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DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD FOR ESTIMATION OF VORAPAXAR BY UPLC-MS/MS: APPLICATION TO A PHARMACOKINETIC STUDY

Tammisetty Mohan Rao *[1], CH. Balasekhara Reddy [2] and P. Srinivasa Babu [3]

Jawaharlal Nehru Technological University [1], Kakinada - 533003, Andhra Pradesh, India.
Vagdevi College of Pharmacy [2], Gurajala - 526415, Andhra Pradesh, India.
Vignan Pharmacy College [3], Vadlamudi, Guntur - 522213, Andhra Pradesh, India.

Keywords:

Vorapaxar, Rabbit plasma, UPLC-ESI-MS/MS, Bioanalysis

Correspondence to Author:
Tammisetty Mohan Rao

Research Scholar,
Jawaharlal Nehru Technological University, Kakinada - 533003, Andhra Pradesh, India.

E-mail: mohanjntuk.sch@gmail.com

ABSTRACT: The validated analytical method was applied for estimation of vorapaxar in aqueous and human plasma with vorapaxar-D5 as an internal standard by using UPLC-ESI-MS/MS. The chromatographic separation was achieved with 5mM Ammonium Formate Buffer (pH: 4.0): Methanol: Acetontrile, (40:30:30, % v/v) using the Pursuit XRs-100Å, C18, 4.6 × 50 mm, 10 µm. The total analysis time was 3.5 min and flow rate was set to 0.2 ml/min. The mass transitions of vorapaxar and vorapaxar-D5 obtained were m/z 591.4→447.2 and 498.6→447.2. The standard curve shows correlation coefficient (r^2) greater than 0.999 with a range of 0.01-200.0 ng/ml using the linear regression model. The developed method was applied successfully for studying the pharmacokinetics of vorapaxar in rabbits.

INTRODUCTION: Vorapaxar is a reversible antagonist of the protease-activated receptor-1 (PAR-1) expressed on platelets, but its long half-life makes it effectively irreversible. Vorapaxar inhibits thrombin-induced and thrombin receptor agonist peptide (TRAP)-induced platelet aggregation *in-vitro* studies. Vorapaxar does not inhibit platelet aggregation induced by adenosine diphosphate (ADP), collagen or a thromboxane mimetic and does not affect coagulation parameters *ex-vivo*. PAR-1 receptors are also expressed in a wide variety of cell types, including endothelial cells, neurons, and smooth muscle cells, but the pharmacodynamic effects of vorapaxar in these cell types have not been assessed [1-2].

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The chemical name of vorapaxar sulfate is ethyl [(1R, 3aR, 4aR, 6R, 8aR, 9S, 9aS)-9-{(1E)-2-[5-(3¬fluoro-phenyl) pyridin-2-yl]ethen-1-yl}-1-methyl-3-oxododecahy-dronaphtho[2,3-c]furan-6-yl]carbamate sulfate. The empirical formula is $C_{29}H_{33}FN_2O_4 \bullet H_2SO_4$, and its molecular weight is 590.7. Vorapaxar sulfate is a white to off-white solid. Vorapaxar sulfate is freely soluble in methanol and slightly soluble in ethanol, acetone, 2-propanol, and acetonitrile. The chemical structure of Safinamide (SA) and its internal Standard (IS) Safinamide - D4 (SA – D4) was shown in **Fig. 1** [3-4].

Review of Literature: As of now, to our knowledge, a few research studies on Vorapaxar estimation and pharmacokinetics have been reported [5-9] like pharmacokinetics and pharmacodynamics [5], Pharmacokinetics of vorapaxar and its metabolite following oral administration in healthy Chinese and American subjects [6], Determination of a novel thrombin receptor antagonist (SCH 530348) in human plasma by LC-MS/MS [7] with concentration range of

0.1–1000 ng/mL in human plasma samples, Pharmacokinetic Drug–Drug Interaction Between Prasugrel and Vorapaxar [8], Estimation of Vorapaxar with different drugs BY UV-VIS [9].

Literature survey reveals, the reported on LC-MS/MS methods were lack of sensitivity, reducibility and plasma sample cleanup process. Hence, to meet the requirement of Bio-pharmaceutical studies with wider range of dosage forms, it was essential to develop more sensitive and a wider quantitation range of method.

Deuterated Vorapaxar (Vorapaxar-D_5**)** was used as internal standards to improve the prediction and accuracy of the method as well as the robustness of the quantification against matrix effect.

The aim of the method is to develop a validated analytical method for estimation of Vorapaxar in rabbit plasma samples as per the International Conference on Harmonization (ICH) guidelines (Q2(R1)) [10-13] by UPLC-MS/MS. To increase the column life by removing interferences due to plasma protein denaturation in this Agilent Captiva Filter Cartridges (3mL capacity with 0.45µm) were used during plasma sample extraction process while employing the protein precipitation technique. The developed method was simple, accurate, specific and economical for the estimation of Vorapaxar and Vorapaxar-D_5 as internal standard (ISTD) by UPLC-MS/MS. The method was successfully applied to a pharmacokinetic study of Vorapaxar in rabbits.

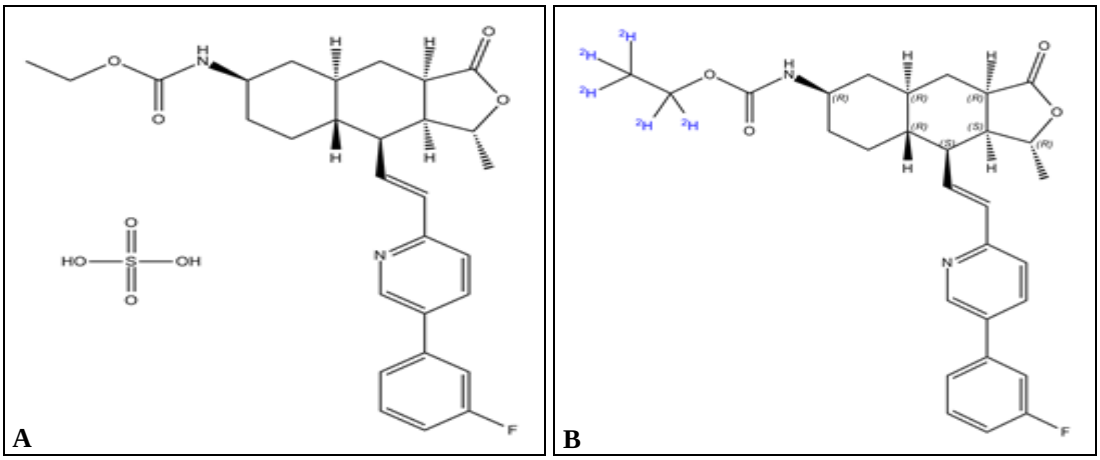

FIG. 1: CHEMICAL STRUCTURES OF A) VORAPAXAR SULFATE B) VORAPAXAR-D5

MATERIALS AND METHODS:

Materials:

Chemical Resources: Vorapaxar (VP) (ALSACHIM), Vorapaxar-D_5 (VPIS) (ALSACHIM), HPLC grade Methanol, Acetontrile (J. T. Baker, Phillipsburg, NJ, USA), Ammonium Formate, Formic acid (Merck Pvt. Ltd, Worli, Mumbai), Ultra pure water (Milli - Q system, Millipore, Bedford, MA, USA), Screened rabbit plasma (K_2EDTA) (Bioneeds, Banglore). The chemicals and solvents were used in this study analytical and HPLC grade.

Instrument Resources: Chromatographic separation was performed on a QSight® Triple Quad UPLC-ESI-MS/MS system (Perkin Elmer) Combined with QSight LX50 UHPLC, data acquisition and processing were accomplished using Simplicity™ 3Q software. Micro balance (ME5 model Sartorius), variable range micro pipette (Eppendorf), Autosampler vials, variable size glass bottles, graduated measuring cylinders, volumetric flasks (Borosil), Ultrasonic bath (Pharmatek Scientifics), Vortexer (Spinix), Refrigerator (LG). Pipette tips 10 µL-1000 µL were employed in the present investigation.

Methods:

Preparation of Standard Stock Solution: Standard stock solution of Vorapaxar (1.0 mg/mL) and Vorapaxar-D_5 (1.0 mg/mL) were prepared by accurately weighing about 10 mg and transferring in to 10 mL volumetric flask and dissolved in Acidified Methanol. All stock solutions were stored in refrigerated conditions (2-8 °C) until analysis.

Preparation of Acidified Methanol: Transferred 1.0mL of formic acid in to 1000.0mL of Methanol (HPLC). Filtered through 0.45µ membrane disc filter and sonicated to degas.

Preparation of 5mM Ammonium Formate Buffer (pH: 4.0): Weighed about 0.315g of ammonium formate, transferred to 1000.0mL volumetric flask and makeup to volume (1000.0mL) with ultra pure water. Finally, pH of the solution was adjusted to pH: 4.0 with formic acid and filtered through 0.45µ membrane disc filter and sonicated to degas.

Preparation of mobile Phase: The mobile phase used was 5mM Ammonium Formate Buffer (pH: 4.0): Methanol: Acetontrile, (40:30:30, % v/v) and the mobile phase was filtered through 0.45µ membrane filter and sonicated before use.

Preparation of 0.1% Formic Acid in Acetonitrile (Extraction Solvent): Transferred 1.0mL of formic acid in 1000.0mL of Acetonitrile (HPLC grade) and sonicated before use.

Preparation of Internal Standard Spiking Solution: The Vorapaxar-D_5 (internal standard) spiking solution (50.00 pg/mL) was prepared from standard stock solution of Vorapaxar-D_5 (1000.00 µg/mL) in mobile phase (5mM Ammonium Formate Buffer (pH: 4.0): Methanol: Acetontrile, (40:30:30, % v/v)). Internal standard spiking solution (Vorapaxar-D_5) was stored in refrigerated conditions (2-8°C) until analysis.

Preparation of Rabbit Plasma: Blood from six healthy rabbits with average weights of 2 kg was placed in the tube treated with heparin anticoagulant. The cells of blood were removed by centrifugation at 3000 r.p.m. for about 10 min. The supernatant was collected, bottled and stored below -70 ºC.

Standard Calibration (CC) and Quality control (QC) Samples Preparation: Standard stock solutions of SP (1.0 mg/mL) were spiked to drug-free screened rabbit plasma to obtain concentration levels of 0.01, 0.05, 0.5, 1.0, 5.0, 10.0, 20.0, 40.0, 80.0, 100.0 and 200.0 ng/mL for analytical standards and 0.010 ng/mL (LLOQ), 0.030 ng/mL (LQC), 110.0 ng/mL (MQC) and 180.0 ng/mL (HQC) for quality control (QC) standards, and stored in the freezer at -30 ºC until analysis. The aqueous standards were prepared in a mobile phase solution (5mM Ammonium Formate Buffer (pH: 4.0): Methanol: Acetontrile, (40:30:30, % v/v) and stored in the refrigerator at 2-8 ºC until analysis.

Method Development: For developing the method, a systematic study of the effect of various factors were undertaken by varying one parameter at a time and keeping all other conditions constant. Method development consists of selecting the appropriate mass parameters and choice of stationary and mobile phase, internal standard, extraction solvent. The following studies were conducted for this purpose.

Selection of Internal Standard: For selection of internal standard; clopidogrel, prasugrel, ticagrelor were tried with optimized mobile phase and column conditions. Finally, Vorapaxar-D_5 (VPIS) was selected as IS (internal standard) due to its compatibility with analyte chromatographic conditions. VP and VPIS were eluted at 1.62 min ± 0.05 and 0.56 min ± 0.05 min, with a total run time of 3.5 min for each sample.

Optimization of Mass Spectroscopic Parameters: The pure drug of Vorapaxar (VP) and Vorapaxar- D_5 ($VPIS_)$ were prepared in acidified methanol (50.00 pg/mL) and injected with a flow rate of 5µL/min into positive ion mode mass spectrometer for optimization of mass parameters like source temperature, IS, heater gas, nebulizer gas, curtain gas, CAD gas (all gas channels were purged with ultra high pure nitrogen gas), EP, DP, CE, FP and CXP were optimized. Analysis was performed using MRM positive ion mode with mass transitions of m/z (amu) 591.4→447.2 and 498.6→447.2 for VP and VPIS. The mass spectra's of parent and product ions were depicted in **Fig. 2**.

Optimised Chromatographic Conditions: Several systematic trials were performed by varying stationary phases and mobile phase ratios to achieve ideal chromatographic conditions. The chromatographic separation was achieved with 5mM Ammonium Formate Buffer (pH: 4.0): Methanol: Acetontrile, (40:30:30, % v/v) using the Pursuit XRs-100Å, C_{18}, 4.6 × 50 mm, 10 µm gave the best peak shape and low baseline noise was observed. The total analysis time was 3.5 min and flow rate was set to 0.2 ml/min. The temperature was set to 40 °C for the column oven. The sample volume for the injection into mass spectrometry was adjusted to 10µl for better ionization and chromatography.

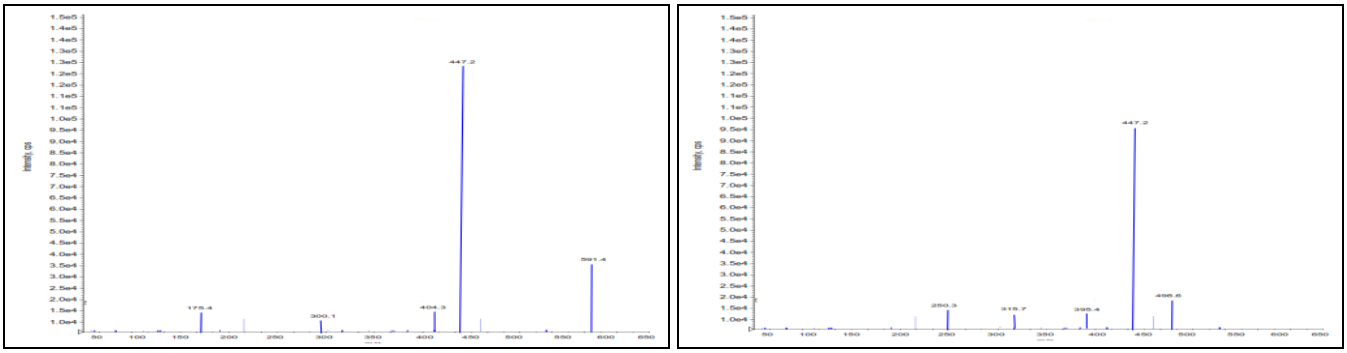

FIG. 2: PARENT ION MASS SPECTRA (Q1) AND (Q3) OF VORAPAXAR & VORAPAXAR-D5

Development of Extraction Method: Various organic solvents were optimized to extract VP and VPIS. After a series of trials, protein precipitation (PPT) was selected as appropriate due to high recovery efficiency and matrix free interference.

Sample Extraction and Cleanup Procedure (Sample Preparation): To each labeled polypropylene tube 100 µL of IS (50.00 pg/mL) was mixed with the 100 µL screened plasma spiked sample and 2ml of 0.1% formic acid in acetonitrile was added, vortexed for 10 min and centrifuged at 2000 rpm for 15 min at 25 °C. The supernatant was withdrawn, filtered using Agilent Captiva Filter Cartridges of 3mL capacity with 0.45µm pore size. The filtrate was dried using lyophiliser. To the residue was reconstituted in 150µl of mobile phase and injected in to was added and respective samples were injected into UPLC-ESI-MS/MS for analysis. After completion of several systematic trials, a sensitive, precise and accurate UPLC-MS/MS method was developed for the analysis of Vorapaxar & Vorapaxar- D_5 in rabbit plasma samples.

Method Validation: The developed method was validated over a linear concentration range of 0.010–200.0 ng/ml. The validation parameters include selectivity and specificity, LOQ, Linearity, precision and accuracy, matrix effect, recovery, stability (freeze–thaw, auto sampler, bench top, long term) was evaluated under validation section.

Selectivity and Specificity: Ten lots of blank plasma samples were analyzed out of which six lots free from interference were selected for assessing the selectivity and specificity. The endogenous/ potential interfering peak areas for blank samples must be less than 20% of the LLOQ peak area of Doravirine retention time and less than 5% for Vorapaxar- D_5 retention time.

Limit of Quantification (LOQ): Six LLOQ standards were prepared in screened plasma lot along with IS (50.00 pg/mL) and signal to noise ratio (S/N) was calculated using analyst software.

Linearity: Calibration standards were prepared to obtain linearity range of 0.01, 0.05, 0.5, 1.0, 5.0, 10.0, 20.0, 40.0, 80.0, 100.0 and 200.0 ng/mL and assayed in five replicates on five different days.

Precision & Accuracy: One set of calibration standards and one set contains four different concentrations of quality control standards of 0.010 ng/mL (LLOQ), 0.030 ng/mL (LQC), 110.0 ng/mL (MQC) and 180.0 ng/mL (HQC) concentrations were prepared in screened plasma and analyzed each quality control (QC) standards in six replicates on the same day (Intra day) and five different days (Inter day).

Matrix Effect: Six extracted blank plasma samples in three replicates were spiked with the un-extracted concentration of mid QC (110.00 ng/ml) and compared with un-extracted standards of the same concentration.

Recovery: The recovery of samples was performed by protein precipitation method. The extraction recovery was determined in sex tuplicate by comparing the extracted QC standards with un-extracted QC standards at three different concentrations of low (0.030.00 ng/ml), medium (110.0 ng/ml), high (180.0 ng/ml).

Stability Studies:

Bench Top Stability (Room Temperature Stability, 30 h): Six replicates of spiked low and high concentrations (BT stability samples) were set aside at ambient temperature up to 30 h. Samples were processed and compared with newly prepared low and high concentrations (comparison samples).

Freeze and Thaw Stability (after 3rd cycle at -30 °C): Six replicates of low and high concentrations (FT stability samples) were frozen at -30 °C and subjected to three freeze-thaw cycles of 24, 36 and 48 h (-30 °C to room temperature) and compared with newly prepared low and high concentrations (comparison samples).

Autosampler Stability/ Processed Stability (2-8°C, 48 h): Six replicates of low and high concentrations (AS stability samples) were stored in auto-sampler up to 48 h at 2-8 °C. Stability samples were compared with newly prepared low and high concentrations (comparison samples).

Long-term Stability (-70°C, 90 Days): After completion of the stability period stored at -70 °C (90 days) six replicates of low and high concentrations (LT stability samples) were compared with newly prepared low and high concentrations (comparison samples).

Pharmacokinetic Application:

Selection of Animals: The present study was applied to bioavailability test formulation. The study protocol was approved by the institutional animal ethics committee (IAEC) and conducted as per the ethical guidelines laid down by CPCSEA, New Delhi, India and Bioneeds Bangalore. Six New Zealand albino male rabbits with body weight of 2.1kg were kept in individual cages and maintained at 25° C for 10 days prior to experiment. Standard diet and water *ad libitum* were given to them.

Study Design: Vorapaxar, 1.0 mg/2.1 Kg of body weight of test formulation of Vorapaxar (Equivalent dose of 20 mg Vorapaxar tablet), was administered orally in a single dose. All studies were performed after keeping rabbits for overnight fasting. A polyethylene catheter was inserted into the ear vein of each rabbit to collect blood samples. Blood samples of 0.3ml to 0.5ml collected into heparinized vacutainer tubes at 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12, 24 and 36 h. These samples were centrifuged immediately at 3500 rpm and 4°C temperature for 10 min.

Plasma samples were taken and stored at -30 °C until assay. The valsartan mean plasma concentration *vs*. time curve was depicted in **Fig. 5** and **Table 4**.

Pharmacokinetic Analysis: The plasma concentrations were used to construct pharmacokinetic profiles by plotting drug concentration–time curves. To determine the pharmacokinetic parameters like peak plasma concentration (Cmax), time to reach peak plasma concentration (C_{max}), area under the concentration–time curve (AUC), elimination rate constant (Kel) and elimination half-life (t_{max}) were calculated by a non-compartmental statistic model using Win Non-Lin 5.1 software (Pharsight, USA).

Plasma Vorapaxar concentration-time profiles were visually inspected, and Cmax and Tmax values were determined. The AUC_{0-t} was obtained by the trapezoidal method. The $AUC_{0-\infty}$ max was calculated up to the last measureable concentration, and extrapolations were obtained using the last measureable concentration and the terminal elimination rate constant (Ke) was estimated from the slope of the terminal exponential phase of the plasma of the Vorapaxar concentration–time curve (by means of the linear regression method). The terminal elimination half-life ($t_{1/2}$) was then calculated as 0.693/Ke. The results were depicted in **Table 4**.

RESULTS AND DISCUSSION:

Method Development: The main aim of this work was to develop a rapid, selective and sensitive analytical method including an efficient and reproducible sample clean-up step for quantitative analysis of Vorapaxar in in rabbit plasma samples.

The MS optimization was performed by direct injecttion of Vorapaxar and Vorapaxar–D5 into the mass spectrometer. The mass parameters were optimized to obtain better ionization of Vorapaxar and Vorapaxar–D5 molecules. The full scan spectrum was dominated by protonated molecules [M+H] m/z 591.4 and 498.6 for Vorapaxar and Vorapaxar–D5, and the major fragment ions observed in each product spectrum were at m/z 447.2 and 447.2, respectively.

In optimizing the chromatographic conditions, the ammonium Formate buffer solution (pH: 4.0) was adopted in the mobile phase of the UPLC in order to suppress the tailing phenomena of chromatographic peaks of Vorapaxar and Vorapaxar–D5. Besides, the concentration of the

ammonium Formate buffer solution (pH: 4.0) was investigated and the concentration of 5mM Ammonium Formate Buffer (pH: 4.0) made the chromatographic peaks sharp and symmetric with Pursuit XRs-100Å, C_{18}, 4.6 x 50 mm, 10 µm at flow rate of 0.6ml/min. The acceptable retention and separation of Vorapaxar and Vorapaxar–D5 was obtained by using an elution system of 5mM Ammonium Formate Buffer (pH: 4.0): Methanol: Acetontrile, (40:30:30, % v/v) as the mobile phase. The UPLC-MS/MS method described here satisfies the requirement of routine analyses since it has a short run time (3.5 min). The column oven temperature was kept at a constant temperature of about 40 °C. Injection volume of 10 µl sample was adjusted for better ionization and chromatography.

Prior to loading the sample for LC injection, the co-extracted proteins should be removed from the prepared solution. For this purpose, we initially tested different extraction procedures like protein precipitation (PPT), liquid–liquid extraction (LLE) and solid phase extraction (SPE). We found less recovery in LLE method for the drug and IS. Further, we tried with PPT and SPE and found that PPT was suitable for extraction of the drug and IS. We tried several organic solvents (Acetontrile, methanol, diluted acids) individually as well with combinations in PPT to extract the analyte from the plasma sample. In our case, the Acetontrile and 0.1% Formic acid combination served as a good extraction solvent. High recovery and selectivity were observed in the PPT method. These optimized detection parameters, chromatographic conditions and extraction procedure resulted in reduced analysis time with accurate and precise detection of Vorapaxar in rabbit plasma.

Method Validation:

Selectivity and Specificity, Limit of Quantification (LOQ): No significant response was observed at retention times of Vorapaxar and Vorapaxar–D5 in blank plasma as compared to LLOQ and blank with IS samples. The limit of quantification for this method was proven as the lowest concentration of the calibration curve which was proven as 10.0 pg/ml. Represent chromatograms were shown in **Fig. 3** and **4**.

Linearity: Linearity was plotted as a peak area ratio (Vorapaxar peak area / Vorapaxar–D5 peak area) on the y-axis against Vorapaxar concentration (pg/ml) on the x-axis. Calibration curves were found to be consistently accurate and precise for Vorapaxar over a linearity range of 0.01 to 200.00 ng/ml. The correlation coefficient was greater than 0.9990 for Vorapaxar. The % CV was less than 15% and mean %accuracy was ranged between 97.80 – 102.60%. Results were presented in **Table 1** and **Fig. 5**.

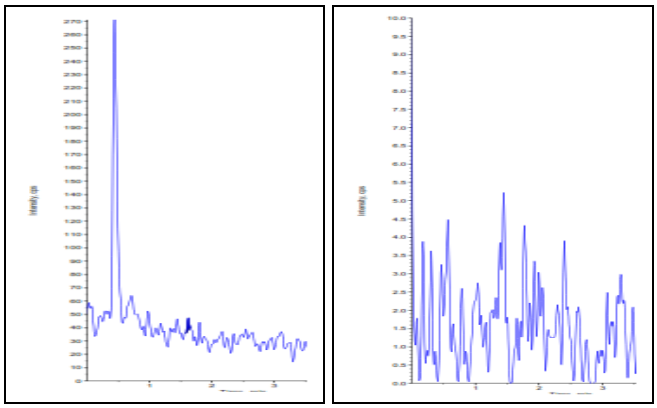

FIG. 3: BLANK PLASMA CHROMATOGRAM OF INTERFERENCE FREE VORAPAXAR AND VORAPAXAR-D5. (Chromatograms shows extracted plasma sample interference-free from matrix at vorapaxar and vorapaxar-D5 retention times)

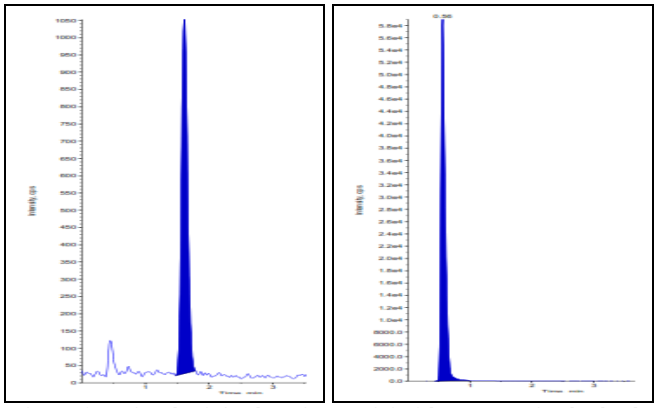

FIG. 4: CHROMATOGRAM OF LLOQ SAMPLE VORAPAXAR AND VORAPAXAR-D5. (Vorapaxar (Peak-1) with a retention time of 1.62 min and vorapaxar-D5 (Peak-2) with a retention time of 0.56 min, respectively)

TABLE 1: CALIBRATION CURVE DETAILS OF VORAPAXAR

Spiked plasma Concentration (ng/ml)	Concentration measured (ng/ml) (Mean ± S.D)	%CV (n = 5)	% Accuracy
0.01	0.01 ± 0.001	1.48	102.60
0.05	0.05 ± 0.002	3.77	98.04
0.5	0.5 ± 0.001	0.56	101.29
1.0	1.0 ± 0.04	4.05	97.80
5.0	5.0 ± 0.04	0.89	100.98
10.0	10.0 ± 0.14	1.34	102.76
20.0	20.13 ± 0.11	0.54	100.66
40.0	39.67 ± 0.49	1.23	99.18
80.0	79.99 ± 1.20	1.50	99.99
100.0	100.18 ± 0.70	0.70	100.18
200.0	199.68 ± 0.67	0.34	99.84

SD is Standard deviation, % CV is Coefficient of variation.

Precision and Accuracy: Intra and inter batch %accuracy for Vorapaxar was ranged between 100.02-100.22 and 97.30-100.81. % CV is 0.12-1.89 and 1.76-5.69. Results are presented in **Table 2**.

Recovery: The mean % recovery for LQC, MQC, HQC samples of Vorapaxar were 96.25%, 97.93%, 99.25%, respectively. The overall mean % recovery and % CV of Vorapaxar across QC levels is 97.84% and 2.69%. For the Vorapaxar-D5 (internal standard) the mean % recovery and %CV is 95.27% and 8.51%.

Matrix Effect: No significant matrix effect found in different sources of rabbit plasma tested for Vorapaxar and Vorapaxar-D5. The %CV was found to be 9.87. The result of a post-column infusion experiment indicated no ion suppression or enhancement at the retention time of the analyte and IS as evident from the flat baseline. There was no ion suppression and enhancement observed at retention time of the analyte and IS.

Stability (Freeze–Thaw, Auto Sampler, Bench Top, Long Term): Stock solution stability was performed to check stability of Vorapaxar and Vorapaxar-D5 in stock solutions prepared in methanol and stored at 2-8 °C in a refrigerator. The freshly prepared stock solutions were compared with stock solutions prepared before 9 days. The % change for Vorapaxar and Vorapaxar-D5 were both less than 5%, indicating that stock solutions were stable at least for 9 days. Bench top and autosampler stability for Vorapaxar was investigated at LQC and HQC levels. The results revealed that Vorapaxar was stable in plasma for at least 30 h at room temperature and 48 h in an autosampler. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with Vorapaxar at LQC and HQC levels did not affect their stability. The long-term stability results also indicated that Vorapaxar was stable in a matrix up to 90 days at -70 °C. Quantification of the Vorapaxar in rabbit plasma samples were subjected to three freeze–thaw cycles (−70°C to room temperature), autosampler (processed), room temperature (Benchtop), long-term stability details were shown in **Table 3**.

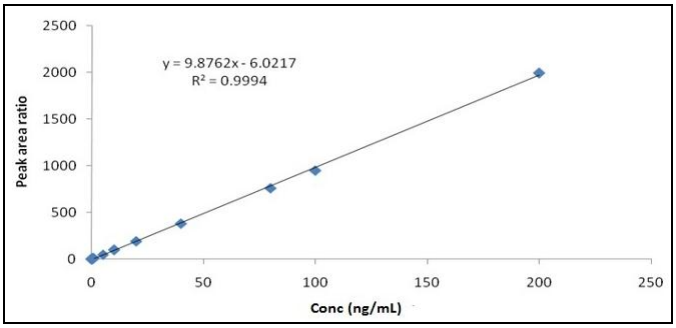

FIG. 5: CALIBRATION CURVE OF VORAPAXAR

TABLE 2: PRECISION AND ACCURACY (ANALYSIS WITH SPIKED SAMPLES AT THREE DIFFERENT CONCENTRATIONS) OF VORAPAXAR

Spiked Plasma Concentration (ng/ml)	Within-run (Intra-day)			Between-run (Inter-Day)		
	Concentration measured (n = 6; ng/ml; mean ± S.D)	% CV	% Accuracy	Concentration measured (n = 6; ng/ml; mean ± S.D)	% CV	% Accuracy
0.03	0.031 ± 0.001	1.89	100.22	0.029 ± 0.001	5.69	97.39
110.00	110.17 ± 0.40	0.36	100.15	108.70 ± 2.05	1.89	98.82
180.0	180.03 ± 0.21	0.12	100.02	181.46 ± 3.20	1.76	100.81

SD is Standard deviation, % CV is Coefficient of variation

TABLE 3: STABILITY STUDIES OF VORAPAXAR IN PLASMA SPIKED SAMPLES

Spiked Plasma concentration (ng/ml)	Room temperature Stability 30 h		Processed sample Stability 48 h		Long term Stability 90 days		Freeze and thaw stability Cycle (48 h)	
	Concentration measured (n = 6; ng/ml; Mean ± S.D)	%CV (n = 6)	Concentration measured (n = 6; ng/ml; Mean ± S.D)	%CV (n = 6)	Concentration measured (n = 6; ng/ml; Mean ± S.D)	%CV (n=6)	Concentration measured (n = 6; ng/ml; Mean ± S.D)	%CV (n = 6)
0.01	0.01 ± 0.001	4.61	0.01 ± 0.001	1.12	0.01 ± 0.001	4.52	0.01 ± 0.001	7.57
180.0	180.89 ± 0.44	0.24	180.78 ± 2.32	1.28	180.37 ± 1.07	0.59	181.07 ± 0.48	0.26

SD is Standard deviation, % CV is Coefficient of variation

Application of the Method: The above validated method was used in the determination of Vorapaxar in plasma samples for establishing the pharmacokinetics of a single 1.0 mg/kg body weight (equivalent dose to 20 mg tablet) in 6 healthy rabbits by oral route. Typical plasma concentration versus time profiles is shown in **Fig. 6**. All the plasma concentrations of Vorapaxar were within the standard curve region and retained above 0.01 ng/mL (LOQ) for the entire sampling period **Table 4**.

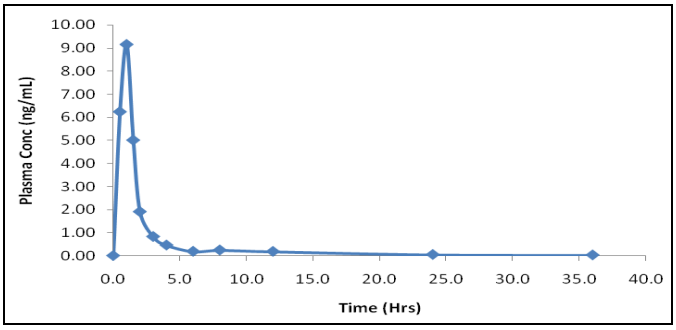

FIG. 6: MEAN PLASMA CONCENTRATIONS *vs*. TIME GRAPH OF VORAPAXAR AFTER ORAL ADMINISTRATION OF 1.0 mg/2.1 kg IN MALE RABBITS

TABLE 4: MEAN PHARMACOKINETIC PARAMETERS OF VORAPAXAR IN RABBIT PLASMA AFTER ORAL ADMINISTRATION OF 1.0 mg/ 2.1 kg IN MALE RABBITS

Pharmacokinetic parameters	Calculated value
AUC_{0-t} (ng x h/mL)	16.50
C_{max} (ng/mL)	9.17
$AUC_{0-\infty}$ (ng x h/mL)	17.22
T_{max} (h)	1.0
Kel (h^{-1})	1.04
$t_{1/2}$ (h)	1.5

$AUC_{0-\infty}$: area under the curve extrapolated to infinity;
AUC_{0-t}: area under the curve up to the last sampling;
C_{max}: the maximum plasma concentration;
T_{max}: the time to reach peak concentration; and
K_{el}: the apparent elimination rate constant.

CONCLUSION: The method exhibited excellent performance in terms of selectivity, linearity, accuracy, precision, recovery, robust, stability in various matrices. This method was successfully applied to pharmacokinetics of rabbit plasma. The method is sensitive enough for quantitative detection of the analyte in biological samples by UPLC-MS/MS. In addition, the reported method has a short analysis run time and deuterated internal standard, an advantage over previously reported methods. The proposed method can thus be used for routine analysis, quality control, stability studies and suitable for therapeutic drug monitoring (pharmacokinetic or bioequivalence studies) of pharmaceutical tablets containing Vorapaxar.

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