Original Article

Full Factorial Experimental Design For Development And Validation Of Rp-Hplc Method For Estimation Of Apixaban In Bulk And Pharmaceutical Formulations

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Abstract

According to International Conference on Harmonization (ICH Q8 [R2]) guidelines, QbD approach was implemented for Chromatographic method development and its validation. The research work demonstrated that RP-HPLC is valid for the determination of assay of Apixaban. Experiment was carried out for varying three parameters were extracted by using principal component analysis. Chromatographic separation is achieved on a C-18 column. In development of RP-HPLC method factors like Mobile Phase (Acetonitrile, Methanol, Water, Buffer), mobile phase composition (10-30% v/v) and pH range (5-7) are critical to maintain. Hence Central Composition factorial design was used as screening model and applied as optimization model for the interaction and quadratic effects of three factors. Optimization was done according to desirability value. Optimized mobile phase was water: acetonitrile (10:90), 7 pH at maximum and Wavelength 277nm. Retention time, asymmetric factor and theoretical plates were found to be 2.9 min, 0.83, 3931 respectively. Optimised standard curve showed a regression coefficient is 0.999. The method was validated as per ICH guidelines.

Keywords: RP-HPLC Method, Apixaban, Quality by Design, Central Composition factorial design.

1. INTRODUCTION

Apixaban is use for serious blood clotting; belong in class of Anticoagulant Drug. It is a phenylpiperidine skeleton of heterocyclic compound, having molecular formula is $C_{25}H_{25}N_5O_4$ and molecular weight is 459.5 g/mol (Fig.1). Apixaban inhibits the coagulation factor which prevents clotting of blood.

In this study, we have estimated drug by using Central Composite design and gave statistical approach. The main objective of this study was to develop robust RP-HPLC method for routine analysis. Design of Experiment is done by the Design Expert software

Present research has its future application in bioanalysis of Apixaban to estimate or study of pharmacokinetics.[1-9]

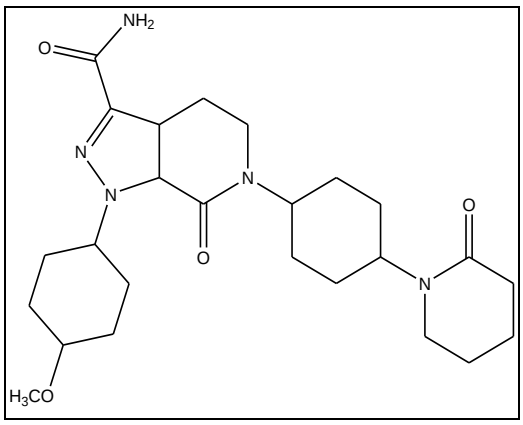

Fig. 1 Structure of Apixaban

2. MATERIAL AND METHOD

2.1 Material

Apixaban is use as an anticoagulant and its formulation is tablets Eliquis: 2.5 mg. Manufactured by Bristol-Myers Squibb S.R.L, Marketed by Pfizer Limited. Chemical is used as Acetonitrile (HPLC Grade), Methanol (HPLC Grade), Potassium Dihydrogen Phosphate, Sodium Hydroxide, Distilled Water and instruments were as follows HPLC (JASCO), pH Meter (Chemiline), Balance (Shimadzu), Sonicator (Rolex).

METHOD

Preliminary Analysis of Drug

Color and texture of Apixaban was compared with reported characters mentioned in drug bank. Solubility was determined in various solvents like water, methanol, ethanol and Acetonitrile. UV analysis was carried out by scanning the solution of Apixaban at 200-400 nm.

Design of Experiment

Column by doing various trials as follow

Design Expert 8 Software has different facility to develop experiment such

- ✓ Central Composition Method

Selected Factorial design was Central Composition Method due to it has flexibility to change/add/delete any parameter at any time when our experiment is going on.it provide facility to give standard run at one time at only one mobile phase.

There are four mobile phases selected

- ✓ Acetonitrile: Water
- ✓ Acetonitrile: phosphate buffer
- ✓ Methanol: Water

MISCELLANEOUS FACTORIAL DESIGN CAN PICK UP ONE MOBILE PHASE. SO, SELECT EACH MOBILE PHASE ONE BY ONE.

- ✓ Acetonitrile: Water
- ✓ Change pH Range: 5 -7
- ✓ Change Mobile phase proportion Range: 10-30%

When all above ranges put in Central Composition Method. It gave 8 runs at different pH, Mobile phase proportion. Followed same procedure for each mobile phase. column C-18 has four mobile Phases with 8 runs for each mobile phase. Column gives its 24 runs. After completion of all trails software give one optimize best value for each column. Optimization means finding an alternative with the most cost effective or highest achievable performance under the given constraints, by maximizing desired factors and minimizing undesired ones. In comparison, maximization means trying to attain the highest or maximum result or outcome without regard to cost or expense. Practice of optimization is restricted by the lack of full information, and the lack of time to evaluate what information is available. Find the "sweet spot" where you meet all your specifications at minimal cost.[8]

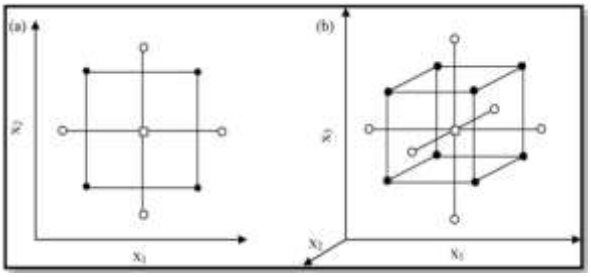

Fig. 2. Central composite designs for the optimization of: (a) two variables (α=1.41) and (b) three variables
Chromatographic Conditions Suggested by Software for each mobile phase

Table 1 Chromatographic Conditions Suggested by Software

Mobile Phase	Mobile Phase	Retention time(min)	Asymmetry	pH	Flow rate	Theoretical Plates
Buffer + ACN (10:90)	10	2.886	0.820	6	1	3931.68
Water +ACN (18.68:92.42)	18.68	3.368	1.382	6.06	1	2009.58
Water + Methanol (10:90)	10	2.980	1.136	7	1	2649.11

Preparation of mobile phase

90 ml of HPLC grade Methanol was added to 10ml of Water i.e. in 90: 10 v/v proportions. The pH was adjusted to 6.5 with Trimethylamine and orthophosphoric acid. The solution was filtered through 0.45µ membrane filter and then sonicated in Sonicator bath for 10 min.

Preparation of stock solutions of Apixaban

Stock solution was prepared by dissolving 10 mg Apixaban in Methanol and then diluted with Methanol in 10 ml of volumetric flask to get concentration of 1000 µg/ml. From the resulting solution 0.1 ml was diluted to 10 ml with Methanol to obtain concentration of 10 µg/ml of Apixaban and labelled as standard stock Apixaban.

Selection of detection wavelength

From the standard stock solution further dilutions were done using Methanol and scanned over the range of 200-400 nm and the spectra were overlain. It was observed that drug showed considerable absorbance at 277 nm.

3. RESULT AND DISCUSSION

3.1. Trials Given By Design Expert Software

Standard concentration of Apixaban was taken 20 µg/ml. Miscellaneous Factorial design gave 8 run at different pH, Solvent proportion Six Solvent Combination with 8 runs for each Solvent Combination. Software gives its 24 runs (Table 2).

Table 2 Trials given by software

Sr. No.	Mobile Phase (for aqueous phase)	pH
1	20	5.79
2	10	6
3	30	6
4	5.86	6.5
5	34.14	6.5
6	10	7
7	30	7
8	20	7.21

3.2. Optimization

Optimization Result Spectral Result of Acetonitrile: Water

Table 3 HPLC Result of Acetonitrile: Water

Sr. No.	Solvent Ratio	pH	Retention time	Area
1	20:80	5.79	3.05	99171.75
2	10:90	6	2.9	689489.658
3	70:30	6	4.1	98259
4	5.86:95.14	6.5	3.1	21803.375
5	34.14:65.86	6.5	3.2	123733.57
6	10:90	7	2.9	102804.5
7	70:30	7	3.05	122375.5
8	20:80	7.21	3.1	92750

Spectral Result of Methanol: Water

Table 4 HPLC Result of Methanol: Water

Fig. No.	Solvent Ratio	pH	Retention time	Area
1	20:80	5.79	3.2	910789
2	10:90	6	3.1	56812
3	70:30	6	3.7	895216
4	5.86:95.14	6.5	4.9	807709.5
5	34.14:65.86	6.5	3.05	640418.25
6	10:90	7	3.992	637696.500
7	70:30	7	9.317	44937.500
8	20:80	7.21	9.933	1551611.250

Spectral Result of Acetonitrile: Phosphate Buffer

Table 5 HPLC Result of Acetonitrile: Phosphate Buffer

Fig. No.	Solvent Ratio	pH	Retention time	Area
1	20:80	5.79	2.95	197000.5
2	10:90	6	3.05	1243682
3	70:30	6	2.54	1481877.5
4	5.86:95.14	6.5	2.925	748835
5	34.14:65.86	6.5	3.05	1555080.5
6	10:90	7	2.95	522874
7	70:30	7	2.74	4542030.8
8	20:80	7.21	3.05	1115780

Optimized trials suggested by software based on desirability value

This methodology is initially based on constructing a desirability function for each individual response. The scale of individual desirability function ranges between i= 0, for completely undesirable response and i =1, for fully desired response. Selection of trial was based on maximum desirability value. Therefore, first trial which was having desirability one (i=1) selected for method optimization (Table 6).

Table 6 Optimized trials suggested by software based on Desirability value

Sr. no.	Amount of water	pH	asymmetry	Retention time	Theoretical plates	Desirability
1	**10**	**7**	**0.82**	**2.9**	3931.68	**1.0**
2	11.01	7	0.88	3.01	3826.41	0.9

Optimized chromatographic conditions

Mobile phase: Water: ACN (10:90 v/v), pH of buffer: 7, Analytical column: C_{18} column Waters X Bridge (4.6× 250mm id. particle size 5µm), UV detection: 277nm, Injection volume: 20 µL, Flow rate: 1.00 mL min $^{-1}$, Temperature: Ambient, Run time: 6 min.

Effect of independent variables

Effect of independent variables on retention time (Y_1):

The equation for response surface quadratic model is as follows

Y_1 = +3.09 +0.053 *A + 2.089E-003* B- 0.053 * A * B

Where, X_1= A, X_2 = B

A graphical representation of amount of Water (A) and pH (B) (Fig. 3). An increase Amount of Water in resulted in decrease in retention time (Y_1), while decrease in pH resulted in increase in retention time (Y_1). Combination of amount of water and pH showed decrease in response.

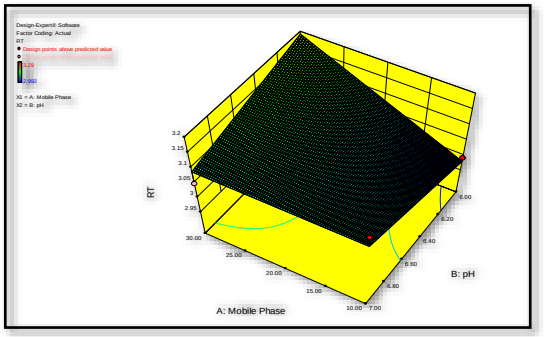

Fig. 3. Three-dimensional plot for Retention time as a function of pH and Mobile Phase (for water)

Fit summary: Quadratic model was suggested by the software.
ANOVA: ANOVA of developed Full three level factorial model for retention time (Y_1).
Values of"Prob F"(p- value) less than 0.0500 indicate model terms are significant.
In this case A, B, AB,are significant model terms (Table 7).

Table 7 Significance of *p* value on model terms of Retention time

Model terms	*p* value	Effect of factor	Remarks
A (X_1)	**0.0382**	+0.053	Significant
B (X_2)	**0.0146**	2.089E-003	Significant
AB (X_1X_2)	0.1941	0.053	Insignificant
Overall model	**0.0128**	-	**Significant**

Effect of independent variables on Asymmetry (Y_2):

The model for response Y_2 (Asymmetry) is as follows:

Y_2 = +1.26 +0.15 * A -0.027 * B

Where, X_1= A, X_2 = B

a graphical representation of amount of Water (A) and pH (B) (Fig. 4), An increase Amount of Water in resulted in decrease in Asymmetry(Y_2), while decrease in pH resulted in decrease in Asymmetry(Y_2).

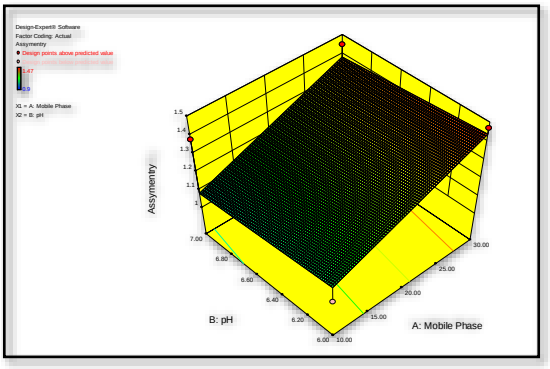

Fig. 4. Three-dimensional plot for Asymmetry as a function of pH and Mobile Phase (for water)

Fit summary: Response Surface Linear Model was suggested by the software.
ANOVA: ANOVA of developed CCD model for Asymmetry (Y_2).
Values of "Prob > F" (p- value) less than 0.0500 Indicate model terms are significant.
In this case A, B are significant model terms (Table 8).

Table 8 Significance of *p* value on model terms of Asymmetry

Model terms	*p* value	Effect of factor	Remarks
A (X_1)	**0.0070**	+0.15	Significant
B (X_2)	**0.0602**	-0.027	Insignificant
Overall model	0.0159	-	**Significant**

For response Y_2, factor X_1 was having synergistic effect with *p* value 0.0070. Therefore, we can conclude that increment in amount of Water was responsible for increase in Asymmetry. factor X_2 was having antagonistic effect with *p* value 0.0602. Therefore, we can conclude that decrease in pH was responsible for increase in Asymmetry.

Effect of independent variables on Theoretical Plates (Y_3):

The model for response Y_3 (theoretical plates) is as follows:

Y_3 = +2019.29 -409.04 * A -220.78 * B

Where, X_1= A, X_2 = B

A graphical representation of amount of Water (A) and pH (B) (Fig. 5).

An increase amount of Water and pH showed antagonistic effect on response (Y_3) individually.

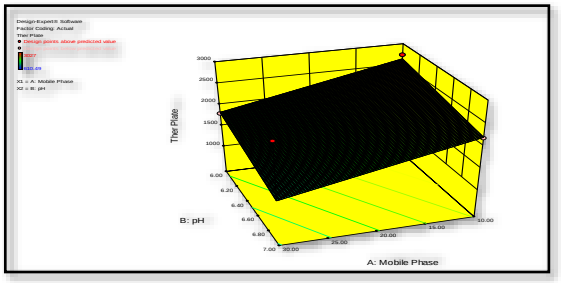

Fig. 5. Three-dimensional plot for Theoretical Plates as a function of pH and Mobile Phase (for water)

Fit summary: Quadratic model was suggested by the software. ANOVA: ANOVA of developed CCD model for Theoretical Plates (Y_3).

Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. For response Y_3, factor pH of Water was having antagonistic effect with *p* value 0.0475. Therefore, we can conclude that increment in pH of buffer was responsible for decrease in theoretical plates (Table 9).Amount of Water was responsible for significant decrease in theoretical plates with significant *p* value of 0.021.

Table 9 Significance of *p* value on model terms of Theoretical plates

Model terms	*p* value	Effect of factor	Remarks
A (X_1)	**0.021**	-409.04	Significant
B (X_2)	0.0475	-220.78	Significant
Overall model	**0.0346**	-	**Significant**

Effect of independent variables on Area(Y_4):

The model for response Y_4 (Area) is as follows:

Y_4= +5.149E+005 -4.932E +005* A +45067.99 * B

Where, X_1= A, X_2 = B

A graphical representation of amount of Water (A) and pH (B) (Fig 6). An increase amount of Water and pH showed Synergistic effect on response (Y_4) is increases.

Fit summary: Quadratic model was suggested by the software

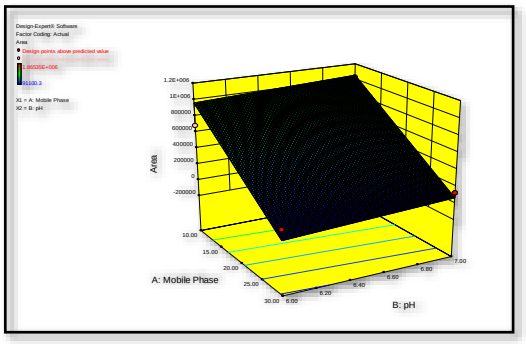

Fig. 6. Three-dimensional plot for Area as a function of pH and amount Mobile Phase (for water)

ANOVA: ANOVA of developed CCD model for Area (Y_4). Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant. In this case A, B are significant model terms. For response Y_4, factor amount of Water was having antagonistic effect with *p* value 0.0255. Therefore, we can conclude that increment in pH of water was responsible for decrease in Area. pH of Water was responsible for significant increase in Area with significant *p* value of 0.0428 (Table 10).

Table No. 10 Significance of *p* Value on Model Terms of Area

Model Terms	*p* Value	Effect of Factor	Remarks
A (X_1)	**0.0255**	-4.932E +005	Significant
B (X_2)	0.0428	+45067.99	Significant
Overall model	**0.0438**	-	**Significant**

4. ANALYTICAL VALIDATION

The HPLC Validation as per ICH Guidelines (ICH, 1996; ICH, 2005) of Optimized result of Apixaban is at 7 pH, Mobile Phase of Methanol: Water (70:30) at Maximum Wavelength 277nm.

4.1 Linearity

Appropriate aliquots of standard Apixaban stock solutions (100µg/ml) were taken in different 10 ml volumetric flask and resultant solution was diluted up to the mark with Methanol to obtain final concentration of 5-50µg/ml (Fig. 8). These solutions were injected into chromatographic system. The chromatograms were obtained and peak area was determined for each concentration of drug solution and given in Table 11. Calibration curve of Apixaban was constructed by plotting peak area vs applied concentration of and regression equation was computed (Fig 7). The slope, intercept, and correlation coefficient were also determined and are shown in Table 12. The results show that an excellent correlation exists between peak area and concentration of drugs within the concentration range which are presented in table 11.

Table 11 Linearity Result of Apixaban

Sr. No.	Concentration (µg/ml)	Peak Area
1	5	99251
2	10	265291
3	15	489038
4	20	609222
5	25	781977
6	30	919950
7	35	1139333
8	40	1281841
9	45	1474717
10	50	1688599

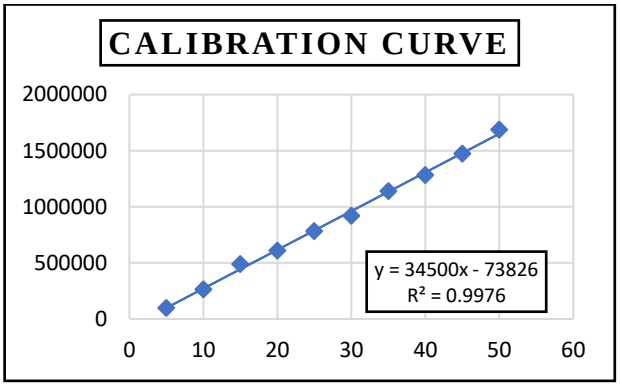

Fig. 7 Calibration curve of Apixaban

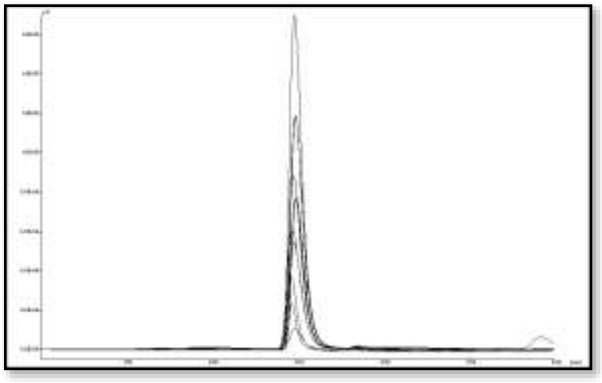

Fig. 8 Overlain of Apixaban

4.2 System Suitability

System-suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (Rt), number of theoretical plates (N) and tailing factor (T) were evaluated for six replicate injections of the drug at a concentration of 20 µg/ml. The results which are given in Table 12 & 13.Values were within acceptable limits.

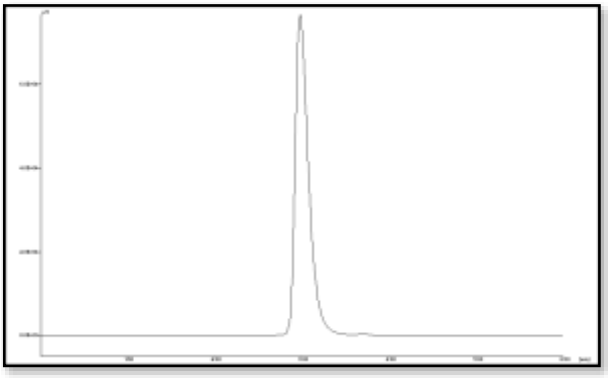

Fig. 9. Chromatogram of Apixaban tablet [Concentration 20ug/ml]

Table 12 Characteristic parameters of Apixaban for the proposed HPLC method.

Sr. No.	Parameter	Result
1	Calibration range (µg/ml)	5-50
2	Detection wavelength (nm)	277
3	Solvent (Acetonitrile: Water)	90:10
4	Regression equation (y*)	y = 34500 x + 73826
5	Slope (b)	34500
6	Intercept (a)	73826
7	Correlation coefficient(r2)	0.9976
8	Limit of Detection (µg/ml)	1.97
9	Limit of Quantitation (µg/ml)	6.81

Table 13 System suitability studies of Apixaban by HPLC method.

Sr. No.	Properties	Values
1.	Retention time	**2.9 ± 0.68**
2.	Area	**3485 ± 260**
3.	Asymmetry	**0.83**

4.3 Specificity

Chromatogram of Apixaban showed peak at a retention time of 2.910 min. The Retention time of Apixaban was 2.910 ± 0.0078min. The wavelength 277 nm was selected for detection because; it resulted in better detection sensitivity for the drug shown in Table 14. The peak for Apixaban from the tablet formulation was shown in Fig. 9.

Table 14 Specificity of Apixaban by HPLC method

Concentration	API Area	Tablet Area
SD	7343.136	11158.89
RSD	1.193671	1.799327

4.4 Sensitivity

The sensitivity of measurement of Apixaban by use of the proposed method was estimated in terms of the limit of detection (LOD) and the limit of quantification (LOQ). The LOD and LOQ were calculated by the use of signal to noise ratio. In order to estimate the LOD and LOQ values, the blank sample was injected six times and the peak area of this blank was calculated as noise level. The LOD was calculated as three times the noise level, while ten times the noise value gave the LOQ. LOD and LOQ were found to be 1.97 and 6.81 respectively.

4.5 Precision

Demonstration of precision was done under two categories. The injection repeatability (System Precision) was assessed by using six injections of the standard solution of Apixaban and the % RSD of the replicate injections was calculated. In addition, to demonstrate the precision of method (Method Precision), six samples from the same batch of formulation were analysed individually and the assay content of each sample was estimated. The average for the six determinations was calculated along with the % RSD for the replicate determinations. Both the system precision and method precision were subjected for inter-day, intra-day variations as reported in Table 15.

Table 15 Intraday and Interday Precision of Apixaban at 277

Conc.	Intraday Peak Area			Interday Peak Area		
	0 min	1 hr	2 hr	1 day	2 days	3 days
SD	10509.11	9059.885	9104.389	9160.36	8800.404	7204.549
RSD%	1.651157	1.483296	1.516534	1.440278	1.429603	1.205665

4.6 Accuracy

Recovery studies by the standard addition method were performed with a view to justify the accuracy of the proposed method. Previously analysed samples of Apixaban (20 µg/ml) were spiked with 80, 100, and 120 % extra Apixaban standard and the mixtures were analysed by the proposed method.
Standard deviation of the % recovery and % RSD were calculated and reported in Table 16.

Table 16 Accuracy of Apixaban at 277 nm.

Sr. No.	Concentration	Peak Area	Found Conc.	recovery%
1	80	589886	15.94	99.68
2	80	596357	16.140	100.87
3	80	589954	15.95	99.69
4	100	725684	19.96	99.81
5	100	725687	19.96	99.81
6	100	731284	20.12	100.64
7	120	865458	24.09	100.39
8	120	861258	23.97	99.88
9	120	861654	23.98	99.92

4.7 Robustness

Robustness is a measure of capacity of a method to remain unaffected by small, but deliberate variations in the method conditions, and is indications of the reliability of the method. A method is robust, if it is unaffected by small changes in operating conditions. To determine the robustness of this method, the experimental conditions were deliberately altered at three different levels and retention time and chromatographic response were evaluated. One factor at a time was changed to study the effect. Variation of mobile phase composition (Acetonitrile: Water and Acetonitrile: buffer) and mobile phase flow rate by 0.9 ml/min (0.8 and 1 ml/min) had no significant effect on the retention time and chromatographic response of the 20 µg/ml solution, indicating that the method was robust. The results are shown in Table 17.

Table 17 Robustness of Apixaban at 277 nm

Concentration	Peak Area	
	Acetonitrile: Water	Acetonitrile: Buffer
SD	8292.065	6585.284
RSD	1.330392	1.103759

5. Conclusion

For routine analytical purpose, it is always necessary to establish methods capable of analysing huge number of samples in a short time period with due accuracy and precision.

A very few analytical methods appeared in the literature for the determination of Apixaban includes HPLC, HPTLC and UV- Visible spectrophotometric methods. In view of the above fact, some simple analytical methods were planned to develop with sensitivity, accuracy, precision and economical. In the present investigation HPLC method (Using Quality by Design) for the quantitative estimation of Apixaban in bulk drug and per ICH guidelines pharmaceutical formulations has been developed. HPLC methods were validated as and results of Linearity, Precision, Accuracy, Specificity, System suitability and Robustness pass the limit. The proposed high-performance liquid Chromatographic method has also been evaluated over the accuracy, precision and robustness and proved to be convenient and effective for the quality control of Apixaban. Developed method was found simple and cost effective for the quality control of Apixaban.

Moreover, the lower solvent consumption leads to a cost effective and environmentally friendly Chromatographic procedure. Thus, the proposed methodology is rapid, selective, requires a simple sample preparation procedure, and represents a good procedure for Apixaban.

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