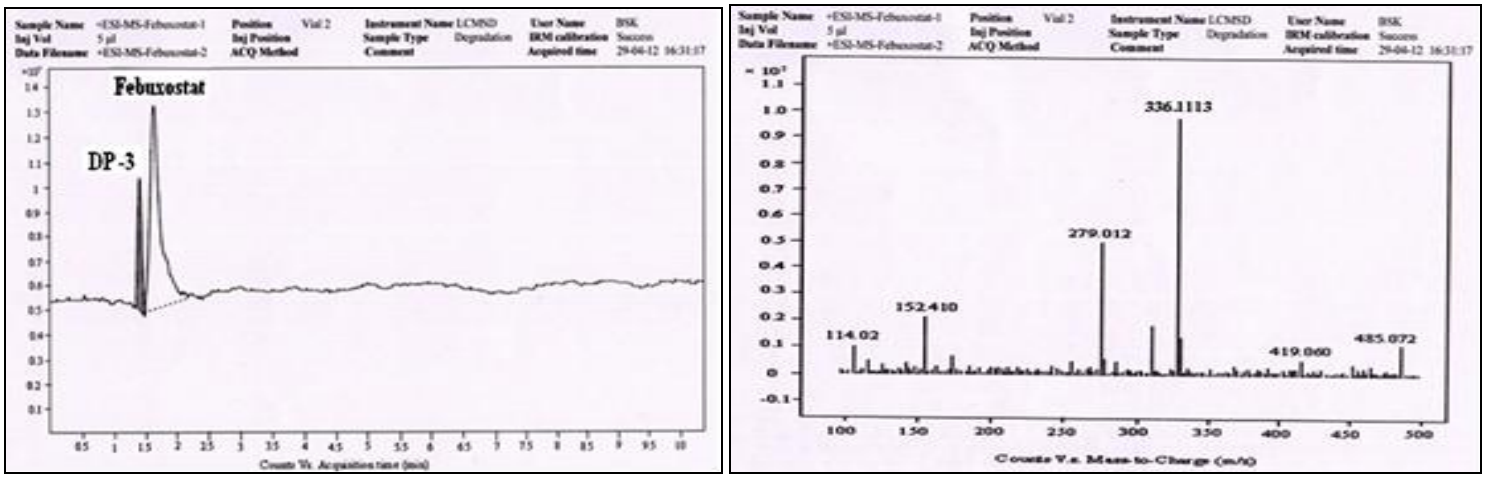


C

FIGURE 8: SHOWS (A) LCMS CHROMATOGRAM OF FEBUXOSTAT WITH 2N HCl FOR 18 HR IN REFLUX. (B) ESI POSITIVE MODE MASS SPECTRA OF DP-1 AND (C) ESI POSITIVE MODE MASS SPECTRA OF DP-2.

Among the two degradation product formed during acid hydrolytic condition, DP-1 showed the prominent peak at m/z ratio 279 as M+1 peak (Fig. 8) indicating breakdown of ether group through acid hydrolysis.

Second degradation product formed during acid hydrolytic condition, showed the prominent peak at m/z ratio 261 as M+1 peak (Fig. 8) indicating breakdown of ether group through acid hydrolysis.



A B

FIGURE 9: IT SHOWS LCMS CHROMATOGRAM(A) AND ESI POSITIVE MODE MASS SPECTRA OF FEBUXOSTAT WITH DP-3 UNDER WATER FOR 24 HR IN REFLUX (B)

Degradation product formed during Neutral hydrolytic condition, DP-3 showed the prominent peak at m/z ratio 336 as (M+1) peak (Fig. 9) indicating breakdown of Cyanide group through hydrolysis to acidic group.

From the mass chromatograms, the probable structures of further fragments from the degradation products can be determined and the pathway was shown in **Fig. 10**.

In case of Neutral hydrolysis and Base hydrolysis, the retention time of the degradation products were same, only catalytic effect was shown by NaOH. So, in Neutral and Basic hydrolytic condition, degradation products formed may be structurally similar.

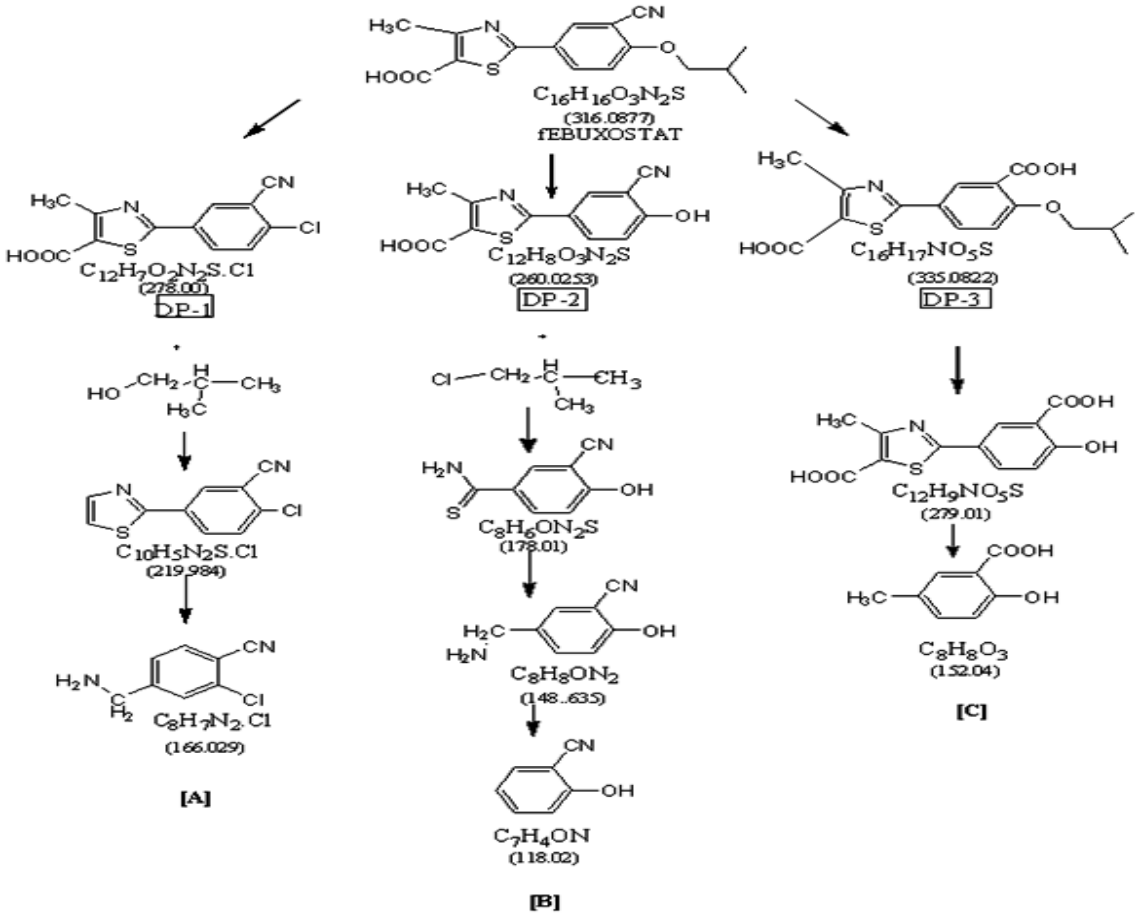
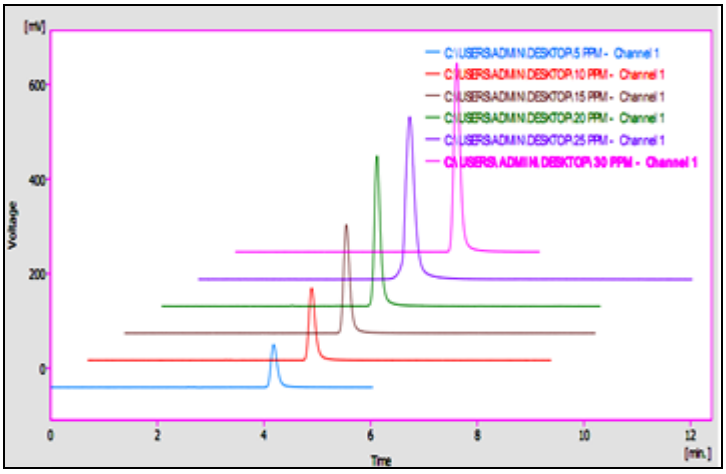


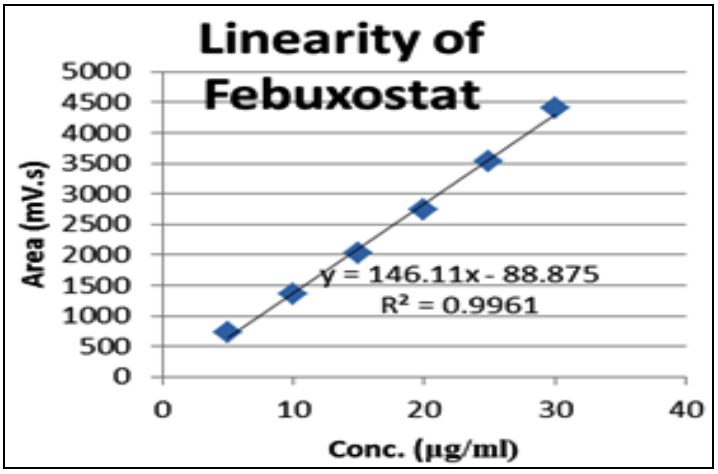
FIGURE 10: SHOWS DEGRADATION PATHWAY FOR FEBUXOSTAT. [A] AND [B] PROBABLE DEGRADATION PRODUCTS AND ITS FRAGMENTS IN ACID HYDROLYTIC CONDITION. [C] PROBABLE DEGRADATION PRODUCTS AND ITS FRAGMENTS IN BASIC AND NEUTRAL HYDROLYTIC CONDITION

Validation of Stability indicating method: Linearity was found to be in the range of **5-30 µgml^{-1}** (**Fig. 11**) in triplicate into the HPLC system keeping the injection volume constant. The recovery of added drug was found to be in the range of **99.17-101.99%** (Table 1). Intraday precision of the method was checked by repeating the studies on same day within different time period and interday precision on three different days (Table 2, 3). Specificity of the method towards the drug was studied by determination of purity for drug peak in a mixture of stressed samples using PDA detector.

Robustness testing was done by changing the mobile phase flow rate and composion of the mobile phase (Table 4). Final result for degradation study was shown in **Table 5**.



A



B

FIGURE 11: SHOWS CHROMATOGRAM FOR LINEARITY OF FEBUXOSTAT(A) AND CLIBRATION CURVE (B).

TABLE 1: SHOWS ACCURACY OF FEBUXOSTAT

Level	Replicate	Mean Area	Amount of			Recovery		
			Drug taken (µg/ml)	Drug added (µg/ml)	Found (µg/ml)	% Recovery	Mean	%RSD
0%	1	1389.124	10	0	10.1857	101.85	101.37	0.4751
	2	1375.985	10	0	10.0893	100.89		
	3	1382.679	10	0	10.1384	101.38		
Level 1 (50%)	1	1967.864	10	5	14.9817	99.87	101.20	1.1454
	2	2009.547	10	5	15.2990	101.99		
	3	2004.893	10	5	15.2636	101.75		
Level 2 (100%)	1	2682.36	10	10	20.1222	100.61	101.10	0.4423
	2	2698.357	10	10	20.2422	101.21		
	3	2705.674	10	10	20.2971	101.48		
Level 3 (150%)	1	3496.587	10	15	24.8226	99.29	99.51	0.4914
	2	3492.674	10	15	24.7948	99.17		
	3	3524.268	10	15	25.0191	100.07		

TABLE 2: SHOWS RESULTS OF REPEATABILITY (N=6)

Sr. No.	Febuxostat
1	1363.797
2	1389.124
3	1375.985
4	1382.679
5	1377.198
6	1381.357
Mean	1378.357
SD	8.5155
RSD	0.6178

TABLE 3: SHOWS RESULTS OF INTRA-DAY AND INTER-DAY PRECISION (N=3)

Febuxostat (µg/ml)	Intraday precision		Interday Precision	
	Mean ± SD	%RSD	Mean + SD	%RSD
10	10.1033±0.1123	0.1124	9.975±0.1484	1.4879
15	15.1066±0.1106	0.7321	15.025± 0.1206	0.8028
20	20.0133±0.1450	0.7246	19.9616±0.1489	0.7460

TABLE 4: SHOWS RESULTS OF ROBUSTNESS DATA.

Condition	%RSD	%Recovery	% Difference in Assay
LIMIT	NMT 2.0 %	98 to 102%	NMT 2%
Flow rate (1.1ml/min)	0.7	99.35	0.6
Flow rate (0.9ml/min)	0.9	98.94	0.4
M.P. composition(94:6% v/v)	0.5	100.29	1.1
M.P. composition(94:6% v/v)	1.04	101.18	0.8

TABLE 5: SHOWS RESULTS OF DEGRADATION STUDY

Degradation condition	Rt of degradation product	Time (hr)	Conc. (µg/ml)	% Potency	% Degradation	Remarks
0.1 N NaOH (Refluxing)	3.3 min	0	20	100	36.89	Labile
		1	12.62	63.11		
2 N HCl (Refluxing)	2.6, 6.8 min	0	20	100	24.99	Very stable
		6	15.00	75.01		
Distilled water (Refluxing)	3.3 min	0	20	100	23.16	Stable
		18	15.36	76.84		
Dry Heat (80°C)	---------	0	20	100	0	Very stable
		8 week	20	100		
Oxidation (10% H2O2 in dark)	---------	0	20	100	0	Stable
		18 hr	20	100		

Degradation Kinetic study: The data for the undegraded drug that is, absorbance and area at particular temperature were determined. The linear relationship (**Fig. 12**) between the log% undegraded against time indicated first-order degradation. Since the hydrolysis was performed in a large excess of HCl (2N and 5N), therefore it follows a pseudo-first order reaction rate [10]. which is the term used when two reactants are involved in the reaction but one of them is in such a large excess (HCl) that any change in its concentration is negligible compared with the change in concentration of the other reactant (drug).

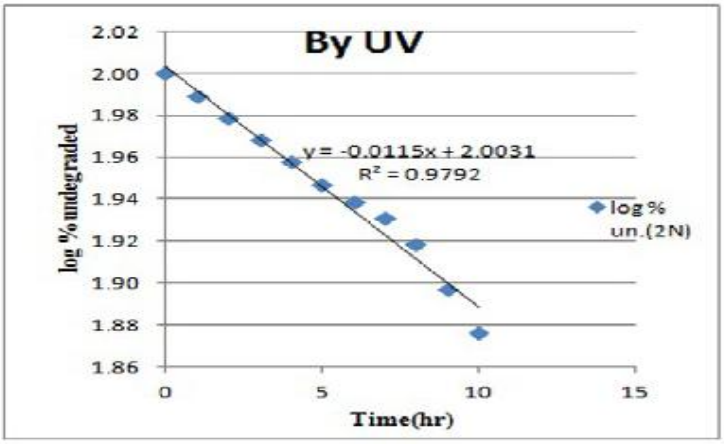
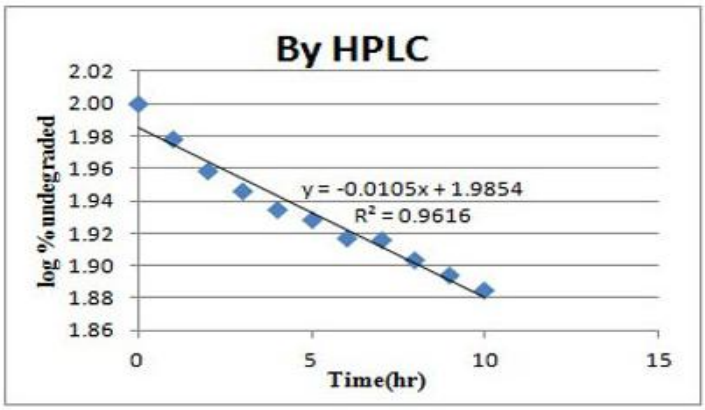


FIGURE 12: SHOWS FIRST ORDER PLOT OF THE HYDROLYSIS OF FEBUXOSTAT IN 2N HCl AT 80°C



Different parameters that affect the rate of the reaction were studied. Overlay Spectra for degradation were taken by both UV and HPLC methods (**Fig. 13**). The temperature dependence of Febuxostat degradation was studied by conducting the reaction at different temperatures using different concentrations of the acid solution (**Fig. 14**). At each temperature the rate constant and $t_{1/2}$ were calculated then the log of the rate constant was plotted against the reciprocal of

the temperature in Kelvin (Arrhenius plot, **Fig. 15**) to demonstrate the effect of temperature on the rate constant. Rate constant for the 1st order reaction can be determined from the following equation.

k = (2.303/t) * log (a/a-x)

Where, k = Rate constant, t = Time in hr, a = Initial concentration of drug, a-x = Concentration of drug after time t.

It was concluded that as the temperature increased, rate of hydrolysis increased with a decrease in the $t_{1/2}$ (**Table 3**). Also, the energy of activation was determined by calculating the rate constant from the following equation [13].

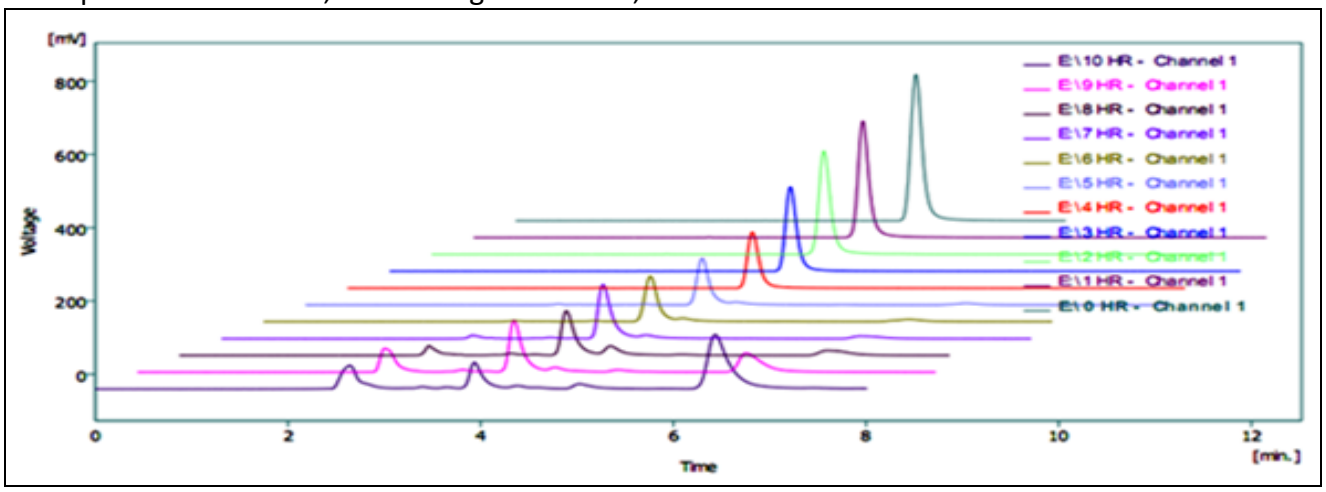
$$\log \frac{k_2}{k_1} = \frac{E_a}{2.303R}\left(\frac{T_2 - T_1}{T_1 T_2}\right)$$

where "E_a" is the activation energy, "T_1" and "T_2" are the two temperatures in Kelvin, "R" is the gas constant, and "k_1" and "k_2" are the rate constants at the two temperatures used.

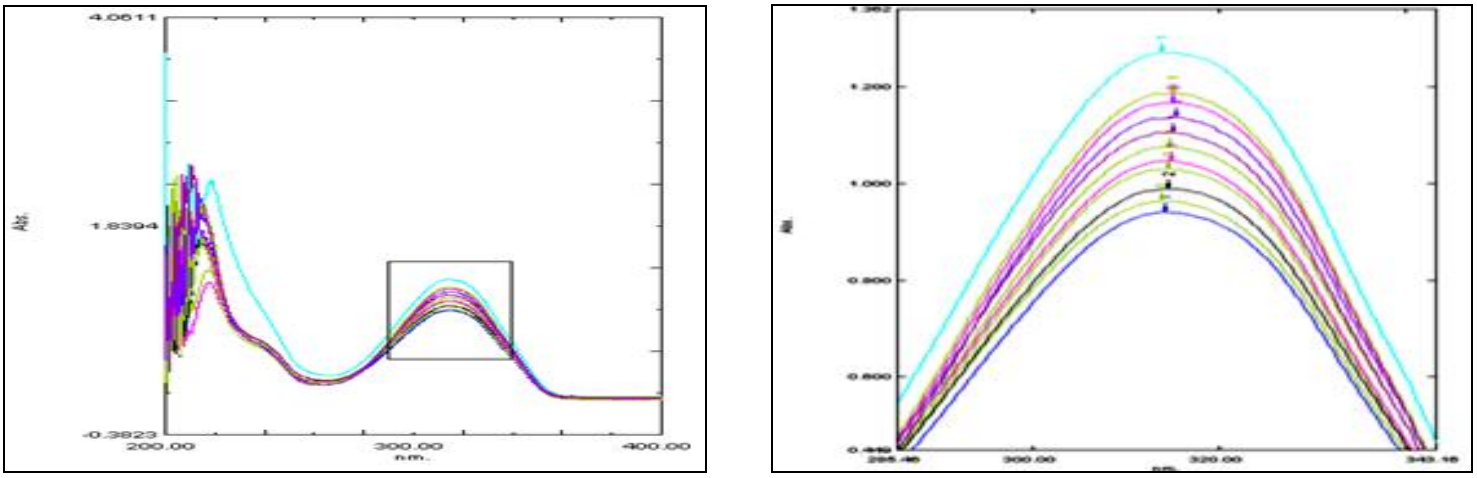
The calculated "E_a" was found to be 10.11 $KJmol^{-1}$ by HPLC and 12.09 $KJmol^{-1}$ by UV. Halflife of the drug is calculated from the equation,

$t_{1/2}$ = 0.693/k

Another factor that affects the rate of the reaction is the acid strength of HCl, thus different normalities were used to study the hydrolysis reaction. The rate of hydrolysis increased with an increasing HCl concentration as well as with increase in temperature (**Table 6**). In conclusion, the acid hydrolysis of Febuxostat was found to follow a pseudo first order reaction rate. Also the reaction rate increases with increase in the temperature and the strength of the acid solution.



A



A B

FIGURE 13: SHOWS OVERLAY SPECTRA OF FEBUXOSTAT IN 2N HCL AT 80°C BY HPLC(A) AND UV (B)

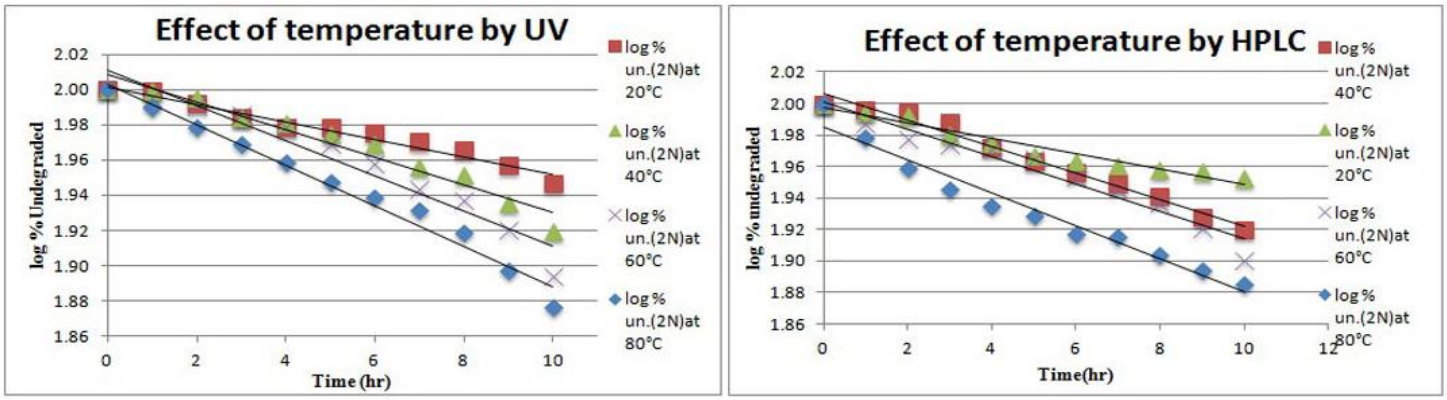


FIGURE 14: SHOWS FIRST ORDER PLOT OF THE HYDROLYSIS OF FEBUXOSTAT WITH 2N HCl AT DIFFERENT TEMPERATURES

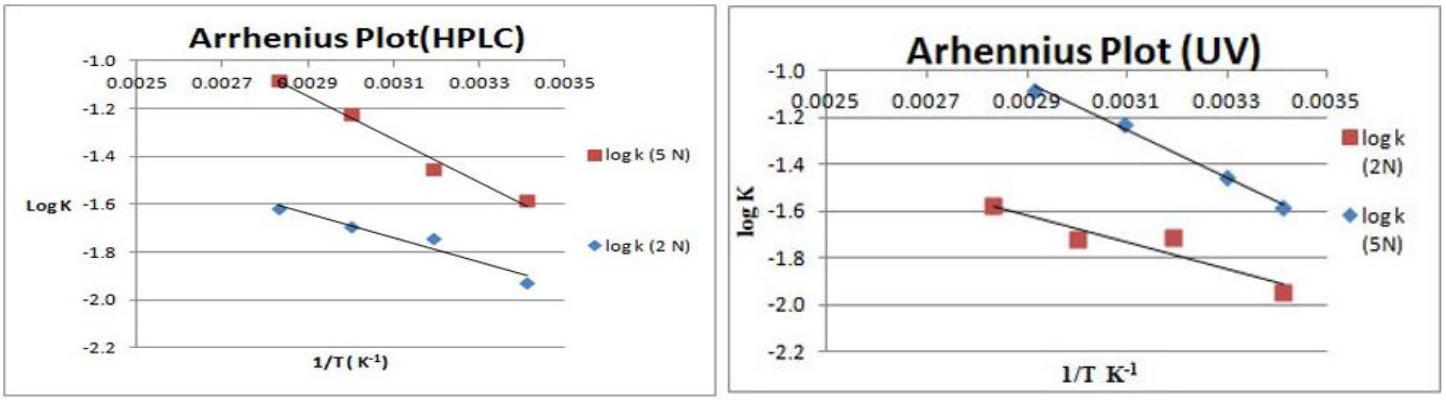


FIGURE 15: SHOWS ARRHENIUS PLOT FOR HYDROLYSIS OF FEBUXOSTAT WITH 2N AND 5N HCl AT DIFFERENT TEMPERATURES

TABLE 6: SHOWS KINETIC DATA OF FEBUXOSTAT ACID DEGRADATION.

Normality of HCl	Temperature (°C)	K (hr $^{-1}$)		Half life (hr)	
		HPLC	UV	HPLC	UV
2N	20	0.0120	0.0114	57.84	60.66
	40	0.0182	0.0196	37.99	35.33
	60	0.0204	0.0193	33.97	35.86
	80	0.0243	0.0265	28.56	26.10
5N	20	0.0260	0.0262	26.60	26.48
	40	0.0352	0.0376	19.69	18.43
	60	0.0597	0.0458	11.60	15.14
	80	0.0830	0.0830	08.35	08.26

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