

# A Validated LC-MS/MS Method for the Estimation of Apixaban in Human Plasma

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## ABSTRACT

A high performance liquid chromatography mass spectrometric method for the estimation of apixaban in human plasma has been developed and validated using apixaban <sup>13</sup>CD<sub>3</sub> as an internal standard (IS). The extraction of analyte and IS was accomplished by liquid-liquid extraction technique. The method has been validated over a concentration range of 1.00 ng mL<sup>-1</sup> to 301.52 ng mL<sup>-1</sup>. Chromatographic separations was achieved using Thermo Beta basic-8, 100 mm x 4.6 mm, 5μ, column eluted at flow rate of 1.0 mL minute<sup>-1</sup> with 1:1 splitted post column with mobile phase Acetonitrile: Ammonium formate buffer pH 4.2 (70:30 v/v). The overall run time of method was about 3.0 min. with elution times of apixaban and its internal standard apixaban <sup>13</sup>CD<sub>3</sub> at around 1.2 min. The multiple reaction monitoring transitions were set at 460.2 > 443.2 (m/z) and 464.2 > 447.4 (m/z) for apixaban and apixaban <sup>13</sup>CD<sub>3</sub> respectively. The calibration curves were linear (r<sup>2</sup> ≥ 0.99) over the range of 1.0-301.52 ng mL<sup>-1</sup> with lower limit of quantitation validated at 1.0 ng mL<sup>-1</sup>. Extraction recoveries were >98 % for both apixaban and its stable labeled IS apixaban <sup>13</sup>CD<sub>3</sub>. The within run and between run precisions were within 0.70%-6.98%, while accuracy ranged from 89.2 to 107.2%.

## INTRODUCTION

Apixaban is a potent, oral, reversible, direct and highly selective active site inhibitor of Factor-Xa. It does not require antithrombin III for antithrombotic activity. Apixaban inhibits free and clot-bound Factor-Xa, and prothrombinase activity. Activation of Factor-X to Factor-Xa (FXa) via the intrinsic and extrinsic pathway plays a central role in the cascade of blood coagulation. Apixaban has no direct effects on platelet aggregation, but indirectly inhibits platelet aggregation induced by thrombin. By inhibiting Factor-Xa, apixaban prevents thrombin generation and thrombus development (Mueck *et al.*,

2011; Mueck *et al.*, 2013; Matchar *et al.*, 2002; Gadisseur *et al.*, 2004; Shenker *et al.*, 2012). Preclinical studies of apixaban in animal models have demonstrated antithrombotic efficacy in the prevention of arterial and venous thrombosis at doses that preserved hemostasis. There is a clear correlation between plasma apixaban concentration and degree of anticoagulant effect. The maximum effect of apixaban on pharmacodynamic parameters occurs at the same time as C<sub>max</sub>. Apixaban is extensively metabolized via CYP3A4 and is a substrate for P-glycoprotein (Barrett *et al.*, 2010; Wang *et al.*, 2010; Wang *et al.*, 2006). It has multiple routes of elimination. Approximately 50-55% of apixaban is eliminated in the feces and 20-25% is excreted in the urine. Earlier publications have described methods for determination of apixaban in biological matrix using liquid chromatography tandem mass spectrometry (LC-MS/MS) methods (Zhang *et al.*, 2016; Baldelli *et al.*, 2016; Noguez *et al.*, 2016; Blaich *et al.*, 2015; Schmitz *et al.*, 2014; Gous *et al.*, 2014; Delavenne *et al.*, 2013). Many places UPLC have been used for separation.

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In this study, we attempted to develop a simple and rapid method for the determination of apixaban in human plasma using a HPLC MS/MS method to evaluate the oral pharmacokinetics of apixaban tablets. Compared with previous methods, the present method has the following advantages: less plasma was required, sample preparation was simpler, and the analysis time was shorter using normal HPLC conditions making it suitable to analyze large number of samples.

## EXPERIMENT

### Chemicals and reagents

The reagents/materials used during analysis include ammonium formate (AR grade), acetonitrile (HPLC grade), orthophosphoric acid (HPLC grade), formic acid (HPLC grade), methyl tert. butyl ether (HPLC grade), methanol (HPLC grade), ethyl acetate (HPLC grade) and water (Milli-Q/HPLC grade). Apixaban and apixaban  $^{13}\text{CD}_3$  with purity > 98% were purchased from Simson Pharma, Mumbai, (Fig.1a and1b). Blank human  $\text{K}_2\text{EDTA}$  plasma was purchased from blood bank.

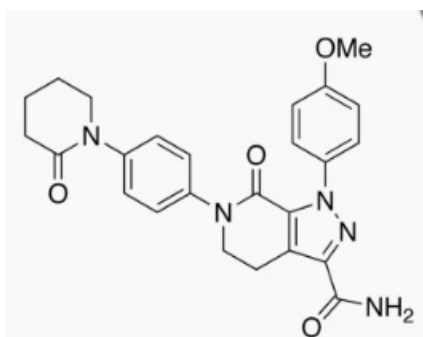


Fig. 1a: Chemical structure of Apixaban.

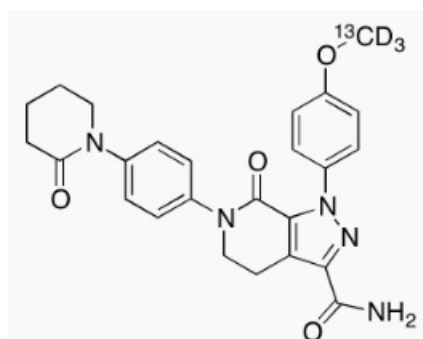


Fig. 1b: Chemical structure of Apixaban  $^{13}\text{CD}_3$ .

### Apixaban stock solution

Approximately 5 mg of apixaban was weighed and transferred to 5 mL volumetric flask. It was dissolved and made up to the mark with HPLC grade methanol to make approximately  $1.0 \text{ mg mL}^{-1}$  stock solution. This stock solution was transferred in a reagent bottle with appropriate label and stored at  $2^\circ\text{C}$  to  $8^\circ\text{C}$ . This solution was used within 8 days from the date of preparation.

Further dilutions of apixaban were prepared in Methanol: MilliQ water, 50:50 v/v for spiking into plasma.

### Apixaban $^{13}\text{CD}_3$ (IS) stock solution

Approximately 2 mg of apixaban  $^{13}\text{CD}_3$  was weighed and transferred to 2 mL volumetric flask. It was dissolved and made up to the mark with HPLC grade methanol to make approximately  $1.0 \text{ mg mL}^{-1}$  stock solution. This stock solution was transferred in a reagent bottle with appropriate label and stored at  $2^\circ\text{C}$  to  $8^\circ\text{C}$ . This solution was used within 8 days from the date of preparation. Further stock dilutions of apixaban  $^{13}\text{CD}_3$  was prepared in Methanol: MilliQ water, 50:50 v/v solution.

### Ammonium formate buffer (pH $4.2 \pm 0.05$ )

About 126.12 mg of ammonium formate was weighted and transferred into 1000 mL volumetric flask. It was dissolved with Milli-Q water/HPLC grade water and volume was made up to the mark. It was then sonicated in an ultrasonicator for 5 to 10 minutes. The pH was adjusted to ( $4.2 \pm 0.05$ ) with diluted formic acid. The buffer was used within 4 days from the date of preparation.

### Rinsing Solution

A mixture of acetonitrile and Milli-Q water was prepared in the volume ratio of 70:30 v/v as rinsing solution. It was sonicated in an ultrasonicator for 5 to 10 minutes. The solution was used for auto sampler rinsing to avoid carryover.

### Mobile Phase

A mixture of acetonitrile and ammonium formate buffer pH ( $4.2 \pm 0.05$ ) was prepared in the volume ratio of 70:30 v/v as mobile phase. It was sonicated in an ultrasonicator for 5 to 10 minutes. This was used within 4 days from the date of preparation.

### Calibration Curve Standards and Quality Control Samples

Calibration curve standards of apixaban concentrations ranging from  $1.0 \text{ ng mL}^{-1}$  to  $301.52 \text{ ng mL}^{-1}$  were prepared by spiking appropriate dilutions of working stock solution in pooled blank plasma having  $\text{K}_2\text{EDTA}$  as anticoagulant. The five levels of QC samples of apixaban concentrations ranging from  $1.0 \text{ ng mL}^{-1}$  (LLOQ QC),  $2.77 \text{ ng mL}^{-1}$  (LQC),  $61.58 \text{ ng mL}^{-1}$  (M1QC),  $123.16 \text{ ng mL}^{-1}$  (MQC) and  $246.31 \text{ ng mL}^{-1}$  (HQC) were prepared by spiking appropriate dilutions of stock solution in pooled blank  $\text{K}_2\text{EDTA}$  plasma. Spiking solution was not used more than 5% of total plasma volume. These samples were stored in deep freezer below  $-50^\circ\text{C}$  until use.

### Plasma sample extraction

The spiked plasma samples were retrieved from the deep-freezer and thawed in a water bath at room temperature. The samples were extracted using liquid-liquid extraction technique. The thawed samples were vortexed to ensure complete mixing of the contents. A  $50 \mu\text{L}$  of IS dilution mixture (approximately 800.0

ng mL<sup>-1</sup> of apixaban (<sup>13</sup>CD<sub>3</sub>) was taken in pre-labeled polypropylene tubes except in blank samples wherein 50 µL of dilution solution was added to compensate. A 250 µL of sample was added to it and vortexed. This was followed by addition of 250 µL of ortho-phosphoric acid and 250 µL of formic acid and vortexed again followed by addition of 2 mL organic solvent mixture of ethyl acetate and methyl tertiary butyl ether (MTBE) in ratio of 70:30 and further vortexed for about 5 minutes. It was centrifuged for 4 min at about 4090 rcf maintained at temperature 5°C. After centrifuge 1 mL of supernatant was taken out into pre-labeled glass tubes. The supernatant was dried at 50°C under a stream of nitrogen. The dried residue was reconstituted with 0.5 mL of solution consisting of acetonitrile and MilliQ water in ratio of 70:30 and transferred into disposable autosampler glass vials. Samples were analysed on AB Sciex API 3000 LC-MS/MS system using Turbo ion spray in positive mode.

### Equipment and software

Shimadzu HPLC equipped with dual pump, auto sampler and column oven. Mass spectrometer AB SCIEX API 3000 LC-MS/MS and data acquisition system Software Analyst Version 1.6.2 were used for the quantitative determination. The chromatograms were acquired using software Analyst version 1.6.2. The slopes, intercepts and goodness of fit were determined by linear regression analysis using the ratios of analyte/IS peak areas of the calibration curve standards. A weighting factor of 1/x<sup>2</sup> (1/ concentration<sup>2</sup>) was used in the calculation of the linear regression line and the concentrations of QC samples were calculated.

### Chromatographic Conditions

Analytes were separated on a Thermo Beta basic- 8, 100 mm x 4.6 mm, 5µ, column maintained at temperature of 40°C. An isocratic flow-rate of 1.0 mL minute<sup>-1</sup> with 1:1 splitted post column with mobile phase Acetonitrile: Ammonium formate buffer (70:30 v/v) was used for chromatographic separation. The pH of ammonium formate buffer was adjusted to (4.2 ± 0.05) with

diluted formic acid. Following 10µL injection analytes were separated. The overall run time was 3.0 min. A mixture of acetonitrile and Milli-Q water in the volume ratio of 70:30 was used as needle wash solution to avoid carry over between two injections.

### Mass Spectrometry

Mass spectrometric detection was performed on an Applied Biosystems Sciex (Concord, Ontario, Canada) API 3000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface. ESI ionization was performed in the positive ion mode. The tandem mass spectrometer was operated at unit resolution in the selected reaction monitoring mode (SRM). The multiple reaction monitoring transitions were set at 460.2 > 443.2 (m/z) and 464.2 > 447.4 (m/z) for apixaban and apixaban <sup>13</sup>CD<sub>3</sub> respectively (Fig. 2a,b,c,d).

The mass spectrometric conditions were optimized for apixaban and apixaban <sup>13</sup>CD<sub>3</sub> by continuous infusion of the standard solution at the rate of 10 µL min<sup>-1</sup> using a Harvard infusion pump. The ion source temperature was maintained at 550°C.

The ion spray voltage was set at 5500 V. The curtain gas (CUR) was set at 6 psi and the collision gas (CAD) at 8 psi. The optimal collision energy (CE) was 35 V. The following parameters of ion path were used as the most favorable ones: declustering potential (DP) at 50 V and entrance potential (EP) at 10 V. Focusing potential (FP) was maintained at 120V. The quantification was performed via peak area ratio. Data acquisition and processing were accomplished using the Applied Biosystems Analyst version 1.6.2 software. Calibration curves were generated using peak area ratios of the components to internal standards versus the known concentrations with a linear regression equation of 1/concentration<sup>2</sup>.

$$y = mx + b$$

Where, y = peak area ratio of apixaban to apixaban <sup>13</sup>CD<sub>3</sub> (IS), m = slope of the calibration curve, x = concentration of apixaban in ng mL<sup>-1</sup>, b = y-axis intercept of the calibration curve.

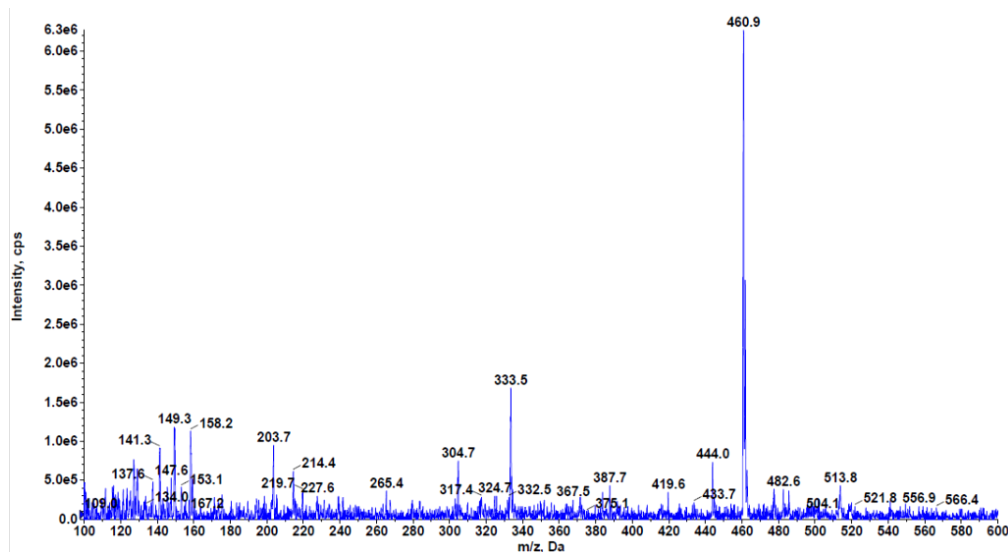


Fig. 2a: Mass spectra of Apixaban.

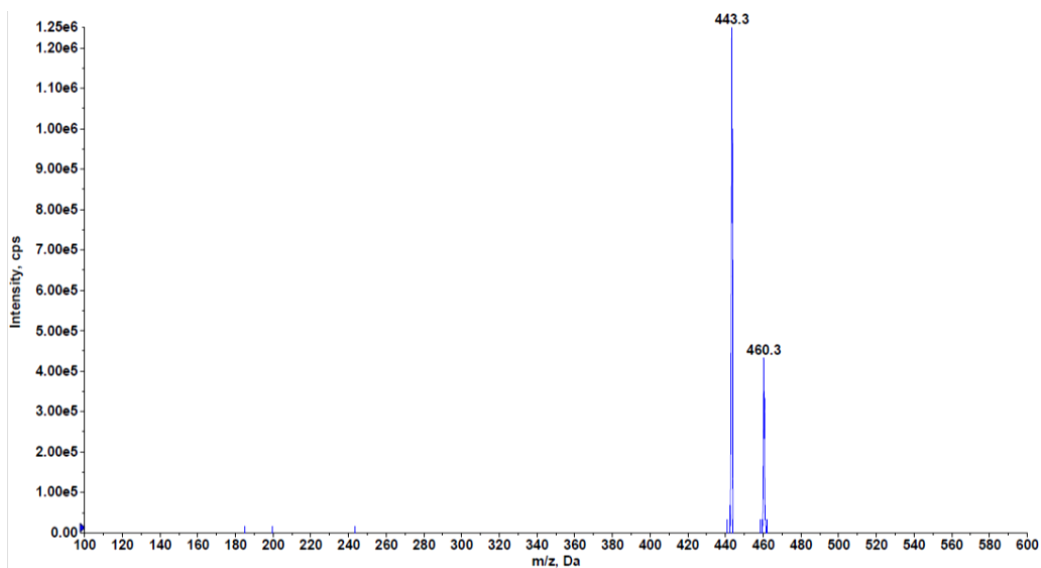


Fig. 2b: Product ion spectra of Apixaban.

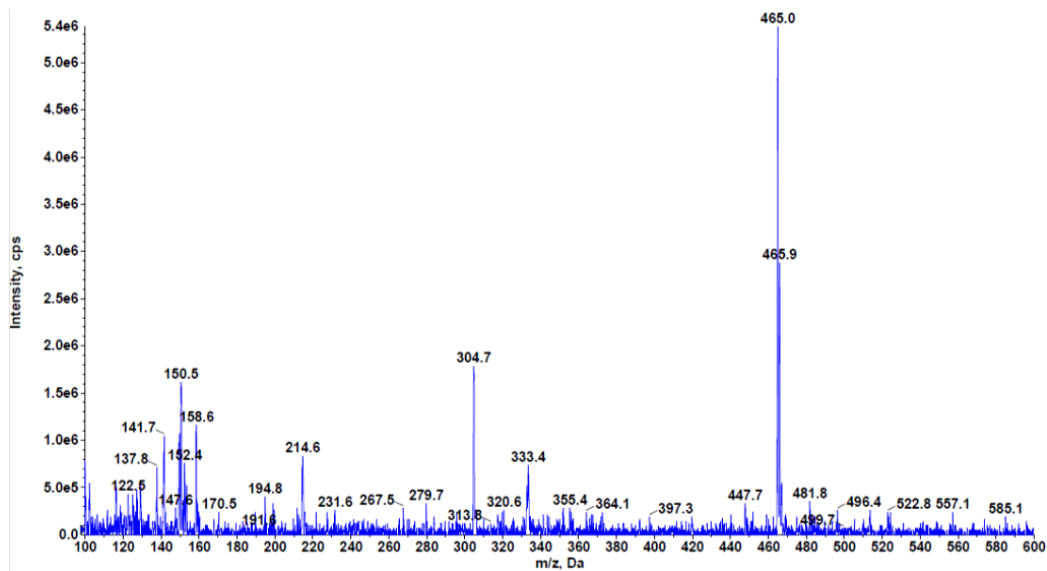


Fig. 2c: Mass spectra of Apixaban  $^{13}\text{CD}_3$ .

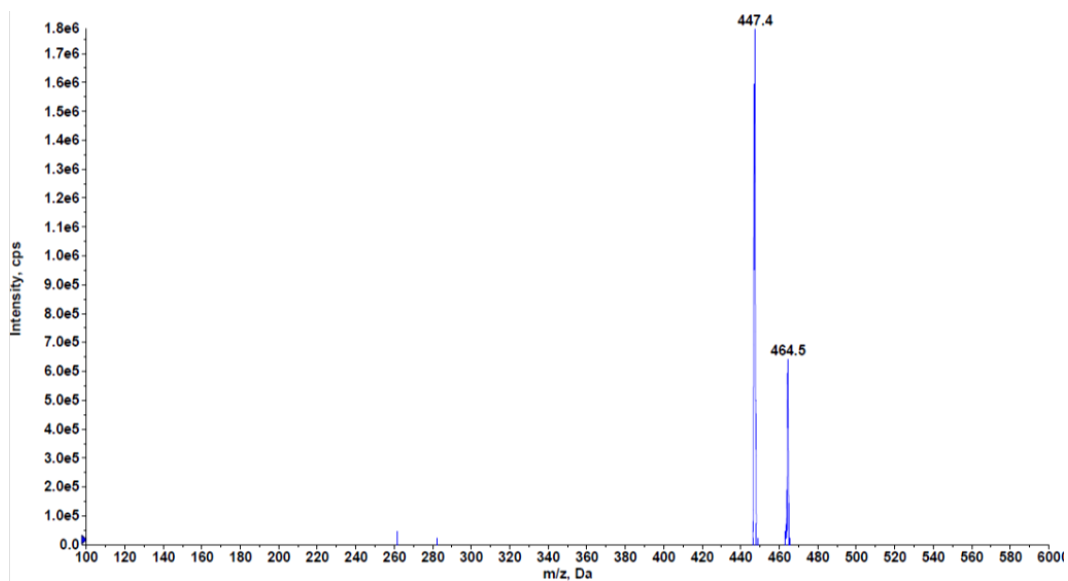


Fig. 2d: Product ion spectra of Apixaban  $^{13}\text{CD}_3$ .

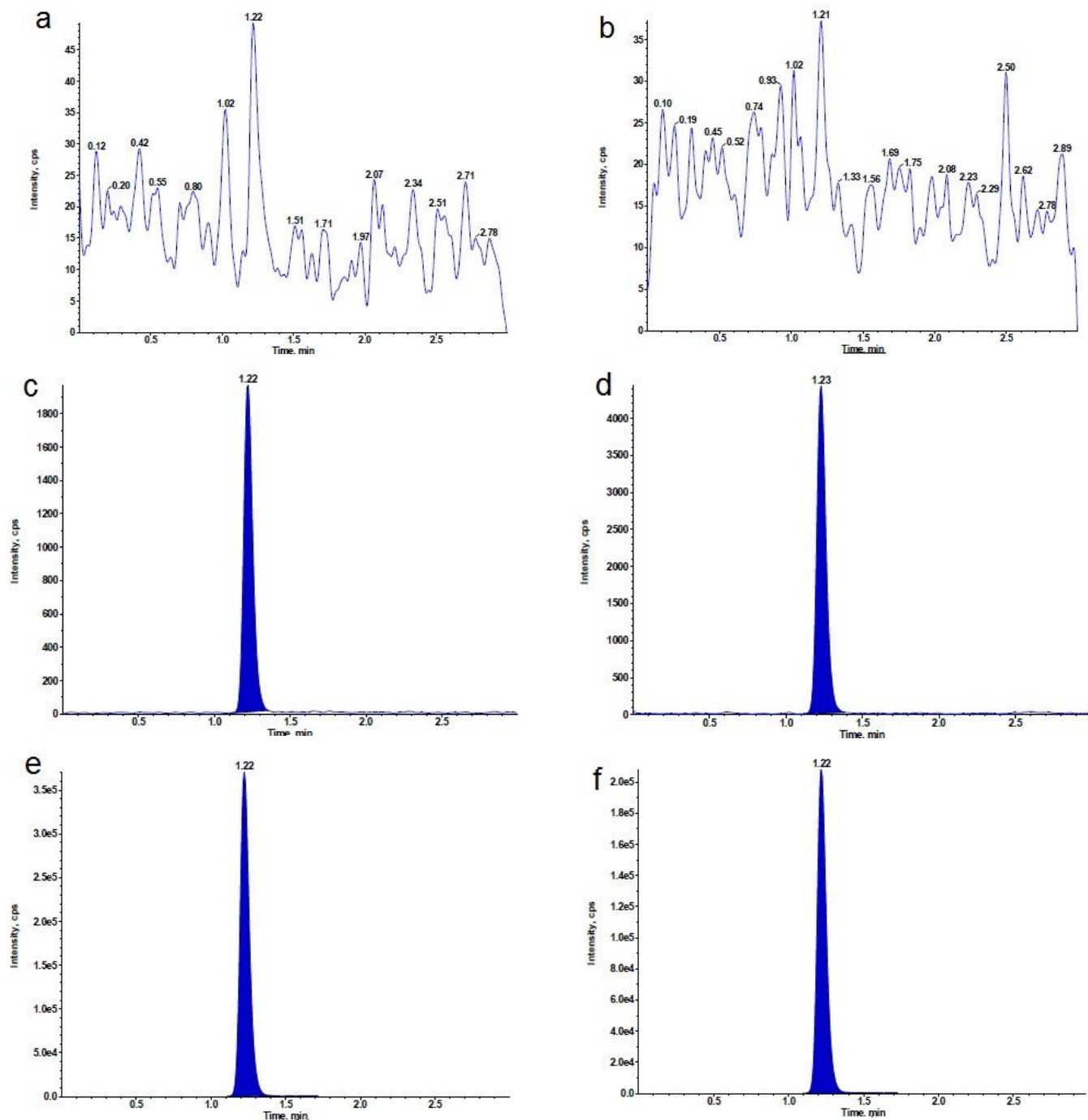
### Method Validation

The HPLC-MS/MS method was validated in accordance with the Guidance for industry, Bioanalytical method validation, as specified by US FDA (US FDA., 2001). The potential presence of endogenous contaminating compounds that may interfere with the analytical assay was determined by analyzing blank human K<sub>2</sub>EDTA plasma samples of ten different individual lots. Representative chromatogram of extracted blank plasma sample is given in (Fig. 3a, 3b). No significant interference from endogenous

components was observed at retention time of analyte and IS in all the human plasma batches screened.

### Sensitivity

The lowest limit of quantification (LLOQ) was set at the concentration of 1.0 ng mL<sup>-1</sup> (Fig. 3c). Six replicates of blank plasma were spiked at a concentration of 1.0 ng mL<sup>-1</sup>. The precision and accuracy at LLOQ were found to be 3.72 % and 93.80 % respectively.



**Fig. 3:** Representative chromatograms a. Blank (Apixaban), b. Blank IS (Apixaban <sup>13</sup>CD<sub>3</sub>), c. Extracted LLOQ 1.0 ng/mL, d. Extracted LQC 2.77 ng/mL, e. Extracted HQC 246.31 ng/mL, f. Extracted IS Apixaban <sup>13</sup>CD<sub>3</sub>.

### Matrix Factor

Ten different lot of blank plasma were taken, extracted as per extraction method described above and spiked with LQC and HQC equivalent aqueous concentrations after extraction, thus achieving unextracted samples. Responses of these unextracted samples were compared with spiked extracted LQC and HQC samples to calculate matrix factor. No significant matrix effect was observed at low and high concentration levels.

### Recovery

The peak areas of extracted LQC, MQC and HQC samples of apixaban and peak areas of extracted MQC samples of IS apixaban  $^{13}\text{CD}_3$  were compared against the peak areas of respective unextracted QC samples. The overall mean recovery of apixaban and IS apixaban  $^{13}\text{CD}_3$  was found to be 98.53 % and 101.18 % respectively.

## RESULTS AND DISCUSSION

### Linearity and Precision and Accuracy

A regression equation with a weighting factor of  $1/(\text{concentration}^2)$  was judged to produce the best fit for the concentration-detector response relationship for apixaban in human plasma. The representative calibration curve for regression analysis is illustrated in (Fig. 4). Coefficient of determination ( $r^2$ ) was greater than 0.999 in the concentration range from  $1.0 \text{ ng mL}^{-1}$  to  $301.52 \text{ ng mL}^{-1}$  (Table 1).

The precision of the assay was measured by the percent coefficient of variation over the concentration range of LLOQ QC, LQC, M1QC, MQC and HQC samples respectively, during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the LLOQ QC, LQC, M1QC, MQC and HQC samples to their

respective nominal values, expressed in percentage (Fig. 3). Within batch precision ranged from 0.70 % to 3.72 % and the within batch accuracy ranged from 89.24 % to 107.20 %. Intra-day precision ranged from 0.98 % to 3.27 % and the intra-day accuracy ranged from 89.60 % to 94.30 %. Between batch/inter day precision ranged from 5.22 % to 6.98 % and the between batch /inter day accuracy ranged from 93.03 % to 98.60 % (Table 2a & 2b).

### Stability Studies

#### Standard stock solution stability

Room temperature stock solution stability of apixaban. The standard stock solution of apixaban was prepared. Stock solution was kept on the bench at room temperature for 24 hrs. Dilution was prepared from stock solution as stability stock and a fresh dilution was prepared as comparison stock. Room temperature stock solution stability of apixaban was carried out by injecting six replicates from the above prepared stock dilutions of apixaban at room temperature. The stability was found to be 100.56%.

#### Freeze-Thaw Stability

The stability of apixaban in human plasma was determined during 3 freeze-thaw cycles. Six sets of QC (LQC & HQC) samples were analyzed after FT-3 cycles. Six sets of freshly spiked QC (LQC & HQC) samples were prepared on the day of experiment and injected along with the freeze-thaw QC samples and quantified against the freshly spiked CC standards.

The precision ranged from 1.53 % to 2.62 % and accuracy ranged from 100.97 % to 102.09 %. The freshly spiked CC standards and QC samples were found within the acceptance criteria (Table 4).

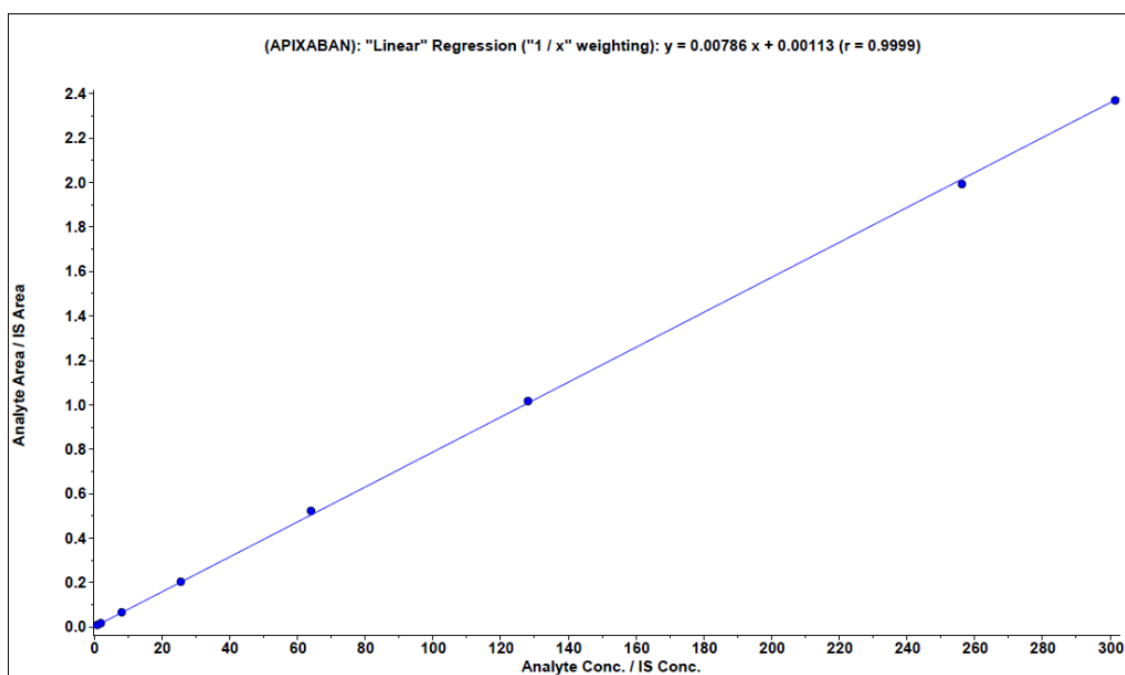


Fig. 4: Apixaban linearity curve over the range of  $1.0 \text{ ng/mL}$  to  $301.52 \text{ ng/mL}$

**Table 1:** Back calculated concentrations of calibrators for Apixaban.

Nominal Concentration of Apixaban	Back calculated concentration (mean $\pm$ SD) n=3	RSD (%)	Accuracy (%)
1.00	0.993 $\pm$ 0.0058	0.58	99.30
2.00	2.033 $\pm$ 0.0289	1.42	101.65
8.20	8.307 $\pm$ 0.0751	0.90	101.30
25.63	25.543 $\pm$ 0.0231	0.09	99.66
64.07	64.393 $\pm$ 1.2573	1.95	100.50
128.15	128.153 $\pm$ 0.6204	0.48	100.00
256.29	253.737 $\pm$ 3.2080	1.26	99.00
301.52	298.070 $\pm$ 0.7202	0.24	98.86

**Table 2a:** Within-batch accuracy (% of nominal concentration) and precision (% RSD) of Apixaban in human plasma.

QC i.d	QC Nominal Concentration (ng mL <sup>-1</sup> )	Within Batch (n=6)	
		Precision (RSD, %)	Mean Accuracy (%)
LLOQ	1.0	3.72	93.80
Low	2.77	1.28	89.96
Medium1	61.58	1.06	92.45
Medium	123.16	1.65	91.98
High	246.31	0.77	91.73

**Table 2b:** Within-day and between days accuracy (% of nominal concentration) and precision (% RSD) of Apixaban in human plasma.

QC i.d	QC Nominal Concentration (ng mL <sup>-1</sup> )	Within same day (n=12)		Different days (n=18)	
		Precision (RSD, %)	Mean Accuracy (%)	Precision (RSD, %)	Mean Accuracy (%)
LLOQ	1.0	3.27	94.30	6.98	98.60
Low	2.77	1.45	89.60	5.53	93.03
Medium1	61.58	0.98	92.04	5.47	95.57
Medium	123.16	1.23	91.87	5.22	95.20
High	246.31	1.07	91.54	5.24	94.90

**Table 3:** Bench top and post preparative (auto injector) stability.

QC i.d	QC Nominal Concentration (ng mL <sup>-1</sup> )	Stability after 6 h at room temperature (Bench top stability, n=6)			Stability after 53 h at 10 ° C (post preparative, n=6)		
		Mean Concentration found (ng mL <sup>-1</sup> )	Precision (RSD, %)	Mean Accuracy (%)	Mean Concentration found (ng mL <sup>-1</sup> )	Precision (RSD, %)	Mean Accuracy (%)
Low	2.77	2.833	1.27	102.27	2.833	1.86	100.72
High	246.31	249.252	1.23	101.19	249.252	0.92	101.01

### Short Term Room Temperature (Bench top) Stability for 6 hrs

Short-term room temperature stability was determined by using six sets of QC (LQC and HQC) samples at room temperature. Six sets of freshly spiked QC (LQC and HQC) samples were prepared on the day of experiment and injected along with the stability QC samples and quantified against the freshly spiked CC standards. Apixaban was found to be stable up to 6 hrs. The precision ranged from 1.23 % to 1.27 % and the accuracy ranged from 101.19 % to 102.27 % (Table 3).

### Auto sampler stability for 53 hrs

In assessing the auto sampler stability, six sets of QC samples (LQC and HQC from PA Batch: 03) were processed and placed in the auto sampler. They were injected after a period of 53 hrs. Six sets of freshly spiked QC (LQC and HQC) samples were prepared on the day of experiment and injected along with the

stability QC samples and quantified against the freshly spiked CC standards. The results demonstrate that the processed samples were stable up to 53 hrs. The precision ranged from 0.92 % to 1.86 % and accuracy ranged from 100.72 % to 101.01 % (Table 3).

### Long Term Stability data (below -50°C) for 199 days

The stability of apixaban, for plasma samples stored below -50°C was generated for 199 days by quantifying six sets of QC samples (LQC and HQC). Six sets of freshly spiked QC (LQC and HQC) samples were prepared on the day of experiment and injected along with the stability QC samples and quantified against the freshly spiked CC standards. The precision of the calculated concentrations of QC samples ranged from 0.97 % to 2.26 % and accuracy ranged from 92.71 % to 94.56 %. The freshly spiked CC standards and QC samples were found within the acceptance criteria (Table 4).

**Table 4:** Long term and freeze thaw stability of Apixaban.

QC i.d	QC Nominal Concentration (ng mL <sup>-1</sup> )	Stability after three freeze thaw cycles			Long term stability of Apixaban at -50 °C after 156 days		
		Mean Concentration found (ng mL <sup>-1</sup> )	Precision (RSD, %)	Mean Accuracy (%)	Mean Concentration found (ng mL <sup>-1</sup> )	Precision (RSD, %)	Mean Accuracy (%)
Low	2.77	2.797	2.62	100.97	2.568	2.26	92.71
High	246.31	251.460	1.53	102.09	232.908	0.97	94.56

**Table 5:** Table Dilution Integrity.

Sample ID	Nominal Concentration (ng mL <sup>-1</sup> )	Mean Back Calculated Concentration n=6 (ng mL <sup>-1</sup> )	Precision (RSD, %)	Mean Accuracy (%)
2xDilution	513.15	499.620	0.94	97.36
4xDilution	513.15	508.703	0.79	99.13

### Dilution Integrity

Dilution integrity samples were prepared by spiking about 1.7 times the highest standard concentration of apixaban (301.52 ng mL<sup>-1</sup>). Six sets of dilution integrity samples were processed by diluting them twice and another six sets were processed by diluting them four times. These dilution QC samples were analyzed along with CC standards and were calculated using 2x and 4x dilution factor respectively. The precision and accuracy for a dilution factor of 2 was found to be 0.94 % and 97.36 % respectively. Similarly, the precision and accuracy for a dilution factor of 4 were found to be 0.79 % and 99.13 % respectively (Table 5).

### CONCLUSION

In this study, we described a sensitive and selective high performance liquid chromatography–tandem mass spectrometry method for the analysis of apixaban in human plasma. Validation of the method in selected conditions shows that the method is selective and precise with linear response of mass spectrometer. The liquid-liquid extraction procedures produced clean chromatograms and high and reproducible recovery was obtained for investigated compound. The method has been found suitable to support pharmacokinetic studies.

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**Declaration of conflict of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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