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Development and Validation of an New High Performance Liquid Chromatography Method for Determination of Apixaban Isomers

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Abstract: The purpose of this research study is to develop a novel, simple, precise, accurate and economical method for determination of Apixaban API isomers. Apixaban API has three isomer as ortho, meta and para. In reverse phase and normal phase chromatography it was very difficult to separate these isomer; hence new chiral technique was adopted. This chromatographic method was developed on chiralpak IA column (250×4.6×5μm) with isocritic technique. The detection of isomeric impurities were observed at wavelength 290nm. This analytical method was validated as per ICH guideline and regression analysis showed R value (correlation coefficient) > 0.999 for Apixaban API and its isomeric impurities. A solution of Apixaban in dichloromethane was found stable up to 48 hrs. The degradation study was done within the given guidelines prescribed by ICH. The method is validated for Linearity, Accuracy and Precision.

Keywords: Apixaban, NP-HPLC, Isocritic, Validation, Chiralpak IA Column

1. Introduction

Apixaban is chemically known as 1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1-yl) phenyl]-4, 5, 6, 7-tetrahydro-1H-pyrazolo [3, 4-c] pyridine-3-carboxamide is a Anti - coagulant drug, a direct inhibitor of factor X, also used in the prevention of venous thrombo embolism. Apixaban is recommended by the National Institute for Health and Clinical Excellence for the prevention of stroke and systemic embolism in people with non-valvular atrial fibrillation and at least one of the following risk factors: prior stroke or transient ischemic attack, age 75 years or older, diabetes mellitus, or symptomatic heart failure [1].

2. Objectives of the Study

The literature survey reveals that few analytical methods have been reported for Apixaban and its related compounds including spectroscopic methods, high performance liquid chromatography (HPLC) methods [7-11]. Our objective is to develop and validate new normal phase HPLC method for determination of Apixaban and its isomer. The proposed NP-HPLC method utilizes economical solvent system having advantage like better retention time, peak sharp and symmetric nature. In reverse phase Chromatographic these impurities are not well separated from the product hence new chiral technique was developed. This method was validated according to ICH Guidelines.
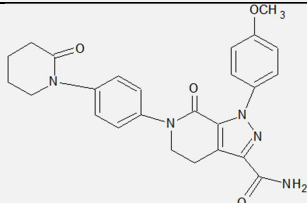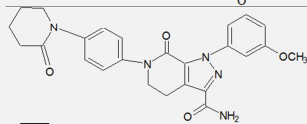
3. Instrumentation

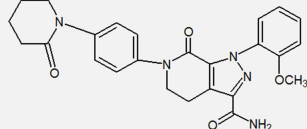Waters, Alliance 2695 series HPLC system comprising a Quaternary pump, an auto-sampler, a thermostat column compartment, a solvent cabinet with gasser along with photo diode array (PDA) 2998 and ultraviolet (UV) 2487 detectors were used for separation and detection. Data acquisition and calculations were carried out using Waters Empower3 software (Milford). Sartorius (Germany) analytical balance was used for weighing material.

4. Materials and Reagent

Apixaban sample, working standard and its related substances working standard were received from Analytical Research and Development department of Indoco Research Centre (Navi Mumbai). HPLC grade n- Hexane, Toluene, Methanol, Ethanol and Dichloromethane were purchased from Merck (India).

***Table 1.** Chemical name of Apixaban and its isomers.*

Sr No.	Component Name	Chemical Name	Structure
1	Apixaban	1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1-yl) phenyl]-4, 5, 6, 7-tetrahydro-1H-pyrazolo [3, 4-c] pyridine-3-carboxamide.	
2	Apixaban Meta isomer	1-(2-methoxyphenyl)-7-oxo-6-(4-(2-oxopoperidine-1-yl) phenyl]-4, 5, 6, 7-tetrahydro-1H-pyrazole (3, 4-c) pyridine-3-carboxamide	
3	Apixaban Ortho isomer	1-(3-methoxyphenyl)-7-oxo-6-(4-(2-oxopoperidine-1-yl) phenyl]-4, 5, 6, 7-tetrahydro-1H-pyrazole (3, 4-c) pyridine-3-carboxamide.	

4.1. Chromatographic Condition and Measurement Procedure

4.1.1. Mobile Phase

Mix 65 volume of n-Hexane, 15 volume of Toluene, 10 volume of Methanol and 10 volume of Ethanol and degas by sonication for 2 mins.

4.1.2. Diluent

Methanol

4.1.3. Preparation of Blank

Transfer 5 ml Dichloromethane into 10 ml volumetric flask and make up with diluent.

***Table 2.** Chromatographic Conditions.*

Column	Chiralapk IA (250mm x 4.6 mm, 5 μm)
Column Temperature	25°C ± 2°C
Flow Rate	1.0 mL/min
Injection Volume	30 μL
Detector Wavelength	290 nm
Run Time	60 minutes
Retention Time	Apixaban (APX) about 32 minutes,
Needle wash	Dichloromethane

4.2. Preparation of Solutions

4.2.1. System Suitability Solution

Transfer about 5 mg of Apixaban working standard, Apixaban ortho impurity and Apixaban meta impurity into 10 mL volumetric flask, add 5 ml dichloromethane and dissolve in and make upto the mark with diluent.

4.2.2. Reference Solution (a)

Transfer about 60 mg of Apixaban working standard into 10 mL volumetric flask, dissolve in about 5 mL of dichloromethane and make upto the mark with diluent.

Transfer 1 mL of above solution to 100 mL volumetric flask and make upto the mark with diluent. Further transfer 1 mL of above solution into 10 mL volumetric flask and make upto the mark with diluent.

4.2.3. Test Solution

Transfer about 60 mg of Apixaban sample into 10 mL volumetric flask, dissolve in about 5 mL of dichloromethane and make upto the mark with diluent.

***Table 3.** Injection sequence.*

SI#	Description	No. of Injections
1	Blank	1
2	System suitability solution	1
3	Blank	1
4	Reference solution (a)	5
5	Test solution	2

4.2.4. Procedure

Equilibrate the HPLC system with the initial composition until a steady baseline is obtained. Inject Blank, System suitability solution and reference solution (a). Ensure that all the system suitability parameters meet the requirements. Inject test solution as per injection sequence and record the chromatograms. Make blank correction if necessary.

***Table 4.** Peak name with Retention Time and Relative Response Factor.*

Sr. No	Peak Name	Relative Retention Time	Relative Retention Factor
1	Apixaban	1.00	1.00
2	Ortho impurity	0.58	0.55
3	Meta impurity	0.90	0.80

4.3. System Suitability [3]

4.3.1. Acceptance Criteria

Resolution: The resolution between the peaks due to meta impurity and Apixaban in the chromatograms obtained with

system suitability solution should not be less than 1.5.

%RSD: The percent relative standard deviation of three replicates for the peak due to Apixaban in the chromatograms obtained with reference solution (a) should not be more than 5.0.

4.3.2. Calculation

Calculate impurity content by formula given below:

$$\% \text{ Ortho/Meta impurity} = \frac{AI \times WS \times 1 \times 1 \times 1}{AR \times WT \times 100 \times 10 \times RRF} \times P$$

Where,

AI = Average peak area for respective impurity in test solution.

AR = Average peak area of Apixaban in reference solution (a).

WS = Weight in mg of Apixaban working standard taken for reference solution (a) preparation.

WT = Weight in mg of Apixaban sample taken for test solution preparation.

P = Potency of Apixaban working standard (%).

4.3.3. Analytical Method Validation

The developed method is subjected to analytical method validation, which is conducted according to International Council for harmonization (ICH) guidelines. The parameters with which analytical method is validated are specificity, limit of detection and limit of quantification, linearity, accuracy, precision [4-6].

4.4. Specificity

Specificity is capability of the method to measure the analyte response in presence of impurities. The typical chromatograms of blank Solution, System suitability solution, Reference Solution (a), and Impurities Spike Solution are given from figure 1 to figure 4 respectively. The results indicate that all impurities are well separated under the current chromatographic conditions. There was no interference of peak from blank solution and samples solution within retention time of impurities obtained. Peak purity for Apixaban and its impurities were passing. For retention time of each impurity and its peak purity refer table no.5
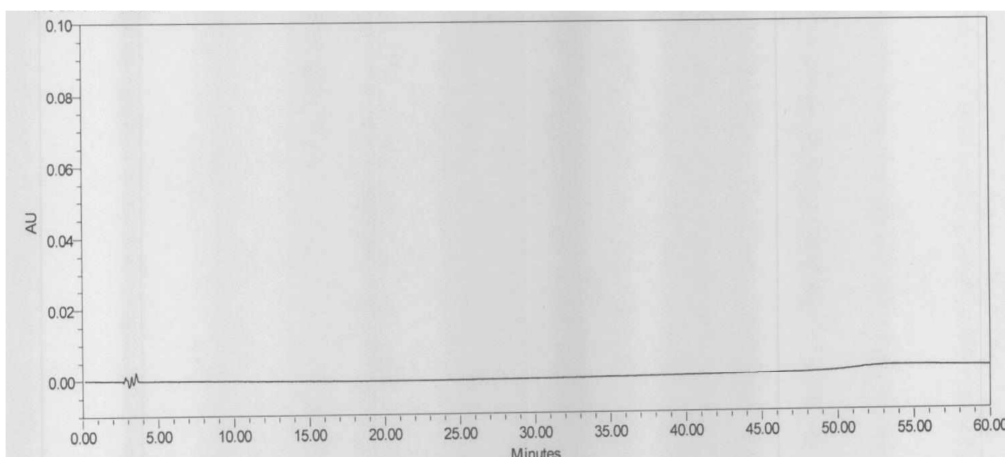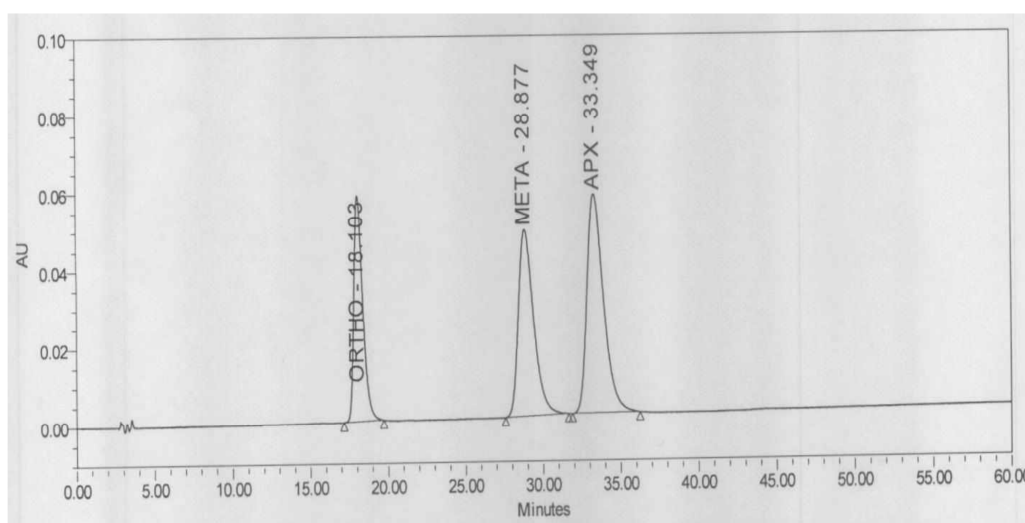

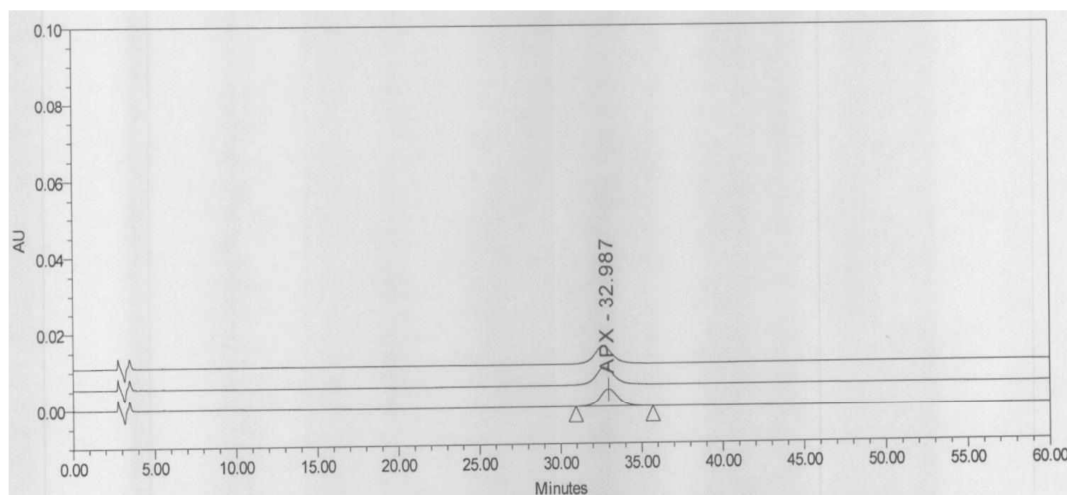
Figure 1. *Blank.*

Figure 2. *System suitability solution.*

Figure 3. *Reference Solution (a).*
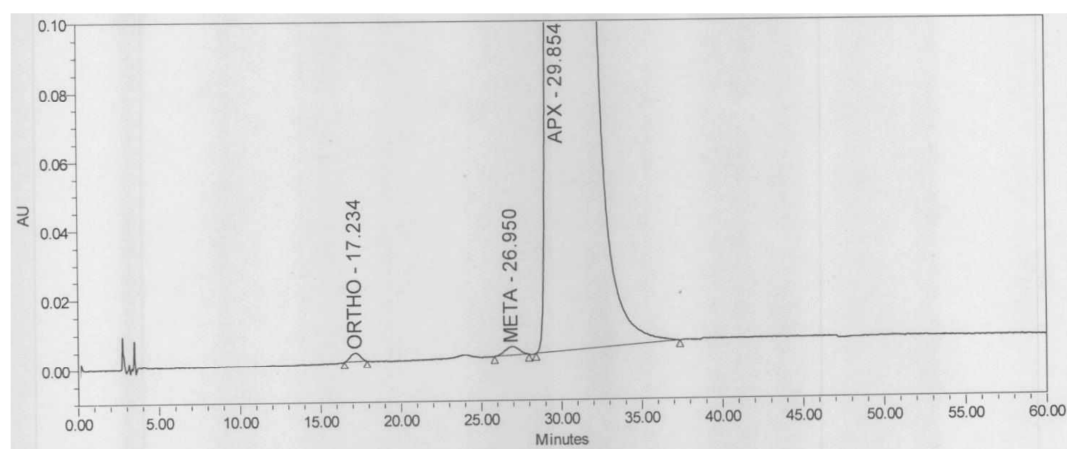

Figure 4. *Impurities spiked in sample.*

Table 5. *Peak purity and RT Ratio for Apixaban and its impurities.*

Sr. no	Peak name	RT	RT Ratio	Purity Angle	Purity Threshold
1	Apixaban	32.0	1.00	0.22	1.18
2	Ortho impurity	18.56	0.58	0.19	1.17
3	Meta impurity	28.8	0.90	0.22	1.16

4.5. Limit of Detection and Limit of Quantification

Series of standard solution of Apixaban and its impurities were prepared and injected in concentration ranging from 50% to 500%. Limit of detection (LOD) and Limit of quantification (LOQ) was calculated based on residual standard deviation of regression line and slope. Both calculated LOD and LOQ were well within limit. The LOQ is below 0.057% for impurities and Apixaban API. For details of LOQ and LOD refer Table.

Table 6. *LOD and LOQ.*

Sr No.	Name of Impurity	LOD (%)	LOQ (%)
1	Apixaban	0.019	0.057
2	Ortho impurity	0.018	0.055
3	Meta impurity	0.011	0.032

4.6. Linearity

Series of linearity solution of Apixaban and its impurities were prepared from 50% to 500% of test concentration. Linearity curves were drawn by plotting the peak areas of Apixaban and its impurities against its corresponding concentration of linearity solution. Observed regression coefficient was greater than 0.998 and % y-intercept was less than 5.0%

Table 7. *Linearity table and its R^2 values Apixaban.*

Slope	2265608.63
Intercept	19532.23
Co-relation Coefficient (R^2)	0.9993
% Y-Intercept	2.77

Apixaban

Figure 5. *Linearity graph of Apixaban.*

Table 8. *Linearity table and its R^2 values of Meta Impurity.*

Slope	1757553.28
Intercept	-17795.47
Co-relation Coefficient (R2)	0.9998
% Y-Intercept	-3.37

Meta Impurity

Figure 6. *Linearity graph of Meta Impurity.*

Table 9. *Linearity table and its R^2 values of Ortho Impurity.*

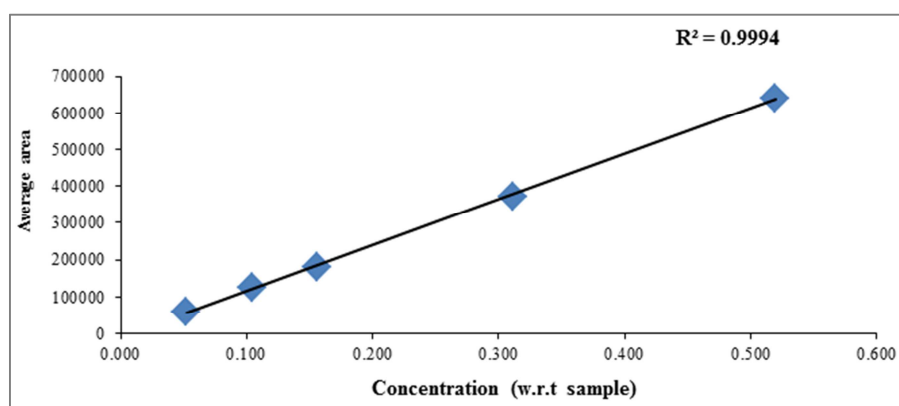|	
Slope	1239296.68
Intercept	-7260.83
Co-relation Coefficient (R^2)	0.9994
% Y-Intercept	-4.02

Ortho Impurity

Figure 7. *Linearity graph of Ortho Impurity.*

4.7. Accuracy

Accuracy of method is calculated and established by carrying out recovery studies of impurities. The test solution was spiked with impurities solution at specific limit level concentration 100%, 150%, 300%. Each spiked test solution was analyzed for recovery study of impurities. Recovery established is between 100% to 105%.

Table 10. Recovery of impurities at 100%.

Sr. No.	Imp Name	Area of 0.10% Level	Theoretical imp Added %	% of IMP Observed	% of Recovery
1	Ortho Impurity	103945	0.09	0.09	103.15
2	Meta Impurity	147724	0.09	0.09	100.78

Table 11. Recovery of impurities at 150%.

Sr. No.	IMP Name	Area of 0.15% Level	Theoretical imp Added %	% of IMP Observed	%of Recovery
1	Ortho Impurity	160337	0.13	0.14	104.56
2	Meta Impurity	226362	0.13	0.14	101.49

Table 12. Recovery of impurities at 300%.

Sr. No.	IMP Name	Area of 0.30% Level	Theoretical imp Added %	% of IMP Observed	%of Recovery
1	Ortho Impurity	319004	0.27	0.28	104.88
2	Meta Impurity	444756	0.27	0.27	100.52

4.8. Precision

System precision was carried out by analyzing six injections of reference solution (a) of Apixaban API at limit level concentration. Relative standard deviation for peak area of Apixaban was calculated and found to be 0.33%.

4.9. Robustness

Robustness was studied by making small but deliberate changes in optimized method conditions and evaluating the effect on resolution between ortho impurity, meta impurity and Apixaban. The mobile phase flow rate was changed by±0.1 units from 1.0 to 0.9 and 1.1 ml/min.

4.10. Solution Stability

The solution stability of Apixaban and its impurities in related substances method was evaluated by leaving a spiked solution in tightly capped volumetric flask at room temperature for 48 hrs and analysis of all its impurities content was done at interval of 12 hrs.

5. Result and Discussion

Since most of the impurities are non-polar in nature, various chiral columns were screened. The parameters like tailing factor and theoretical plates were recorded during the study. From obtained data Chiralpak IA (250 x 4.6 x 5μm) was found suitable for analysis.

All impurities were prepared at 100 ppm and their UV-visible spectra was acquired. The Apixaban and all its impurities has good and satisfactory response at 290nm. Hence detection at 290nm was selected for method-development.

Retention time were confirmed by injecting working standards of Apixaban, Meta and ortho impurities. For System Suitability, the resolution between Apixaban and Meta imp should be not less then 1.5. Relative Standard Deviation for five replicate injections peak due to Apixaban should not be more then 5%.

6. Conclusion

The analytical method validation for Apixaban by normal Phase HPLC was carried out by performing the parameters such as specificity, limit of detection and limit of quantification, linearity, accuracy, precision, robustness and solution stability. All the data has been compiled and found to be satisfactory. Hence, method developed for normal Phase HPLC can be suitably used for analysis of Apixaban isomer in pharmaceutic industries.

Acknowledgements

Authors' Contributions

This work was carried out in collaboration between all authors. All available literature search and regulatory requirement author AG and RP designed the study. Authors DP and KG wrote the protocol and authors PT carried out the experiment along with KG. All author involved in statistical analysis and writing the first draft of manuscript and subsequent revision. All the authors read and approved the final manuscript.

References

[1] Indian Pharmacopoeia, volume III, Ministry of Health and Family Welfare Government of India. Published by Indian Pharmacopoeia Commission, Ghaziabad, 2014.

[2] The British Pharmacopoeia, the British Pharmacopoeia Commission, London. 2012; Vol. II.

[3] The United state Pharmacopoeia, the United state Pharmacopoeia Commission, America. 2012.

[4] ICH Harmonized Tripartite Guideline, Validation of analytical procedure text and methodology Q2 [R1]. In International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use, 2005.

[5] AM K, Vidhate MB, Ingale MR. Analytical method development and validation for estimation of apixaban by RP-HPLC. Indian Drugs, 2017; Vol. 54 (4); 76-79.

[6] Sun YY, Zhao YL, Duan MM, Wang DK, Yu ZG. Determination of related substances in apixaban by HPLC. Journal of Shenyang Pharmaceutical University. 2017 (1): 7.

[7] Mahendra B, Sundari KH, Vimalakkannan T. Method developed for the determination of apixaban by using UV spectrophotometric. International Journal of Research In Pharmaceutical Chemistry and Analysis. 2019 Jul 29; 1 (3): 83-7.

[8] El-Bagary RI, Elkady EF, Farid NA, Youssef NF. Validated spectrofluorimetric methods for the determination of apixaban and tirofiban hydrochloride in pharmaceutical formulations. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 2017 Mar 5; 174: 326-30.

[9] Bhavesh R. Sharma Chainesh N. Shah, A Recent Research Review- Development and Validation with liquid chromatography, J Pharm Sci Bioscientific Res. 2016. 6 (5): 723-727.

[10] Rajan ST, Eswaraiah S, Venkatesh M, inventors; Msn Laboratories Limited, assignee. Intermediate and polymorphs of 1-(4-methoxypheny1)-7-oxo-6-[4-(2-oxopiperidin-1-yl) phenyl]-4, 5, 6, 7-tetra hydro-1H-pyrazolo [3, 4-c] pyridine-3-carboxamide and process thereof. United States patent US 9,718,779. 2017 Aug 1.

[11] Vemula Archana Reddy, Method development and validation of apixaban in bulk and tablet dosage form by RP-HPLC. Journal of Pharmacreations Vol-5 (1) 2018 [01-24]

[12] D. Shravan Kumar, Dr. M. Ajitha, Mahesh Rajendra Awate, K. S. L. Harika, stability indicating assay method development and validation of apixaban tablets by RP-HPLC. J Sci Res Pharm, 2017; 6 (Suppl 1): 71-75.