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Development and Validation of Stability Indicating UPLC Method for Determination of Related Impurities of Apixaban Tablets

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Abstract: **Apixaban is an Anti-coagulant which prevents or reduces the coagulation of blood manifested by prolonging the clotting time. In the present study, a rapid, sensitive and economical UPLC method was developed and fully validated for analysis of Apixaban and its impurities (MPD4, APIX1B, N-Formyl and Methyl ester) in pharmaceutical finished product. Challenging task of separation of highly polar and non-polar impurities simultaneously with good resolution by development of accurate and sensitive UPLC method. The final UPLC method was developed by using Fortis Speed Core C18 column (150 mm × 4.6 mm, 2.6 µm). The temperature of column compartment was maintained at 40 °C. A gradient elution at a flow rate of 0.5 mL/min was employed and detection was carried out at 235 nm. Injection volume of 3 µL was employed. Eluent A : Mixture of 10 mM potassium dihydrogen phosphate, pH adjusted to 5.0 and Methanol in the ratio (90:10 v/v respectively) and eluent B : mixture of 10 mM Potassium dihydrogen phosphate, pH adjusted to 5.0, Acetonitrile, Methanol in the ratio (20:20:60 v/v/v). The validation parameters were inaccordance with FDA and ICH specifications, showing accuracy, precision, specificity, robustness and linearity from 0.1 – 2.5 µg/mL for the Apixaban and its impurities. The limit of detection and limit of Quantitation were Acid impurity and MPD4 impurity (LOD: 0.002%, LOQ: 0.007%), APIX1B impurity (LOD: 0.003%, LOQ: 0.009%), Apixaban (LOD: 0.002%, LOQ: 0.005%), N-formyl impurity (LOD: 0.001%, LOQ: 0.003%), Methyl ester impurity (LOD: 0.001%, LOQ: 0.002%) respectively. The validated method is simple, rapid, linear, precise, robust and method is suitable for Quality control applications.**

Keywords: **UPLC method, Apixaban Tablets, Atrial fibrillation.**

1. INTRODUCTION

Anticoagulants as the name indicates are the substances which prevent or reduce the coagulation of blood manifested by prolonging the clotting time. With the rise in various life style diseases, the need for Oral anticoagulation therapy has increased being relevant for cardiac complications like Atrial fibrillation, Venous thromboembolism, Myocardial infarction, etc,.[1] Recent important developments have improved the clinical outcomes with this therapy and led to a dimensional increase in the use of this therapy by improving safety [2]. One such anticoagulant which got FDA approval in the recent past is apixaban. It is orally bioavailable, highly selective and direct acting/reversible factor Xa inhibitor for prevention and treatment of thromboembolic diseases [3]. The chemical structure of the apixaban and its related impurities were shown in Figure- 1A-1F.

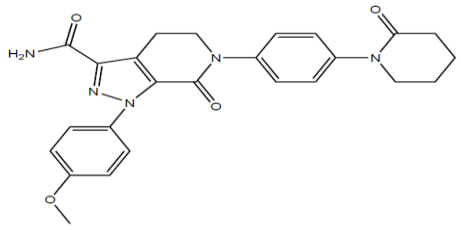

Figure-1A-Apixaban

Chemical name: 1-(4-Methoxyphenyl)-7-oxo-6-(4-(2-oxopiperdin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo(3,4-c)pyridine-3-carboxamide.

Molecular formula: $C_{25}H_{25}N_5O_4$

Molecular Wight: 459.4971 g/mol.

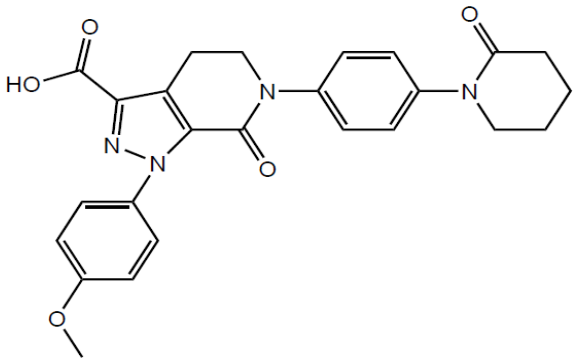

Figure -1B-Acid impurity

Chemical name: 1-(4-Methoxyphenyl)-7-oxo-6-(4-(2-oxopiperdin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo(3,4-c)pyridine-3-carboxylic acid.

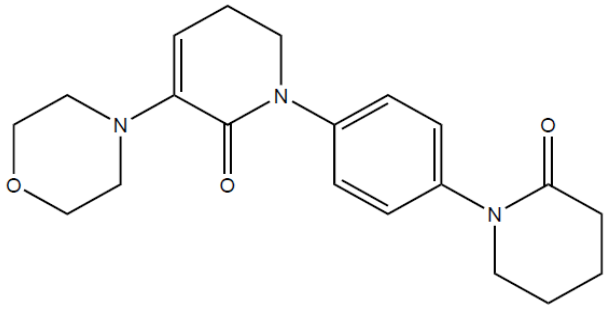

Figure -1C-MPD-4

Chemical name: 3-morpholino-1-(4-(2 oxopiperdin-1-yl)-5,6-dihydropyridin-2(1h)-one.

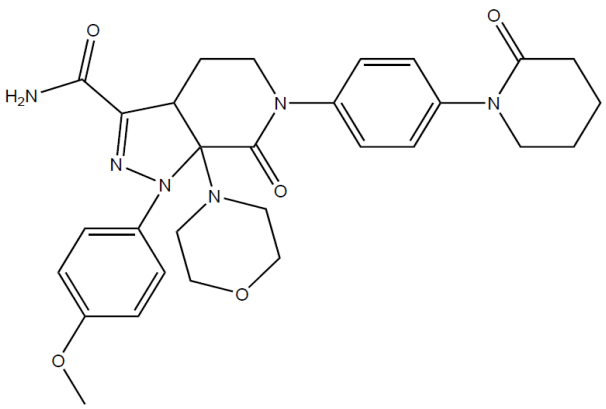

Figure -1D-APX 1B

Chemical name: 1-(4-Methoxyphenyl)-7a-morpholino-7-oxo-6-(4-(2-oxopiperdin-1-yl)phenyl)-3a,4,5,6,7,7a-hexahydro-1H-pyrazolo(3,4-c)pyridine-3-carboxamide.

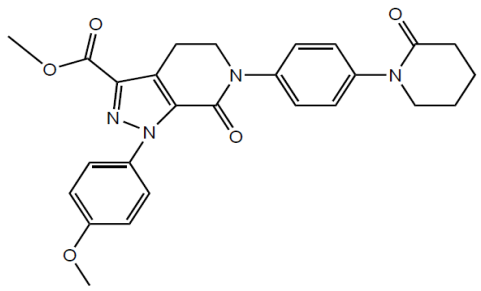

Figure -1E-Methylester

Chemical name: Methyl 1-(4-Methoxyphenyl)-7-oxo-6-(4-(2-oxopiperdin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo(3,4-c)pyridine-3-carboxylate.

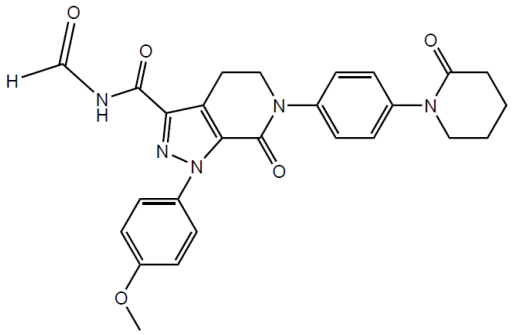

Figure -1F-N-Formyl impurity

Chemical name: N-formyl-1-(4-Methoxyphenyl)-7-oxo-6-(4-(2-oxopiperdin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo(3,4-c)pyridine-3-carboxamide.

Literature reports include methods for determination of apixaban in biological matrix like human plasma by LC-MS/MS technique[4-7] Stability-indicating assay methods for determination of apixaban like HPLC[8-9], LC-MS[10] and Spectrophotometric [11] were also reported. Moreover simultaneous quantitative screening method for apixaban along with other ingredients using LC-MS was also reported. Among the reported methods Prabhune et al.[8] and Landge et al.[9] concluded that apixaban is susceptible to acid and alkali hydrolysis only. However, among all the reported analytical methods separation and quantification of process related impurities of Apixaban is not available. Therefore an attempt was made to develop and validate an UPLC based method for determination of Apixaban in presence of its process impurities. It can be foreseen for its application to the quality control analysis and determinations of adulterants.

The current method was developed and validated for process as well as degradation impurities of Apixaban. For closely related impurities method, robustness plays a significant role. This method was covered the all method variations (Flow, column temperature, pH of buffer, and Organic variation in Mobile phases), whereas literature method covers flow and column temperature variations. Filter study is also a key step for filtering the samples during the analysis to avoid the load of column. This method was established for filter study with two filters (PTFE and Nylon). Based on all the considerations this method is effective than the literature reports for Quality control applications.

2. EXPERIMENTAL

2.1 Chemicals and Reagents:

Apixaban (> 99.5%) purity was received as a gratis sample by Dr. Reddy's Laboratories, (Hyderabad, India), Acid impurity (> 97.96%), MPD-4 (> 99.1%), APX-1B (> 99.1%), Methyl ester (> 91.9 %), N-Formyl Impurity (> 97.1 %) purity were kindly provided by MSN Pharma, (Hyderabad, India), HPLC grade Acetonitrile and Methanol were purchased from Merck (Mumbai, India). Chemicals like Potassium dihydrogen phosphate, Potassium chloride-GR was purchased from SD fine chemicals, (Hyderabad, India. Tablets of apixaban were purchased from local market. Milli-Q water (16.2 MΩcm) was obtained using Milli-Q system (Millipore, Billerica, MA).

2.2 Equipment and Chromatographic conditions:

All the experiments were performed using an Aquity UPLC-H Class system from Waters (Waters, Milford, MA, USA). The low pressure mixing system was equipped with a pumping device, auto sampler, column oven and UV - PDA detector. The quaternary solvent delivery pump was able to work up to pressure of 1000 bar and include an optional six-port solvent selection valve, allowing the selection of up to 6 different buffers and organic modifiers in any combination. The auto sampler is a flow through needle (FTN) injection system. The column oven can work up to 90 ^{o}C. The UV-PDA possesses a 0.5 µL flow cell. The chromatograms were analysed using Empower software and the separation was performed on a Fortis Speed Core C18 column (150 mm × 4.6 mm, 2.6 µm).

2.3 Preparation of solutions:

2.3.1 Standard solution of Apixaban:

Mixture of Water and Acetonitrile (1:1 v/v respectively) was prepared and used as a diluent. Stock standard solution-1 of 500 ppm of Apixaban was prepared by accurately weighing of 50 mg of Apixaban, transferring it into a 100 mL calibrated volumetric flask and adding 80 mL diluent. The flask was sonicated to dissolve and filled to volume with diluent. The stock standard solution-2 was prepared by diluting 5 mL of Stock standard solution-1 to 100 mL diluent to obtain a solution having concentration of 25 ppm of Apixaban. The reference standard solution was prepared by diluting 4 mL of Stock standard solution-2 to 100 mL diluent to obtain a solution having concentration of 1 ppm of Apixaban.

2.3.2 Impurity stock solution:

Accurately weighed 2.5 mg of each impurity (Acid impurity (> 97.96%), MPD-4 (> 99.1%), APX-1B (> 99.1%), Methyl ester (> 91.9 %), N-Formyl Impurity (> 97.1 %)), transferring it into a 20 mL calibrated volumetric flask and adding 15 mL diluent. The flask was sonicated to dissolve and filled to volume with diluent.

2.3.3 Peak identification solution:

Dilute 1 mL of impurity stock solution into 50 mL of volumetric flask and diluted to volume with standard stock solution-1.

2.3.4 Sample solution:

For sample analysis, 20 tablets (5 mg and 2.5 mg tablets) were weighed and transferred in to a 100 mL volumetric flask, 80 mL of diluent was added and the flask was sonicated for 40 minutes with intermittent shaking. The flask was filled to volume with diluent in order to obtain a solution having concentration of 500 ppm of Apixaban.

Centrifuged the solution at 5000 rpm for 10 minutes.

2.3.5 Excipient solution:

Excipient solutions were prepared by transferring a homogeneous mixture of the placebo mixture with the sample quantitative composition as the pharmaceutical formulations into a 100 mL volumetric flask, 80 mL of diluent was added and the flask was sonicated for 40 minutes with intermittent shaking, flask was filled to volume with diluent.

Centrifuged the solution at 5000 rpm for 10 minutes.

2.4 Method development:

The chromatographic method was developed to yield an adequate analytical performance in a short running time. The optimal conditions were determined by investigating the effects of mobile phase, pH, mobile phase composition and column temperature on the separation.

2.5 Method validation:

Method validation was performed by following ICH and FDA specification for system suitability, stability, specificity, linearity, precision. Accuracy, detection limit, quantitation limit and robustness.

2.5.1 System suitability:

System suitability testing was carried out by injecting three times of reference standard solution of Apixaban at 1 ppm. According to USP, the USP symmetry factor must be not more than 2.0, theoretical plates must be not less than 30000 and relative standard deviation for three replicate standards must be not more than 10.0%.

2.5.2 Specificity:

Specificity of the developed method was evaluated by forced degradation of test samples. The forced degradation conditions are Acid degradation: 1N HCl and reflux for 2 hours at 80 C, Base degradation: 0.5 N NaOH and reflux for 10 hours at 80° C, Oxidative stress: 15% H_2O_2 maintained at room temperature for 24 hours, Neutral hydrolysis: 5 mL water and reflux for 24 hours at 80° C. Photolytic degradation: Sample exposed to light in a photostability chamber for 1.2 lux hours. The respective samples were quenched and centrifuged at 5000 rpm for 10 minutes and the collected filtrate was injected into UPLC.

2.5.3 Detection and Quantitation limit:

The detection limit (DL) and Quantitation limit (QL) of the method was obtained from the equations:

DL= 3(SD/a)

QL= 10 (SD/a)

Where SD is the standard deviation of the y-intercepts and a is the slope of the calibration curves in the linearity study.

2.5.4 Linearity:

The Linearity was evaluated by the analysis of standard solutions of Apixaban and is impurities preparing series of solutions in the concentration range of 0.1 – 2.5 µg/mL (0.02% to 0.40% of test concentration). Curves were generated by plotting the average peak area of respective standard versus concentrations. From the obtained results, the calibration equation (y=ax+b) were calculated for each standard. From the calibration plots, relative response factors were calculated for impurities against to Apixaban standard.

2.5.5 Accuracy:

The accuracy of the method was evaluated by addition of five different amounts of Apixaban and its impurities to placebo mixtures. The amounts were equivalent to 0.02% to 1.0% of test concentration (0.10 to 5.00 ppm). At each level, asmples were prepared in triplicate and the recovery percentage was determined.

2.5.6 Precision:

The precision of an analytical method expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision was established by spiking the impurities at 0.2 % level with respect to the test concentration of 500 ppm in sample solution for the six times. The RSD of peak areas (n=6) was evaluated.

2.5.7 Solution stability:

The stability of the analytical solution was checked on benchtop and refrigerator (2-8°C) by preparing standard and impurities spiked sample at 0.2% test concentration level. The stability of standard and sample was established by calculating the correlation for the standard and % impurity difference for the sample for 96 hours.

2.5.8 Robustness:

The robustness of an analytical method is a measurement of its capacity to remain unaffected by small but deliberate variations in method parameters. The robustness of the UPLC method is very crucial to verify the reliability of a study with respect to deliberate variations in method parameters. It was evaluated by analysing data after verifying the column temperature (30 ± 5.0 °C), flow rate (0.5 ± 0.1), pH of the mobile phase (5.0 ± 0.2), Organic (Methanol) composition in Mobile phase-A (10%) and Organic (Acetonitrile) composition in Mobile phase-B (10%) using chromatographic conditions. The sample spiked with impurities at 0.2% test concentration level was used in these experiments. RRT's were evaluated as part of experiment at each variable condition.

2.5.9 Quantification of Apixaban impurities:

The relative response factors for Apixaban impurities were determined from the solution containing Apixaban and all the impurities from linearity concentration 0.1-2.5 µg/mL. The accurate weight percentage of the impurity present in Apixaban sample was calculated using its RRF value and peak response. The percentage area obtained from the area normalized method was divided by corresponding RRF value to determine accurate amount of each impurity.

3. RESULTS AND DISCUSSION

3.1 Method Development:

3.1.1 Selection of diluent:

Literature review indicated that apixaban have low solubility in aqueous media in the pH range from 1.2 to 7.4. Whereas with the combination of organic solvent increases the solubility. Based on best solubility and adaptivity acetonitrile: water (50:50 % v/v) was selected and considered for development.

3.1.2 Selection of wavelength:

The UV spectra of the drug exhibited two maxima at 220 nm and 278 nm. The overlay spectra of all the impurities and drug showed optimum response at 235 nm. Hence the wavelength of 235 nm was used for initial developmental trials.

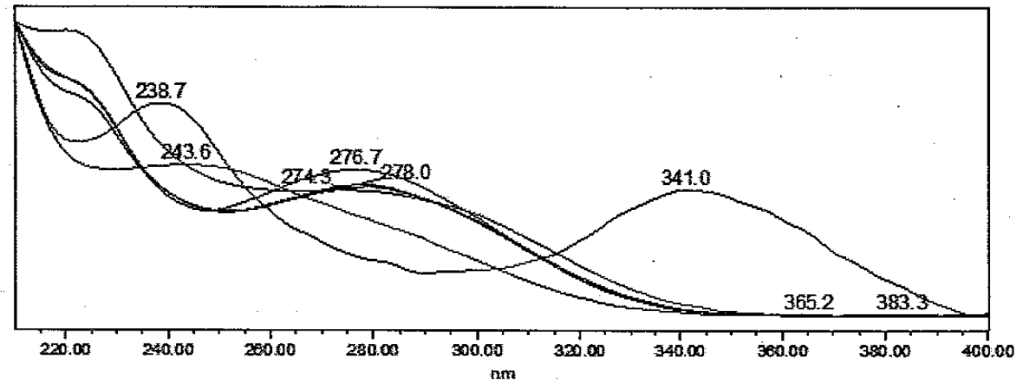

Figure-2: UV plot

3.1.3 Selection of buffer for mobile phase:

The physico-chemical properties of the drug reveal that the drug is strongly acidic (pKa 13.12) and strongly basic (pKa -1.6). The mobile phase selection was made based on characteristics of the drug without any interference with UV cut-off range. Thus potassium dihydrogen phosphate 10 mM pH-5.0 was selected for initial developmental trials.

3.1.4 Selection of column:

Literature reports highlight the usage of Ascentis Express C18 column 100 X 4.6 mm, 2.7 µm for the separation of nine impurities related to the drug. Hence the initial trials were carried using Agilent XDB C18, 100 X 4.6 mm, 1.8 µm column was selected. By considering the baseline inconsistency and separation of close related impurity, Fortis Speed Core C18-3, 150 mm X 4.6 mm, 2.6 µm was selected. In connection with this the selected column is suitable in the pH range 2-9, hence it is considered for the initial developmental trials.

3.2 Developmental trials:

1. Based on the above selected parameters developmental trials were conducted in gradient mode (0-10 minutes (100% A), 10-15 minutes (30-70% B), 15-25 minutes (70% B), 25-30 minutes (70-0% B), 30-35 minutes (100% A)) using a mobile phase-A consisting of pH 5.0 phosphate buffer: Acetonitrile (80:20) and mobile phase-B consisting of pH 5.0 phosphate buffer: Acetonitrile (20:80), water : Acetonitrile (1:1) as diluent and by using the Agilent XDB C18, 100 X 4.6 mm, 1.8 µm column at temperature of 35°C with flow rate of 0.5 mL/min at chromatographic detection of 235 nm. It resulted in co-elution of APIX-1B impurity and Apixaban, baseline was not smooth and found to be distorted after 18 minutes.

2. By considering the co-elution of impurity and Apixaban, another trail was conducted by changing the length of column. Hence Fortis Speed Core C18-3, 150 mm X 4.6 mm, 2.6 µm column was selected and trail was performed by using the same conditions as mentioned in the first trail. It resulted in no improvement of co-eluting peak with main peak.

3. By considering the co-elution and baseline was not smooth, organic composition of Mobile phase-A was changed to pH 5.0 phosphate buffer: Methanol (90:10) and mobile phase-B consisting pH 5.0 phosphate buffer: Methanol (20:80), with gradient of 0.0 – 10 minutes (25-50 % B), 10 – 20 minutes (50 % B), 25 – 30 (50-70 % B), 30 – 32 (70 % B), 40 –

42 (70 – 25 % B) and 32 – 40 (25 % B) at flow rate of 0.5 mL/min and keeping all trial-1 parameters constant experiment was performed, resulted baseline was not smooth and co-elution of peak with main peak found satisfactory.

Likewise trials were conducted using different gradient programmes, parameters etc., each trial resulted in better understanding and lead to the optimization phase.

Results of relative response factor:

The relative response factor (RRF) of Acid impurity, MPD-4, APX-1B, N-Formyl and Methyl ester Impurities with respect to Apixaban were found to be 0.80, 0.62, 0.73, 1.22 and 0.79 respectively.

3.3 Optimised method:

The final UPLC analysis was performed using Fortis Speed Core C18 column (150 mm × 4.6 mm, 2.6 µm). The temperature of column compartment was maintained at 40 ^{o}C. A gradient elution at a flow rate of 0.5 mL/min was employed and detection was carried out at 235 nm. Injection volume of 3 µL was employed. Eluent A : Mixture of 10 mM potassium dihydrogen phosphate, pH adjusted to 5.0 and methanol in the ratio (90:10 v/v) and eluent B : mixture of 10 mM potassium dihydrogen phosphate, pH adjusted to 5.0, acetonitrile, methanol in the ratio (20:20:60 v/v/v). Run time of 50.0 minutes with a gradient elution: 0.0 – 10 minutes (30 - 40 % B), 10 – 25 minutes (40 – 45 % B), 25 – 35 (45-70 % B), 35 – 40 (70 % B), 40 – 42 (70 – 30 % B) and 42 – 50 (30 % B) was used. The retention time for main peak was found to be 23.4 minutes and resolution between APX-1B and unknown found to be 1.6. Good separation was observed for co-elution of unknown peak before retention time of N-formyl impurity (Rs – 2.2). Similarly very good separation was observed for co-elution of unknown peak before retention time of acid impurity (Rs – 6.5). The final chromatogram with a neat separation of all compounds is shown in Figure-3.

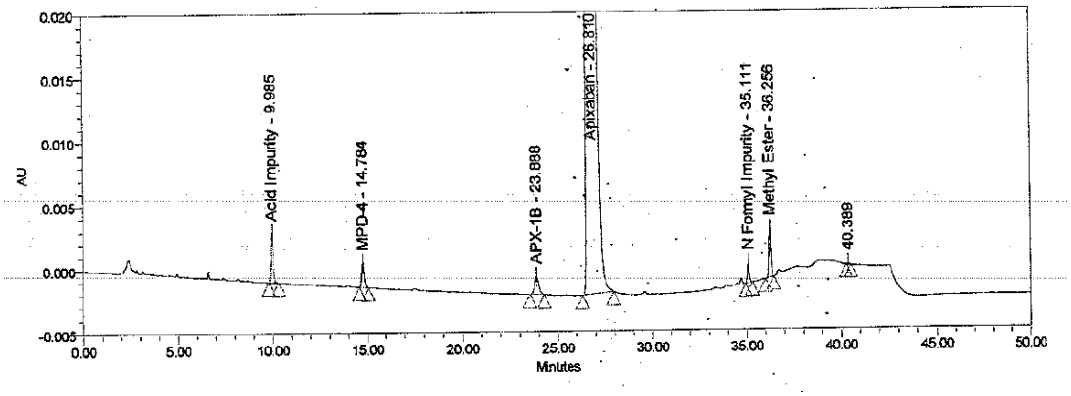

Figure-3: Spiked sample chromatogram

3.4 Method validation:

3.4.1 System suitability testing:

A representative chromatogram of system suitability testing is shown in Figure. 4. The following results were found: USP tailing factor of 1.1, theoretical plates of 74601 and % RSD for three replicate injections of 0.4. Thus all parameters are in agreement with the USP recommendations.

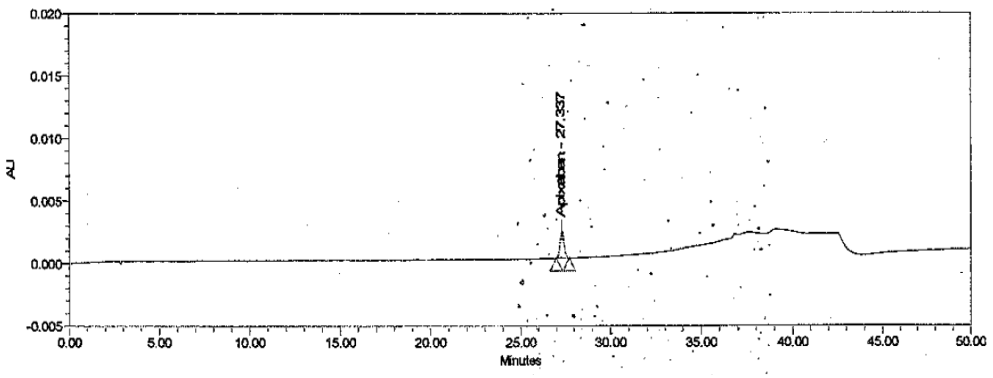

Figure-4: Standard chromatogram

3.4.2 Specificity:

Specificity of the developed method was evaluated by forced degradation of test samples. The forced degradation were analysed using UPLC. The results of forced degradation condition and evaluations are represented in Table 1 & 2. Represented chromatograms are shown in the Figure-5 to Fiure-15. Results indicated that the drug is susceptible for acid (21.6%) and base degradation (1.16 %). It is also observed that no interference was found from diluent, placebo, and any of degradation peaks at the retention time of apixaban and all its impurities.

Table-1: Forced degradation study

Sample name	Condition	% Degradation	% Assay	Actual mass= % Degradation +% Assay	% Mass balance= Actual mass * 100/% Assay in control sample
Control	NA	0.15	98.7	98.85	NA
Acid degradation	1N HCI, 80°C, 2 Hr	21.66	79.4	101.06	102.2
Base degradation	0.5N NaOH, 80°C, 10 Hr	1.16	95.1	96.3	97.4
Peroxide degradation	15% H2O2, 24 Hr benchtop	0.14	98.3	98.4	99.6
Water degradation	Water, 80°C, 3 Hr	0.14	103.3	103.4	104.6
Photo (open)	1.2 million lux Hrs	0.14	101.4	101.5	102.7
Photo (close)	1.2 million lux Hrs	0.14	102.3	102.4	103.6
Thermal degradation	105°C, 5 days	0.14	101.9	102.0	103.2

Table-2: Results of forced degradation studies of Apixaban sample.

Name	RRT	Control	Acid Stress	Base Stress	Peroxide Stress	Water Stress	Photo Stability (Open)	Photo Stability (Close)	Heat Stress
Acid Impurity	0.37	0.04	1.98	0.35	0.05	0.06	0.05	0.05	0.05
MPD4	0.55	ND	ND	0.05	ND	ND	ND	ND	ND
APIX-1B	0.89	ND	ND	ND	ND	ND	ND	ND	ND
N-Formyl	1.31	0.04	ND	ND	ND	ND	0.04	0.04	0.04
Methyl ester	1.35	0.01	0.01	ND	ND	0.01	ND	ND	ND
Unknown-1	0.20	ND	0.02	ND	ND	ND	ND	ND	ND
Unknown-2	0.27	ND	0.40	0.27	0.02	ND	ND	ND	ND
Unknown-3	0.32	ND	ND	ND	ND	ND	ND	ND	ND
Unknown-4	0.42	ND	ND	0.06	ND	ND	ND	ND	ND
Unknown-5	0.47	ND	ND	ND	ND	ND	ND	ND	ND
Unknown-6	0.52	ND	ND	ND	0.02	0.02	ND	ND	ND
Unknown-7	0.64	ND	ND	0.02	ND	ND	ND	ND	ND
Unknown-8	0.66	ND	ND	0.02	ND	ND	ND	ND	ND
Unknown-9	0.77	ND	19.16	0.29	ND	0.03	ND	ND	ND
Unknown-10	0.95	ND	ND	0.30	ND	ND	ND	ND	ND
Unknown-11	1.10	0.02	ND	0.03	0.02	ND	0.02	0.02	0.02
Unknown-12	1.19	ND	ND	ND	ND	ND	ND	ND	ND
Unknown-13	1.24	ND	ND	ND	ND	ND	ND	ND	ND
Unknown-14	1.28	ND	ND	0.03	0.03	ND	0.03	0.03	0.03

Unknown-15	1.34	ND	0.03	ND	ND	ND	ND	ND	ND
Unknown-16	1.40	ND	0.02	ND	ND	0.03	ND	ND	ND
Unknown-17	1.54	ND	0.02	ND	ND	ND	ND	ND	ND
Total	NA	0.15	21.66	1.16	0.14	0.14	0.14	0.14	0.14

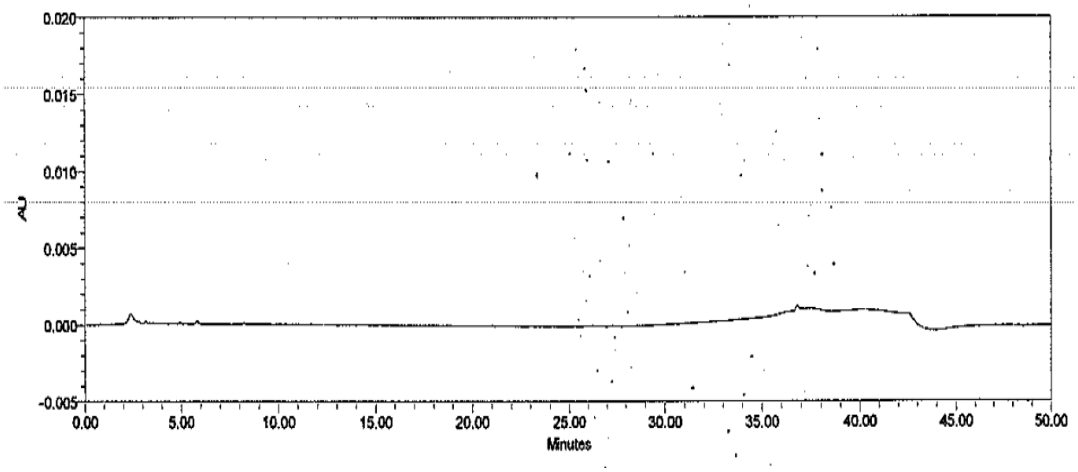

Figure-5: Diluent chromatogram

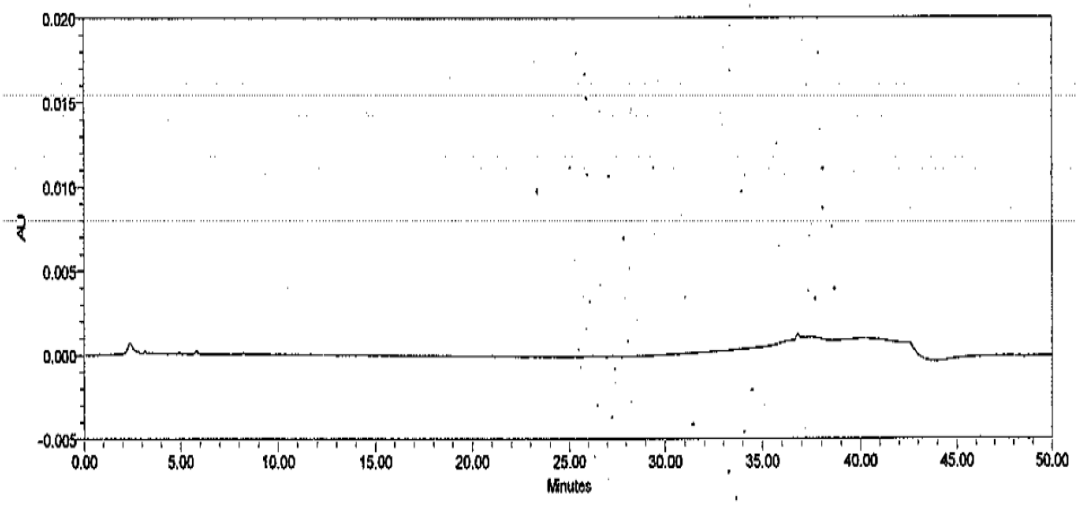

Figure-6: Placebo chromatogram

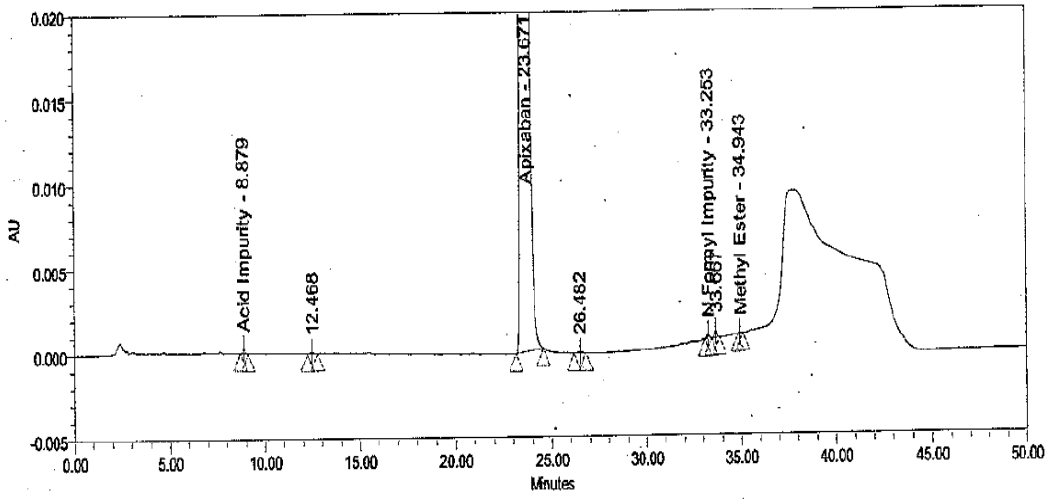

Figure-7: Unspiked test chromatogram

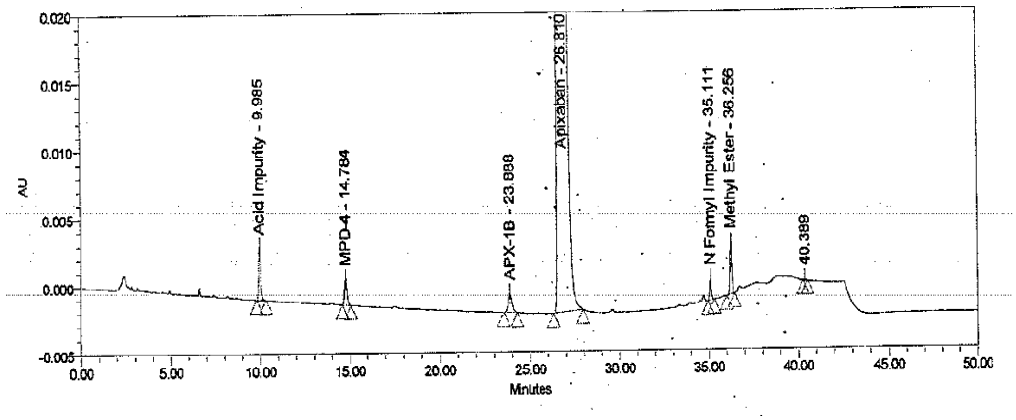

Figure-8: Peak Identification chromatogram

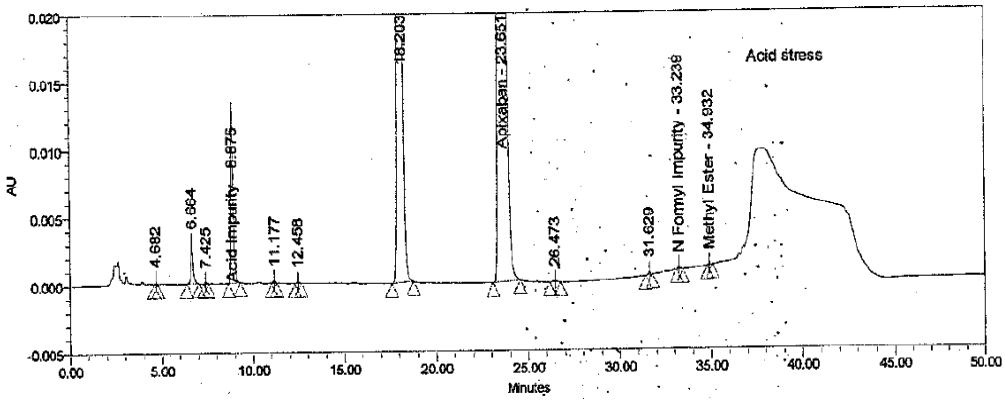

Figure-9: Acid Stress chromatogram

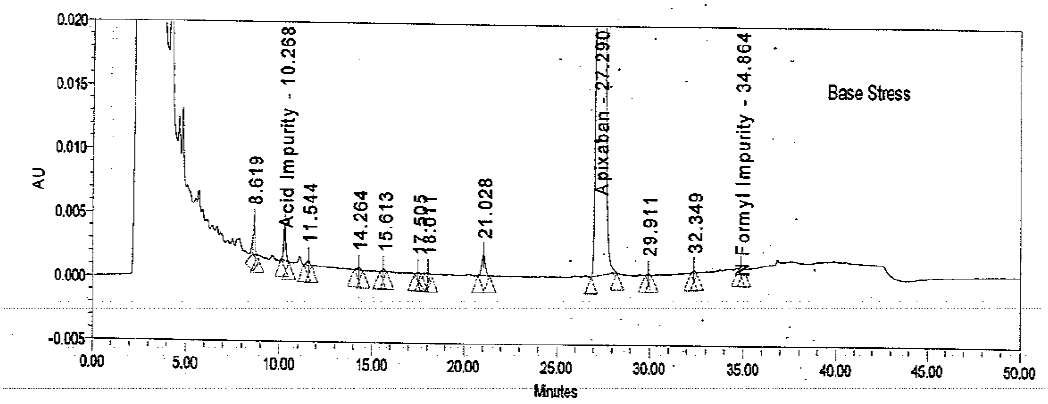

Figure-10: Base Stress chromatogram

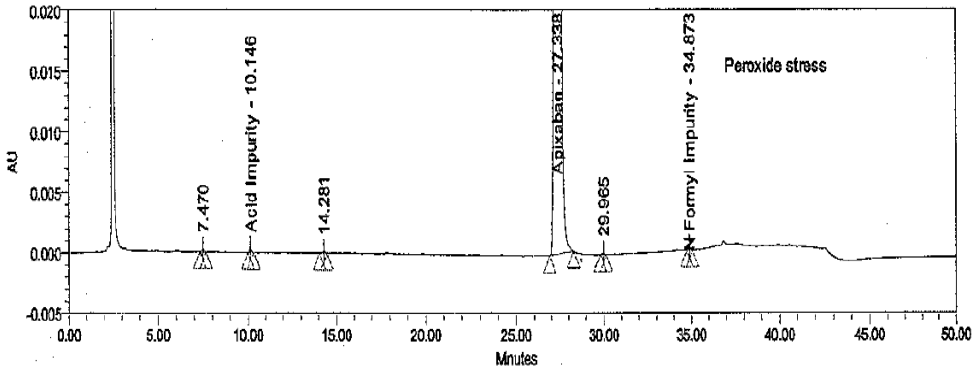

Figure-11: Peroxide Stress chromatogram

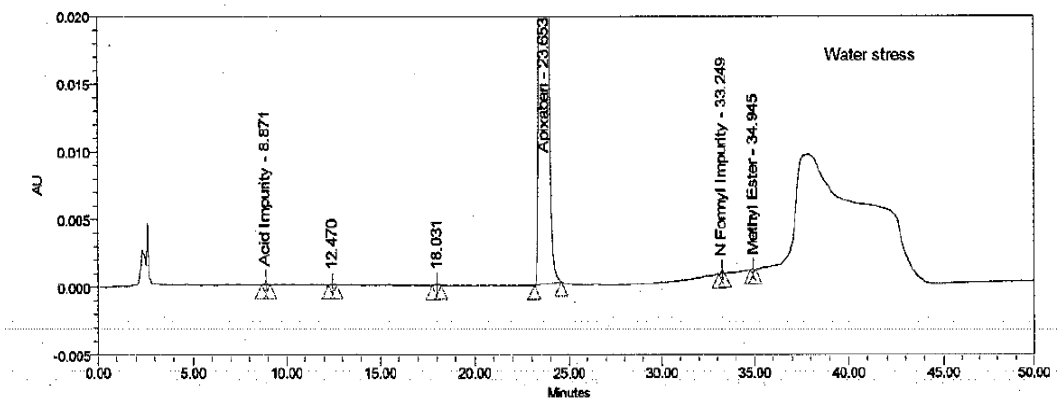

Figure-12: Water Stress chromatogram

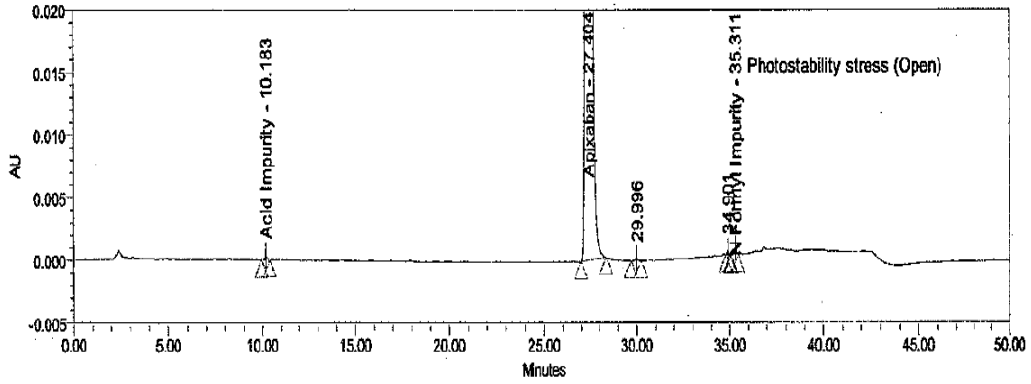

Figure-13: Photo stability Stress (open) chromatogram

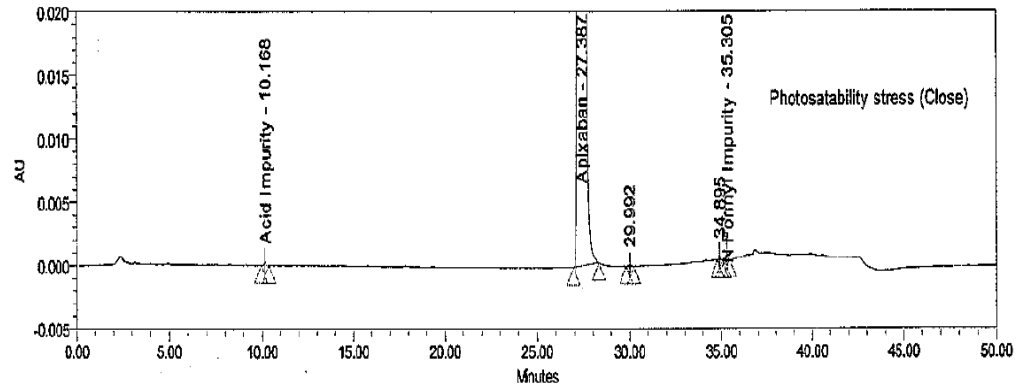

Figure-14: Photo stability (close) Stress chromatogram

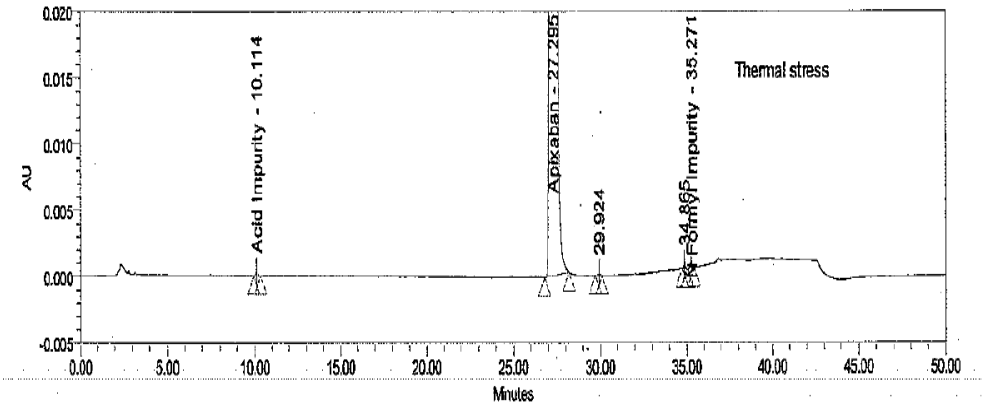

Figure-15: Thermal Stress chromatogram

3.4.3 Detection limit and Quantitation limit:

The Detection limit and Quantitation limit of the UPLC method were established by preparing series of solutions of Apixaban and its impurities. Results were summarized in the table-3.

Table-3: Results of Establishment of LOD and LOQ

Name of Impurity	% Impurity	
	LOD	LOQ
Acid impurity	0.002	0.007
MPD4 impurity	0.002	0.007
APIX1B impurity	0.003	0.009
N-formyl impurity	0.001	0.003
Methyl ester impurity	0.001	0.002
Apixaban	0.002	0.005

3.4.4 Linearity:

Linearity of the developed method was evaluated by preparing series of solutions of Apixaban and impurities. The response was plotted against concentration in the tested range and the co-relation coefficient for all impurities and Apixaban was found to be more than 0.99. The results of the linearity were represented in the Table-4.

Table-4: Results of Linearity

Name of Impurity	**Correlation coefficient**	**Slope**	**% Y-intercept**
Acid impurity	0.9999	11984.7	0.02
MPD4 impurity	0.9999	9216.7	-0.37
APIX1B impurity	1.0000	10923.9	-0.61
N-formyl impurity	0.9999	18249.4	-0.10
Methyl ester impurity	0.9999	11820.5	-0.74
Apixaban	1.0000	14944.8	-0.72

3.4.5 Precision:

The precision of an analytical method expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision was established by spiking the impurities at 0.2 % level with respect to the test concentration of 500 ppm in sample solution. Results are summarized in Table 5.

Table-5: Results of method precision (n=6).

Sample Name	**Acid Impurity**	**MPD4**	**APIX-1B**	**N-Formyl Impurity**	**Methyl Ester**
Prep-1	0.294	0.246	0.228	0.255	0.241
Prep-2	0.287	0.242	0.224	0.25	0.237
Prep-3	0.285	0.241	0.224	0.249	0.236
Prep-4	0.285	0.241	0.223	0.244	0.238
Prep-5	0.287	0.241	0.222	0.242	0.237
Prep-6	0.284	0.237	0.223	0.243	0.236
Mean	**0.287**	**0.241**	**0.224**	**0.247**	**0.238**
% RSD	**1.27**	**1.19**	**0.94**	**2.04**	**0.79**

3.4.6 Accuracy:

The accuracy of the established method was evaluated by measuring the recovery after spiking the drug with impurities at 0.02%, 0.1 %, 0.2 %, 0.4 %, 0.5 % and 1.0 % of test concentration level. The calculated percentage of each impurity at 0.1 %, 0.2%, 0.5% and 1.0% level indicating the suitability of the developed method in quantifying the concentration of Apixaban impurities in pharmaceutical tablets. Results are summarized in Table 6.

Table-6: Results of Accuracy

Name of impurity	Average % Recovery at each level					
	0.02%	0.1%	0.2%	0.4%	0.5%	1.0%
Acid impurity	100.6	102.3	103.5	103.3	103.9	104.2
MPD4 impurity	100.7	97.2	97.9	98.8	101.9	100.9
APIX1B impurity	96.7	98.1	99.3	98.9	103.2	101.3
N-formyl impurity	106.2	101.9	98.8	97.8	110.2	108.1
Methyl ester impurity	101.9	101.1	101.6	102.0	108.6	107.8

3.4.7 Solution stability:

The stability of the Apixaban standard solution and sample with impurities at spiked at 0.2 % level stored at room temperature and at 2-8. The similarity factor for standard solution at different time interval with respect to initial area was between 95-105 %. The percentage difference in impurity results at different time interval with respect or initial result was within the limit. It can be concluded that both standard and sample is stable for 96 hours on room temperature and refrigerated condition (2-8°C).

3.4.8 Robustness:

The robustness of an analytical method is a measurement of its capacity to remain unaffected by small but deliberate variations in method parameters. The robustness of the UPLC method is very crucial to verify the reliability of a study with respect to deliberate variations in method parameters. The RRT's evaluation of impurities at each variable condition confirmed that the robustness of the method, since resolution of all peaks was not much affected after variation of all parameters, which indicates that the method is robust within the defined working region. Results are shown in Table 7.

Table-7: Results of robustness study performed with impurities spiked at 0.2 % level to the sample.

Parameter	RRT for impurities				
	Acid impurity	MPD4	APIX-1B	N-Formyl	Methyl ester
As such	0.37	0.57	0.90	1.44	1.59
Column Temp 35 ˚C	0.37	0.55	0.89	1.37	1.42
Column Temp 45 ˚C	0.37	0.59	0.91	1.49	1.56
Flow 0.4 mL	0.38	0.58	0.91	1.34	1.39
Flow 0.6 mL	0.36	0.56	0.89	1.50	1.56
Buffer pH 4.8	0.40	0.55	0.89	1.32	1.36
Buffer pH 5.2	0.37	0.55	0.89	1.33	1.38
MP-A (90:9 v/v)$	0.39	0.57	0.93	1.20	1.23
MP-A (90:11 v/v)$	0.37	0.56	0.89	1.38	1.42
MP-B (18:20:60 v/v)#	0.37	0.56	0.90	1.36	1.41
MP-B (22:20:60 v/v)#	0.38	0.58	0.91	1.39	1.45

$ - Minor Component in mobile phase-A is methanol.

- Minor Component in mobile phase-B is Acetonitrile.

3.4.9 Filter study:

Suitability of filter study was established against the centrifuged and 0.45 µm PTFE, 0.45 µm Nylon filtered samples which spiked impurities at 0.2% level. Area difference between centrifuged sample and filter sample for each impurity was calculated and found no much difference in impurity areas in filter samples and also found no other peaks observed in the filter samples. The results of Filter study are presented in Table 8.

Table-8: Results of robustness study performed with impurities spiked at 0.2 % level to the sample.

Name of Impurity	Area of impurity				
	Centrifuge	Nylon	% diff	PTFE	% diff
Acid impurity	17007	16916	0.54	16897	0.65
MPD4	11083	11024	0.53	11063	0.18
APX1B	12079	11935	1.19	12052	0.22
N-Formyl	21793	21637	0.72	21826	0.15
Methyl ester	13840	13801	0.28	14097	1.86

3.5 Analysis of pharmaceutical samples:

To demonstrate applicability of the developed method several different lots of Apixaban samples have been tested. Sample of Apixaban was prepared at test concentration, i. e, 500 ppm in diluent and injected in to equilibrated UPLC system. Area percent of impurities was obtained by area normalized method and the actual percentage of each known impurity was calculated by applying RRF value. The results of Apixaban formulation samples are presented in Table 9.

Table-9: Results of analysis of different lots Apixaban formulated samples

Name of impurity	Content of impurities in different formulations	
	Apixaban tablets 5 mg	Apixaban tablets 2.5 mg
Acid impurity	0.0566	0.0542
MPD4	BQL	BQL
APIX-1B	BQL	BQL
N-Formyl	0.0701	0.0706
Methyl ester	0.0250	0.0223
Highest unknown	0.0349	0.0342
Total	0.2081	0.1939

4. CONCLUSION

A sensitive, rapid, suitable, precise, linear, accurate and robust UPLC method has been developed for the determination of Apixaban impurities in pharmaceutical tablets. All the impurities were well resolved. All parameters meet the acceptance criteria for method validation according to the FDA and ICH specifications and method shows suitability, precision, accuracy, specificity, linearity and robustness. This developed method was provided better applications inters of robustness and filter compatibility studies than the literature reports. The validated method is rapid, sensitive, and economical and may be used to quantify the Apixaban impurities in a short analysis time and with well separation which shows an advantage method. The developed method can be applied for routine analysis to quantify the process as well as degradation impurities in quality control laboratories.

REFERENCES

[1] Walsh, P. N. Oral anticoagulant therapy. *Hospital Practice*, 1983, 18, 101-120.

[2] Ansell, J., Hirsh, J., Dalen, J., Bussey, H., Anderson, D., Poller, L., & Matchar, D. Managing oral anticoagulant therapy. *Chest Journal*, 2001, 119, 22S-38S.

[3] Raghavan, N., Frost, C. E., Yu, Z., He, K., Zhang, H., Humphreys, W. G., & Zhang, D. Apixaban metabolism and pharmacokinetics after oral administration to humans. *Drug metabolism and disposition*, 2009, 37, 74-81.

[4] Delavenne, X., Mismetti, P., & Basset, T. Rapid determination of apixaban concentration in human plasma by liquid chromatography/tandem mass spectrometry: application to pharmacokinetic study. *Journal of pharmaceutical and biomedical analysis*, 2013, 78, 150-153.

[5] Gous, T., Couchman, L., Patel, J. P., Paradzai, C., Arya, R., & Flanagan, R. J. Measurement of the direct oral anticoagulants apixaban, dabigatran, edoxaban, and rivaroxaban in human plasma using turbulent flow liquid chromatography with high-resolution mass spectrometry. *Therapeutic drug monitoring*, 2014, 36, 597-605.

[6] Pursley, J., Shen, J. X., Schuster, A., Dang, O. T., Lehman, J., Buonarati, M. H., & Arnold, M. E. LC–MS/MS determination of apixaban (BMS-562247) and its major metabolite in human plasma: an application of polarity switching and monolithic HPLC column. *Bioanalysis*, 2014, 6, 2071-2082.

[7] Schmitz, E. M. H., Boonen, K., den Heuvel, D. J. A., Dongen, J. L. J., Schellings, M. W. M., Emmen, J. M. A., & Kerkhof, D. Determination of dabigatran, rivaroxaban and apixaban by ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) and coagulation assays for therapy monitoring of novel direct oral anticoagulants. *Journal of Thrombosis and Haemostasis*, 2014, 12, 1636-1646.

[8] Prabhune, S. S., Jaguste, R. S., Kondalkar, P. L., & Pradhan, N. S. (2014). Stability-indicating high-performance liquid chromatographic determination of apixaban in the presence of degradation products. *Scientia pharmaceutica*, 2014, 82, 777-786.

[9] Landge, S. B., Jadhav, S. A., Dahale, S. B., Solanki, P. V., Bembalkar, S. R., & Mathad, V. T. Development and validation of stability indicating RP-HPLC method on core shell column for determination of degradation and process related impurities of apixaban—an anticoagulant drug. *American Journal of Analytical Chemistry*, 2015, 6, 539.

[10] Secrétan, P. H., Sadou-Yayé, H., Aymes-Chodur, C., Bernard, M., Solgadi, A., Amrani, F., & Do, B. A comprehensive study of apixaban's degradation pathways under stress conditions using liquid chromatography coupled to multistage mass spectrometry. *RSC Advances*, 2015, 5, 35586-35597.

[11] Tantawy, M. A., El-Ragehy, N. A., Hassan, N. Y., & Abdelkawy, M. Stability-indicating spectrophotometric methods for determination of the anticoagulant drug apixaban in the presence of its hydrolytic degradation product. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 2016, 159, 13-20.