Research Article

RP-HPLC Method Development and Validation for the Quantitative Determination of Potential Impurities of Apixaban

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ABSTRACT

Quality Control is an integral part of pharmaceutical drug development. Various instrumental methodologies are developed to quantify the impurities that are developed during the stages of manufacturing, storage and transportation of pharmaceutical products. HPLC is one of the most reliable analytical methodologies to detect production and stress related impurities, to quantify them and to establish assay of active ingredient so as to pursue regulatory compliance. Here we communicate a novel method developed for impurity profiling with RP-HPLC. This study was to develop and evaluate the reverse phase high performance liquid chromatography (RP-HPLC) method for the quantitative determination of potential impurities of Apixaban active pharmaceutical ingredient. The method uses Puratis C18 column (250 × 4.6mm, 5µm) with mobile phase A consisted, 0.1% TFA in water and mobile phase B consisted acetonitrile with a gradient programme. The column temperature was maintained at 25°C and the detection was carried out at 280 nm. Results and Discussions: Efficient and reproducible chromatographic separation was achieved on C18 stationary phase in gradient elution profile. The newly developed HPLC method was validated according to ICH guidelines considering six impurities to demonstrate precision, linearity, accuracy and robustness of the method. The developed HPLC method was found to be accurate and sensitive. The correlation coefficient values are greater than 0.99 for Apixaban and its six impurities. Detection limit and quantitation limit was 0.31ppm and 0.96ppm respectively for Apixaban, indicating the high sensitivity of the newly developed method. Accuracy of the method was established based on the recovery obtained between 94.2% and 108.5% for all impurities. The result of robustness study also indicates that the method is robust and is unaffected by small variation in chromatographic conditions. The developed HPLC method provides reliable, reproducible, accurate and sensitive for the quantification of Apixaban related substances. This newly developed method has been validated as per regulatory requirements and has shown acceptable precision, accuracy and adequate sensitivity. This method can be used for the routine analysis of Apixaban active pharmaceutical ingredient related substances.

Keywords: Apixaban; Impurities; RP-HPLC; Validation.

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INTRODUCTION

The proposed study aims to develop efficient HPLC methods for API. The investigation includes the method development for analyzing API and their impurities. This will include the drug molecules from various therapeutic areas and their impurity. These pharmaceuticals may develop impurities at various stages of their development, transportation and storage, which must be detected and quantitated. The impurities are classified as process related impurities, degradation products, enantiomers, genotoxic impurities, etc. Various chemical and instrumental methods are developed at regular intervals, which are involved in the estimation of drugs for purity along with impurity profiling. Though several instruments are available for assay of drugs, HPLC is the general instrumentation technique. The main advantage of HPLC along with the drug purity profiling is that the content of process related impurities or stress degradant impurities present in minor quantities can also be determined simultaneously. In addition, great attention has been received using HPLC methods for the determination of drugs because of their importance in the quality control. Ease of operation, availability and its ability to provide quality control of bulk drugs and their formulations, HPLC is the unique, versatile, universal instrument and well utilized by the researchers. Identified impurities were characterized by mass spectrometry (MS) & Infrared spectroscopy (IR) and subsequently structural elucidation were performed with the help of nuclear magnetic resonance (NMR) spectroscopic technique.

Apixaban is an anticoagulant medication used to treat and prevent blood clots and to prevent stroke in people with nonvalvular atrial fibrillation. Specifically it is used to prevent blood clots following hip or knee replacement and in those with a history of prior clots. It is used as an alternative to warfarin and does not require monitoring by blood tests. It is taken by mouth.

Apixaban is a highly selective, orally bioavailable, and

reversible direct inhibitor of free and clot-bound factor Xa. Factor Xa catalyzes the conversion of prothrombin to thrombin, the final enzyme in the coagulation cascade that is responsible for fibrin clot formation. Apixaban has no direct effect on platelet aggregation, but by inhibiting factor Xa, it indirectly decreases clot formation induced by thrombin.

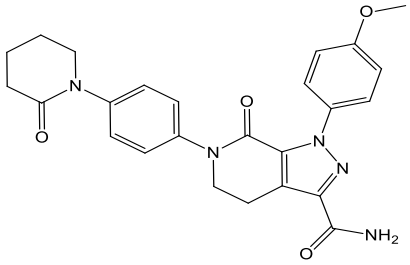


Figure 1: Chemical structure of Apixaban (AXN)

Molecular weight = 459.50g/mol

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

Several analytical methods have been reported to determine Apixaban in bulk drug, formulation and in biological matrices. These methods include high performance liquid chromatography (HPLC) [1-11,15-19,21,22] liquid chromatography tandem mass spectrometry (LC/MS) [12] and Ultra high-performance liquid chromatography (UHPLC-MS/MS) [13-14] and spectrophotometry [20]. Extensive literature survey reveals that no HPLC methods have been reported for the analysis of Apixaban drug and its related substance. Hence it was felt necessary to develop an accurate, rapid and sensitive HPLC method for the determination of Apixaban and its impurities.

METHODS

Reagents and Chemicals

Samples of Apixaban and standards of Imp-1, Imp-2, Imp-3, Imp-4 , Imp-5 and Imp-6 (Table 1) were received from Analytica Chemie Inc, Bangalore, India. HPLC grade methanol and acetonitrile was purchased from Rankem, Mumbai, India. Deionized water was prepared using a Milli-Q plus water purification system from Millipore (Bedford, MA, USA). Analytical reagent grade of trifluoroacetic acid, sodium hydroxide, hydrogen peroxide and hydrochloric acid were purchased from Merck India Limited

The LC method development and validation were done using Waters 2695 Alliance separations module HPLC equipped with Waters 2996 Photo diode array detector. The data were collected and the peak purity of the Apixaban peak was checked using Empower software.

Column selection

The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. Silica-based packing materials dominate in applications for RP separations in the pharmaceutical industry. The vast majority of RP LC separations take place on column that contain C_{18} bonded stationary phases due to their stability, retentivity and reproducibility. In addition, these hydrophobic ligands provide the desired separation most of the time. However, screening several different types of stationary phases during method development for a particular separation is often useful because different columns usually have different selectivity for components in a sample.

Several experiments were conducted to get a baseline resolution between Apixaban and impurities.

Water miscible organic solvent.

Use of RP_{18} column with a 250 mm length × 4.6mm ID column and 5µm particle size, use of 0.1% trifluoro acetic acid in water as mobile phase A and acetonitrile as mobile phase-B was significant in achieving the desired resolution of Apixaban and its impurities. After several trials for gradient profile, chromatographic conditions were finalized as described under section chromatographic conditions.

Chromatographic conditions

The chromatographic separations were achieved on Puratis Eximius, C18 column (250 mm length × 4.6 mm ID with 5µm particle size. Mobile phase A consisted, 0.1% Trifluoroacetic acid in water and mobile phase B consisted acetonitrile with a gradient programme (T_{min}A:B) $T_0$95:05, $T_2$95:05, $T_{15.0}$5:95, $T_{17.0}$5:95, $T_{18.0}$95:5, $T_{20.0}$95:5. The column temperature was maintained at 25˚C and the detection was carried out at 280 nm. The flow rate was set to 1.0 mL/min. The test concentration was about 100 µg/mL and the injection volume of 10µL. A degassed mixture of acetonitrile and water (8:2) was used as diluent for standard and sample preparations.

Method development

Buffers are generally recommended to control the pH stability of the mobile phase. Buffers like ammonium acetate, dipotassium hydrogen orthophosphate, potassium hydrogen phosphate, diammonium hydrogen orthophosphate, ammonium hydrogen phosphate, and its combination were studied for HPLC method development. The principle difference in the behavior of acetonitrile and methanol is that where acetonitrile forms a thick multi-molecular adsorbed layer on the surface of reverse phase adsorbent (C_1-C_{18} and phenyl phases), while methanol is adsorbed only in monomolecular fashion. This brings a principal difference in the analyte retention mechanism in these two hydro-organic systems.

Preparation of stock solutions for method validation

A test preparation of 100 µg/mL of Apixaban API sample was prepared by dissolving in diluent. Stock solutions 1 of impurities was prepared by accurately weighing 12.5 mg each of Imp-1, Imp-2, Imp-3, Imp-4, Imp-5 & Imp-6 in diluent and made up to 25 mL with diluent. Solution 2: Transfered 5 mL of each Individual impurity stock solution

1 into a 25 mL volumetric flask and made up to volume with diluent. Impurity standard solution was prepared by taking 5ml of Impurity solution 2 and made up to 10 mL volume in a volumetric flask using diluent of each impurity.

Stock solutions 1 of Apixaban was prepared by dissolving 25 mg made up to 25 mL with diluent. Solution 2: Transferred 5.0 mL of the stock solution 1 into a 25 mL volumetric flask and made up to volume with diluent. Apixaban standard solution was prepared by taking 5ml of stock solution 2and made up to 10ml volume in a volumetric flask.

Sample preparation for forced degradation studies

Stress study is a complementary part of stability testing wherein influence of environmental factors like pH, temperature, humidity, oxygen and light are evaluated on a drug substance and products. Stress testing of the drug substance was performed as per ICH guidelines Q1 (R2) and it can help to identify the likely degradation products, which can in turn help to establish the degradation pathways, the intrinsic stability of the molecule and specificity of the proposed method. Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. Acid hydrolysis was performed in 1N HCl at 30 °C for 24hrs. The study in basic solution was carried out in 1N NaOH at 30 °C for 24hrs. For study in neutral solution, the drug dissolved in water and was kept at 40 °C for 24hrs. Oxidation studies were carried out at ambient temperature in 10% hydrogen peroxide for 24hrs. Samples were withdrawn subjected to LC analysis after suitable dilution (100 µg/mL) to evaluate the suitability of the proposed method to separate Apixaban from its degradation products. The excess of acid or base in volumetric flasks were neutralized and made up to the volume with diluent. Corresponding blank solutions were prepared following the sample procedure without apixaban sample. Thermal degradation was done at 80°C for 24 hrs on the solid sample. Photodegradation studies were carried out according to option 2 of Q1B in ICH guidelines. Photolytic degradation was performed by keeping 150 mg of sample in loss on drying (LOD) bottles in a photo stability chamber model TP 090G (Thermo Lab equipments Pvt. Ltd., Mumbai, India). One bottle was covered with lid and then with aluminium foil (dark control) whereas another bottle (photolytic exposed sample) was covered with lid to get a minimum exposure of 24 hours for light and 200 Wh/m2 for ultraviolet region. 0.1 mg/mL samples were prepared for thermal degradation and photolytic degradation samples.

RESULTS AND DISCUSSION

Method development

The determination of the suitability of a HPLC method is based upon the level of development. However, at a minimum HPLC method should provide baseline separation of starting materials, desired products, known impurities, and expected by-products. The chromatographic conditions should also be chemically compatible with the analytes. The main objective of the HPLC method development for Apixaban was to achieve efficient separation of impurities and a short run time method.

Selection of wavelength

The optimum wavelength of detection is the wavelength that gives the highest sensitivity for the significant related substances and minimizes the difference in response factors between those of the active pharmaceutical ingredient and the related substances. Apixaban and its impurities give good detector response at 280 nm, therefore the final absorption wavelength for detection was chosen at 280 nm.

Mobile phase selection

In reverse phase chromatography, the mobile phase consists of an aqueous buffer and a non-UV active organic solvent.

Results of forced degradation

Apixaban was found to be stable under stress conditions such as thermal, photolytic and hydrolysis conditions. The major degradation products of apixaban under these conditions were Imp-2. The developed method was able to separate other impurities generated during the oxidation, acid & base hydrolysis from apixaban and other known impurities. Significant degradation of the drug substance was observed under acidic stress conditions leads to a major degradation product at RRT 0.85 & 1.10 & basic stress conditions leads to a major degradation product at RRT 1.05 & 1.10. LC/MS analysis was carried out to identify this degradation product of apixaban using Agilent 6140 single quadrupole mass spectrometer. Acid hydrolysis stressed sample shown in the chromatogram, indicating that the degradation products formed between apixaban and hydrochloric acid, base hydrolysis stressed sample shown in the chromatogram indicating that the degradation products formed between apixaban and sodium hydroxide. Chromatograms of forced degradation study have been depicted in Figure 2 and degradation studies are depicted in Table 3.

Degradation studies and peak purity test results derived from PDA detector and LC/MS confirmed that the apixaban peak was homogenous. The method was found to be specific in the presence of Imp1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6 and their degradation products confirmed the stability indicating power of the newly developed method.

Method Validation

The newly developed method was validated for sensitivity, linearity, precision and accuracy, robustness and system suitability according to ICH guidelines 23. Validation study was carried out for Imp-1, Imp-2, Imp-3, Imp-4, Imp-5 & Imp-6. The system suitability and selectivity were checked by injecting 100 µg/mL of Apixaban solution containing 50µg/mL of all impurities monitored throughout the validation. Method validation results are summarized in

Table 2.

Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection and limit of quantitation were determined for Apixaban and for each of the related substances as per ICH $Q2R_1$ guideline. The LOD and LOQ for Imp-1, Imp-2, Imp-3 , Imp-4,Imp-5 and Imp-6 and Apixaban were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively by injecting a series of diluted solutions with known concentration. The limit of detection and the limit of quantitation for Imp-1, Imp-2, Imp-3, Imp-4, Imp-5 & Imp-6 and Apixaban were about 0.25, 0.40, 0.33, 0.36, 0.51, 0.11 & 0.32ppm, 0.76, 1.22, 1.01, 1.08, 1.54, 0.34 & 0.97ppm respectively.

Linearity and range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to concentration of the analyte in the sample. A linearity test solution for related substance method was prepared by diluting the impurity stock solution to the required concentrations. The correlation coefficient obtained was greater than 0.99 for all impurities. The result showed an excellent correlation between the peak and concentration of all impurities. The range of the method was from 4 to 14 µg/mL of the analyte concentration (100 µg /mL).

Precision

Precision of the method was studied for method precision and intermediate precision. Method precision was checked by injecting six individual preparations of (100 µg/mL) Apixaban spiked with 50µg/mL of each impurity. In the intermediate precision study, the similar procedure of method precision was carried out on different days. % RSD of areas of each impurity was within 5.0, confirming good precision at low level of the developed analytical method.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found.

The accuracy of the method was evaluated in triplicate 50%level (0.10mg/mL), 100% level (0.21mg/mL) and 150% level (0.30mg/mL). The percentage recovery of all impurities in drug substance has been calculated. Chromatogram of Apixaban spiked with six impurities was depicted in Figure 2.

Robustness

To determine the robustness of the method, experimental conditions were deliberately changed and the resolution between closely eluting peaks were evaluated. Close observation of analysis results of deliberately changed chromatographic conditions viz; flow rate (0.2±0.05 mL/min), 2% gradient composition variation and column temperature (25± 2 °C) shown no significant change in relative retention time for all impurities in spiked sample illustrating the robustness of the method. Robustness results are summarized in Table 4.

Solution stability and mobile phase stability

The solution stability of Apixaban and its potential impurities was carried out by leaving both spiked and unspiked sample solutions in tightly capped HPLC vials for 72 h in an auto sampler. Content of each impurity was determined against freshly prepared standard solution. No significant changes were observed in the content of any of the impurities. The solution stability and mobile phase stability experiment data confirms that the sample solutions and mobile phase used during related substance determination were stable for at least 72 hours.

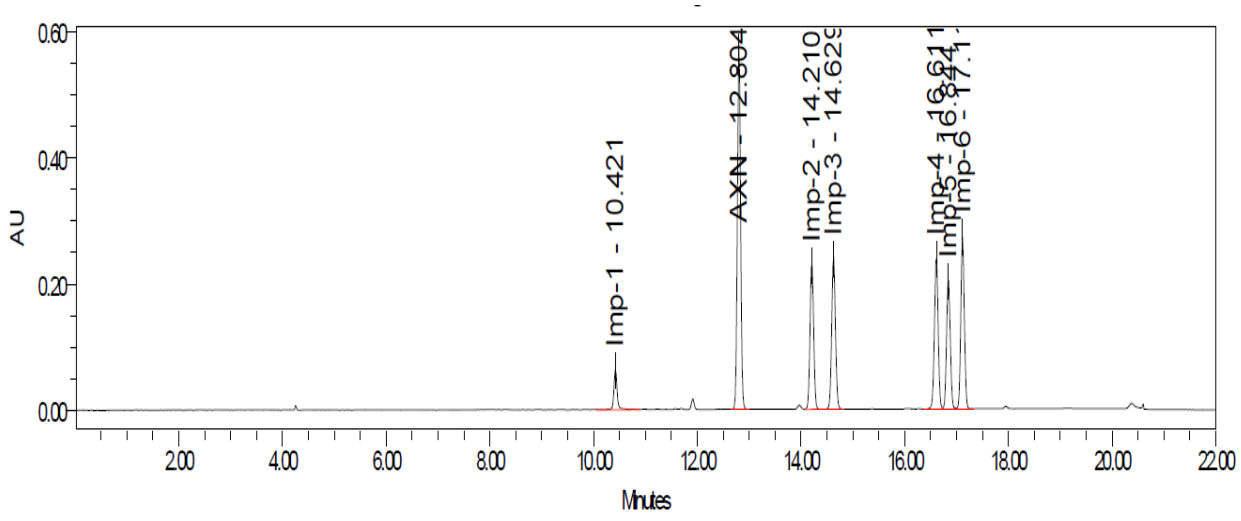


Figure 2: Chromatogram of Apixaban spiked with impurities

Table 1: Potential impurities of Apixaban

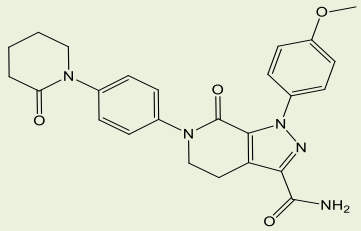
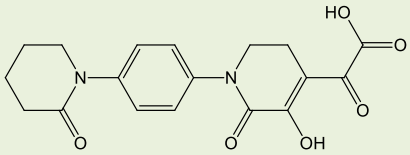
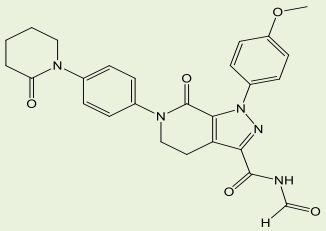
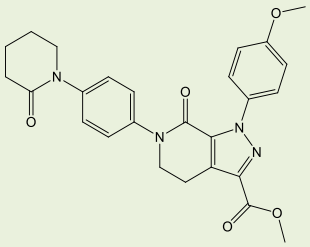
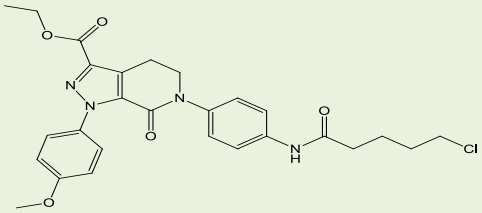
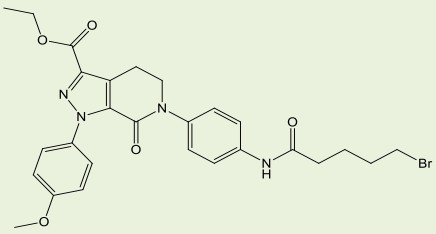
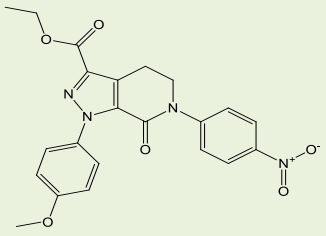
Name	CAS no	IUPAC name	Structure	Mol wt (g/mol)
AXN	[503612-47-3]	1-(4-Methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1-yl)phenyl]-4,5-dihydropyrazolo[3,4-c]pyridine-3-carboxamide		459.50
Imp-1	[1609409-54-2]	1,2,5,6-Tetrahydro-3-hydroxy-α,2-dioxo-1-[4-(2-oxo-1-piperidinyl)phenyl]-4-pyridineacetic acid		358.35
Imp-2	[1351611-14-7]	N-Formyl-4,5,6,7-tetrahydro-1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxo-1-piperidinyl)phenyl]-1H-pyrazolo[3,4-c]pyridine-3-carboxamide		487.51
Imp-3	[1074365-84-6]	Methyl 4,5,6,7-tetrahydro-1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxo-1-piperidinyl)phenyl]-1H-pyrazolo[3,4-c]pyridine-3-carboxylate		474.51
Imp-4	[1421823-20-2]	Ethyl 6-(4-(5-chloropentanamido)phenyl)-1-(4-methoxyphenyl)-7-oxo-4,5,6,7-tetrahydro- 1H-pyrazolo[3,4-c]pyridine-3-carboxylate		525.0
Imp-5	[881386-12-5]	Ethyl 6-[4-(5-bromopentanoylamino)phenyl]-1-(4-methoxyphenyl)-7-oxo-4,5-dihydropyrazolo[3,4-c]pyridine-3-carboxylate		569.45
Imp-6	[536759-91-8]	Ethyl-1-(4-methoxyphenyl)-6-(4-nitrophenyl)-7-oxo-4,5,6,7-tetrahydro-1h-pyrazolo[3,4-c] pyridine-3-carboxylate		436.42

Table 2: Method validation summary report

Parameter	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6	AXN
System suitability							
RT	10.4	14.2	14.6	16.6	16.8	17.1	12.8
RRT	0.81	1.10	1.14	1.29	1.31	1.33	
Rs	-	1.2	3.4	1.6	1.9	2.2	2.3
N	188519	238329	230679	297717	299503	344477	216930
T	1.2	1.0	1.0	1.0	1.0	1.0	1.0
Linearity							
r^2	0.9953	0.9987	0.9991	0.9991	0.999	0.9994	0.9996
Slope	4567.5	21364.5	22586.9	23599.2	18396.1	18772.8	23271.4
Detection limit (ppm)	0.25	0.40	0.33	0.35	0.50	0.11	0.31
Quantitation limit (ppm)	0.76	1.22	1.00	1.07	1.54	0.34	0.96
Precision							
% RSD (n 6)	2.1	0.28	0.34	0.58	0.19	0.63	0.48
Repeatability (intraday)							
% RSD (n 6)	1.1	0.06	0.09	0.03	0.20	0.22	0.18
Intermediate precision (interday)							
% RSD (n 6)	3.5	1.1	1.1	1.3	1.0	1.2	1.2
Accuracy at 50% level (n 3)							
Amount added (mg/ml)	0.0384	0.0416	0.0428	0.0406	0.0448	0.0544	0.10
Amount recovered (mg/ml)	0.0416	0.0414	0.0449	0.0426	0.0456	0.0568	0.10
% Recovery	99.12	108.45	99.53	105.05	102.59	104.48	99.12
Accuracy at 100% level (n 3)							
Amount added (mg/ml)	0.0768	0.0832	0.0857	0.0812	0.0888	0.1088	0.2155
Amount recovered (mg/ml)	0.0783	0.0783	0.0867	0.0821	0.0877	0.1084	0.2123
% Recovery	101.95	94.21	99.53	101.01	98.63	99.69	98.52
Accuracy at 150% level (n 3)							
Amount added (mg/ml)	0.1152	0.1248	0.1286	0.1219	0.1334	0.1632	0.3008
Amount recovered (mg/ml)	0.1169	0.1183	0.1304	0.1245	0.1328	0.1645	0.2970
% Recovery	101.55	94.81	101.42	100.83	99.57	102.13	98.76

Table 3: Degradation studies report

Stress condition	Time(hrs)	Temp(°C)	% Assay of active substance	% Area
Acid Hydrolysis (1N HCl)	24	30	92.37	98.32
Basic Hydrolysis (1N NaOH)	24	30	93.87	96.76
Oxidation (10% H_2O_2)	24	30	99.21	99.91
Hydrolysis (30 °C)	24	40	99.26	99.27
Thermal	24	80	99.69	99.17
Photolytic	24	30	99.04	99.47

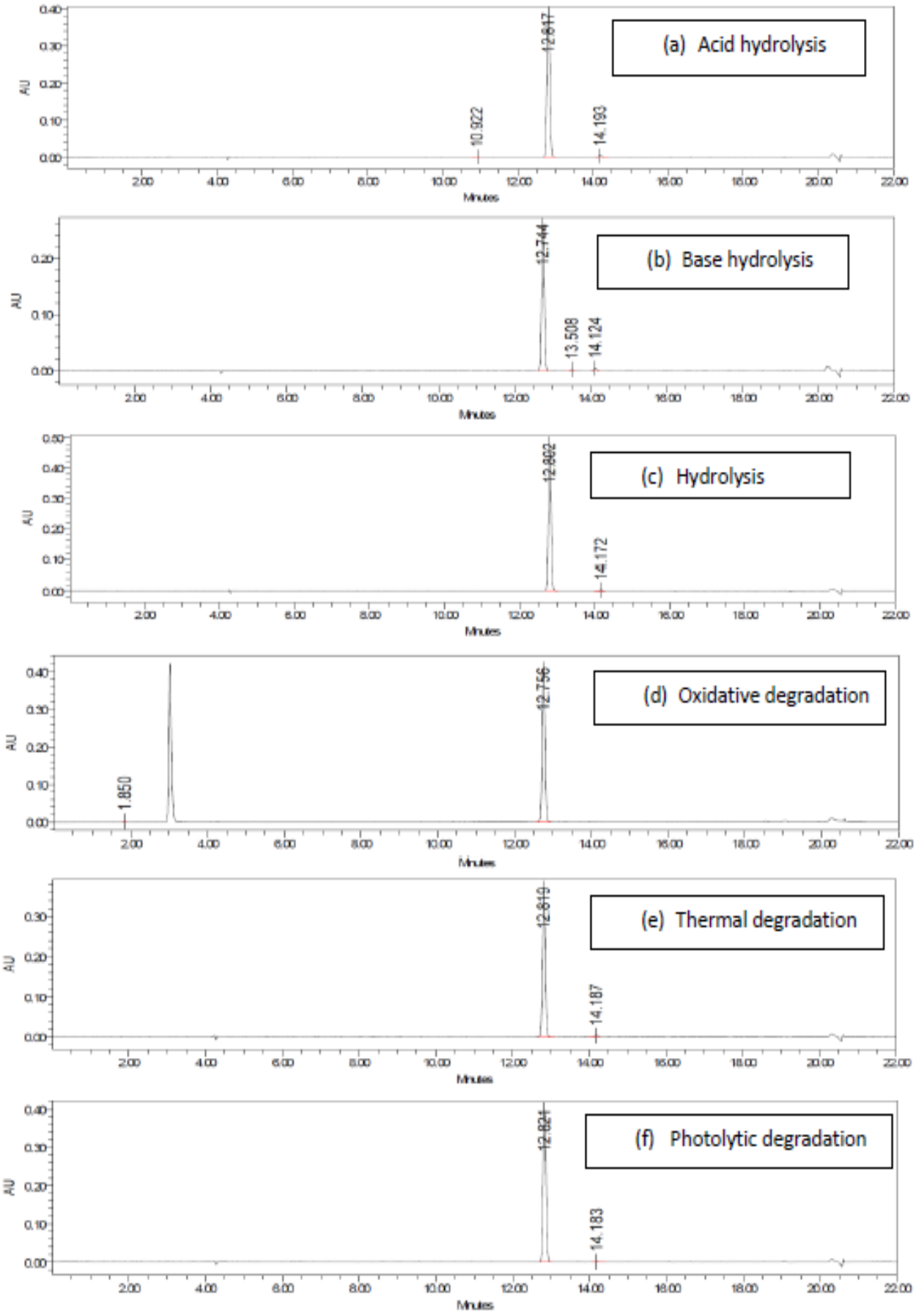


Figure 3: Typical chromatogram of Apixaban under stress conditions: (a)acid hydrolysis, (b)base hydrolysis, (c)hydrolysis, (d)oxidative degradation, (e)thermal degradation and (f) photolytic degradation

Table 4: Robustness study report

Chromatographic Condition			
Column oven temperature (°C)	**Flow rate (ml/min)**	**Composition of A:B (%)**	**% Assay of active substance**
23	1.0	95:5	99.8
25	1.0	95:5	100.3
27	1.0	95:5	99.9
25	0.8	95:5	100.0
25	1.0	95:5	100.3
25	1.2	95:5	99.9
25	1.0	93:7	99.9
25	1.0	95:5	100.3
25	1.0	97:3	99.7

CONCLUSION

The developed HPLC method provides reliable, reproducible, accurate and sensitive for the quantification of Apixaban and impurities. This newly developed method has been validated as per regulatory requirements and has shown acceptable precision, accuracy and adequate sensitivity. This method can be used for the routine analysis of Apixaban active pharmaceutical ingredient impurities.

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