International Journal of Pharmacy and Pharmaceutical Sciences

Print ISSN: 2656-0097 | Online ISSN: 0975-1491 Vol 11, Issue 8, 2019

Original Article

METHOD DEVELOPMENT AND VALIDATION OF SELECTIVE AND HIGHLY SENSITIVE METHOD FOR DETERMINATION OF APIXABAN IN HUMAN PLASMA USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Received: 05 May 2019 Revised and Accepted: 04 Jul 2019

ABSTRACT

Objective: The present research work aims to develop and validate a selective and highly sensitive method for the determination of apixaban in human plasma using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Methods: 200 µl of sodium heparin plasma samples were acidified and clean-up was performed by using solid-phase extraction (SPE). Apixaban 13C D3 was used as an internal standard (deuterated) to lower the relative matrix effects and a single step SPE was employed for sample clean up. 10 µl of SPE eluent was loaded onto Hypersil Beta Basic C18, 100×4.6 mm, 5 µ column for highly selective chromatographic separation using an isocratic mobile phase. 2 mmol ammonium acetate in water and acetonitrile were delivered by using a quaternary low-pressure gradient pump without premixing at a minimum flow rate of 0.50 ml/min.

Results: LC-MS/MS method was successfully developed and validated to demonstrate the lowest detection limit of 0.05 ng/ml and a linear dynamic range from 1-250 ng/ml with r^2>0.99. Method development and validation results proved that the method is selective and highly sensitive for the determination of apixaban in human plasma using LC-MS/MS.

Conclusion: Current method can be applied for both therapeutic drug monitoring (TDM) and pharmacokinetic (PK) study analysis.

Keywords: Human Plasma, Liquid Chromatography-Tandem, Mass spectrometry

DOI: http://dx.doi.org/10.22159/ijpps.2019v11i8.33948

INTRODUCTION

Apixaban is a direct oral anticoagulant used in the management of thromboembolism and has gained importance as an alternative replacement to the vitamin K antagonists such as warfarin [1]. Therapeutic dose of apixaban results in nanogram level plasma concentrations. Therefore, it is evident to use a validated method for the estimation of apixaban in biological matrices like human plasma either for pharmacokinetic (PK) study analysis [2] or for therapeutic drug monitoring (TDM). LC-MS/MS is the only advanced technology that allows highly sensitive and selective-high throughput analysis even with low sample volumes. Moreover, it requires minimal and simple sample preparation procedures. Though several methods are available for determination of apixaban in human plasma or serum on LC-MS/MS [3-12], they were applied either for PK study analysis or for TDM but not for both. Therefore, the objective of the current study was to develop and validate a highly sensitive method with the lowest possible sample volumes, so that the same method can be further used for TDM also apart from PK study analysis.

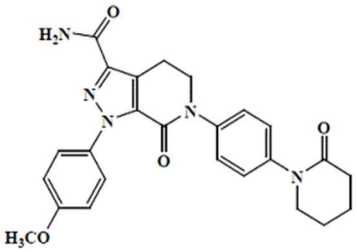

Fig. 1: Structure of apixaban

Apixaban is chemically described as 1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1-yl) phenyl]-4,5,6,7-tetrahydro-1H-pyrazolo [3,4c] pyridine-3-carboxamide. Its molecular formula is $C_{25}H_{25}N_5O_4$, which corresponds to a molecular weight of 459.50. Apixaban has the following structural formula [13].

MATERIALS AND METHODS

Reagents and chemicals

Methanol (gradient grade), acetonitrile (gradient grade), orthophosphoric acid (guaranteed reagent grade) was purchased from Merck. Ammonium acetate (reagent grade) was purchased from Sigma Aldrich. Water (liquid chromatography-mass spectrometry grade) was used in-house from Milli Q system. Apixaban and apixaban 13C D3 were purchased from Vivan life sciences.

Preparation of standard solutions and quality control samples

Standard solutions of apixaban (100 µg/ml) and apixaban 13C D3 (100 µg/ml) were prepared in methanol. Intermediate stock solutions of both analyte and internal standard (10 µg/ml) were prepared in diluent (50% methanol in water) along with standard internal dilution (40 ng/ml). Nine level calibrators and four-level controls were prepared in human plasma containing sodium citrate as anticoagulant ranging from 1-250 ng/ml and 1-125 ng/ml respectively.

Sample preparation

50 µl of internal standard was added to 200 µl pre-spiked plasma samples and 50 µl of diluent has been added to the blank samples in prelabelled micro vials. Samples were vortexed to mix well. Samples were pretreated with 500 µl of 0.10% orthophosphoric acid, vortexed to mix. Apixaban and apixaban 13CD 3 were subjected to

SPE using celerity deluxe (bed weight 30 mg, volume 1 ml-DVB LP) cartridges purchased from Orochem India Pvt Ltd., Cartridges were conditioned and equilibrated with 0.90 ml of methanol followed by 0.90 ml of water. Pretreated plasma samples were dispensed onto the cartridges and were washed with 0.90 ml of water. Cartridges were allowed to dry under the stream of nitrogen gas and were washed with 0.90 ml of 20% methanol in water (twice) before drying again. Analyte and internal standard were eluted with 0.75 ml of acetonitrile and the eluent was collected onto prelabelled HPLC vials. 10 µl of the eluent from each sample was injected on the LC-MS/MS system.

Instrumentation and analytical conditions

Above mentioned extraction procedure was optimized during method development and it was validated using ultimate 3000 HPLC system interfaced with a TSQ Endura triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc). Heated electrospray-ionization source (HESI) was operated in the positive mode. Chromatographic separations were performed using a Hypersil beta basic C18 (100×4.6 mm inner diameter, 5 µ particle size; Thermo Fisher Scientific Inc) at a column temperature of 40 ⍰. 2 mmol ammonium acetate in water and acetonitrile were used as mobile phase in pump A and pump B respectively without premixing. Isocratic program conditions were optimized with the composition of phase A ranging from 50 to 20% out of which 20% phase A and 80% phase B gave optimal results at a flow rate of 0.50 ml/min without a splitter. Apixaban and apixaban 13C D3 were selectively resolved on the reverse phase column at 2.35 min with a total run time of 4.00 min.

Optimized mass spectrometer parameters used for apixaban analysis were mentioned below: sheath gas 50 (arb), auxiliary gas pressure 25 (arb), capillary temperature 300 °C, Q2 gas pressure 1.50 m Torr, ion spray voltage 3500 V, and vaporizer temperature 300 °C. Selected reaction monitoring (SRM) transitions for quantification were m/z 460.10→443.05 for apixaban and 464.22→447.06 for apixaban 13C D3 respectively. Representative chromatograms and a calibration curve of apixaban obtained during method validation are shown in fig. 2 and 3:

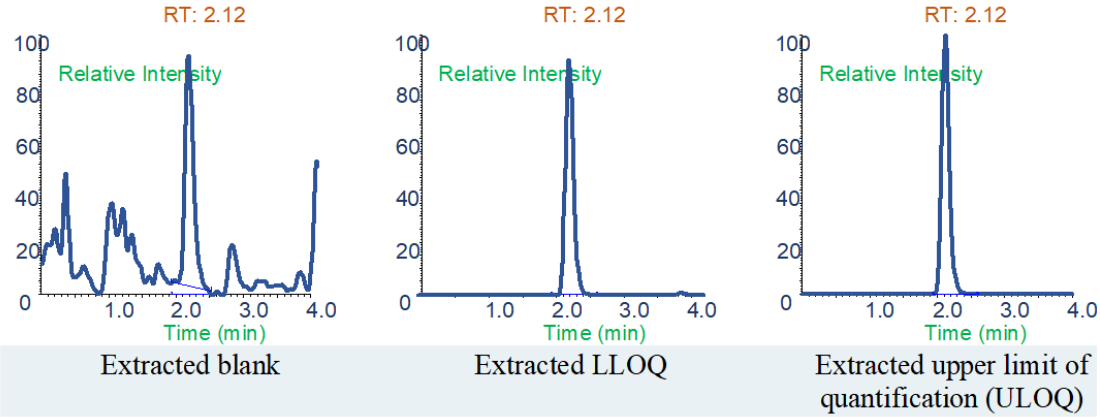

Fig. 2: Representative chromatograms of apixaban

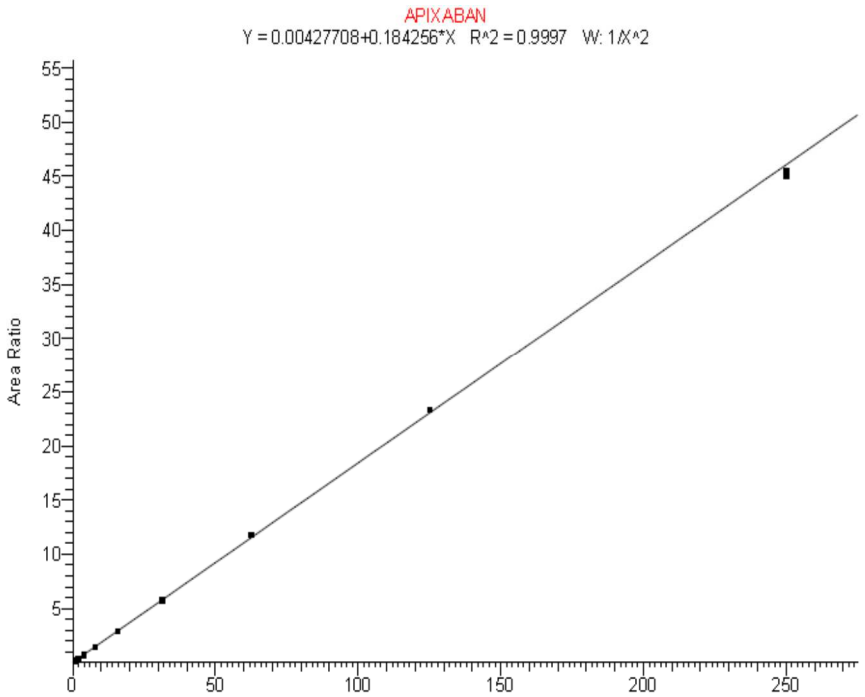

Fig. 3: Calibration curve of apixaban in human plasma from 1-250 ng/ml

RESULTS

Method validation

During validation, the method has been validated for selectivity, linearity, precision and accuracy (PA), recovery and stability studies as per current United States food and drug administration (USFDA) recommendations [14].

5 Precision and accuracy batches (includes ruggedness and stability PA batch) were analyzed with the calibration curve ranging from 1-250 ng/ml. A straight-line equation (y=mx+c) with $1/x^2$ weighting factor has been used to quantify the back-calculated concentration of the calibrators and the coefficient of determination (r^2) was greater than 0.99 in all 5 batches. Summary of back-calculated concentrations and calibration curve parameters from all 5 PA batches were mentioned below in table 1-2.

Table 1: Precision and accuracy

Standard name	CS-1	CS-2	CS-3	CS-4	CS-5	CS-6	CS-7	CS-8	CS-9
Nominal concentration (ng/ml)	**0.98**	**1.95**	**3.91**	**7.81**	**15.63**	**31.25**	**62.5**	**125**	**250**
P and A-01	0.98	1.91	3.91	7.81	15.66	13.3	64	126.8	249.56
P and A-02	0.97	2.01	3.5	7.56	14.6	31.2	66.6	130.26	251.69
P and A-03	1.02	2	3.99	8.01	16.52	33.25	60.53	125.03	255.33
P and A-04	0.95	1.86	3.55	7.95	15.02	31.03	63.26	126.36	251.03
P and A-05	1.1	1.99	3.86	8.01	14.96	30.13	60.24	124.57	248.6
mean±SD	1.00±0.06	1.95±0.07	3.76±0.22	7.87±0.19	15.35±0.76	27.78±8.17	62.92±2.63	126.60±2.24	251.24±2.59
% CV	5.92	3.43	5.88	2.42	4.94	29.43	4.18	1.77	1.03
% Nominal	102.78	100	96.26	100.73	98.25	88.9	100.68	101.28	100.5

Mean statistical data are expressed as mean±SD [n=5]

Table 2: Calibration curve parameters summary

Result table ID	Slope	Y-Intercept	Regression coefficient [r^2]
P and A-01	0.0043	0.18	1.000
P and A-02	0.0033	0.16	0.999
P and A-03	0.0030	0.17	0.999
P and A-04	0.0043	0.17	1.000
STABILITY P and A	0.0041	0.17	0.999

Specificity and selectivity of the method were assessed in 6 different lots of human plasma containing sodium citrate as an anticoagulant. Hemolyzed and lipidemic (each lot) were also used for evaluation of selectivity of apixaban. % interference in blank was found to be 0.89% when compared against the lower limit of quantification (LLOQ) area of apixaban. Results were presented below in table 3.

Table 3: Specificity and selectivity

Apixaban				Apixaban ^{13}CD3		
Matrix Lot no	**Area in Blank Matrix at analyte RT**	**Area of LLOQ**	**%Interference at the retention time of Apixaban**	**Area in Blank Matrix at IS RT**	**Area of LLOQ**	**% Interference at the retention time of Apixaban 13 CD3**
LOT 1	872	84476	1.03	100	444921	0.02
LOT 2	649	86521	0.75	80	468670	0.02
LOT 3	573	85961	0.67	120	459203	0.03
LOT 4	921	88753	1.04	104	440031	0.02
LOT 5	953	92546	1.03	98	441722	0.02
LOT 6	687	95685	0.72	112	465281	0.02
LOT 7 (HEMOLYZED)	1012	102346	0.99	150	445281	0.03
LOT 8 (LIPIDEMIC)	986	110251	0.89	126	452281	0.03

Intra-day precision and accuracy was evaluated in 6 replicates of quality control (QC) samples at LLOQ, low (LQC), middle (MQC) and higher (HQC) levels over one PA batch and was found to be between 0.78-1.03 % and 98.49-101.29 respectively over a range of 0.98-125 ng/ml. Intra-day precision and accuracy results were presented in table 4.

Table 4: Intra-day precision and accuracy

QC concentration	LLOQ QC	LQC	MQC	HQC
	0.98	3.95	31.25	125
Mean±SD (n=6)	0.96±0.01	3.89±0.03	31.52±0.25	126.61±1.10
% CV	1.03	0.85	0.78	0.87
% Nominal	98.7	98.49	100.87	101.29

Mean statistical data are expressed as mean±SD [n=6]

Inter-day precision and accuracy experiments were evaluated in 4 batches at the same levels mentioned above and the results were found to be between 2.48-4.29 % and 97.72-102.36 %, respectively. Results of Inter-day precision and accuracy were tabulated in table 5.

Table 5: Inter-day precision and accuracy

QC	LLOQ QC	LQC	MQC	HQC
Nominal concentration	0.98	3.95	31.25	125
mean±SD (n=24)	0.95±0.03	4.05±0.17	31.50±1.02	126.89±3.15
% CV	2.67	4.29	3.25	2.48
% Nominal	97.72	102.36	100.82	101.51

Mean statistical data are expressed as mean±SD [n=24]

Matrix effect was studied for both apixaban and apixaban 13C D3 in eight lots of plasma (6 normal, 1 hemolyzed and 1 lipidemic plasma). Internal standard (IS) normalized matrix factor was calculated as a ratio of the response ratio of post extracted spiked sample upon aqueous sample at both HQC and LQC concentration levels and mean IS normalized matrix factor was found to be 1.00.

Results of IS normalized matrix effect experiment were provided below in table 6.

Table 6: Matrix effect experiment

QC	Response ratio of post extracted spike sample	Response ratio of aqueous standard	Matrix factor	QC	Response ratio of post extracted spike sample	Response ratio of aqueous standard	Matrix factor
	0.190	0.189	1.004		0.727	0.729	0.997
	0.191	0.190	1.006		0.717	0.707	1.014
	0.188	0.190	0.990		0.733	0.702	1.044
LQC	0.190	0.190	1.002	**HQC**	0.72	0.693	1.040
	0.189	0.188	1.008		0.694	0.727	0.955
	0.190	0.188	1.014		0.684	0.713	0.960
	0.190	0.187	1.014		0.722	0.724	0.997
	0.190	0.190	0.998		0.709	0.720	0.984
Mean IS normalized matrix factor of LQC [mean±SD (n=8)]			1.00±0.01	Mean IS normalized matrix factor of HQC [mean±SD (n=8)]			1.00±0.03
% CV			0.8	% CV			3.3

Mean statistical data are expressed as mean±SD [n=8]

Mean recovery of apixaban was obtained by calculating the response ratio of extracted and aqueous samples at LQC, MQC, HQC levels and was found to be 99.22% and 95.25% for apixaban and apixaban ^{13}C D3 respectively. Results of recovery experiment were presented in table 7.

Table 7: Recovery of apixaban

QC	Response of extracted sample	Response of Unextracted sample	% Recovery
LQC (n=6)	3291664	3322320	99.03
MQC (n=6)	2468748	2491740	99.49
HQC (n=6)	9874992	9966960	99.13
Mean recovery (mean±SD)			99.22±0.24
% CV			0.24

Mean statistical data are expressed as mean±SD [n=6x3]

Stability experiments in the biological matrix were conducted for bench top (10.0 h), freeze-thaw 4th cycle (at-50 °C and at-20 °C), auto-sampler (48 h), wet extract (32 h 30 min) and long-term storage (at-50 °C) and results were mentioned below in table 8-9

Table 8: Stability experiments in biological matrix

Comparison QC details	Mean±SD (n=6)	% CV	% Nominal
Freshly spiked LQC	4.12±0.16	3.91	103.97
Freshly spiked HQC	125.95±4.83	3.83	100.75
Stability QC details	**mean±SD (n=6)**	**% CV**	**% Nominal**
Autosampler stability-LQC (48 h)	4.25±0.25	5.78	107.38
Autosampler stability-HQC (48 h)	128.54±2.13	1.66	102.82
FT 4th Cycle LQC (-50 °C)	4.14±0.16	3.86	104.6
FT 4th Cycle HQC (-50 °C)	133.27±2.90	2.18	106.61
FT 4th Cycle LQC (-20 °C)	4.22±0.16	3.84	106.43
FT 4th Cycle HQC (-20 °C)	133.22±3.00	2.25	106.56
Wet extract stability LQC (32 h 30 min)	3.90±0.10	2.6	98.38
Wet extract stability HQC (32 h 30 min)	126.94±2.77	2.18	101.54
Bench top stability (LQC) (10 h)	3.60±0.33	9.08	90.74
Bench top stability (HQC) (10 h)	139.27±9.77	7.01	111.41

Mean statistical data are expressed as mean±SD [n=6]

Table 9: Long term matrix stability

Comparision QC details	Mean±SD (n=6)	% CV	% Nominal
Freshly spiked LQC	3.95±0.12	3.15	99.58
Freshly spiked HQC	132.31±9.28	7.01	105.84
Stability QC details	**mean±SD (n=6)**	**% CV**	**% Nominal**
LTMS LQC-100 d	3.42±0.31	9.08	86.2
LTMS HQC-100 d	119.65±4.59	3.83	95.71

Mean statistical data are expressed as mean±SD [n=6]

Sensitivity (Limit of detection or LOD) was performed by injecting six replicates of the extracted sample prepared at a concentration of 50 pg/ml under the same chromatographic conditions and the results were found to be accurate and precise. Summarized data of the experiment is presented in table 10.

Table 10: Limit of detection

Actual concentration of LOD	Mean concentration-pg/ml [mean±SD (n=6)]	% CV	% Nominal
50 pg/ml (n=6)	47.88±1.83	3.83	95.77

Mean statistical data are expressed as mean±SD [n=6]

Reinjection reproducibility was evaluated by re-injecting the entire PA batch, which was earlier subjected to analysis. Samples were kept in autosampler for 26 h 30 min before subjecting the samples for reinjection. Results were provided below in table 11.

Table 11: Reinjection reproducibility

Sample name	Mean±SD (n=6)	% CV	% Nominal
LLOQ QC 0.98 pg/ml	0.93±0.06	6.31	94.98
LQC 3.95 pg/ml	4.12±0.15	3.68	104.22
MQC 31.25 pg/ml	32.41±1.34	4.14	103.71
HQC 125.00 pg/ml	125.76±2.00	1.59	100.61

Mean statistical data are expressed as mean±SD [n=6]

Dilution integrity was checked in the dilution quality control sample diluted up to 2 and 5 times respectively. Samples were processed under freshly prepared calibration curve standards. Results of the dilution integrity experiment were presented below in table 12.

Table 12: Dilution integrity

Dilution integrity	Dilution factor: 2 concentration	Dilution Factor: 5 concentration
	375	**375**
	Observed concentration (ng/ml)	
mean±SD (n=6)	374.35±3.75	365.46±12.83
%CV	1	3.51
%Nominal	99.83	97.46

Mean statistical data are expressed as mean±SD [n=6], Ruggedness experiment was performed by injecting a freshly prepared PA batch on a different column. Results of the experiment were provided below in table 13.

Table 13: Ruggedness

QC concentration	LLOQ QC	LQC	MQC	HQC
	0.95	**4.11**	**31.13**	**126.43**
mean±SD (n=6)	0.03±3.30	0.04±0.89	1.49±4.77	3.11±2.46
% CV	97.56	104.09	99.61	101.14
% Nominal	0.95	4.11	31.13	126.43

Mean statistical data are expressed as mean±SD [n=6]

DISCUSSION

The current study has been successfully validated as per USFDA guidelines [14]. Validation parameters and acceptance criteria of the results are mentioned below in table 14 where the requirements for assessment of the data has been clearly defined.

Table 14: Validation parameters and their acceptance criteria

S. No.	Parameter	Acceptance criteria
1	Linearity (includes ruggedness and stability)	Minimum 5-point standards are required for building a calibration curve Two consecutive standards should not fail First and last calibration standard should not fail At least 75 % of the calibration curve standards should be with the acceptable limits for accuracy and precision % Accuracy and precision should be within 85-115 % for all standards except LLOQ QC. % Accuracy and precision should be within 80-120 % for LLOQ QC
2	PA [(inter-day and intra-day) and (stability studies)]	% Accuracy and precision should be within 85-115 % for all QCs except LOQ QC. % Accuracy and precision should be within 80-120 % for LOQ QC. At least 67 % of the quality control samples should be within specified criteria for precision and accuracy At least 50 % quality control samples should meet the criteria specified for accuracy and precision
3	Specificity and selectivity	% interference at the retention time of the analyte in the blank sample should not be more than 20 % of the peak area of analyte % interference at the retention time of the internal standard in the blank sample should not be more than 5 % of the peak area of the internal standard
4	Matrix effect	Mean matrix factor and is normalized matrix factor should be between 0.85-1.15
5	Recovery	No as such criteria defined for % recovery. The precision obtained for mean and global recovery should be with in±15 %
6	Dilution integrity (DI)	Precision and accuracy of the DI QCs should be within 85-115 %
7	Limit of detection (LOD)	Precision and accuracy of the sensitivity samples should be within 85-115 %

On the basis of the results obtained, all experiments were found to be within acceptance criteria that were defined above in table 14. While assessing the linearity of the calibration curves, % nominal of the standard concentrations was found to be between 88.91 to 102.78. Linearity study results were given in table 1-2. Inter-day and intra-day QC data were tabulated in table 4-5 and the data represented do not contain any outliers. Method was found to be more selective and specific, without any matrix effect and the results were presented in table 3 and 6. Mean extraction recoveries were found to be 99.22±0.24 and the results were given in table 7.

During method comparison, it was observed that only few methods employed a single step sample extraction [2, 5, 10, 12] by using protein precipitation. But protein precipitation is not preferred by many due to improper sample cleanup. Techniques like turbulent flow liquid chromatography with high-resolution mass spectrometry was used [11], but the setup requires highly skilled manpower and it is a costly setup. The current method was developed and validated by using a simple and single-step solid phase extraction procedure which is relatively cost-effective, highly specific and sensitive. Further sensitivity of the method was successfully evaluated till the lowest detection level i.e., 50 pg/ml, whereas the limit of detection in the reported literatures [2-13] is not less than 1 ng/ml. Results of LOD were presented in table 10.

CONCLUSION

The developed method is highly sensitive, selective and it is a key differentiator with a LOD of 50 pg/ml employing just 200 µl of the plasma sample. This was achieved due to the proper cleanup of the samples using a single-step solid-phase extraction of acidified plasma samples. With the ability to analyze over 360 samples per day this method is not only suitable for PK study analysis but can be validated and used for TDM also provided, the sample quantity must be further reduced as the single sample in clinical diagnostics is required for performing many other additional tests. This kind of method transfer is possible as a recent study [12] conducted on a set of blood samples from 116 patients treated with new oral anti-coagulant reveals that "the results of both specific dilute thrombin time (dTT) tests for dabigatran provided the same results as the activated partial thromboplastin time (aPTT) screening test in comparison with LC-MS/MS as a reference." The results of both specific dTT tests for dabigatran provided the same results as the activated partial thromboplastin time aPTT screening test in comparison with LC-MS/MS as a reference. Hence, the objective of the current research work is fulfilled as it can be applied to conduct PK study analysis as well as TDM.

AUTHORS CONTRIBUTIONS

Dr. Cheepurupalli Prasad and Rajaram S Patil were involved in supervision and guidance of the proposed research work. Chaitanya Krishna Atmakuri is involved in the design, development and implementation of the current research work. Dr. Karunakranth Dharani played his role as an expert advisor for the current research work.

CONFLICT OF INTERESTS

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome

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