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A VALIDATED LCMS/MS METHOD FOR ESTIMATION OF RIVAROXABAN IN HUMAN PLASMA

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ABSTRACT

A LCMS/MS method was developed and validated for estimation of rivaroxaban in human plasma. A simple LLE method was used for sample preparation. An isocratic method with a flow rate of 1.0ml/min was used to elute the sample from a C_8 column and analyzed using positive atmospheric ionization mode in LCMS/MS API 4000. Rivaroxaban D4 was used as an internal standard. The method was linear in the range of 0.5 - 609.3ng/ml and the lower limit of quantification was 0.5 ng/ml. Assay accuracy and the precision were in the range of 87.5 – 112.6% and 0.7 – 10.9%, respectively. Recovery was 69.7%. This method was successfully used in a bioequivalence study.

Keywords: Rivaroxaban, Isocratic, Reversed phase, LCMS/MS, validation and Anticoagulant.

INTRODUCTION

Treatment and prevention of thrombosis and thromboembolism involves a long-term oral anticoagulant (OAC) therapy. Warfarin, the vitamin K antagonist, has been the standard OAC for several decades, but recently, the newer agents known as 'NOACs' are now available for long-term use. Rivaroxaban (Xarelto, Janssen Pharmaceuticals, Titusville, New Jersey, USA), the factor Xa (FXa) inhibitor and a member of NOAC family, has been approved by FDA and other regulators for stroke prevention in non-valvular atrial fibrillation, treatment of venous thromboembolism (VTE) and VTE prophylaxis after elective major joint replacement surgery[1]. Molecular weight of rivaroxaban is 436 g mol^{-1}. It is almost insoluble in water and exhibits high plasma protein binding (92–95%) in humans, with serum albumin being the main binding component. Its bioavailability is around 100% when taken with food[2]. Highly selective binding of rivaroxaban to the S1 and S4 pockets of a serine endopeptidase responsible for the potency of factor Xa inhibition explains its mechanism of action[3]. It reversibly inhibits free and clot-bound factor Xa, thus preventing the conversion of prothrombin to thrombin and subsequent fibrin clot formation[2].

Few chromatographic methods are available in the literature for estimation of rivaroxaban in plasma[3-12]. In HPLC –UV method the sample preparation is very tedious and relatively costlier because of the use of SPE cartridges[3]. In other HPLC method, the sample preparation was done by simpler precipitation method; however, the limit of quantification is only 4.5µg/ml[4]. On the other hand, the sensitivity is dramatically increased in LCMS/MS method. In the method developed by Rohde[5], the calibration range was 0.5 -500 ng/mL, and the processed human plasma sample volumes were 0.2 mL. Total run time for each injected sample was 5 min. Korostelev et al.[6] developed a method for simultaneous estimation of dabigatran and rivaroxaban in human plasma using LC-MS/MS. The calibration range was 2.5-500 ng/mL and total run time was 5 min. A surface-assisted laser desorption/ionization mass spectrometry method described by Cheng et al.[7] used dispersive liquid-liquid micro-extraction involving use of toxic reagents such as tetrahydrofuran, chloroform and dimethyl sulphoxide for sample preparation. In method reported by Schellings et al.[8], total run time

was 4.75 min. Srinivas Reddy et al.[9] developed and validated a high throughput LC-MS/MS method for the quantification of rivaroxaban. The calibration range was 2-500 ng/mL and run time was 2 min; however, solid phase extraction was used for sample of plasma sample (100 µL). Varga et al[10] developed a LCMS/MS method with lower limit of quantification of rivaroxaban 24.00 ng/mL. However, this method has a considerable shorter run time (1.7 min). The method developed by Derogis et al[11] used simple protein precipitation technique as sample preparation method. Although lower limit of quantification was 2 ng/mL in this method, the volume of plasma used was more (200 µL).

In view of the above, we developed a sensitive method in the range of 0.5 – 500 ng/ml. In this method LLE was used and lower limit of quantification was 0.5 ng/mL. This method is validated as per FDA guidelines[13] and used successfully in a trial for determination of pharmacokinetics parameters in healthy volunteers.

MATERIALS AND METHODS

Both the analyte (Rivaroxaban; purity: 99.98%) and the internal standard (Rivaroxaban D4; purity: 99.09%) were purchased from Vivan Life sciences, India.

Other chemicals including methanol (HPLC-grade), acetonitrile, formic acid and ammonium acetate of highest purity grade were purchased locally. Water (Milli Q purified; Millipore, Milford, MA) was used throughout the study.

Plasma lots used for the experiments were either collected in-house or procured from the Deccan's Pathological Lab's, Hyderabad.

Preparation of analyte and internal standard solutions

Rivaroxaban stock solution (200 µg/mL) was prepared in acetonitrile. Concentration was corrected using its potency and actual amount weighed. The stock solution was then diluted with diluent (acetonitrile:Milli-Q-water: 50:50 v/v) to concentration ranges of 24.9 ng/mL to 30000.0 ng/mL.

Similarly, stock solution of rivaroxaban D4 (200 µg/mL) was prepared in acetonitrile and then diluted to 100 ng/mL using diluent (methanol:Milli-Q-water: 50:50 v/v) after correcting the concentrations of the stock solution of rivaroxaban D4 as mentioned above.

Preparation of calibration standards

20 µL of the diluted samples of rivaroxaban was added to 980 µL of K_2EDTA pooled plasma to obtain a concentration range about 0.5 ng/mL to 600.0 ng/mL. All these bulk spiked samples were stored at about -20°C in aliquot of 200 µL.

Preparation of Quality Control Samples

To obtain the concentration ranges of 25.3-23200.0 ng/mL, stock solution of rivaroxaban was diluted with 50% acetonitrile in water. 20 µL of respective diluted solution was added into the mixture of 980 µL of K_2EDTA plasma to obtain final concentration range of 0.5-464.0 ng/mL for rivaroxaban.

Sample preparation

50 µL of internal standard (rivaroxaban D4) was added to all RIA vials except blank. 100 µL of sample was then added to each labeled RIA vials followed by addition of 100 µl of 100 mM di-sodium hydrogen phosphate dihydrate to respective RIA vials. After mixing by vortex, 2 mL of TBME (Tertiary butyl methyl ether) was added to all vials. Capped all vials and then placed on vibramax at 2500 RPM for 10 mins. They were centrifuged at 4000 RPM for 5 mins at about 4°C. 1.6 mL of supernatant was then transferred to a fresh RIA vial. After drying at 40°C in nitrogen evaporator, 0.5 mL of mobile phase (5mM Ammonium acetate in 0.1% Formic acid::Acetonitrile; 60:40, v/v) was added to reconstitute the sample and vortexed. The reconstituted samples were transferred to labeled HPLC vials and placed in the auto-sampler.

Chromatography

25 µL of sample was injected on a reversed phase column (Zorbax SB C8, 4.6 x 100mm, 3.5µm). The temperature of column oven was set at 50^0C. The sample was analyzed on API 4000 Mass spectrometer (Applied Biosystems, USA) attached to Waters UPLC by using mobile phase (5mM Ammonium acetate in 0.1% Formic acid::Acetonitrile; 60:40, v/v). Flow rate was 1 mL/min with a splitter. The run time was 3.5 minutes.

Mass Spectrometry

Atmospheric pressure ionization (API) interface operated in positive ionization mode was used for the multiple reaction monitoring (MRM). Diluted stock solution of analyte and internal standard were infused to optimize the operational conditions (**Table 1**).

Unit resolution was set for quadrupoles Q1 and Q3. MRM transitions were monitored as m/z 436.0→ 144.9 for rivaroxaban whereas for rivaroxaban D4, MRM transitions were 440.0→ 144.9.

Sample concentrations were calculated by linear regression analysis using the analyst software 1.6.2. Data was processed by peak

area ratio. The concentration of unknown was calculated from the equation (Y= mX+ c) using regression analysis of spiked plasma calibration standards with reciprocal of the square of the drug concentration ($1/X^2$).

RESULTS AND DISCUSSION

Method Development

Samples clean–up methods play an important role in developing a sensitive and selective LC–MS/MS method for determination of very low concentration levels of pharmaceutical compounds present in biological samples. Commonly used samples clean–up methods are protein precipitation (PPT), liquid–liquid extraction (LLE) and solid-phase extraction (SPE), LLE was the method of choice because of insolubility of rivaroxaban in water which facilitates its extraction by organic solvent in LLE method. Although protein precipitation method is a simple method, chances of matrix effect cannot be ruled out. SPE technique which is otherwise a good technique is not cost effective due to an additional cost of a specific cartridge. LLE method used here for rivaroxaban provided clean samples, gave good and reproducible recoveries of both analyte and IS. It was also a very robust method.

The extraction recovery of analyte was calculated as follows

Recovery (%) =
[peak area of analyte extracted from plasma sample / peak area of analyte extracted from aqueous sample] x 100

The mean recoveries across QC levels (with precision) were 69.7% (2.3%) for rivaroxaban and 74.3% for rivaroxaban D4 (IS) (n=6).

An isocratic method is developed for the quantification of rivaroxaban in K_2EDTA human plasma. The total run time including recalibration of the column was 3.5 min. The retention times for rivaroxaban and rivaroxaban D4 were 2.35 min and 2.34 min, respectively.

Method Validation

Following parameters were validated in this method as per FDA Guidelines[13].

Specificity and Selectivity

Analyte at LLOQ concentration and intended concentrations of internal standard were spiked to eight individual human plasma lots which included one haemolysed and one lipemic lot. These samples were processed for determination of specificity and selectivity. There was no interference observed at the retention times of analyte and internal standard when compared to peak responses in blank lots. (**Figure 1 & 2**) This indicates the specificity of the method. The method was selective also as signal to noise ratio was found to be more than 59.9 which is 12 times more than the acceptable limit of > 5.

Linearity and Sensitivity

An eight-point calibration curve was prepared using rivaroxaban concentrations ranging 0.5 - 600.0 ng/mL. The linearity of calibration curve was determined by plotting peak-area ratio (y) of analyte to internal standard against the nominal concentration ratio of analyte to internal standard. Excellent linearity was achieved with correlation coefficients greater than 0.99 for all validation batches (**Figure 3**). The accuracy of each calibration point which ranges between 97.8 – 101.1%, was obtained after back calculation of concentrations of calibration standards.

Six processed LLOQ samples were injected along with a 'Precision and Accuracy' batch to determine the sensitivity of this method. Mean precision and accuracy for rivaroxaban at the LLOQs were 4.2% and 104.8% respectively. The LLOQ of the method is 0.5 ng/mL. Limit of detection was 0.13 ng/mL. This method is therefore quite sensitive even for a pharmacokinetic study. Signal to noise ratio was 36.5 (12 times more than the required one) at the limit of detection of 0.13 ng/mL. This method is therefore capable to quantify even lower concentration of rivaroxaban. This is very important when plasma volume is a challenge like in the case of pediatric patients. This further widens the application of this method.

Precision and accuracy

Precision and accuracy were determined by injecting a set of calibration curve samples and quality control samples. The correlation coefficient of calibration curve was more than 0.99(r) as required by FDA guidelines. The accuracy and precision were 98.6 – 102.7% and < 1.3 which were within acceptable limits.

Six replicate analyses of QC samples at four different concentrations – Lower Limit Of Quantification Quality Control (LOQQC), Low Quality Control (LQC), Middle Quality Control (MQC) and High Quality Control (HQC), were used to determine precision and accuracy for intra- and inter-day batches for all analytes. The concentrations for rivaroxaban were 0.5 ng/mL, 1.4 ng/mL, 217.3 ng/mL and 482.8 ng/mL for LOQQC, LQC, MQC and HQC respectively. **Table 2 (a & b)** shows the results of precision and accuracy of quality control samples. The intra-day and inter-day

precisions for both freshly spiked and bulk spiked QC samples were within 6.2%. Similarly, the assay accuracy for both freshly spiked and bulk spiked samples was in the range of 96.5 – 112.6% of the nominal values. Assay accuracy was calculated by using the formula [(mean observed concentration) / (spiked concentration)] x 100%. Relative standard deviation (RSD) was used for evaluation of precision.

Matrix effect

The interfering compounds present in matrix can lead sometimes to erroneous results either by strengthening or reducing the detection of analyte of interest. Since dosing regimens are being optimized based on pharmacokinetic profiles and is dependant on accurate determination of drug plasma concentrations, such miscalculations can lead to errors in determining optimum dosing regimens and in extreme cases failure of a drug in clinical trials.

To determine the matrix effect, eight blank matrix lots from different sources were processed. These included one haemolysed and one lipemic lot. 100 µL of blank plasma from each lot was processed as mentioned in sample preparation. Rivaroxaban (either at LQC or HQC level) and known concentration of internal standard (rivaroxaban D4) were added to each of the processed plasma in order to prepare 'Post extracted samples' (presence of matrix).

Similarly, the solutions of rivaroxaban either at LQC or HQC level containing same concentration of IS as above were prepared and considered as 'aqueous samples' (absence of matrix). Six replicates of each aqueous sample along with post extracted samples of LQC or HQC were injected.

Mean analyte and IS area responses of the aqueous sample were compared with respective analyte and IS area responses of each post extracted sample. Matrix effect was calculated using the formula: Matrix effect (%) = A_2/ A_1 x 100 (%), where A_1= response of aqueous concentrations and A_2 is response of post-extracted concentrations.

Average (n=8) matrix factor at LQC level was 95.9% with a CV of 2.7% whereas at HQC level it was 95.5% with a CV of 0.8%. These are within the accepted limit (% CV ≤15) (**Table 3**).

Dilution integrity

Since diluted samples were used for quantification, the integrity of dilution is required to be monitored. Dilution integrity was evaluated after spiking interference free human plasma with 2 times of HQC concentration of rivaroxaban (i.e. 2 x 467.579 ng/mL = 935.15 ng/mL). These spiked plasma were diluted either 2 fold (2T) or 4 fold (4T) with interference-free human plasma. These samples (six replicates of each dilution) were processed and then analyzed against a set of freshly spiked calibration standards. The mean accuracy and precision were 102.34% and 0.6% for 2T and 107.0% and 1.5% for 4T (data not shown).

Carry – over Effect

The cleaning ability of wash solvent (Acetonitrile: Milli-Q-water :: 50:50v/v) used for rinsing the injection needle and port was evaluated to avoid any carry–over of injected sample in subsequent runs. The order of placing samples was: LLOQ of individual analyte, blank plasma, upper limit of quantitation (ULOQ) of individual analyte and blank plasma. There were no carry over observed during the experiment (data not shown).

Stability

Both aqueous and matrix based samples were subjected for stability evaluations. Short-term and long-term stabilities for aqueous solutions were determined as follows

a) Stability in aqueous solution

i) Short – Term stock solution stability (STSS)

STSS was performed to ensure that the analyte and IS stock solution and working solution are stable at room temperature. Stock solutions of both analyte and IS were prepared separately and named as stability stock. Analyte at LQC and HQC level were prepared from the stability stock solution and marked as stability working solution. Both stability stock and stability working solutions were stored at 25^0C for 17 h (for stock solution) and 23 h (for working solution). Just before injection, fresh stock solutions of analyte and IS were diluted to LQC and HQC concentrations of analyte and intended concentration of IS. LQC and HQC samples (both stock and working solutions) and diluted IS solutions were injected in six replicates and the results were compared with those obtained from the freshly prepared LQC and HQC solution of analyte or the IS. No significant differences were noticed indicating that analyte were stable at 25^0C (**Table 4 a & b**). For IS, stability were 103.6% and 104.4% after 17 h and 25 h, respectively (data not shown). Criteria accepted for the ratio of mean response for stability samples is 85-115%.

ii) Long term stock solution stability (LTSS)

LTSS was performed to ensure that the analyte and IS stock solution and working solution are stable at its stored conditions in 2-8^{0}C. The stock solution stability was proved for 20 days and working solution stability was proved for 09 days for analyte and IS respectively.

Stock solutions of both analyte and IS were prepared separately and named as stability stock. From the stability stock solution, stability working solutions at LQC & HQC concentrations and intended concentration of IS were prepared by dilution and stored at 2-8^{0}C for 9 days. However, stability stock solutions were kept at 2-8^{0}C for 20 days.

Mean area responses of stored stock/working solutions were then compared against respective freshly prepared stock/working solutions. Similarly, mean area response for internal standard was also compared. Stabilities for rivaroxaban working solutions after 9 days were 99.79% (LQC), 99.10% (HQC) and 99.69% for rivaroxaban D4 (data not shown) were well within accepted limit (90 – 110%). This indicated that both analyte and internal standard working solutions were stable for 9 days at 2-8 ^{0}C (**Table 4 a& b**). Stabilities of rivaroxaban and IS after 20 days were 96.70% (LQC) and 102.19% (HQC). IS stock stability was100.38%(data not shown).

b) Stability in human plasma

Stabilities of rivaroxaban in human plasma were determined as follows.

i) Bench-top stability

Six aliquots of each analyte in K_2EDTA containing human plasma (at LQC and HQC concentrations) from the -70^{0}C were allowed to thaw unassisted at room temperature (25^{0}C) for 7.5 h. A set of freshly prepared calibration standards was processed with these LQC and HQC samples. The stabilities for LQC and HQC samples were 99.70% for LQC and 96.20% respectively.

ii) Freeze thaw stability

After 4 freeze thaw cycles, the stability of rivaroxaban were 99.83% for LQC and 96.30% for HQC.

iii) In-injector stability

The stability for LQC and HQC samples kept in auto-sampler at 10^{0}C for about 46.5 h were 95.57% and 91.83% respectively. IS stability was found to be 110.93%.

iv) Wet extract stability

The stability of rivaroxaban after 5 h at 25°C was 100.16% for LQC and 97.16% for HQC. As per FDA, accepted range for all the stability studies mentioned above is that the mean concentration for stability samples should be 85-115% of the mean concentration of freshly prepared samples. Thus the analyte was stable during the analysis process.

v) Dry extract stability

Six sets each of LQC and HQC samples (bulk spiked) were processed as per analytical test procedure till before reconstitution step and stored in dry state at 2-8°C for 50hrs.

The samples retrieved from the cooling cabinet were reconstituted as per the analytical test procedure and analyzed against a set of freshly prepared (freshly spiked, unfrozen) calibration standards and six aliquots each of freshly prepared (freshly spiked, unfrozen) LQC and HQC samples. The percentage mean concentration for stability samples should be 85-115% against mean concentration of comparison samples.

The dry extract samples of rivaroxaban stored at 2-8°C were stable for 50hrs. The stability for LQC and HQC were found to be 95.48% and 92.94% respectively.

vi) Processed sample stability

Six sets each of LQC and HQC samples (bulk spiked) were processed as per analytical test procedure and stored at 2-8°C for 52hrs. The samples were retrieved from the cooling cabinet after 52hrs and were analyzed against a set of freshly prepared (freshly spiked, unfrozen) calibration standards and six aliquots each of freshly prepared (freshly spiked, unfrozen) LQC and HQC samples. The percentage mean concentration for stability samples should be 85-115% against mean concentration of comparison samples. The processed sample stability of rivaroxaban stored at 2-8°C was stable for 52hrs. The stability for LQC and HQC were found to be 94.55% and 92.94% respectively.

Results of all stability studies in human plasma were presented in **Table 5 and 6**.

viii) Lipemic and Hemolysis effect

Six replicates each of freshly spiked LQC and HQC in hemolysed and lipemic plasma were processed and analysed against freshly spiked calibration curve standards in normal plasma. 2% of hemolysed plasma was used for the hemolysis effect experiment. The volunteer's (triglyceride level more than 400mg/dL) blood was collected and the separated plasma was used for the lipemic effect experiment. In hemolysed plasma, the precisions were 2.40% and 1.52% and the accuracies were 101.31%

and 95.67% for LQC and HQC, respectively. In case of lipemic plasma, the precisions were 2.19% and 3.25% and the accuracies were 101.07% and 94.90% for LQC and HQC, respectively. Acceptable limits as per FDA guidelines[13] are ±15%. Hence there were no effect of hemolysis or lipemia on determination of rivaroxaban by this method. (**Table 6**)

ix) Stability in human blood

Stability of rivaroxaban in whole human blood was also determined to find out whether rivaroxaban was stable for the time period before the plasma was separated from the whole blood by centrigugation. Analyte solutions were prepared at LQC and HQC levels by dilution of stock solution. Similarly, analyte solutions (at LQC and HQC levels) in whole blood were prepared by adding the required amount of stock solution directly to the blood. The spiked blood samples were kept on ice bath for 1 hr and plasma was separated by centrifugation at 3500rpm for 10mins before processing further as mentioned under sample preparation. Internal standard was added just before processing. All samples were then analyzed in LCMS as mentioned earlier. % stabilities of rivaroxaban were found to be 101.27% (LQC), 101.00% (HQC) for 1 hr in whole blood respectively. Whole blood stability was found to be within acceptance limits i.e. the mean area ratio of the stored analyte samples should be within ±15% of freshly processed analyte samples at LQC and HQC levels (data not shown).

Extended precision and accuracy run

This test was performed by processing and analyzing one set of bulk spiked calibration standards and 30 each of bulk spiked LQC, MQC and HQC samples as a batch. Results of precision and accuracy were presented in **Table 7**. This batch size test was performed for 100 samples and found to be within the acceptance limit. The precision was 2.48% for LQC, 0.95% for MQC and 0.83% for HQC. The accuracy was 106.31% for LQC, 100.05% for MQC and 98.24% for HQC.

Concomitant drug effect

The precisions of rivaroxaban in presence of commonly used compounds like Caffeine, Nicotine, Chlorpheniramine, Ondansetron, Ranitidine, Paracetamol, Cetirizine and Heparin was 4.1%, 2.6%, 2.6%, 1.4%, 3.2%, 0.8%, 3.7% and 6.6% respectively.

The accuracies of rivaroxaban in presence of the above mentioned drugs was 101.9%, 103.9%, 104.2%, 106.7%, 105.5%, 105.0%, 107.3% and 110.0% respectively which are well within the acceptable limit (±15%) (data not shown).

All these drugs were used at reported Cmax level concentrations. There was no interference observed in blank matrix processed with spiked concomitant drug for both analyte and internal standard.

CONCLUSIONS

A relatively simple, fast, sensitive and specific LC–MS/MS method for determination of rivaroxaban in human plasma was described here. Liquid-liquid extraction technique for this relatively non-polar molecule was utilized. This offers consistent and reproducible recoveries with insignificant interference and matrix effect. On the top of it, this method was also user friendly and cost-effective compared to the reported LCMS/MS method which involved SPE technique[9]. As per FDA guideline[13], deuterated rivaroxaban was used as an internal standard for developing and validating this method. It demonstrated that the method was reproducible, sensitive and suitable for high-throughput sample analysis. The run time of this method was only 3.5 min which was lower than many reported methods[5,6,8]. Moreover, lower limit of quantification was 0.5 ng/mL which was much lower than reported methods [6,9-11]. Unlike Rohde[5] and Derogis et al[11] the lower limit of quantification was achieved by using 100 µL plasma samples. On the top of it, the detection limit of this method is 0.13 ng/mL with signal to noise ratio >12 times of acceptable limit. This means that the plasma volume can be further lowered if necessary. Due to the high sensitivity of this method, it can even be used for analysis of pediatric samples where sample volume is always a challenge. In addition, no interferences of hemolytic or lipemic plasma samples for rivaroxaban measurement by LCMS/MS was also demonstrated. This method has been successfully used for bioequivalence studies and has the potential for routine therapeutic drug monitoring.

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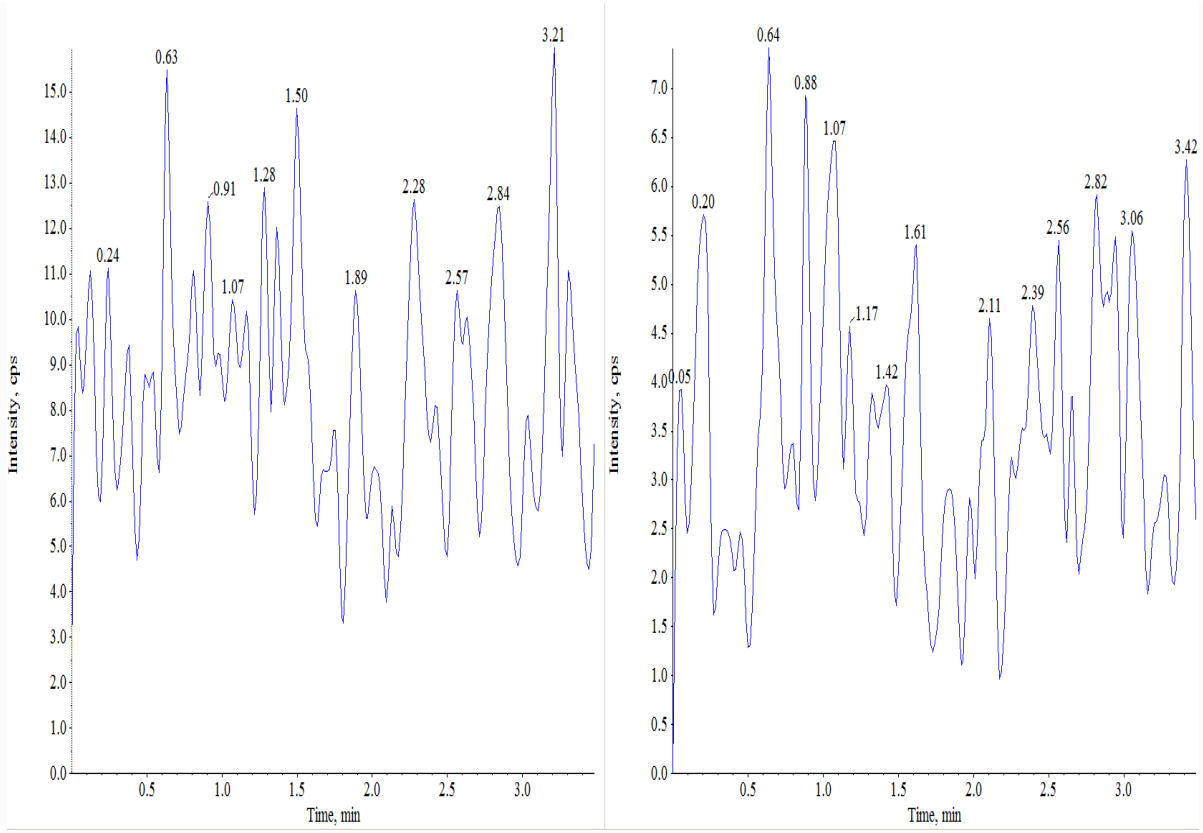

Fig. 1: Chromatogram of extracted blank

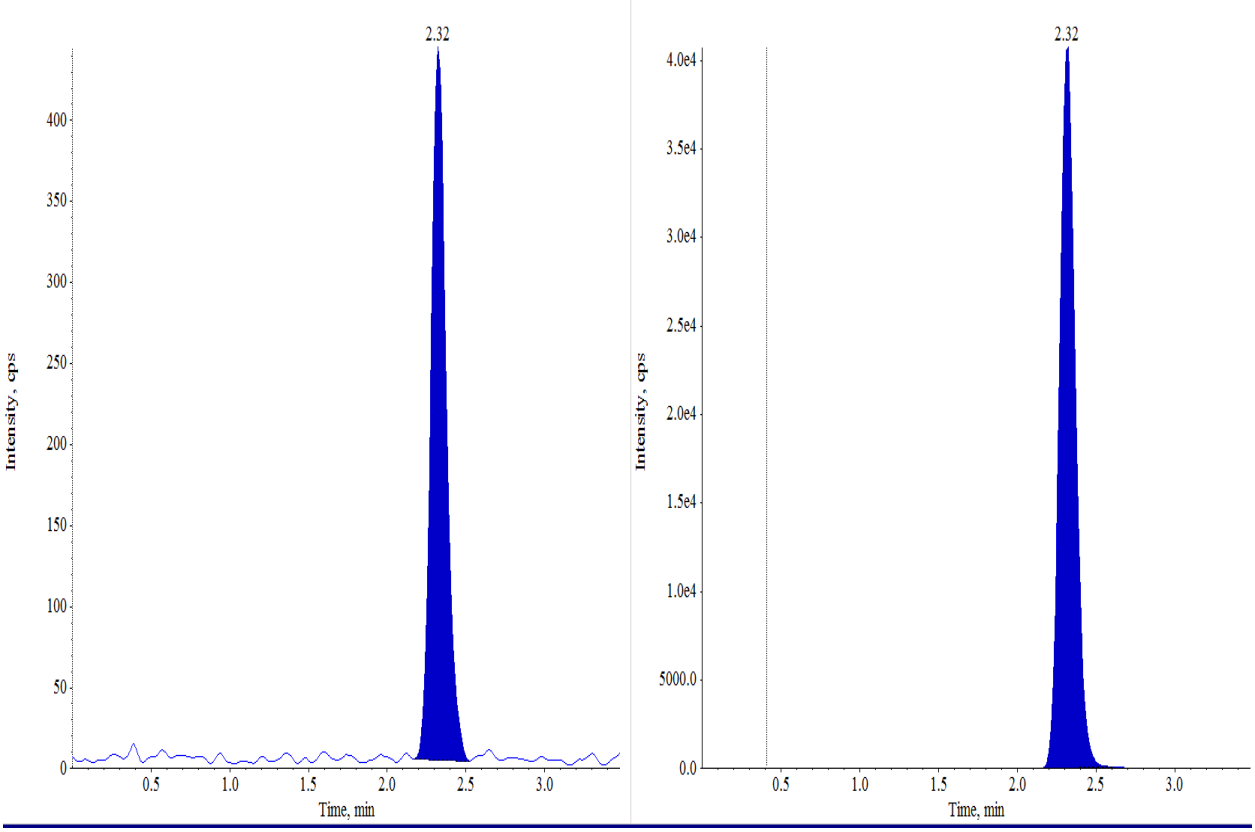

Fig. 2: Chromatograms of rivaroxaban (LLOQ) and IS

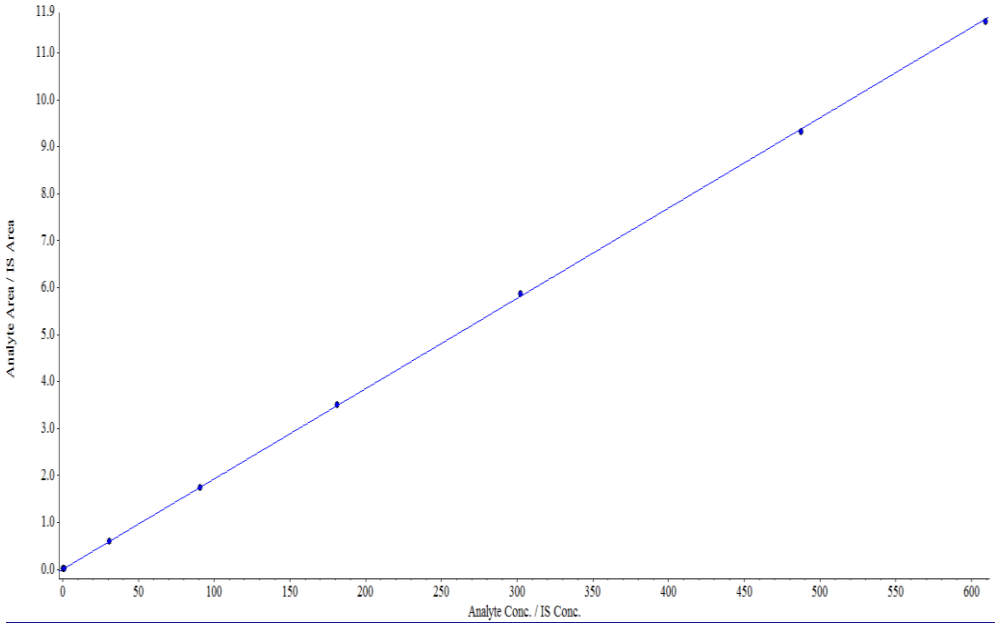

Fig. 3: Calibration curve of rivaroxaban

Table 1: MS parameters optimized for analysis

Analyte/IS	Declustering Potential (DP) (V)	Entrance Potential (EP) (V)	Collision Energy (CE) (V)	Collision Cell Exit Potential (CXP) (V)	Collision activated dissociation (CAD) (psi)	Dwell Time (ms)	Ion source voltage (V)	Curtain gas flow (CUR) (psi)
Rivaroxaban	90.00	10.00	35.00	9.00	8.0	400	5500	30
Rivaroxaban D4	90.00	10.00	35.00	9.00	8.0	400	5500	30

Table 2a: Intra-day and inter-day accuracy and precision for the determination of rivaroxaban in freshly spiked human plasma

Sample ID	LOQQC (Nominal Conc. 0.52 ng/ml)			LQC (Nominal Conc 1.41 ng/ml)			MQC (Nominal Conc 217.26 ng/ml)			HQC (Nominal Conc 482.80 ng/ml)		
	Mean calculated Conc (pg/ml)	Mean accuracy (%)	% CV	Mean calculated Conc (pg/ml)	Mean accuracy (%)	% CV	Mean calculated Conc (pg/ml)	Mean accuracy (%)	% CV	Calculated Conc (pg/ml)	Mean accuracy (%)	% CV
PA - 1	0.56	106.7	4.9	1.49	105.9	4.6	224.72	103.4	3.2	478.19	99.0	2.9
PA – 2	0.53	100.4	2.8	1.43	101.6	2.5	209.67	96.5	3.7	472.83	97.9	0.7
PA – 3	0.59	111.8	6.2	1.58	112.6	2.9	223.42	102.8	2.2	515.31	106.7	0.7
Inter-day	0.56	106.3	6.3	1.50	106.7	5.3	219.27	100.9	4.2	488.78	101.2	4.2

Table 2b: Intra-day and inter-day accuracy and precision for the determination of rivaroxaban in bulk spiked human plasma

Sample ID	LOQQC (Nominal Conc. 0.52 ng/ml)			LQC (Nominal Conc 1.39 ng/ml)			MQC (Nominal Conc 215.09 ng/ml)			HQC (Nominal Conc 467.58 ng/ml)		
	Mean calculated Conc (pg/ml)	Mean accuracy (%)	% CV	Mean calculated Conc (pg/ml)	Mean accuracy (%)	% CV	Mean calculated Conc (pg/ml)	Mean accuracy (%)	% CV	Calculated Conc (pg/ml)	Mean accuracy (%)	% CV
PA – 4	0.56	108.4	3.9	1.48	106.3	1.8	215.67	100.3	1.3	459.12	98.2	0.9
PA – 5	0.57	109.2	4.1	1.45	104.3	3.2	214.84	99.9	1.7	455.06	97.3	1.1
PA – 6	0.54	104.0	3.4	1.46	104.5	2.1	216.53	100.7	0.9	458.62	98.1	1.4
Inter-day	0.56	107.2	4.1	1.46	105.0	2.4	215.68	100.3	1.3	457.60	97.9	1.1

Table 3: Matrix effects for rivaroxaban in human plasma

Matrix ID	LQC			HQC		
	Area in absence of matrix	Area in presence of matrix	Matrix factor	Area in absence of matrix	Area in presence of matrix	Matrix factor
PL_1207	13330	12867	98.3	4066379	3887325	95.2
PL_1208	12892	12868	98.3	4105080	3938838	96.4
PL_1210	13373	12737	97.3	4094589	3883556	95.1
PL_1213	13111	12562	95.9	4090786	3907626	95.7
PL_1214	12637	12227	93.4	4067267	3945570	96.6
PL_1215	13220	12870	98.3	4085111	3891341	95.3
LPL_1209		12024	91.8		3889115	95.2
HPL_1224		12280	93.8		3853497	94.3
Average	13094	12554	95.9	4084869	3899608	95.5
SD	283	337	2.6	15426	30306	0.7
%CV	2.2	2.7	2.7	0.4	0.8	0.8

Table 4a: Short and long –term stability of rivaroxaban aqueous solution at LQC

Short-term stability for LQC						Long-term stability for LQC					
Stock solution at 25^{0}C for 17h			Working solution at 25^{0}C for 23h			Stock solution at 2-8^{0}C for 20 days			Working solution at 2-8^{0}C for 9 days		
Average area		% Stability	Average area		% Stability	Average area		% Stability	Average area		% Stability
Stock solution	Fresh solution		Working solution	Fresh working solution		Stock solution	Fresh solution		Stock solution	Fresh solution	
12357.5	12190.2	101.0	12042.0	12190.7	98.4	11746.3	12075.5	96.7	11313.5	10761.8	105.4

Table 4b: Short and long –term stability of rivaroxaban aqueous solution at HQC

Short-term stability for HQC						Long-term stability for HQC					
Stock solution at 25^{0}C for 17h			Working solution at 25^{0}C for 23h			Stock solution at 2-8^{0}C for 20 days			Working solution at 2-8^{0}C for 9 days		
Average area		% Stability	Average area		% Stability	Average area		% Stability	Average area		% Stability
Stock solution	Fresh solution		Working solution	Fresh working solution		Stock solution	Fresh solution		Stock solution	Fresh solution	
3680461	3546332	103.4	3728131	3546332	104.7	3677463	3577630	102.2	3297122	3304068	100.1

Table 5: Stability studies of rivaroxaban in plasma

Parameters (n = 6)	Bench-top stability for 7.5h		Freeze-thaw stability after 4 cycles		In-injector stability for 25h		Wet extract stability for 4h	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
Nominal (ng/mL)	1.4	467.6	1.4	467.6	1.4	467.6	1.4	467.6
Mean calculated conc. (ng/ml)	1.4	448.5	1.4	449.0	1.4	428.2	1.5	453.0
SD	0.01	3.08	0.04	3.11	0.02	5.62	0.04	3.28
%CV	0.7	0.7	2.6	0.7	1.7	1.3	2.6	0.7
% Stability	99.7	96.2	99.8	96.3	95.6	91.8	100.2	97.2

Table 6: Stability studies, Dilution Integrity and Lipemic/hemolysis effects

Parameters (n = 6)	Dry extract stability for 50h		Processed sample stability for 52h		Dilution Integrity		Lipemic effect		Hemolysis effect	
	LQC	HQC	LQC	HQC	2T	4T	LQC	HQC	LQC	HQC
Nominal (ng/mL)	1.4	467.6	1.4	467.6	935.2	935.2	1.4	482.8	1.4	482.8
Mean calculated conc. (ng/ml)	1.4	433.4	1.4	433.4	957.0	1000.8	1.4	458.2	1.4	461.9
SD	0.04	12.21	0.03	2.50	5.39	14.96	0.03	14.88	0.03	6.99
%CV	2.6	2.8	2.4	0.6	0.6	1.5	2.2	3.2	2.4	1.5
% Stability	95.5	92.9	94.6	92.9	102.3	107.0	101.1	94.9	101.3	95.7

Table 7: Extended precision and accuracy of rivaroxaban

LQC				MQC				HQC			
Nominal conc. (pg/mL)	Mean calculated conc. (pg/mL)	Accuracy (%)	% CV	Nominal conc. (pg/mL)	Mean calculated conc. (pg/mL)	Accuracy (%)	% CV	Nominal conc. (pg/mL)	Mean calculated conc. (pg/mL)	Accuracy (%)	% CV
1.4	1.5	106.3	2.5	215.1	215.2	100.0	0.9	467.6	459.3	98.2	0.8

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