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LC-MS/MS ASSAY FOR THE DETERMINATION OF APIXABAN IN HUMAN PLASMA: ITS APPLICATION TO A PHARMACOKINETIC STUDY

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ABSTRACT

Introduction: Apixaban is a novel oral anticoagulant for the management of thromboembolic disease. In the present research work, a novel LC-MS/MS method was developed and validated for the determination of apixaban in human plasma. **Method:** Apixaban 13C-d3 was used as an internal standard (IS). A C18 column with isocratic mobile phase of 0.1% formic acid – methanol (15:85, v/v) used for the separation of extracted analyte. The flow rate was 0.75mL/min. The proposed linearity for apixaban was 1.02–301.3 ng/mL. **Results:** A total of five linearity curves were generated with quality control samples to calculate the precision and accuracy. Variety of validation experiments namely precision and accuracy, linearity, dilution integrity, recovery and matrix effect were well within the acceptablelimits. Also, the stability of analyte was extensively evaluated in plasma as well in extracted samples and results were met the acceptance criteria as per the regulatory guidelines. **Conclusion:** The present LC–MS/MS assay method is simple, rapid and sensitive for the determination of apixaban in human plasma. This is well suitable for pharmacokinetic or bioavailability/bioequivalence application.

KEYWORDS: Apixaban, Human plasma, SPE, LC–MS/MS, Pharmacokinetics.

I. INTRODUCTION

Apixaban is a potent, direct, selective factor Xa inhibitor that was approved on December 28, 2012. It (Eliquis, Bristol-Myers Squibb) is the third novel oral anticoagulant for the management of thromboembolic disease.[1] Apixaban is absorbed throughout the gastrointestinal (GI) tract, with about 55% of absorption occurring in the distal small bowel and ascending colon. The drug is metabolized primarily by cytochrome P450 (CYP) 3A4/5. The recommended apixaban dosage for patients with atrial fibrillation is 5 mg orally twice daily. A reduced dosage of 2.5 mg orally twice daily is recommended for patients with any 2 of the following characteristics: age 80 years or older, body weight 60 kg or less, or SCr 1.5 mg/dL or greater.[2]

Apixaban has multiple elimination pathways, and its pharmacokinetics is not substantially altered by patient age, sex, race, or ethnicity.[3] It has about 50% bioavailability, and approximately 25% is excreted by the kidney Aspirin reduces the risk of stroke in patients with atrial fibrillation by about 20% and is used in treating patients with atrial fibrillation for whom vitamin K antagonist therapy is unsuitable.[4] Chemical structure of apixaban and Apixaban 13C- d3 (IS) is given in Figure 1.

As a result of FXa inhibition, apixaban prolongs clotting tests such as prothrombin time (PT), INR, and activated partial thromboplastin time (aPTT). Apixaban inhibits free and clot-bound FXa and prothrombinase activity. Apixaban has no direct effect on platelet aggregation, but indirectly inhibits platelet aggregation induced by thrombin. By inhibiting FXa, apixaban decreases thrombin generation and thrombus development.[5-7]

A review of literature reveals that few LC-MS/MS methods[8–11] have been reported for determination of apixaban alone or simultaneously along with other drugs in human plasma. These methods are having limitations like' complicated and expensive extraction procedures or long chromatographic run time.

The author Delavenne *et al.,* 2013[8] reported a LC-MS method for the quantification of apixaban in human plasma utilizes protein precipitation (PP) technique for sample preparation. But this technique is not efficient to remove the endogenous compounds such as lipids, fatty acids, and phospholipids completely which is most likely to cause ion suppression. The method was validated in the concentration range of 5-500 µg/L. Recently Wiesen *et al.,* 2017[9] published a method for the simultaneous determination of apixaban,

dabigatran, edoxaban in human plasma by using UHPLC-MS technique. UHPLC methods are more sensitive and rapid compared with conventional HPLC methods. The method employs paramagnetic micro-particles, subsequent magnetic depletion for sample preparation, which is complex with many strengthen method development protocols and required skilled persons. The method was validated in the range of 2-500 ng/mL in plasma. Similarly, Baldelli *et al.*, 2016[10] published a paper for the simultaneous determination of dabigatran, rivaroxaban and apixaban in human plasma. This method was also employed PP technique for sample preparation. The run time was more than 3 min, which may not suitable rapid determination of apixaban. Moreover, these methods are not only for quantitation of apixaban, involves estimation of other anticoagulant drugs namely dabigatran, edoxaban and rivaroxaban. It may create conflicts in final quantification of analyte of interest. Another author Pursley *et al.*, 2014[11] reported LC MS/MS determination of apixaban (BMS-562247) and its major metabolite in human plasma. The method was complex with polarity switching. A monolithic HPLC column was used for the separation of analyte from endogenous components in plasma.

To investigate the safety and tolerability of new formulations of apixaban and/or for comparative bioavailability and bioequivalence studies (BA/BE) of apixaban, it is necessary to estimate the *in vivo* plasma concentrations of apixaban. The reported LC-MS/MS methods have a longer chromatographic run time >4 min, which are not be suitable for high-throughput bioanalysis apixaban and are complex. Thus, the aim of this study was to develop a more specific, selective, sensitive and high-throughput LC-MS/MS method for the determination of apixaban in human plasma (100 µL) using Apixaban 13C-d3 as internal standard. The main advantages of our method are: higher sensitivity, isocratic mobile phase elution and shorter runs time (2.0min). The validated method was successfully applied to a human pharmacokinetic study.

II. EXPERIMENTAL STANDARDS AND CHEMICALS

Apixaban reference sample (99.30% pure) was obtained from Clearsynth Labs Limited, (Mumbai, India). Apixaban 13C- d3 (96.50% pure) was employed as an internal standard and was obtained from Vivan Life Sciences Limited (Mumbai, India). LC-MS grade methanol was purchased from J.T. Baker (Phillipsburg, USA). Analytical grade Formic acid was purchased from Merck Ltd (Mumbai, India). Water used for the LC–MS/MS analysis was

prepared by using Milli Q water purification system procured from Millipore (Bangalore, India) in house.

2.1. LC–MS/MS Instrument and Conditions

An HPLC system (Shimadzu LC-20 AD (Make: Shimadzu Corporation, Japan) consisting of a Zorbax SB C18 column (50 mm × 4.6 mm; 3.5 µM), a binary LC–20AD prominence pump, an auto sampler (SIL–HTc) and a solvent degasser (DGU–$20A_3$) was used for the study. Aliquot of 15 µL of the processed samples were injected into the column, which was kept at 35 ºC. An isocratic mobile phase consisting of a mixture of 0.1% formic acid – methanol (15:85, v/v) was used to separate the analyte from the endogenous components and delivered at a flow rate of 0.75 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantification was achieved with MS–MS detection in positive ion mode for the analyte and the IS using an API-4000, MDS Sciex, Applied Biosystems, (Foster City, CA, USA) equipped with a Turboionspray ™ interface. The optimized chromatographic conditions were listed in Table 1.

2.2 Preparation of Plasma Standards and Quality Controls

Standard stock solution of apixaban and IS (1 mg/mL) were prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in methanol and water (50:50, v/v; diluent). The IS working solution (2000 ng/mL) was prepared by diluting its stock solution with diluent. Calibration samples were prepared by spiking 200 µL of control K_2 EDTA human plasma with the 200 µL working standard solution of the analyte as a bulk, to obtain apixaban concentration levels of 1.02, 2.04, 5.11, 15.03, 30.05, 60.109, 120.22, 180.78, 241.04, 301.30 ng/mL as a single batch at each concentration. Similarly, quality control (QC) samples were also prepared as a bulk based on an independent weighing of standard drug, at concentrations of 1.03 (LLOQ), 3.03 (LQC), 45.24 (MQC1), 150.79 (MQC2) and 226.75 ng/mL (HQC) as a single batch at each concentration. Samples were stored in the freezer at –70 ± 10ºC until analyses.

2.3 Processing of the plasma samples

The samples were thawed at room temperature and vortexed to ensure complete mixing of the contents. 100 µL of the plasma sample was pipetted into pre-labeled RIA vial tubes, 20 µL of internal standard dilution (2000.000 ng/mL of Apixaban 13CD3) was added to it and vortexed, except in blank plasma samples where 20 µL diluent was added and vortexed. Then, 500 µL of 1% formic acid buffer was added and vortex.

The sample mixture was loaded onto Strata X 33µm polymeric sorbent (30 mg/1cc) cartridges that were pre-conditioned with 1.000 mL of HPLC grade methanol followed by 1 mL Milli Q/HPLC grade water (New cartridge for each sample). After applying the maximum pressure, the extraction cartridge was washed with 2 mL of Milli Q/ HPLC grade water (1 mL each time). Then the samples were eluted with 1 mL of mobile phase and transferred to autosampler loading vials and loaded into the auto-sampler.

III. BIOANALYTICAL METHOD VALIDATION

The validation of the above method was carried out as per US FDA guidelines. The parameters determined were selectivity, specificity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity and stability.[12]

IV. PHARMACOKINETIC STUDY DESIGN

A pharmacokinetic study was performed in healthy male subjects (n = 6). The subjects were fasted 12 h before administration of the drug formulation. Blood samples were collected following oral administration of 10 mg Apixaban tablet at pre–dose 0.00 and 0.5, 1, 1.5, 2, 2.33, 2.67, 3, 3.33, 3.67, 4, 4.33, 4.67, 5, 6, 8, 10, 12, 16, 24, 36, 48 and 72 h of post-dose in to 5 mL K2 EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at –70 ± 10 °C till their use. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. Plasma concentration–time profile of apixaban was analyzed by non–compartmental method using WinNonlin Version 5.2.

V. RESULTS AND DISCUSSION

5.1 Mass Spectrometry

Mass parameters were tuned in both positive and negative ionization modes using electrospray ionization source. The intensity response obtained in positive mode was much higher than those in negative ion mode since the analyte and IS having the ability to accept protons. Protonated form of analyte and IS, $[M+H]^+$ ion was the parent ion in the Q_1 spectrum and was used as the precursor ion to obtain Q_3 product ion spectra. The most sensitive mass transition was observed from transition pairs of m/z 460.20 precursor ion to the m/z 77.20 for Apixaban and m/z 464.20 precursor ion to the m/z 78.20 product ion for the IS. The dwell time for each transition was 200 ms. LC–MRM is a very powerful technique for

pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development.

5.2 Method Development

The method development includes mobile phase selection, flow rate, column type and injection volume. 0.1% formic acid and acetonitrile were tried in different volume ratio with buffers like 5mM ammonium acetate, 5mM ammonium formate as well as acid additives like formic acid in varying strength. The presence of a small amount of formic acid in the mobile phase improved the detection of the analyte. It was observed that 0.1% formic acid – methanol (15:85, v/v) as the mobile phase was most appropriate to give best sensitivity, efficiency and peak shape. Zodiac, C18, 50 x 4.6 mm, 3.0 µm column gave good peak shapes and response even at lowest concentration level for the analyte. The mobile phase was operated at a flow rate of 0.75 mL/min. The retention time of analyte and the IS were low enough (0.75±0.3min) allowing a short run time of 2.0 min.

Based on the previous reports, different sample pre–treatment methods were investigated. Initially protein precipitation (PP) was tried using methanol and acetonitrile as precipitating agents resulted matrix effect and poor chromatography of the analyte. Further we done for LLE was carried out using solvents like ethyl acetate, *tert*–butyl methyl ether, diethyl ether and dichloromethane alone and in combination under acidic conditions the observance is Poor extraction efficiency. Thus, SPE was carried out on Orephus C_{18}, Strata X 33µm polymeric sorbent and HLB cartridges on acidic conditions will gave Inconsistent response at LLOQ level and not reproducible. High recovery of apixaban was achieved on Strata X 33µm polymeric sorbent (30 mg/1cc) and thus used for the present study. For LC–MS/MS analysis, use of stable isotope–labeled drugs as internal standards proves to be helpful when a significant matrix effect is possible. Hence, apixaban 13C-d3 was used as an IS for the present work.

5.3 Selectivity and chromatography

Best chromatographic results were obtained with 0.1% formic acid – methanol (15:85, v/v) as the optimized mobile phase. The mobile phase was used in isocratic mode at a flow rate of 0.75 mL/min. The method selectivity was examined by analyzing 6 human plasma (K3-EDTA) lots obtained from six individual sources. As shown in Fig. 1A, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug–free human plasma at the retention time of the analyte and the IS. Similarly, Fig. 1B shows

the absence of direct interference from the IS to the MRM channel of the analyte. Fig. 1C depicts a representative ion–chromatogram for the LLOQ sample (1.02 ng/mL).

5.4 Matrix effect

Matrix effect was checked at LQC and HQC levels in six human plasma lots including hemolytic and lipemic. No significant matrix effect was observed in all the six lots of human plasma for apixaban at low (LQC) and high (HQC) concentrations. The %RSD of apixaban for IS normalized matrix factor at LQC and HQC level was found to be 1.48% and 0.89%, respectively and IS normalized factor was 1.013 for LQC and 1.005 for HQC.

5.6 Linearity, sensitivity, precision and accuracy

The ten–point calibration curve was found to be linear over the concentration range of 1.02–301.3 ng/mL for apixaban. Five acceptable precision and accuracy batches were analyzed in this range. Correlation coefficient (r^2) results were >0.99 for all five linearity batches**.** Sensitivity was tested at a concentration of 1.02 ng/mL which is the lowest level of concentration (LLOQ) At this concentration, the precision and accuracy of apixaban was 7.65% and 98.32%.

The results for intra–day and inter–day precision and accuracy in plasma quality control samples are summarized in Table 2.

5.7 Extraction efficiency

The extraction efficiency of apixaban was determined by comparing the detector response of analyte at three distinct levels of extracted low, medium2 and high-quality control samples with detector response obtained from un-extracted aqueous quality control samples. The mean extraction efficiency of apixaban was 97.22% with a precision ranging from 0.92% to 3.35%. The mean extraction efficiency of internal standard was 95.17% with a precision ranging from 0.96% to 3.98%. The recoveries of analyte and IS was good and reproducible.

5.8 Stability Studies

In the different stability experiments carried out viz. Stock solution stability at room temperature (20±5°C) Stock solution stability at 2 - 8 °C bench top stability (20 h), In-injector stability (75 h) repeated freeze–thaw cycles (4 cycles), Post-processing stability (76 h at 2–8 °C) Re-injection reproducibility (62 h), and long term stability at –70 °C for 65 days the mean % nominal values of the analyte were found to be within ±15% of the predicted

concentrations for the analyte at their LQC and HQC levels (Table 3). Thus, the results were found to be within the acceptable limits during the entire validation.

5.9 Dilution integrity

Dilution integrity (DI) samples were analyzed along with precision and accuracy batch. DI samples were prepared by spiking 1.7 times of ULOQ sample (514.02 ng/mL for two-time dilution and 1030.05 ng/mL for four-time dilution). The diluted samples (2 and 4 times) were processed and analyzed with un-diluted CC samples. QC samples were calculated using suitable dilution factor. Precision and accuracy, for dilution 2 was 0.65% and 99.04%, respectively. Similarly, precision and accuracy, for dilution 4 was 0.99% and 99.18%, respectively.

5.10 Pharmacokinetic study results

To verify the sensitivity and selectivity of this method in a real–time situation, the present method was used to test for apixaban in human plasma samples collected from healthy male volunteers (n = 6). The mean plasma concentrations vs time profile of apixaban is shown in Fig. 2. The maximum concentration (C_{max}) in plasma (146.8±26.26 ng/mL) for apixaban was attained at 3.22±1.45 h (t_{max}) The area under the plasma concentration–time curve from time zero to last measurable time point ($AUC_{0–t}$) and area under the plasma concentration time curve from time zero to infinity time point ($AUC_{0–inf}$) for apixaban were 1482±284 and 1488±291 ng h/mL, respectively. The terminal half–life ($t_{½}$) was found to be 8.10±1.12 h.

Table 1: Optimized LC-MS/MS conditions.

Parameter	Apixaban	Apixaban 13C-d3
Detection	Positive	Positive
Monitoring mode	Multiple reaction monitoring (MRM)	
Ionization mode	Positive ion mode	
m/z	460.20 (parent) and 77.20 (product)	464.20 (parent) and 78.20 (product)
Ion Spray Voltage (ISV)	5500 V	5500 V
Temperature (TEM^{0}C)	500 ^{0}C	500 ^{0}C
Curtain Gas (CUR)	35 psi	35 psi
Collision Gas (CAD)	8 psi	8 psi
GS1	35 psi	35 psi
GS2	40 psi	40 psi
Declustering Potential (DP)	101 V	101 V
Collision Energy (CE)	90 V	90 V
Collision Cell Exit Potential (CXP)	13 V	13 V
Entrance Potential (EP)	10 V	10 V
Dwell time	200 ms	200 ms

Table 2: Precision and accuracy data for apixaban.

Quality control	Run	Concentration found Mean±SD (ng/mL)	Precision (%)	Accuracy (%)
Intra–day (*n*=12)				
LLOQ		0.970 ± 0.09	9.21	94.16
LQC		2.954 ± 0.04	1.48	97.45
MQC1		47.02 ± 0.47	1.00	103.9
MQC2		156.3 ± 1.15	0.74	103.6
HQC		226.5 ± 1.73	0.77	99.89
Inter–day (*n*=30)				
LLOQ		0.995 ± 0.10	9.91	96.54
LQC		2.884 ± 0.08	2.71	95.16
MQC1		46.46 ± 1.32	2.85	102.7
MQC2		154.2 ± 5.08	3.30	102.3
HQC		224.6 ± 4.17	1.86	99.04
Nominal concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 1.03, 3.03, 45.24, 150.79 and 226.75 ng/mL, respectively.				

Table 3: Stability data for apixaban in plasma (*n*=6).

Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Precision (%)	Accuracy/ Stability (%)
Auto-sampler (75 h)	3.16	3.003 ± 0.11	3.76	95.03
	235.68	232.3 ± 3.05	1.32	98.59
Wet extract stability (76 h)	3.16	2.996 ± 0.07	2.50	94.83
	235.68	229.5 ± 0.90	0.40	97.42
Bench top (20 h)	3.16	3.062 ± 0.05	1.79	95.13
	235.68	233.3 ± 1.26	0.54	99.02
freeze and thaw (4 Cycles)	3.16	2.985 ± 0.05	1.68	94.47
	235.68	231.5 ± 3.91	1.69	98.26
Re-injection (62 h)	3.16	3.008 ± 0.04	1.57	95.11
	235.95	227.5 ± 2.01	0.88	96.43
Long–term (65 days)	3.16	2.942 ± 0.13	4.63	93.11
	235.68	231.3 ± 4.42	1.91	98.18

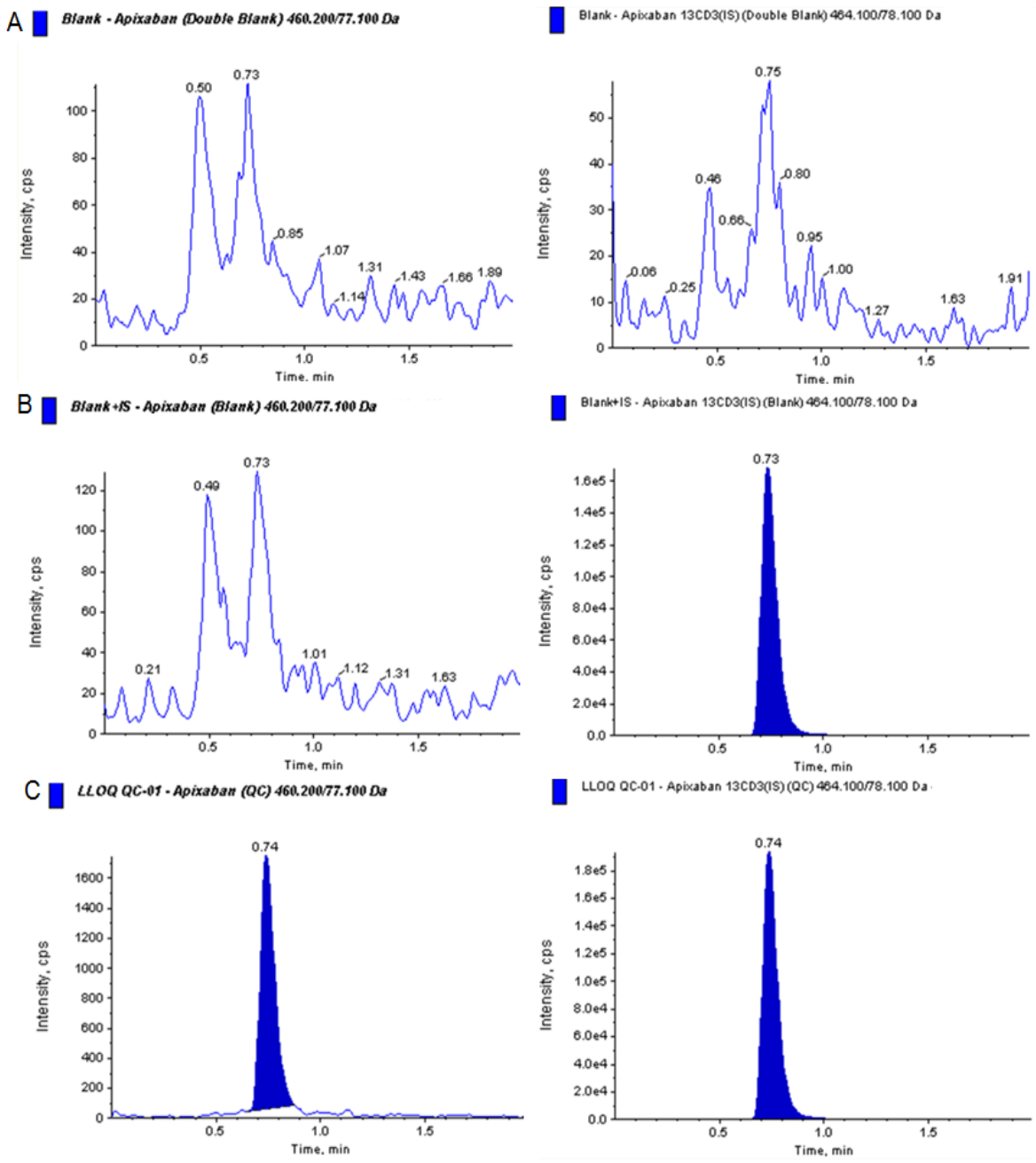

Figure 1: A model MRM chromatogram of apixaban (left panel) and IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), a LLOQ sample along with IS (C).

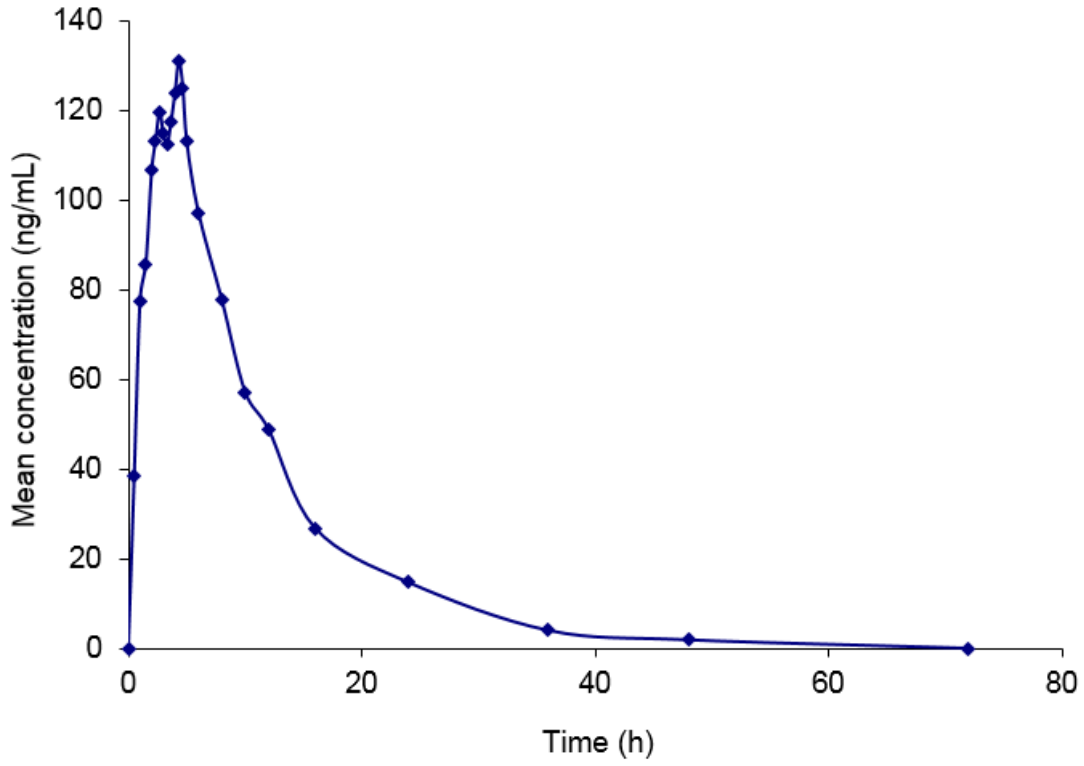

Figure 2: Mean plasma concentration–time profile of apixaban in human plasma following oral administration of apixaban (10 mg) to healthy volunteers (*n*=6).

VI. CONCLUSIONS

In conclusion, the proposed LC-MS/MS method is simple, rapid and sensitive for the determination of apixaban in human plasma. Use of stable labeled isotope as IS helped us to obtain the consistent and reproducible results. The current method has shown acceptable precision and adequate sensitivity for the quantification of apixaban in human plasma samples obtained for pharmacokinetic studies. Also, the method showed no matrix effect and limited variability in recovery between the analyte and IS. The sample preparation with SPE gave high recovery for the analyte. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

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