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RESEARCH ARTICLE

Stability-Indicating, Method, for the Determination, of Rivaroxaban, and its Degradation, Products, using, LC-MS, and, TLC,.

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ABSTRACT:
A validated stability indicating thin layer chromatographic (TLC) and liquid chromatography coupled with mass spectrometry LC/MS methods were developed to analyze Rivaroxaban and its degradation products. Forced degradation studies under stress conditions were carried out in order to establish its stability profile. Stress conditions recommended by the international conference on harmonization (ICH) including oxidative, photolytic, thermal, acidic, and basic hydrolysis were applied. Rivaroxaban found susceptible to acid and base hydrolytic stress conditions. Degradation products were identified isolated and characterized using LC-MS and TLC. Three major degradation products were detected, separated and determined and two of them were further characterized by NMR spectroscopy and FT-IR.

KEYWORDS: Rivaroxaban, LC-MS, TLC, stability indicating, degradation products.

1. INTRODUCTION:
Rivaroxaban Figure 1, chemically known as (S)-5-Chloro-*N*-{2-oxo-3-[4-(3-oxomorpholin-4-yl)phenyl]-1,3-oxazolidin-5-yl-methyl}thiophen-2-carboxamide, is a novel, oral, direct Factor Xa inhibitor developed and marketed by Bayer under the brand name Xarelto®. It was approved for the prevention of venous thromboembolism in adult patients after hip or knee replacement surgery [1-3].

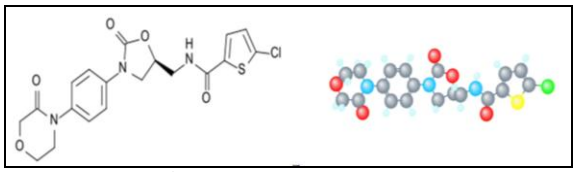


Figure 1 Structure of the Rivaroxaban [4].
Molecular Formula $C_{19}H_{18}ClN_3O_5S$
Molecular Weight 435.9

Chemical stability of the drugs is a major concern during drug development as it impacts the efficacy and safety of the drug. Stability testing provides evidence of the quality of a drug when exposed to the influence of environmental factors such as pH, temperature, humidity and light. The data from such studies enables storage conditions; re-test periods and shelf lives to be established. Stress testing helps to determine the intrinsic stability of the molecule by establishing its degradation pathways [5, 6].

The stability of Rivaroxaban has been studied by the following techniques: ultra performance liquid chromatography (UPLC) coupled with a photodiode array detector [7, 8], HPLC, TLC Densitometry, First derivative and First-derivative ratio spectrophotometry [9]. The stability profile of Rivaroxaban under various stress conditions has been investigated recently in several studies [10-12], nevertheless none of these studies included full isolation or identification of the degradation products. RP-HPLC coupled with tandem mass spectrometry and NMR have been used to characterize the degradation products [13] and further analytical techniques were used in order to investigate

the degradation kinetics of Rivaroxaban including HPLC, TLC-densitometry, LC/MS/MS and UPLC-Q-TOF-MS/MS [14,15].

Since Rivaroxaban is not yet official in any of the pharmacopoeia [16-18], and there are only few studies dealing with its stability profile, the aim of this research was to develop a stability indicating method for determination of Rivaroxaban plus an identification, isolation and characterization of its degradation products (DPs) in order to identify its major degradation pathways, by using techniques of LC-MS, TLC-densitometry, NMR spectroscopy and FT-IR spectral data.

2. MATERIALS AND METHODS:

2.1. Chemicals and reagents:

Rivaroxaban standard was purchased from NIFTY LABS PVT LTD. APIs were obtained from NIFTY LABS PVT LTD, Megafine Pharma (P) Ltd and Nanyangpukang Pharmaceutical CO. LTD. HPLC acetonitrile, methanol, hydrochloric acid, sodium hydroxide, hydrogen peroxide, dimethylsulphoxide-d6 (DMSO-d6) and Toluene were purchased from SIGMA-ALDRICH®. HPLC water was obtained by Siemens Water Technologies LaboStar and Dimethylsulfoxide (DMSO) was obtained from Scharlau.

2.2. Instruments

The chromatographic analysis was performed with SHIMADZU LC-MS prominence system (Shimadzu, Japan) provided with UV-Vis Detector SPD 20A, MS Detector 2020, two pumps A and B: LC/20AD, column oven CTO-20A, manual injector and with Macherey-Nagel Nucleodur C18 column (250 x 4.6 mm, 5µm particle size), System control and data analysis were carried out using LabSolutions CS (Schimadzu, Japan). KNAUER HPLC Smartline system with PDA detector (Germany). TLC aluminum-packed silica gel 60F 254 plates from Merck (Darmstadt, Germany), Camag TLC Scanner II, with computer system and CATS software V.3.12 (Camag). Sartorius sensitive analytical balance (sensitivity of 10^{-4}g).

JEKEN Digital Ultrasonic Cleaner. Bechers, Volumetric flasks, Micropipettes and Glassware of different volumes from Marienfeld Company. Filters PVDF 0.45µm for HPLC purchased from TEKNOKROMA. The photostability study was carried out in a photostability chamber (Sanyo, Leicestershire, UK). The thermal stability study was carried out in a dry air oven (Memmert, Germany). NMR BRUKER 400 MHz Ultra shield TM instrument. FTIR Nicolet 6700 with the Detector DTGS Operating software (OMNIC version 7.3 Thermo Nicolet USA).

2.3. Chromatographic conditions:

The chromatographic separation was performed on C18 column (250 x 4.6 mm, 5µm particle size) at a column temperature of 55°C, The mobile phase A was HPLC grade water and the mobile phase B was acetonitrile, the gradient program of the mobile phase was set as [Time (min)/ Pump B Value (%)] [0.01/15, 15/25, 26/26, 30/40 and 35/100]. The mobile phase was filtered using 0.45 µm disposable filter, and degassed by ultrasonic vibration prior to use. The flow rate of the mobile phase was 1.5 ml/min. The injection volume was 20 µL and the detection was carried out at 254nm.Water and acetonitrile 10:90 (v/v) was used as a diluent. Stock solutions of Rivaroxaban (2.0 mg/ml) was prepared by dissolving 200 mg of Rivaroxaban standard in minimum amount of DMSO and made up to volume with diluents to obtain a concentration of 2.0 mg/ml. The analysis was performed in positive electro-spray ionization mode ESI^+, the ion source voltage was 5000 V, and the source temperature was 450 °C, and the curtain gas flow was 15 psi. This LC-MS method was successfully developed and validated as per ICH guidelines and according to USP 35 guideline recommendations [18, 19].

2.4. TLC-densitometric assay:

Pre-coated TLC-plates, silica gel 60 F254 (20 x 20 cm, 0.25 mm), were used for all determinations. In all experiments, bands were spaced 2.0 cm apart and 1.5 cm from the bottom edge of the plate. A mobile phase composed of Toluene: Methanol: Acetonitrile (50:20:30, v/v/v) was used and detection was carried out at 254 nm. System suitability criteria were determined in order to assess the efficiency of separation. Calibration curve was constructed using a standard series covering a concentration range 3-10µg/band. Assay validation was carried out according to ICH guidelines.

2.5. NMR H^1 and C^{13} spectroscopy:

H^1, C^{13} and Dept135 NMR spectra were recorded in DMSO-d6 at 25°C. The NMR chemical shift values were reported on the δ scale in ppm, relative to TMS (δ = 0.00) as internal standard.

2.6. FT-IR spectroscopy:

The IR spectra for Rivaroxaban, and its degradation products were recorded in the solid state as KBr dispersion, with Range 400-4000nm, the resolution was $4cm^{-1}$, scans were 32.

2.7. Degradation protocols:

The stress conditions employed for degradation studies as per ICH recommendation include photolytic, thermal, oxidation, and hydrolysis with acid and base. Degradation samples were prepared by diluting Rivaroxaban stock standard solution in diluents to obtain a final concentration of (1mg/ml). The photolytic stress

study was performed for 7 days at 200 W h/ m^2 of UV light and 1.2 million lux hours of visible light. The thermal stress study was performed at 105°C for 7 days. The acid, base stress studies were performed with 0.1 N HCI for 72h and 0.1 N NaOH for 72 h at room temperature. The oxidation stress was done with 3% H_2O_2 solution over a period of 72 h at room temperature. All of the stressed samples were quantified against the Rivaroxaban reference standard. Each experiment was performed in triplicate.

3. RESULTS:

3.1. Methods development and validation:

3.1.1. LC-MS method:

The main target of the chromatographic method is to achieve the separation of degradation products from Rivaroxaban after stress conditions employed for degradation studies. The described LC-MS method was successfully developed and validated as per ICH and USP 35 guidelines. It was suitable for the separation of Rivaroxaban from most of the other degradation products and the retention time for Rivaroxaban was 25.6 min Figure 2. The degradation products produced in the forced degradation were well-separated (Resolution > 2.0) from Rivaroxaban, the tailing factor for Rivaroxaban was 0.978, and the theoretical plates was 31115. The method was proved to be linear over the calibration range 300-1000µg/ml and the correlation coefficient was > 0.999. The method was validated in terms of accuracy, precision, repeatability and robustness, the percentage recovery values were 99.98%, 100.13%, 100.09% and 100.12%, respectively and the RSD was 0.41. The Detection limits (DL) and Quantification limits (QL) were 0.402 and 1.326 µg/ml, respectively.

3.1.2. TLC-densitometric method:

Chromatography was performed on 20 cm × 20 cm thin-layer chromatographic plates precoated with silica gel 60 F254. In order to obtain a simple, rapid, reproducible and satisfactory resolution and to avoid peak tailing, optimization was performed with different mobile phase.

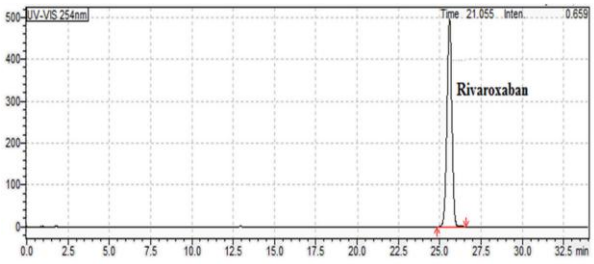
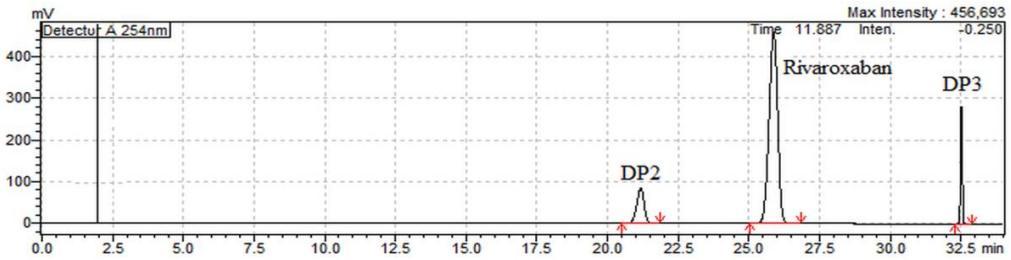


Figure 2 Chromatogram of standard solution of Rivaroxaban.

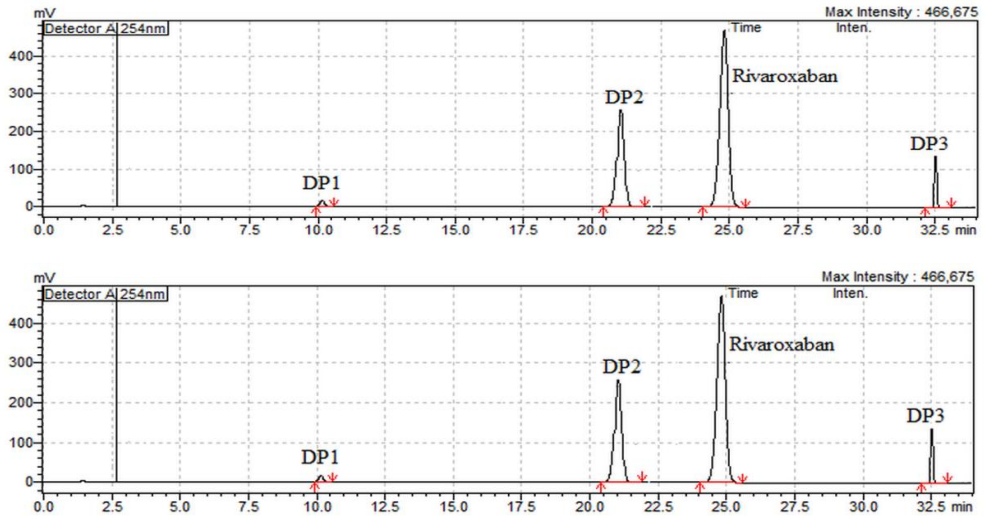
The retention behavior using single non polar toluene and polar (methanol, acetonitrile) solvents was investigated. Standard and degradation solutions were applied 15 mm above the lower edge of the plate. Ascending chromatography to a distance of 180 mm was performed in the TLC chamber previously saturated for 20 min. A satisfactory resolution and separation of Rivaroxaban and its degradation products was obtained with Toluene: Methanol: Acetonitrile (50:20:30, v/v/v). After the development, the plates were dried in ambient air and the separated zones were scanned in the linear reflectance–absorbance mode at 254 nm by means of a Camag TLC Scanner II. The peak areas were used for quantification. The migration distances of Rivaroxaban was 150mm, the recovery of Rivaroxaban ranging from 98.9 to 102.1%, and the RSD values lower than 2% confirms the accuracy of the method. The repeatability of the method was assessed by replicate chromatography applications (n=6) of Rivaroxaban at the three different concentrations. The RSD values obtained from these results are lower than 1.5%. The detection limit (DL) and the quantification limit (QL) were 0.02 µg/band and 0.06 µg/band, respectively.

3.2. Degradation of Rivaroxaban:

Significant degradation of Rivaroxaban was observed in acidic and basic hydrolytic stress conditions meanwhile it was found stable at neutral hydrolysis, thermolysis, photolysis and oxidation. This was consistent with what has been reported recently. In total, five DPs were observed, two DPs were detected in the solutions subjected to acidic hydrolysis and three DPs were detected in the solutions subjected to basic hydrolysis, as shown in Figure 3 and Table1.The degradation products are named "DPn", where n accounts for the elution order.



Acidic Hydrolysis



Basic Hydrolysis

Figure 3 LC-MS Chromatograms of hydrolysis degradation products of Rivaroxaban.

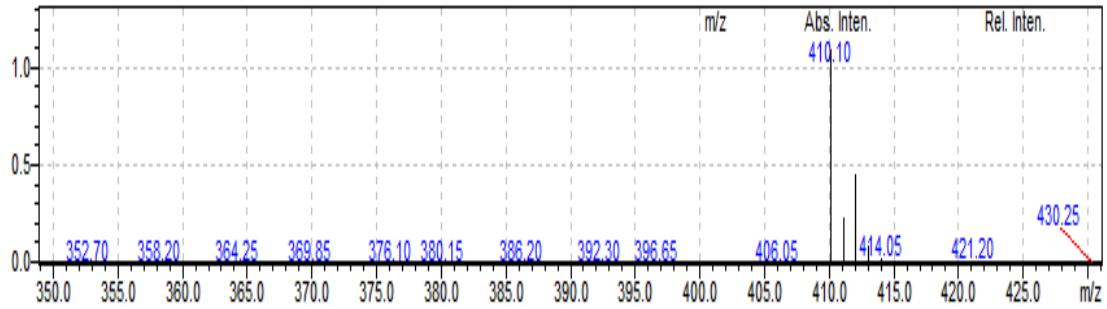
Table 1: Summary of forced degradation studies of Rivaroxaban

Stress condition	**Time**	**Assay of Apixaban (% w/w)**	**Total degradants (% w/w)**	**Mass balance**	**Commentaries**
Rivaroxaban before degradation	-	99.96	0.03	99.99	-
Acid hydrolysis (0.1 N HCl)	72 hours	71.834	27.61	99.44	Degradation accompanied by appearance of DP2 and DP3
Base hydrolysis (0.1 N NaOH)	72hours	63.585	34.83	98.42	Degradation accompanied by appearance of DP1, DP2, and DP3
Neutral hydrolysis	7 days	99.04	0.92	99.96	No degradation occurred
Oxidation (3% H2O2)	72 hours	99.76	0.08	99.84	No degradation occurred
Thermal (105° C)	7 days	99.55	0.32	99.87	No degradation occurred
Photolytic (UV light)	7 days	99.70	0.09	99.79	No degradation occurred
Mass balance = assay %+ sum of all degradants% .					

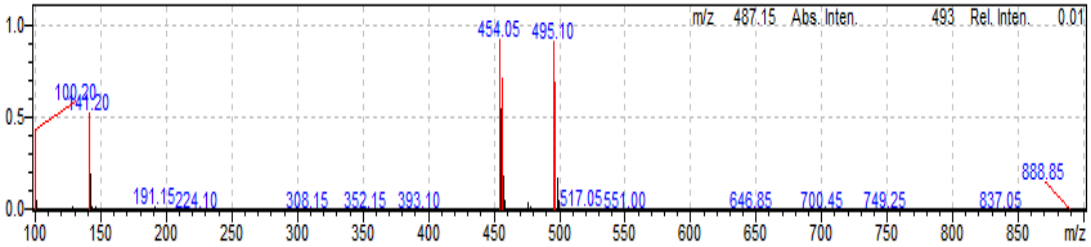
Mass balance (% assay + % total degradation products) of all the stressed samples of Rivaroxaban was obtained in the range of 98.42–99.96%. As shown in Table 1, loss of 28% of Rivaroxaban was observed after 72 hours in acidic conditions, while 35% was recorded after 72 hours in basic conditions. The identification of Rivaroxaban and its degradation products was confirmed by ESI-MS using scan mode from 50-900 mu to produce spectra of molecular weight as shown in Table2. Most possible structures were proposed for DPs by comparing their fragmentation patterns with that of Rivaroxaban. The ESI-MS spectra of Rivaroxaban and DPs are shown in Figure 4

Table 2: $[M+H]^+$m/z of Rivaroxaban and its degradation products.

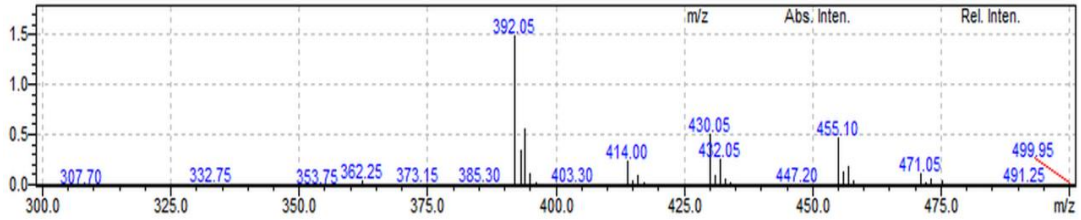
Stress condition	**Peak name**	**Retention time RT(min)**	**$[M+H]^+$m/z**
Acid hydrolysis	DP2	21.060	454.05
	Rivaroxaban	25.717	436.05
	DP3	32.490	392.10
Base hydrolysis	DP1	10.107	410.10
	DP2	21.083	454.05
	Rivaroxaban	25.760	436.10
	DP3	32.193	392.10



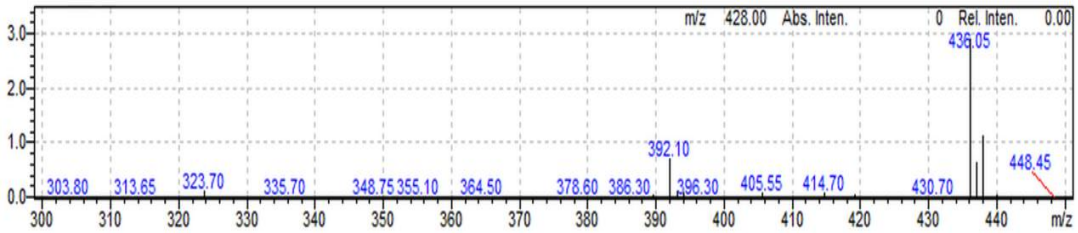
ESI^+-MS spectrum of DP1



ESI$^+$-MS spectrum of DP2



ESI$^+$-MS spectrum of DP3



ESI$^+$-MS spectrum of Rivaroxaban

Figure 4 ESI-MS spectrums of Rivaroxaban and its degradation products

Degraded samples were analyzed using TLC Figure 5. Bands corresponding to degradation products were scratched, extracted using diluent then analyzed using LC/MS in order to reveal the identity of each degradation product. And as has been previously reported, using TLC for preparation and isolation of the degradation products presents a simple and economic approach when compared to semi-preparative HPLC, and the TLC-densitometric analysis results are shown in Figure 6.

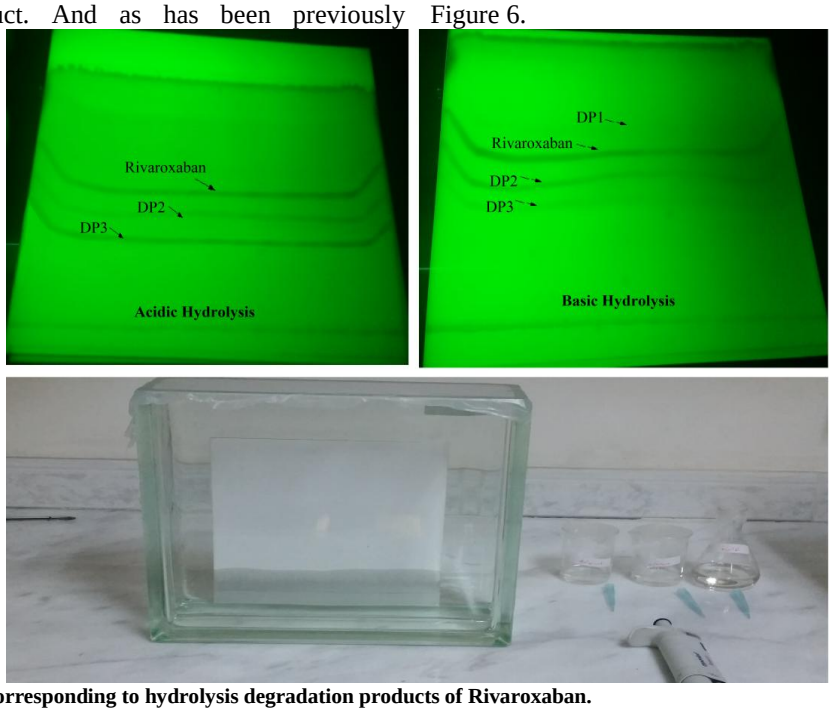


Figure 5 TLC bands corresponding to hydrolysis degradation products of Rivaroxaban.

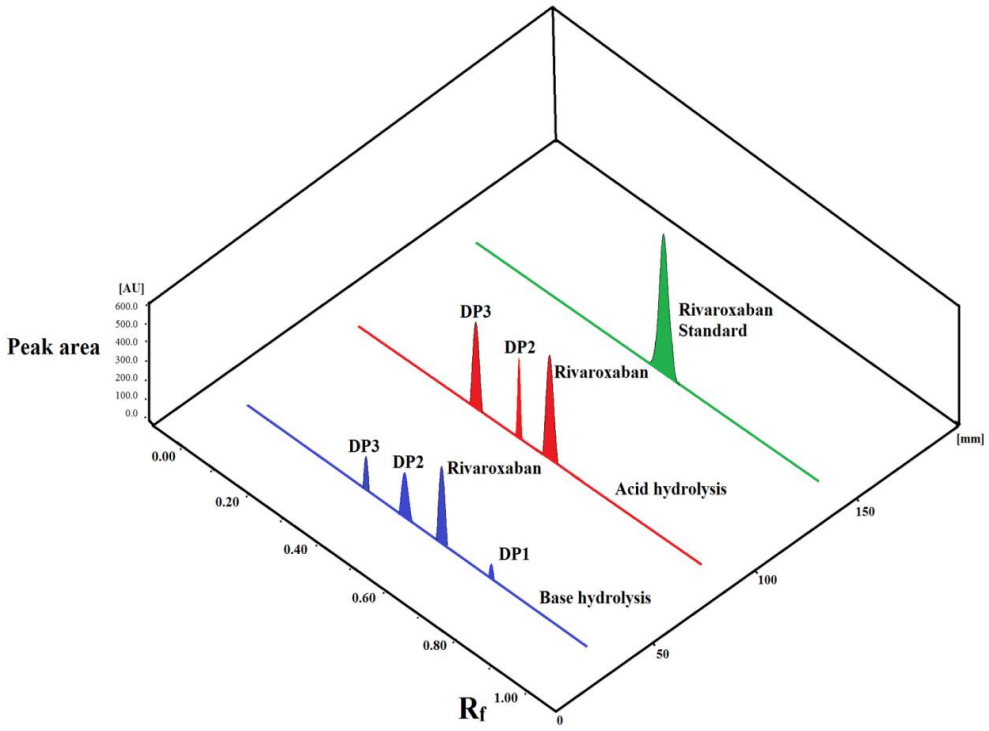
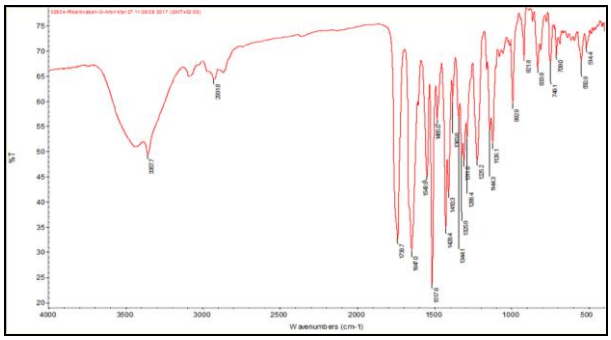
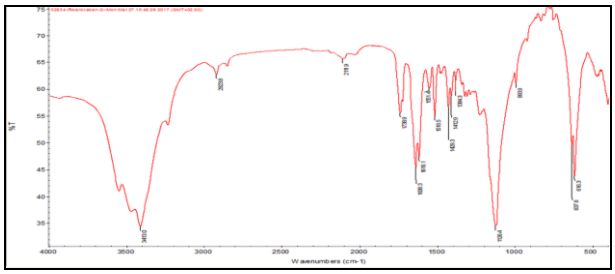


Figure 6 TLC Chromatograms of hydrolysis degradation products of Rivaroxaban.

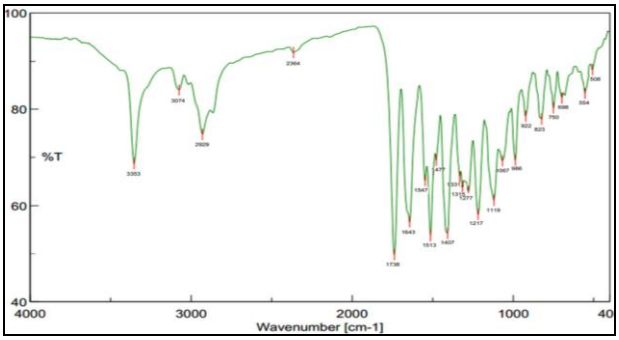
Dp2 and Dp3 which were isolated by TLC were further subjected to NMR and FT-IR studies for structure elucidation using conditions mentioned in Materials and Methods section. Whereas structural elucidation of DPs has been accomplished from NMR, FT-IR and mass spectral data. FT-IR spectra of DP2 and DP3 are shown in Figure 7.



DP2



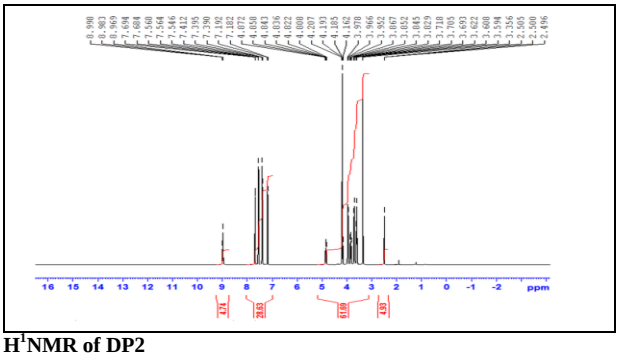
DP3



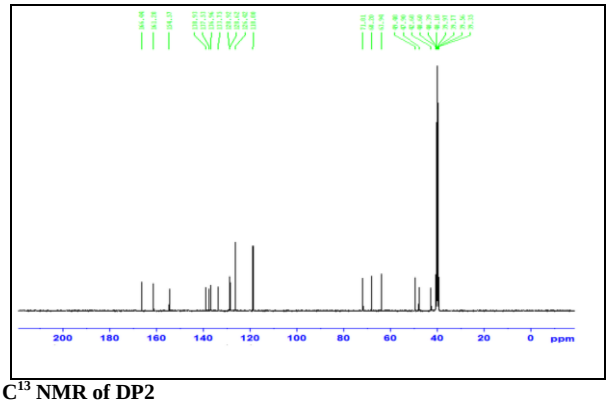
Rivaroxaban

Figure 7 FT-IR spectra of Rivaroxaban and its degradation products.

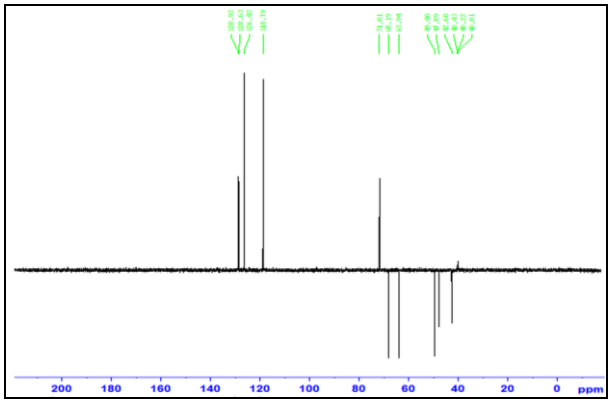
Proton H^1, carbon $C^{13}$and Dept135 NMR spectra of Rivaroxaban degradation products are shown in Figure 8.



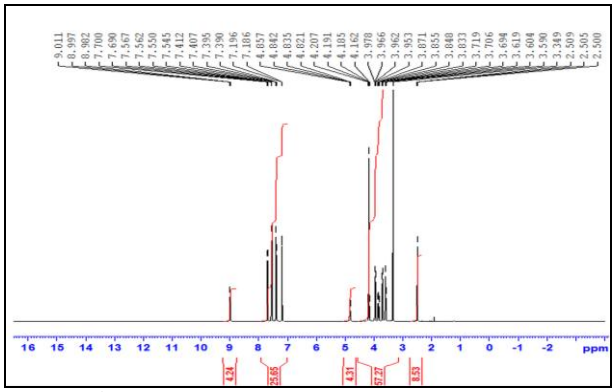
H^1NMR of DP2



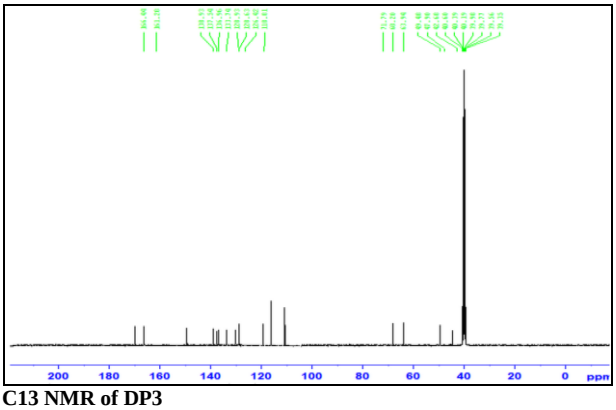
C^{13} NMR of DP2



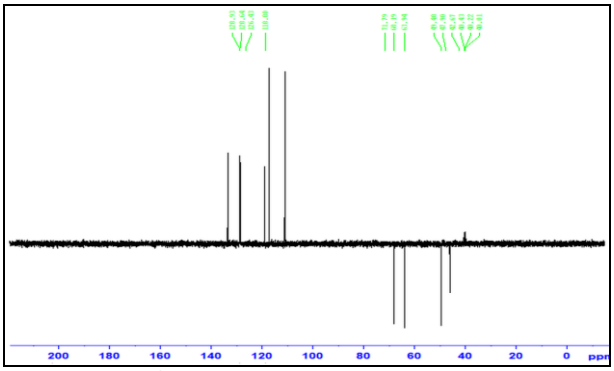
Dept 135 NMR of DP2



H1NMR of DP3



C13 NMR of DP3



Dept 135 NMR of DP3

Figure 8 NMR H^1, $C^{13}$and Dept 135 spectra of Rivaroxaban degradation products.

4. DISCUSSION:

Under influence of various stress conditions as per ICH guidelines, Rivaroxaban was more susceptible to hydrolysis condition than to the other tested conditions; it degraded under stress acid hydrolytic degradation into two DPs while under stress base hydrolytic degradation three DPs were formed. And as shown in Figure 3 and Figure 4 the degradation products DP2 and DP3 were the same in the acidic and basic hydrolysis degradation, they had identical retention time the same $[M+H]^+$in LC method and the same R_f in TLC method, which confirms that they are the same components. These results were proved by NMR and FT-IR spectra. Thus DP2 and DP3 are hydrolysis degradation products which suggest that adding acid to the hydrolysis medium only help to accelerate the degradation and increase the formation of DP3, while the basic conditions also increase the speed of degradation, increase the formation of DP2 and lead to formation of DP1, which only appeared in basic hydrolysis. Both chromatographic methods were able to separate all the degradation products not only from Rivaroxaban but also from each other in stressed samples. Thus, the methods proved to be selective and stability-indicating. The degradation products were successfully characterized using mass spectrometry, NMR and FT-IR.

DP1, appeared only under basic hydrolysis at retention time of 10.1min, with $[M+H]^+$ 410 m/z and its R_f was 0.75 in the TLC method, but unfortunately, within the applied conditions, DP1 could not be separated in a sufficient amount to continue NMR and FT-IR studies. Only DP2 and DP3 were isolated by TLC-densitometric and analyzed by NMR and FT-IR spectra.

DP2, appeared in both acidic and basic hydrolysis at retention time of 21.0min, with $[M+H]^+$ 454 m/z and its R_f was 0.50 in TLC method. And with the aid of the FT-IR and NMR H^1, C^{13} and Dept135 spectra it was suggested that DP2 has a molecular weight 453 and molecular formula $C_{19}H_{20}ClN_3O_6S$. It could be seen from

NMR spectra of DP2 by comparing to Rivaroxaban, that there was an increase in protons (two protons). Additional protons could be due to –OH and –NH formation by the hydrolysis of oxomorpholin ring. FT-IR spectrum shows C=O stretching absorptions at 1738cm^{-1}, N-H stretching absorptions at 1647cm^{-1} and O-H board stretching absorptions at 3367cm^{-1}.According to that it is suggested that the oxomorpholin ring was opened and a carboxylic acid derivative was formed.

For DP3, which appeared also in both acidic and basic hydrolysis at retention time of 32.5 min, with $[M+H]^+$ 392m/z, its R_f was 0.35 in TLC method. The NMR spectra of DP3 shows a decrease in one carbon at 154 δ ppm and the protons remain the same as Rivaroxaban. Dept135 shows that DP3 has four -CH_2 and eight -CH. FT-IR spectrum shows an increase in N-H stretching absorptions at 3413cm^{-1},additional C-N stretching absorptions at 1126cm^{-1}and C=C asymmetric stretch at (616 and 637)cm^{-1}, while there was a clear absorption decrease in C=O stretching absorptions at 1738cm^{-1}. As a result, DP3 could be formed by hydrolysis of oxazolidine ring, where its molecular formula is $C_{18}H_{18}ClN_3O_3S$ and molecular weight is 391.

The above results allowed to propose degradation pathways of Rivaroxaban, and the schematic representations of mechanism of formation of the degradation products under hydrolytic stress are shown in Figure 9.

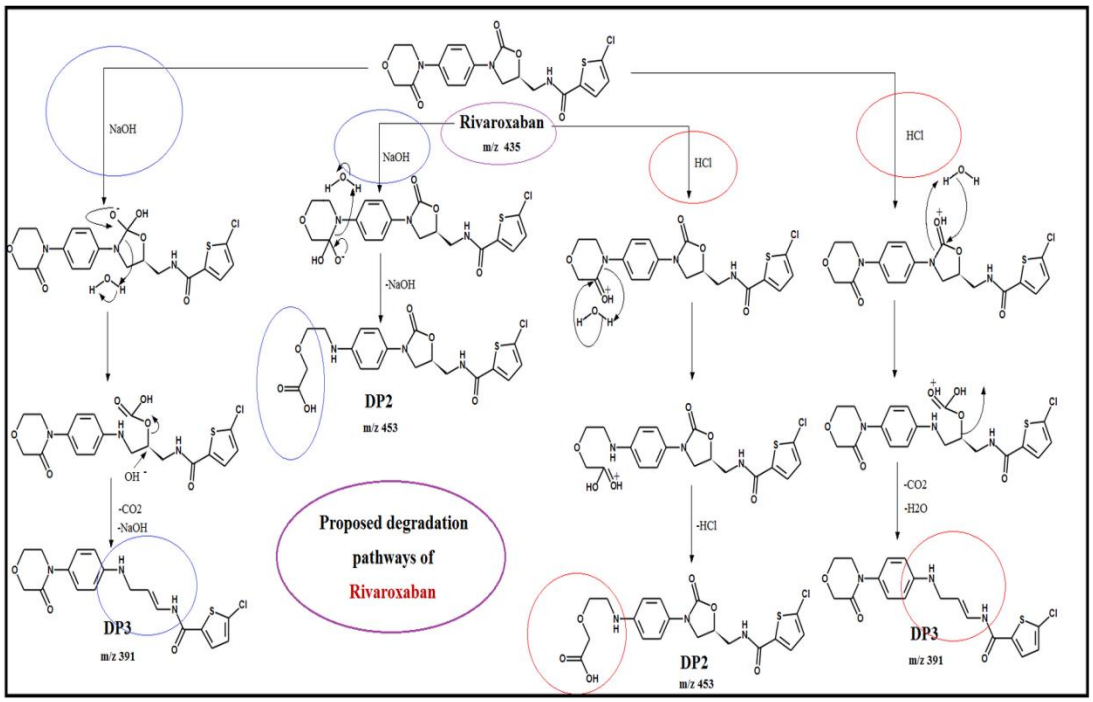


Figure 9 Proposed degradation pathways of Rivaroxaban.

5. CONCLUSION:

The forced degradation behavior of Rivaroxaban was studied as per ICH prescribed guidelines. Three degradation products were formed under stress acid and base hydrolytic conditions as detected by LC-MS and TLC-densitometer. ESI-MS studies were carried out to characterize structures of degradation products. Two of them were isolated by TLC and characterized also by NMR and FT-IR spectroscopy, and it was possible to predict the major degradation mechanisms. A simple, rapid and selective stability indicating LC and TLC methods have been developed and validated for the determination of Rivaroxaban and its degradation products. The developed methods were found to be suitable for the drug quantification as well as for the impurity determination.

6. ACKNOWLEDGEMENT:

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