METHOD DEVELOPMENT AND VALIDATION OF ANTI- COAGULANT DRUG IN PHARMACEUTICAL DOSAGE FORM BY HPLC

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ABSTRACT:

The paper involves the development of a simple, Precise, and sensitive method for estimation of Apixaban in bulk drug and its marketed formulation using the reverse-phase liquid chromatographic method. The separation was achieved on C_{18} Hypersil-BDS Column (250 mm×4.6 mm×5 μm) using mobile phase (Methanol: Acetonitrile: Water: Glacial Acetic Acid)in the ratio of 65:15:20:0.5(v/v) with a run time of 7 minutes and wavelength for estimation ofapixaban was taken as 277 nm. Literature survey reveals that there are very few HPLC methods were available using this composition of mobile phase Hence an attempt has been made to develop an RP-HPLC method for estimation of Apixaban.

The developed method was validated for Accuracy, Precision, Linearity, System Suitability, LODand LOQ, Robustness and Assay. The linearity was found to be in the range of 1-3 μg/ml with correlation coefficient found for linearity is 0.999. The developed and validated RP-HPLC method is applied for the identification of eluted.

KEYWORDS: Apixaban, Anti-Coagulant, HPLC, Method Development, Validation.

1.   INTRODUCTION:

Apixaban is an anticoagulant drug 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 and sold under the brand name "Eliquis "to treat the people with atrial fibrillation (a heart rhythm disorder) to lower the risk of stroke caused by a blood clot. It was invented by Adrei's pharmaceuticals and was developed jointly by Pfizer and Bristol-Myers Squibb.

Apixaban is a selective, reversible, direct inhibitor of factor Xa indicated to reduce the risk of stroke and

systemic embolism in patients with non-valvular atrial fibrillation. "Eliquis" was approved both in US and Europe in Dec 2012 and Jan 2010 respectively. "Eliquis" is also usedafter hip or knee replacement surgery to prevent a type of blood clot called deep vein thrombosis (DVT), which can lead to blood clots in the lungs (pulmonary embolism).[1]

Apixaban is not an official drug in any Pharmacopoeia.[2] Apixaban is a widely prescribed oralimmediate-release anticoagulation treatment used to treat patients with NVAF [3]. The physical appearance of it is a white to pale-yellow powder with melting of 326.53 °C. It has good solubility nature in water and dimethyl sulfoxide [4]. It is non-hygroscopic crystalline powder, with an aqueous solubility of 0.058 mg/mL at 24°C. Apixaban is a non-ionizable compound and its partition coefficient at 24°C is 44.7 (log Po/w = 1.65) at pH 7.4 (n-octanol / aqueous.
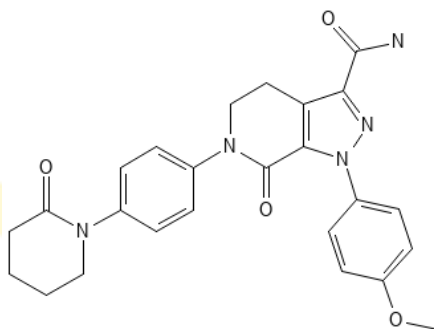

Fig. No: 01 (Chemical Struture of Apixaban)

2.   MATERIALS AND METHODS

2.1 Chemical and Reagents:

Med Koo Biosciences, New York, USA provided a working standard of pharmaceutical-gradeAPI of Apixaban as a gift sample. The marketed Tablet of Apixaban (5 mg) was purchased from a neighborhood pharmacy.

Table No: 01 (Instrumentation and Chromatographic Condition)

PARAMETERS	SPECIFICATION
HPLC	Younglin-HPLC System
Analytes	Apixaban
Mobile Phase	(Methanol: Acetonitrile: Water: Glacial Acetic Acid) 65:15:20:0.5(v/v)
Column	Hypersil-BDS, C 18 (250 mm*4.6 mm, 5 μm)
Flow Rate	1.00 ml/min
Elution Mode	Isocratic
Injection Volume	20μl
λ max	277nm
Retention Time	3.4 min
Run Time	7 min
UV Spectrophotometer	Shimadzu UV1800 SpectrophotometerJapan Corporation)

2.2 Solvents and Chemicals:

- Methanol (gradient grade)

- Acetonitrile (gradient grade)

- Glacial Acetic Acid (gradient grade)

- Water (HPLC Grade)

2.3 Preparation of Standard and Stock Solution:
Weighed accurately 40 mg of Apixaban standard and transfer to 100 ml of volumetric flask, dissolved and diluted up to the mark with help of diluent, shake well sonicate this solution for about 2 min and pass through the 0.45 μm membrane filter.

2.4 Preparation of standard solution:
Pipette out 2 ml from stock solution, transfer to 20 ml volumetric flask, diluted up to the mark with diluent shake well, sonicate for about 2 min, filter through 0.2 μm syringe filter.

2.5 Preparation of Mobile Phase:

Various mobile phase combinations were used on a trial-and-error basis. The appropriate mobile phase solvent used was Methanol (65%); Acetonitrile (15%); Water (20%); Glacial Acetic Acid (0.5%).

2.6 Selection of Wavelenght for Apixaban:

After baseline correction, the UV spectrophotometer scanned with 10 μg/mL working standard solution between 400 to 200 nm against methanol as a blank. The UV-analyst software displayed a maximum wavelength of 277nm.

2.7 Marketed Tablet Test Preparation:

Weighed 10 tablets separately, crush all tablets in mortar and pestle. Weighed tablet powder (API) equivalent to standard concentration and dissolve to 100 ml with the help of diluent and shake well. Sonicate for 2 mins, filter through 0.2 μm membrane syringe filter.

3.   HPLC Method Optimization:

For method optimization various mobile phases were tried in different ratios, such as

1.   Solvent A- Acetonitrile (50%); Solvent B- Water (50%),
2.   Solvent A- Methanol (60%); Solvent B- Water (40%),
3.   Solvent A- Methanol (65%); Solvent B- Water (35%); Solvent C-Glacial Acetic Acid(1%)
4.   Solvent A- Methanol (65%); Solvent B- Water (25%); Solvent C- Acetonitrile (10%);Solvent D- Glacial Acetic Acid (0.2%)

All these mobile phases were unacceptable due to tailing, fronting and no sharpness in the peak.After various trials mobile phase consisting of Methanol: Acetonitrile: Water: Glacial Acetic Acid in ratio (65:15:20:0.5) was selected which gave sharp peaks with no tailing and fronting.The chromatogram of standard Apixaban was shown in **Fig 02.**
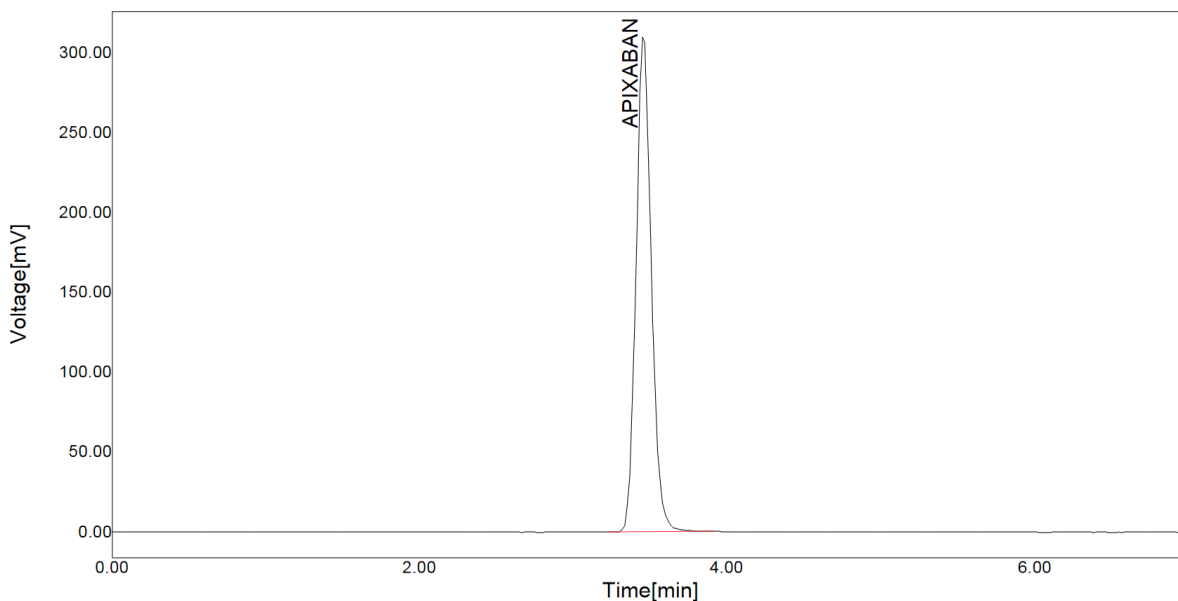
Fig. No: 02 Chromatogram of Final Trail

4. VALIDATION OF DEVELOPED METHOD:

4.1 ACCURACY:

The concentrations used were 80, 100, and 120% to analyze the recovery studies using the standard method. The procedure involved combining 0.8, 1.0, and 1.2 mL of standard solutionwith 0.2 mL of tab solution having10 µg/mL concentration. The % accuracy was determined by using the following formula:

$$\% \, Accuracy = \frac{Mean \ measured \ concentration}{Nominal \ Concentration} \times 100$$

Table No: 02 – Apixaban Accuracy Data

	MEAN % recovery	SD	%RSD (NMT 2)
Accuracy at 80 %	100.64	0.2537	**0.25**
Accuracy at 100 %	99.51	0.4632	**0.47**
Accuracy at 120 %	100.88	0.2327	**0.23**

The mean % recovery of 100.64 to 100.88 was observed and within %RSD between 0.25 to 0.23. All the obtained results were within the range of acceptable limits. [6]

4.2 PRECISION:

The system precision was demonstrated by preparing the standard solution at test concentrationand injected repeatedly six times. [7]

Acceptance Criteria:

%RSD of assay results should be NMT 2.0%. Assay should be in the range of test method. [8]
Precision studies were carried out by injecting six replicate injections of the standard drug mixture on one day. This process is called intraday precision. The results were calculated in terms of %RSD.

Table No: 03- Intraday Precision

Name	Preparation	% ASSAY
Set-1	prep-01	98.78
	prep-02	100.05
Set-2	prep-01	100.40
	prep-02	100.74
Mean		99.99
SD		0.8560
% RSD (NMT 2.0)		0.86

Precision studies were also carried out by injecting six replicate injections of the standard drugmixture on six different days. This process is called interday precision. The results were calculated in terms of % RSD. [9]

Table No: 04- Interday Precision

Name	Preparation	% ASSAY
Day-1	prep-01	98.78
	prep-02	100.05
Day-2	prep-01	100.57
	prep-02	98.86
Mean		99.57
SD		0.8867
% RSD (NMT 2.0)		0.89

4.3 LINEARITY:

A graph of peak area versus concentration (in ppm) was plotted for Apixaban a concentrationrange between 20.05 - 60.15 µg/ml. The linear regression equation and correlation coefficient(R^2) were y 52.106x+15.514 and 0.9996 respectively. [10]

Fig No: 03- Linearity study of Apixaban

Level	Con. (ppm or µg/ml)	Area
1	20.05	1047.3844
2	30.08	1591.8982
3	40.10	2105.5178
4	50.13	2649.6572
5	60.15	3130.3018

4.4 SYSTEM SUITABILITY:

System suitability tests were performed using Apixaban standard and test solutions to checkfor compliance with specified parameters [10].

Table No: 05- System Suitability Parameters

Name	Area	RT (min)	TP (NLT 2000)	TF (NMT 2.0)
Standard _Inj_01	2152.0251	3.483	6070	1.01
Standard_Inj_02	2076.0889	3.467	7009	1.06
Standard_Inj_03	2068.1008	3.467	8662	1.13
Standard_Inj_04	2114.9524	3.517	7340	1.03
Standard_Inj_05	2121.9077	3.467	5153	1.10
Mean	2106.6150	3.480		
SD	34.5711	0.0217		
%RSD (NMT 2.0)	**1.64**	**0.62**		

The plate count and tailing factor results were found to be within the limits.

4.5 LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ):

LIMIT OF DETECTION (LOD):

The limit of detection is the lowest concentration of an analyte that can be detected in a sample but not necessarily quantitated, under the given experimental conditions.

LIMIT OF QUANTIFICATION (LOQ):

It is the lowest concentration of analyte in a sample that can be accurately and preciselyidentified under the given experimental conditions.

LOD and LOQ were determined using the following formulas.LOD = 3.3 ×(SD)/S   LOQ = 10× (SD)/S

Where,

SD = Standard deviation   S = Slope

% Recovery [11]

Table No: 06- LOD and LOQ Data

Level	Con. (ppm or µg/ml)	Area
1	20.05	1047.3844
2	30.08	1591.8982
3	40.10	2105.5178
4	50.13	2649.6572
5	60.15	3130.3018
	Correlation coefficient (r)	0.9998
	STEYX	19.3778
	SLOPE	52.1057
	LOD (µg/ml)	1.23
	LOQ (µg/ml)	3.72

LOD and LOQ observed 1.23 µg/ml and 3.72 µg/ml respectively.

4.6 ROBUSTNESS:

For the parameters like Flow rate, wavelength and the chosen solution was used for a robustness assessment.

% RSD (NMT2) should not be present in the variation. The percentage assay should also fallbetween 98 and 102%. [11]

Table No: 07- Robustness Data

Name	Preparations	%Assay
Robustness changes in method parameters		
Original method parameters	Test prep 1	98.78
Original method parameters	Test prep 2	100.05
Flow rate 0.90 ml/min	Test prep	100.63
Flow rate 1.10 ml/min	Test prep	99.57
Wavelength 275 nm	Test prep	100.6
Wavelength 279 nm	Test prep	101.3
Mean		100.16
SD		0.8920
%RSD (NMT 2)		**0.89**

Robustness examines the effect of operational parameters on the analytical method. [12]

4.7 ASSAY:

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{WT} \times \frac{DT}{DS} \times \frac{P}{100} \times Avg.\ Wt. = mg/tab$$

Where:

AT = Peak Area of medication acquired with test readiness

AS = Peak Area of medication acquired with standard readiness WS = Weight of working standard taken in mg

WT = Weight of test taken in mg

DS = Dilution of Standard arrangement DT = Dilution of test arrangement
P = Percentage virtue of working standard

Table No:08- Preparation of Standard Solution for Apixaban

Test Preparation	Apixaban Wt. of test (mg)	Diluted to (ml)	ml taken	Diluted to (ml)
Preparation-1	39.9	100	2	20
Preparation-2	40.3	100	2	20

Table No:10- Marketed Preparation

Name	Area	RT (min)	% ASSAY
Test solutions 1	2075.6240	3.467	98.78
Test solutions 2	2123.5635	3.500	100.05

CONCLUSION:

The method has been shown to be specific for the determination of % Assay of Apixaban in Apixaban Film Coated Tablet 5 mg. A rapid, user friendly, precise method for determination of the Apixaban in its pharmaceutical dosage form was developed and validated. The Linearity, accuracy, precision, LOD, LOQ, Robustness and %Recovery was within the limits as specified by the ICH guidelines. This method exhibited an excellent performance in terms of sensitivity and speed. This method suitable for the estimation of Apixaban.

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