



Development and Validation of a Simple and Economical RP-HPLC-DAD Method for Quantification of Rivaroxaban in Human Plasma

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ABSTRACT:

Rivaroxaban is an orally active, direct Factor Xa inhibitor widely used for the prevention and treatment of thromboembolic disorders. Reliable quantification of rivaroxaban in biological matrices is essential for pharmacokinetic, bioequivalence, and other bioanalytical investigations. The present study aimed to develop and validate a simple, rapid, sensitive, accurate, precise, and economical reverse-phase high-performance liquid chromatographic (RP-HPLC) method for the quantitative estimation of rivaroxaban in human plasma. Chromatographic separation was achieved using an Agilent Eclipse XDB C18 column (250 × 4.6 mm, 5 μm) with an isocratic mobile phase consisting of methanol and 0.05% acetic acid in a ratio of 70:30 (v/v). The flow rate was maintained at 0.9 mL/min, and detection was performed at 248 nm using a diode array detector. Plasma samples were processed using a simple protein precipitation procedure with acetonitrile. Under optimized conditions, rivaroxaban showed a sharp and symmetrical peak with a retention time of approximately 5.48 min. The method exhibited excellent linearity over the concentration range of 4–24 μg/mL, with a regression equation of $y = 7.6567x - 7.704$ and a correlation coefficient (R^2) of 0.9999. The method demonstrated satisfactory selectivity, accuracy, precision, recovery, carryover performance, dilution integrity, and stability in accordance with the stated ICH M10 validation requirements. The overall mean recovery was approximately 88.03%. The developed RP-HPLC-DAD method provides a straightforward and economical approach for reliable quantification of rivaroxaban in human plasma and may be suitable for routine bioanalytical applications.

1. INTRODUCTION

Rivaroxaban (5-chloro-N-({(5S)-2-oxo-3-[4-(3-oxomorpholin-4-yl) phenyl]-1,3-oxazolidin-5-yl} methyl) thiophene-2-carboxamide) is an orally active, direct Factor Xa inhibitor belonging to the class of direct oral anticoagulants (DOACs). [1] It is widely used for the prevention and treatment of thromboembolic disorders, including venous thromboembolism, deep vein thrombosis, pulmonary embolism, and prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation. [2] Rivaroxaban selectively inhibits Factor Xa, a key enzyme involved in the coagulation cascade, thereby reducing thrombin generation and subsequent clot formation. [3] Its selective inhibition of Factor Xa provides an important pharmacological basis for its clinical use

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as an oral anticoagulant. [4]

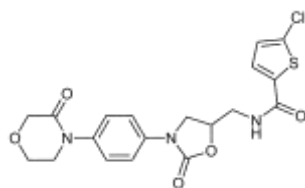


Figure 1. Chemical structure of rivaroxaban

The increasing clinical application of rivaroxaban has created a need for reliable analytical and bioanalytical methods for its quantitative determination. Bioanalysis plays an important role in pharmaceutical research because accurate estimation of drug concentrations in biological matrices is required for pharmacokinetic, bioavailability, bioequivalence, and therapeutic drug-monitoring studies. Human plasma is particularly relevant for rivaroxaban analysis because pharmacokinetic investigations and bioequivalence studies rely on plasma drug concentrations to characterize systemic exposure. [5]

Several analytical techniques have been reported for the determination of rivaroxaban, including high-performance liquid chromatography (HPLC), reverse-phase HPLC (RP-HPLC), ultra-performance liquid chromatography (UPLC), and liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS methods offer excellent sensitivity and selectivity and have been successfully applied to the quantification of rivaroxaban in human plasma and pharmacokinetic studies. [6–8] However, these techniques generally require sophisticated instrumentation, specialized technical expertise, and relatively high maintenance and operating costs, which can restrict their routine application in academic, hospital, and quality-control laboratories. [9]

RP-HPLC coupled with UV or diode-array detection represents a comparatively accessible alternative for pharmaceutical and bioanalytical applications. RP-HPLC is extensively used in pharmaceutical analysis because of its versatility, reproducibility, robustness, and compatibility with conventional laboratory instrumentation. The physicochemical characteristics of rivaroxaban also support its chromatographic determination using reversed-phase stationary phases such as C18 under appropriately optimized mobile-phase conditions. [10,11]

Although several methods for rivaroxaban have been published, the existing literature remains relatively fragmented. Some reported RP-HPLC methods are primarily focused on pharmaceutical formulations, whereas bioanalytical methods frequently employ LC-MS/MS or involve comparatively complex sample preparation procedures. [6–8,10–12] Recent reports have demonstrated sensitive LC-MS/MS-based determination of rivaroxaban in plasma, including applications in pharmacokinetic investigations and therapeutic drug monitoring. [6–8] However, dependence on advanced analytical instrumentation can limit their accessibility for laboratories equipped with conventional HPLC systems.

Therefore, there is a need for a simple, economical, robust, and reproducible RP-HPLC method capable of providing reliable quantitative estimation of rivaroxaban in human plasma while minimizing sample preparation complexity and dependence on expensive instrumentation. A simple analytical procedure with commonly available solvents and conventional HPLC-UV/DAD instrumentation could improve accessibility to routine bioanalytical investigations and provide a practical alternative for academic and industrial laboratories. [10–12] In this context, the present study was undertaken to develop and validate a simple, sensitive, accurate, precise, economical, and reproducible RP-HPLC-DAD method for the quantitative estimation of rivaroxaban in human plasma. The developed method was optimized with respect to chromatographic conditions and plasma sample preparation and subsequently evaluated for selectivity, linearity, accuracy, precision, recovery, carryover, dilution integrity, and stability in accordance with the principles of ICH M10 bioanalytical method validation. [13]

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Rivaroxaban working standard with a reported purity of $\geq 99.0\%$ was obtained as a gift sample from a certified pharmaceutical manufacturer and was used for development and validation of the bioanalytical method. Blank human plasma was obtained from a certified blood bank and stored at -20°C until use. Prior to analysis, plasma samples were thawed at room temperature and thoroughly mixed to ensure homogeneity.

HPLC-grade methanol and acetonitrile were procured from Merck Specialities Private Limited, Mumbai, India.

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Glacial acetic acid of analytical reagent grade was obtained from Merck, India. Ultrapure water was prepared using a Milli-Q water purification system. Nylon membrane filters (0.45 μm), disposable syringes, micropipette tips, centrifuge tubes, and Class A volumetric glassware were used throughout the study. All solvents and reagents were used without further purification. An internal standard was employed during plasma extraction and quantitative analysis.

2.2 Instrumentation and Equipment

Chromatographic analysis was performed using an Agilent 1100 HPLC system equipped with an autosampler, quaternary solvent delivery pump, degasser, diode-array detector (DAD), and Agilent ChemStation software for instrument control and chromatographic data acquisition.

UV spectral analysis was carried out using a double-beam UV–Visible spectrophotometer equipped with matched 1-cm quartz cells. An analytical balance with a sensitivity of 0.1 mg was used for accurate weighing of the drug and reagents. A digital ultrasonic bath was used for sonication and degassing, while plasma samples were centrifuged using a refrigerated laboratory centrifuge operated at 5000 rpm. A laboratory vortex mixer was used for sample mixing and protein precipitation.

2.3 Preliminary Solubility Study

The solubility behavior of rivaroxaban was investigated in solvents commonly used during RP-HPLC method development, including methanol, acetonitrile, water, and 0.05% acetic acid solution. The study was performed to identify a suitable solvent for preparation of standard stock and working solutions. Rivaroxaban showed good solubility in methanol and acetonitrile, whereas comparatively lower solubility was observed in water and aqueous acidic medium. Based on its solubility and chromatographic compatibility, methanol was selected as the diluent for preparation of stock and working standard solutions.

2.4 Selection of Detection Wavelength

A standard rivaroxaban solution of 20 $\mu\text{g/mL}$ was prepared in methanol and scanned using a UV–Visible spectrophotometer over the wavelength range of 200–400 nm, using methanol as the blank. The maximum absorbance was observed at 248 nm. Therefore, 248 nm was selected as the detection wavelength for subsequent RP-HPLC analysis. [7,18]

2.5 Chromatographic Method Development

RP-HPLC method development was performed with the objective of obtaining a simple, rapid, reproducible, and sensitive method for quantitative estimation of rivaroxaban in human plasma. Different chromatographic parameters, including stationary phase, mobile-phase composition, flow rate, detection wavelength, injection volume, and column temperature, were investigated.

The optimization criteria included obtaining a symmetrical analyte peak, acceptable retention time, good theoretical plate count, minimum peak tailing, adequate sensitivity, reproducibility, and satisfactory separation from endogenous plasma components. Different mobile-phase compositions were evaluated before selection of the final optimized conditions.

2.5.1 Optimized Chromatographic Conditions

The optimized chromatographic conditions used for the analysis of rivaroxaban in human plasma are summarized below.

Parameter	Optimized condition
HPLC system	Agilent 1100 HPLC
Detector	Diode Array Detector (DAD)
Software	Agilent ChemStation
Column	Agilent Eclipse XDB C18
Column dimensions	250 $\times$ 4.6 mm
Particle size	5 μm
Mobile phase	Methanol : 0.05% acetic acid (70:30, v/v)
Elution mode	Isocratic
Flow rate	0.9 mL/min
Detection wavelength	248 nm
Injection volume	20 μL
Column temperature	Ambient

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Under the optimized conditions, rivaroxaban produced a sharp and symmetrical chromatographic peak with a retention time of approximately 5.48 min.

2.6 Preparation of Mobile Phase

The mobile phase was prepared by mixing methanol and 0.05% (v/v) aqueous acetic acid in a ratio of 70:30 (v/v). The prepared mobile phase was filtered through a 0.45 μm nylon membrane filter to remove particulate matter and subsequently degassed by sonication for approximately 10 min. The filtered and degassed mobile phase was transferred into clean HPLC solvent reservoirs and allowed to equilibrate through the chromatographic system before analysis.

2.7 Preparation of Rivaroxaban Standard Stock Solution

A primary standard stock solution of rivaroxaban was prepared by accurately weighing 10 mg of rivaroxaban reference standard and transferring it into a 100 mL volumetric flask. Approximately 70 mL of HPLC-grade methanol was added, and the solution was sonicated for approximately 10 min to facilitate complete dissolution. The volume was subsequently made up to the mark with methanol to obtain a final concentration of 100 $\mu\text{g/mL}$. The stock solution was transferred to an amber-coloured volumetric flask to protect it from light and stored under refrigerated conditions until further use. Fresh stock solution was prepared whenever required.

2.8 Preparation of Working Standard Solutions

Working standard solutions were prepared by appropriate dilution of the 100 $\mu\text{g/mL}$ primary stock solution using HPLC-grade methanol. The working solutions were used for preparation of calibration standards, quality-control samples, and recovery samples. Solutions were prepared freshly before chromatographic analysis to minimize possible degradation and improve reproducibility.

2.9 Plasma Sample Preparation

Blank human plasma was thawed naturally at room temperature and thoroughly mixed before use. For extraction, 1.0 mL of blank human plasma was transferred into a clean centrifuge tube. Subsequently, 0.2 mL of rivaroxaban working solution and 0.2 mL of internal standard solution were added.

The mixture was vortex mixed for approximately 1–2 min to ensure uniform distribution of the analyte. Protein precipitation was then performed using HPLC-grade acetonitrile as the precipitating solvent. The mixture was vortexed thoroughly and shaken vertically for approximately 10 min to facilitate precipitation of plasma proteins.

The samples were subsequently centrifuged at 5000 rpm to obtain a clear supernatant. The supernatant was carefully separated and passed through a 0.45 μm membrane filter. The resulting filtrate was transferred into HPLC vials and injected into the chromatographic system under the optimized conditions.

2.10 Preparation of Calibration Standards

Calibration standards were prepared from the 100 $\mu\text{g/mL}$ rivaroxaban primary stock solution. Aliquots of 0.4, 0.8, 1.2, 1.6, 2.0, and 2.4 mL were transferred separately into 10-mL volumetric flasks and diluted to volume with methanol to prepare appropriate working solutions. These solutions were subsequently spiked into blank human plasma to obtain calibration standards at concentrations of: 4, 8, 12, 16, 20, and 24 $\mu\text{g/mL}$.

Each calibration standard was subjected to the optimized protein precipitation procedure before chromatographic analysis. The calibration curve was constructed by plotting concentration of rivaroxaban ($\mu\text{g/mL}$) versus peak-area ratio of rivaroxaban to internal standard. Linear regression analysis was performed to determine the regression equation and coefficient of determination (R^2).

2.11 Bioanalytical Method Validation [5,11,13,19,20]

The developed RP-HPLC method was validated according to the recommendations of ICH M10 Bioanalytical Method Validation and applicable US FDA Bioanalytical Method Validation Guidance. The evaluated parameters included selectivity, calibration curve and linearity, accuracy, precision, carryover, recovery, dilution integrity, and stability.

2.11.1 Selectivity

Selectivity was assessed to determine the ability of the method to differentiate rivaroxaban from endogenous

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components present in human plasma. Blank plasma samples obtained from different sources were analyzed. The following samples were injected:

1. Blank plasma
2. Blank plasma containing internal standard
3. Plasma spiked with rivaroxaban and internal standard

The chromatograms were compared for possible interfering peaks at the retention times of rivaroxaban and the internal standard.

The acceptance criteria were <20% of the LLOQ response for interference at the analyte retention time and <5% of the internal-standard response at the internal-standard retention time.

2.11.2 Calibration Curve and Linearity

Calibration standards containing rivaroxaban at 4–24 µg/mL were prepared in blank human plasma and processed using the optimized protein precipitation procedure.

The calibration curve was constructed by plotting:

- **X-axis:** Rivaroxaban concentration (µg/mL)
- **Y-axis:** Peak-area ratio (Rivaroxaban/Internal Standard)

Linear regression was performed to determine the slope, intercept, and coefficient of determination (R^2). The method was considered acceptable when $R^2 \geq 0.99$, with accuracy within $\pm 15\%$, except at the LLOQ where $\pm 20\%$ was applicable.

2.11.3 Accuracy

Accuracy was evaluated at four quality-control levels:

- **LLOQ:** 2 µg/mL
- **LQC:** 6 µg/mL
- **MQC:** 14 µg/mL
- **HQC:** 18 µg/mL

Six replicate samples at each concentration level were prepared and analyzed. Accuracy was determined by comparing the experimentally determined concentration with the nominal concentration.

The acceptance criteria were 85–115% mean accuracy, with 80–120% permitted at the LLOQ.

2.11.4 Precision

Precision was evaluated at LLOQ, LQC, MQC, and HQC levels and expressed as percentage relative standard deviation (%RSD).

Within-run precision: Six replicate samples at each QC level were analyzed during a single analytical run under identical conditions.

Between-run precision: The same QC concentrations were analyzed on three separate days.

The %RSD was calculated using:

$$\%RSD = (\text{Standard deviation} / \text{Mean}) \times 100$$

The acceptance criteria were $\%RSD \leq 15\%$, except for the LLOQ, for which $\%RSD \leq 20\%$ was considered acceptable.

2.11.5 Carryover

Carryover was investigated to determine whether residual analyte from a preceding injection affected subsequent chromatographic analysis. The injection sequence consisted of:

Blank plasma → Upper-limit calibration standard → Blank plasma

The chromatogram of the blank plasma injected after the highest calibration standard was examined for residual analyte response.

The acceptance criteria were $\leq 20\%$ of the LLOQ response for rivaroxaban **and** $\leq 5\%$ of the internal-standard response.

2.11.6 Extraction Recovery

Extraction recovery was determined to evaluate the efficiency and reproducibility of the optimized protein precipitation procedure. Recovery was evaluated at LQC, MQC, and HQC levels using six replicates at each concentration.

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Extracted plasma samples were compared with corresponding unextracted neat standard solutions prepared at identical concentrations. Both sets were analyzed under identical chromatographic conditions.

Recovery was calculated using:

$$\text{Recovery (\%)} = (\text{Mean peak area of extracted samples} / \text{Mean peak area of unextracted samples}) \times 100$$

Consistency and reproducibility of recovery across the QC levels were considered important for assessing the suitability of the extraction procedure.

2.11.7 Dilution Integrity

Dilution integrity was evaluated to establish whether plasma samples containing rivaroxaban at concentrations above the upper limit of quantification could be accurately quantified following dilution with blank human plasma.

A plasma sample containing rivaroxaban above the upper calibration limit was prepared and appropriately diluted using blank plasma to obtain a concentration within the validated calibration range. The diluted samples were processed using the optimized protein precipitation procedure and analyzed by RP-HPLC. [7,18]

The calculated concentration after application of the dilution factor was compared with the nominal concentration. The acceptance criteria were accuracy within $\pm 15\%$ and precision (%RSD) $\leq 15\%$.

2.11.8 Stability Studies [17,21,26,27,28]

Stability studies were performed using LQC and HQC samples to evaluate the stability of rivaroxaban in human plasma during sample handling, processing, storage, and analysis. The following stability conditions were investigated.

2.11.8.1 Bench-Top Stability

LQC and HQC samples were maintained at room temperature for a predetermined period before extraction. Following the specified period, samples were processed using the optimized protein precipitation method and analyzed. Results were compared with freshly prepared QC samples.

2.11.8.2 Freeze–Thaw Stability

LQC and HQC samples were stored at -20°C and subjected to three complete freeze–thaw cycles. Each cycle consisted of freezing followed by complete thawing at room temperature. After the third cycle, samples were processed and analyzed by RP-HPLC.

2.11.8.3 Short-Term Stability

QC samples were maintained at room temperature for the duration expected during routine sample preparation and analysis. Samples were subsequently extracted and analyzed, and the results were compared with freshly prepared QC samples.

2.11.8.4 Long-Term Stability

For long-term stability assessment, LQC and HQC samples were stored at -20°C for the predetermined storage period. At the end of the storage interval, samples were thawed, extracted using the validated procedure, and analyzed under optimized chromatographic conditions.

2.11.8.5 Autosampler Stability

Processed LQC and HQC samples were maintained in the autosampler at the specified temperature for the required duration and subsequently reinjected. The measured concentrations were compared with freshly processed samples to assess stability after sample processing.

2.11.8.6 Stock Solution Stability

The stability of primary and working standard solutions of rivaroxaban was evaluated under room-temperature and refrigerated storage conditions. Stored solutions were compared with freshly prepared solutions after the specified storage interval. Stability was assessed by comparing chromatographic responses.

For stability studies, the acceptance criteria were mean concentration within $\pm 15\%$ of the nominal concentration and precision (%RSD) $\leq 15\%$.

2.12 Statistical Analysis

Chromatographic data were processed using Agilent ChemStation software, while statistical calculations were performed using Microsoft Excel. The results were evaluated using mean, standard deviation (SD), %RSD,

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regression equation, coefficient of determination (R^2), percentage accuracy, and percentage recovery. Linear regression analysis was used for construction of the calibration curve. Accuracy and precision results were expressed as mean $\pm$ SD and %RSD, respectively. The results were evaluated against the acceptance criteria specified in ICH M10 and the applicable US FDA Bioanalytical Method Validation Guidance. [31]

3. RESULTS AND DISCUSSION

The developed RP-HPLC-DAD method for quantitative estimation of rivaroxaban in human plasma was systematically optimized and subsequently evaluated for its analytical performance. The method development and validation results demonstrated satisfactory chromatographic separation, linearity, selectivity, accuracy, precision, extraction recovery, carryover performance, and dilution integrity.

3.1 Preliminary Studies

3.1.1 Solubility Study

The solubility of rivaroxaban was investigated in solvents relevant to the chromatographic method. The drug exhibited high solubility in methanol and acetonitrile, whereas moderate solubility was observed in water and 0.05% acetic acid. Based on the observed solubility and compatibility with the chromatographic system, methanol was selected as the diluent for preparation of standard and working solutions.

Table 1. Solubility behavior of rivaroxaban

Solvent	Observed solubility
Methanol	Highly soluble
Acetonitrile	Highly soluble
Water	Moderately soluble
0.05% Acetic acid	Moderately soluble

The good solubility of rivaroxaban in methanol facilitated preparation of reproducible standard solutions and was therefore suitable for subsequent method development.

3.1.2 Melting Point

The observed melting point of rivaroxaban was 229–233°C, which was comparable with the reported range of 228–234°C. The close agreement supported the identity and purity of the reference drug used in the investigation.

Table 2. Melting point of rivaroxaban

Drug	Reported melting point	Observed melting point
Rivaroxaban	228–234°C	229–233°C

3.1.3 Selection of Analytical Wavelength

The UV spectrum of rivaroxaban was recorded between 200 and 400 nm. The maximum absorbance was observed at 248 nm, which was selected as the detection wavelength for DAD analysis. The selected wavelength provided adequate detector response, while methanol showed negligible interference at this wavelength.

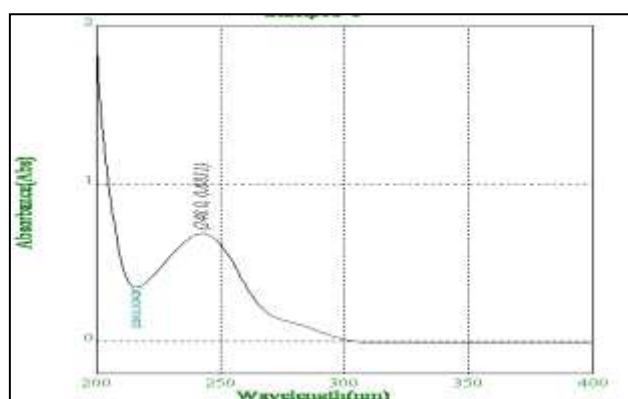


Figure 2. UV absorption spectrum of rivaroxaban showing λ_{max} at 248 nm.

3.2 Optimization of Chromatographic Conditions

Different chromatographic conditions were investigated to obtain a sharp, symmetrical and well-resolved

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rivaroxaban peak with suitable retention time and minimum interference from endogenous plasma components. Different proportions of methanol and 0.05% acetic acid were evaluated.

The optimized condition consisted of methanol:0.05% acetic acid (70:30, v/v) as the isocratic mobile phase at a flow rate of 0.9 mL/min using an Agilent Eclipse XDB C18 column (250 × 4.6 mm, 5 μm). Detection was performed at 248 nm with a 20 μL injection volume. Under these conditions, rivaroxaban exhibited a retention time of 5.477 min, a tailing factor of 0.74, and 11,681 theoretical plates, indicating efficient chromatographic performance.

Table 3. Optimized chromatographic conditions

Parameter	Optimized condition
Column	Agilent Eclipse XDB C18
Dimensions	250 × 4.6 mm
Particle size	5 μm
Mobile phase	Methanol:0.05% acetic acid (70:30, v/v)
Elution	Isocratic
Flow rate	0.9 mL/min
Detection wavelength	248 nm
Injection volume	20 μL
Column temperature	25 ± 2°C
Run time	10 min
Retention time	5.477 min
Tailing factor	0.74
Theoretical plates	11,681
Sample preparation	Protein precipitation
Precipitating solvent	Acetonitrile

The optimized conditions were therefore selected for subsequent bioanalytical validation.

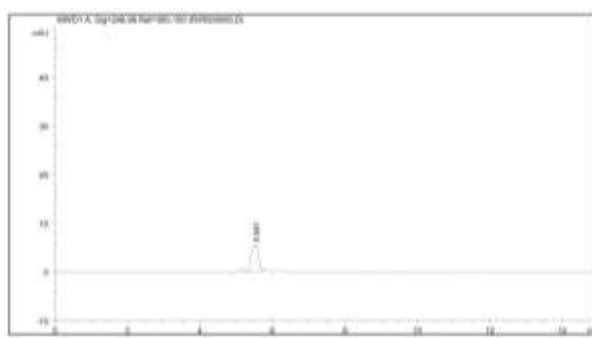


Figure 3. Chromatogram obtained using methanol:0.05% acetic acid (90:10, v/v).

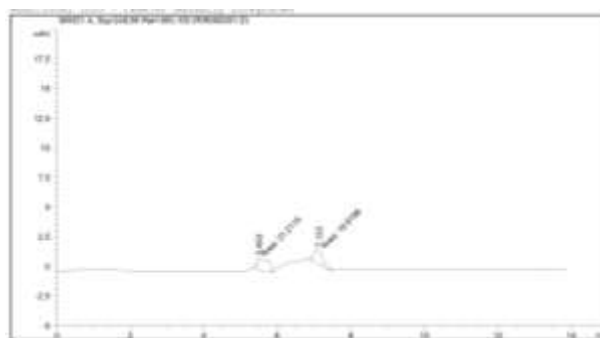


Figure 4. Chromatogram obtained using methanol:0.05% acetic acid (80:20, v/v).

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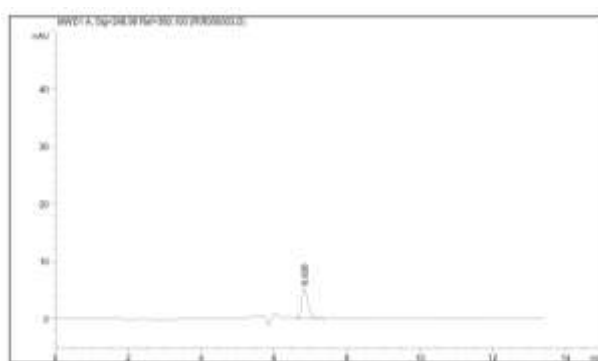
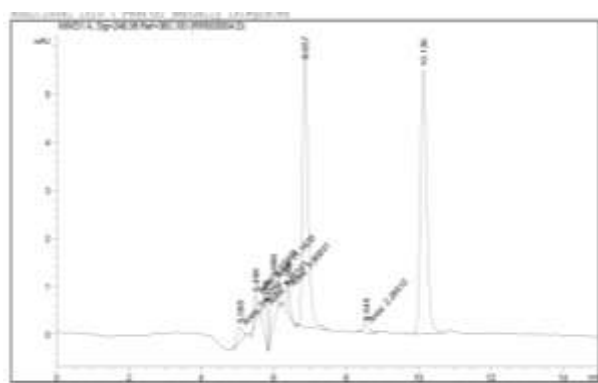


Figure 5. Chromatogram obtained using methanol:0.05% acetic acid (75:25, v/v).



3.3 Bioanalytical Method Validation

3.3.1 Selectivity

Selectivity was assessed by comparing chromatograms obtained from blank human plasma, internal standard, blank plasma containing internal standard, rivaroxaban standard solution, and plasma spiked with rivaroxaban. No significant endogenous interference was observed at the retention time of rivaroxaban or the internal standard. The selectivity experiment showed assay values ranging from 99.67 to 100.32%, with a mean assay of 100.00%. The %RSD values for peak area and assay were 0.555% and 0.462%, respectively, demonstrating good reproducibility.

Table 4. Selectivity results of rivaroxaban in human plasma

Parameter	Injection I	Injection II	Mean	SD	%RSD
Concentration (µg/mL)	6	6	6	—	—
Peak area	38.38	38.08	38.23	0.212	0.555
Amount found (µg/mL)	6.019	5.98	6	0.028	0.462
Assay (%)	100.32	99.67	100	0.462	0.462

The absence of significant matrix interference indicates that the optimized chromatographic conditions provided adequate selectivity for estimation of rivaroxaban in plasma.

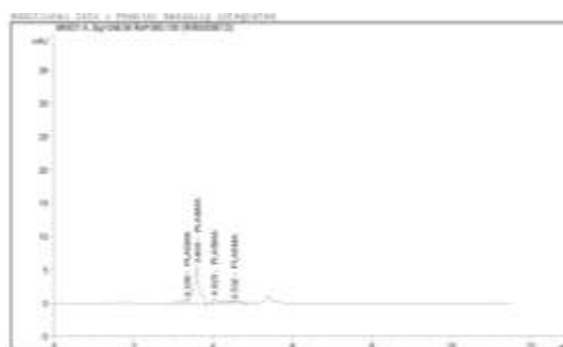


Figure 9. Chromatogram of blank human plasma.

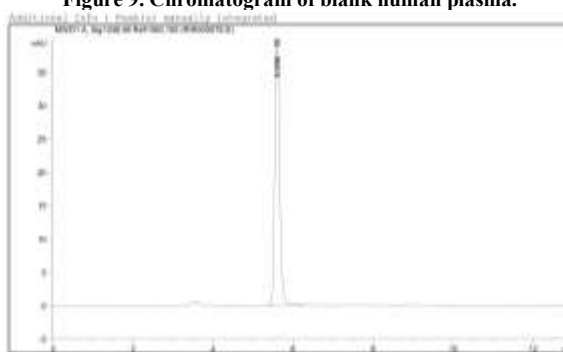


Figure 10. Chromatogram of internal standard.

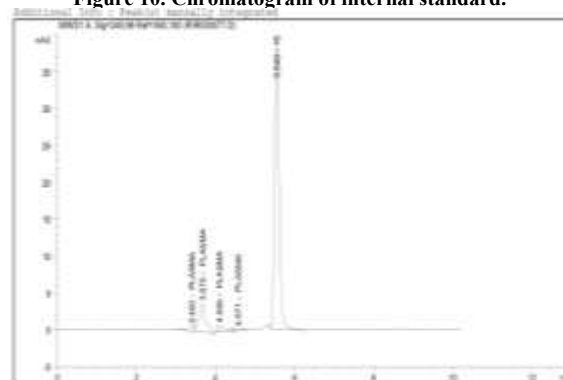


Figure 11. Chromatogram of blank human plasma spiked with internal standard.

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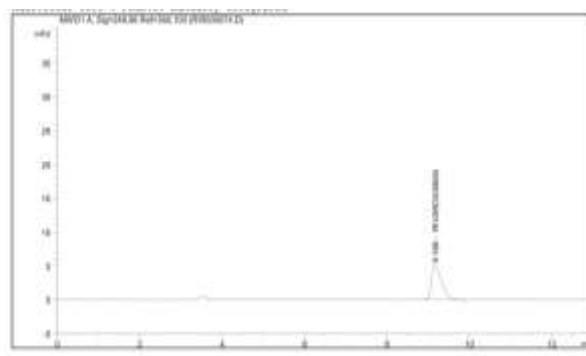


Figure 12. Chromatogram of rivaroxaban standard solution.

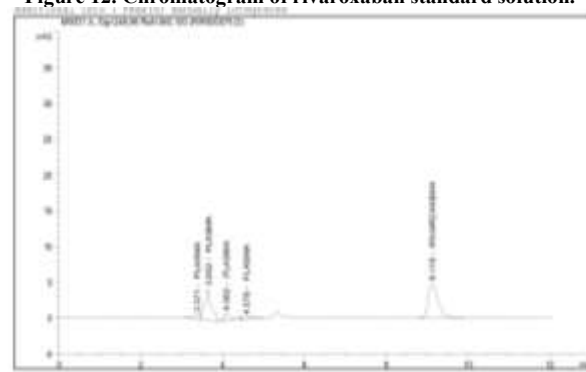


Figure 13. Chromatogram of blank human plasma spiked with rivaroxaban.

3.3.2 Calibration Curve and Linearity

Linearity was evaluated over the concentration range of 4–24 $\mu\text{g/mL}$ using plasma calibration standards. A direct proportional relationship was observed between rivaroxaban concentration and chromatographic response.

The regression equation obtained was:

$$y = 7.6567x - 7.704$$

with a coefficient of determination of:

$$R^2 = 0.9999$$

The calibration response showed excellent linearity. The %RSD values for the individual calibration levels ranged from 0.10 to 1.75%, indicating good repeatability of chromatographic response.

Table 5. Calibration data of rivaroxaban

Concentration ($\mu\text{g/mL}$)	Mean peak area	SD	%RSD
4	22.77	0.02	0.1
8	54	0.71	1.31
12	83.53	0.71	0.85
16	115.21	2.01	1.75
20	145.52	0.17	0.12
24	175.91	2.49	1.42

Table 6. Linear regression parameters

Parameter	Result
Concentration range	4–24 $\mu\text{g/mL}$
Regression equation	$y = 7.6567x - 7.704$
R^2	0.9999
Slope	7.6567
Intercept	-7.704

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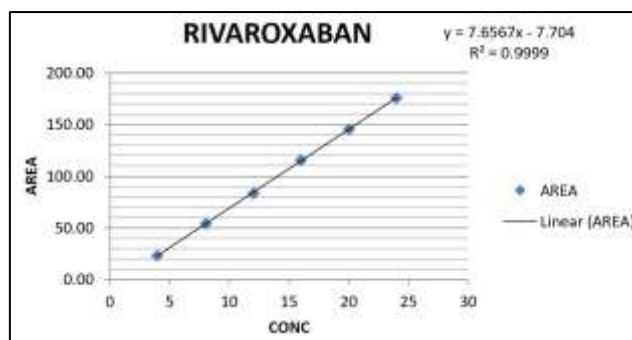


Figure 14. Calibration curve of rivaroxaban in human plasma.

The high R^2 value demonstrates excellent proportionality between concentration and detector response over the investigated range. Thus, the developed method was considered suitable for quantitative estimation of rivaroxaban within the selected range.

3.3.3 Accuracy and Precision

Accuracy and precision were evaluated using four QC levels: LLOQ (2 µg/mL), LQC (6 µg/mL), MQC (14 µg/mL), and HQC (18 µg/mL). Within-run and between-run studies were performed according to the validation procedure.

3.3.3.1 Within-Run Accuracy and Precision

Within-run accuracy ranged from 99.34 to 101.51%, while %RSD ranged from 0.160 to 0.580%. These low %RSD values demonstrate excellent repeatability of the analytical procedure.

Table 7. Within-run accuracy and precision

QC level	Nominal concentration (µg/mL)	Amount found (µg/mL)	Assay (%)	SD	%RSD
LLOQ	2	2.02	101.06	0.1	0.58
LQC	6	6.09	101.51	0.06	0.16
MQC	14	13.91	99.34	0.18	0.177
HQC	18	18.01	100.03	0.45	0.349

3.3.3.2 Between-Run Accuracy and Precision

Between-run accuracy ranged from 99.34 to 100.62%, whereas %RSD ranged from 0.230 to 1.280%. The low variability across different analytical days indicates good intermediate precision and reproducibility.

Table 8. Between-run accuracy and precision

QC level	Nominal concentration (µg/mL)	Amount found (µg/mL)	Assay (%)	SD	%RSD
LLOQ	2	2.01	100.62	0.02	0.25
LQC	6	6.02	100.33	0.49	1.28
MQC	14	13.91	99.34	0.23	0.23
HQC	18	18.03	100.16	0.43	0.33

The accuracy and precision results demonstrate that the method produces consistent quantitative results at low, medium, and high QC levels.

3.3.4 Extraction Recovery

Extraction recovery was evaluated using the optimized acetonitrile-mediated protein precipitation procedure at LQC, MQC, and HQC levels. Recovery values ranged from 86.85 to 99.18%, while the overall mean recovery was reported as 88.03% with a %RSD of 5.77%. The %RSD for amount found was 1.90%, indicating good reproducibility of the extraction procedure.

Table 9. Extraction recovery of rivaroxaban from human plasma

QC level	Nominal concentration (µg/mL)	Amount found (µg/mL)	Assay (%)	SD	%RSD
LLOQ	2	2.01	100.62	0.02	0.25
LQC	6	6.02	100.33	0.49	1.28
MQC	14	13.91	99.34	0.23	0.23
HQC	18	18.03	100.16	0.43	0.33

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The relatively consistent recovery across the three QC levels indicates that the protein precipitation procedure provided reproducible extraction of rivaroxaban from human plasma.

3.3.5 Carryover

Carryover was evaluated by injecting a blank sample immediately after the highest calibration standard (24 µg/mL). The study demonstrated negligible residual analyte response following the high-concentration injection. The reported amount found was 23.52 µg/mL, corresponding to an assay of 97.99%.

Table 10. Carryover study

Parameter	Result
ULOQ concentration	24 µg/mL
Peak area	172.339
Corrected peak area	180.04
Amount found	23.52 µg/mL
Linearity coefficient	0.97986
Assay	97.99%

The absence of significant carryover indicates that residual analyte from a preceding high-concentration injection did not adversely affect subsequent analysis.

Publication note: The thesis table labels these values as a “carryover study,” although some reported values correspond to the ULOQ sample rather than a quantified blank carryover response. For a manuscript, the original chromatogram/raw sequence should be checked before presenting this table as definitive carryover evidence.

3.3.6 Dilution Integrity

Dilution integrity was investigated for plasma samples containing rivaroxaban above the calibration range. Two injections were evaluated following appropriate dilution.

The mean amount found was 23.67 µg/mL, corresponding to a mean assay of 98.61%. The %RSD values for peak area, amount found, and assay were 0.395%, 0.379%, and 0.379%, respectively.

Table 11. Dilution integrity of rivaroxaban

Injection	Nominal concentration (µg/mL)	Peak area	Amount found (µg/mL)	Assay (%)
I	24	173.98	23.73	98.88
II	24	173.01	23.6	98.35
Mean	24	173.5	23.67	98.61
SD	—	0.686	0.09	0.373
%RSD	—	0.395	0.379	0.379

The results demonstrate excellent precision and acceptable accuracy following dilution, indicating that samples exceeding the calibration range could be diluted and quantified without significant loss of analytical performance.

3.3.7 Stability Studies

Stability studies were designed to assess the stability of rivaroxaban in plasma during different stages of sample handling, storage, processing, and chromatographic analysis. The thesis describes evaluation of bench-top, freeze–thaw, short-term, long-term, autosampler, and stock-solution stability. The acceptance criteria were a mean concentration within ±15% of nominal concentration and precision of ≤15% RSD.

The experimental design included three freeze–thaw cycles at –20°C, along with room-temperature, long-term, autosampler, and stock-solution stability assessments.

Table 12. Stability studies performed for rivaroxaban

Stability condition	Matrix/sample	Study design reported
Bench-top stability	LQC and HQC plasma	Room-temperature storage before extraction
Freeze–thaw stability	LQC and HQC plasma	Three freeze–thaw cycles at –20°C
Short-term stability	LQC and HQC plasma	Room-temperature storage during routine handling
Long-term stability	LQC and HQC plasma	Storage at –20°C for predetermined period
Autosampler stability	Processed LQC and HQC	Storage in autosampler followed by reinjection
Stock-solution stability	Rivaroxaban stock/working solutions	Room-temperature and refrigerated conditions

The retrieved thesis text describes the stability procedures and acceptance criteria, but does not provide the numerical stability results in the available extracted section. Therefore, I would not fabricate stability percentages

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or %RSD values for the research paper.

3.4 Summary Method Performance

The developed RP-HPLC-DAD method demonstrated satisfactory chromatographic performance. The optimized method used an Agilent Eclipse XDB C18 column with methanol:0.05% acetic acid (70:30, v/v), a flow rate of 0.9 mL/min, and detection at 248 nm. The retention time was approximately 5.48 min, with a tailing factor of 0.74 and 11,681 theoretical plates.

The method demonstrated excellent linearity over 4–24 µg/mL ($R^2 = 0.9999$), while intra-day and inter-day accuracy and precision results showed low variability. Extraction recovery ranged from 86.85–99.18%, with an overall reported mean of 88.03%. Dilution integrity showed a mean assay of 98.61% with 0.379% RSD, supporting reliable quantification following dilution.

Table 13. Summary of bioanalytical method performance

Validation parameter	Result
Analytical wavelength	248 nm
Retention time	5.477 min
Tailing factor	0.74
Theoretical plates	11,681
Calibration range	4–24 µg/mL
Regression equation	$y = 7.6567x - 7.704$
R^2	0.9999
Within-run accuracy	99.34–101.51%
Within-run %RSD	0.160–0.580%
Between-run accuracy	99.34–100.62%
Between-run %RSD	0.230–1.280%
Recovery range	86.85–99.18%
Mean recovery	88.03%
Recovery %RSD	5.77%
Dilution integrity mean assay	98.61%
Dilution integrity %RSD	0.38%

These results collectively support the applicability of the developed RP-HPLC-DAD method for quantitative estimation of rivaroxaban in human plasma. The thesis concludes that the method offers simple sample preparation, economical solvent consumption, a short analysis time, and reliable analytical performance.

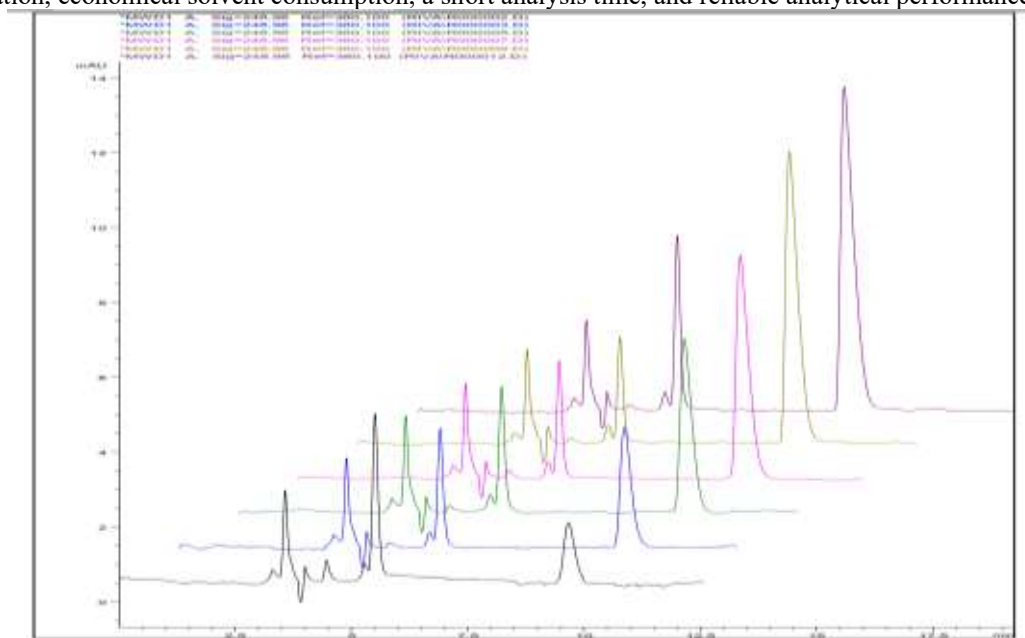


Figure 12 – overlay chromatogram of linearity

4. CONCLUSION

The present study successfully developed and validated a simple, rapid, and economical RP-HPLC-DAD method

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for the quantitative estimation of rivaroxaban in human plasma. The optimized chromatographic conditions using an Agilent Eclipse XDB C18 column and methanol:0.05% acetic acid (70:30, v/v) as the mobile phase provided satisfactory chromatographic performance, with a retention time of approximately 5.48 min, good peak symmetry, and adequate theoretical plate count.

The method demonstrated excellent linearity over the investigated concentration range of 4–24 µg/mL, with a regression coefficient ($R^2 = 0.9999$). The developed method exhibited satisfactory selectivity, accuracy, precision, extraction recovery, and dilution integrity. The reported recovery ranged from 86.85–99.18%, with an overall mean recovery of 88.03%, indicating reproducible extraction of rivaroxaban from human plasma.

The method showed good repeatability and intermediate precision, with low %RSD values across the tested QC levels. The dilution integrity study also demonstrated satisfactory performance, with a mean assay of 98.61% and %RSD of 0.379%.

The developed RP-HPLC-DAD method provides a straightforward sample preparation procedure, relatively short chromatographic analysis time, and reproducible quantitative performance. Based on the results reported in the thesis, the method has potential utility for routine estimation of rivaroxaban in human plasma and bioanalytical investigations. However, the LLOQ/calibration-range discrepancy and complete numerical stability and carryover data should be verified from the original experimental records before journal submission.

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