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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF RIVAROXABAN IN HUMAN PLASMA BY RP-HPLC

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ABSTRACT

A simple, precise, and accurate reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the quantitative estimation of Rivaroxaban in human plasma. The method employed protein precipitation for sample preparation and used a mobile phase composed of acetonitrile and ammonium formate buffer (78:22 v/v) at pH 4.5. Chromatographic separation was achieved on a Symmetry C18 column (250 mm × 4.6 mm, 3.5 µm) with a flow rate of 1 mL/min, and detection was carried out at 251 nm. The retention time of Rivaroxaban was approximately 4.4 minutes. The method demonstrated good linearity in the concentration range of 5–25 µg/mL with correlation coefficients (r^2) exceeding 0.995. Validation parameters including accuracy, precision, recovery, and stability were within the limits established by USFDA guidelines. Stability studies confirmed that Rivaroxaban was stable under various conditions, including freeze-thaw, short-term, long-term, and stock solution storage. The developed method is suitable for pharmacokinetic studies and therapeutic drug monitoring of Rivaroxaban in human plasma.

KEYWORDS: Rivaroxaban, RP-HPLC, Bioanalytical Method, Method Validation, Human Plasma, Protein Precipitation, Acetonitrile, Ammonium Formate Buffer, Linearity, Accuracy, Precision, Recovery, Stability Studies, USFDA Guidelines, Pharmacokinetics etc.

INTRODUCTION

Bio analytical Method Development

Bioavailability

Bioavailability is defined as the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action.

Bioequivalence

Bioequivalence is defined as the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study.

Techniques used in Bioanalysis

The quantitative determination of drugs and their metabolites in biological matrices (bioanalysis) includes a number of steps from sample collection to the final report of the results. The intermediate steps typically include sample storage, sample preparation, separation, identification and quantification of analyte(s). Sample preparation prior to the chromatographic separation has three principal objectives:

- The dissolution of the analyte in a suitable solvent
- Removal of as many interfering compounds as possible
- Pre-concentration of the analyte
- Choice of body fluids

1. Drug concentration in blood, plasma or serum

Measurement of drug concentration (levels) in blood, plasma or serum is the most direct approach to assessing the pharmacokinetics of the drug in the body. Assuming that drug in the plasma is in dynamic equilibrium within tissue and then changes in the drug concentration in plasma will reflect changes in tissue drug concentration.

2. Drug concentration in Tissue

Tissue biopsies are occasionally done for diagnostic purpose, such as identification of malignancy. Usually only small sample of tissue is removed, making estimation of drug concentration difficult. Drug concentration in tissue biopsies may not reflect drug concentration in the other tissue or the drug concentration in all other parts from which the biopsy material has been removed.

3. Drug concentration in Urine

Measurement of drug concentration in urine is an indirect method to ascertain the bioavailability of drug.

The rate and extent of dug excreted in the urine reflects the rate and extent of systemic drug absorption.

4. Drug concentration in Feces

Measurement of drug concentration in feces may reflect that the drug has not been absorbed after oral dose or may reflect that drug has been expelled by biliary secretion after systemic absorption. Fecal drug level estimation is often done in mass balance studies.

5. Drug concentration in Saliva

Oral fluid is the proper term; however word saliva is used commonly. Saliva is a component of oral fluid. Oral fluid is composed of many components and concentrations of drugs typically parallel to those found in blood.

❖ **Sample preparation techniques**

a) Protein precipitation
b) Liquid liquid extraction
c) Solid phase extraction
i. Off - line solid phase extraction
ii. On – line solid phase extraction

1. Protein Precipitation

In protein precipitation, acids or water-miscible organic solvents are used to remove the protein by denaturation and precipitation. Acids, such as trichloroacetic acid (TCA) and perchloric acid, are very efficient at precipitating proteins. The proteins, which are in their cationic form at low pH, form insoluble salts with the acids. A 5– 20 per cent solution of these acids is generally sufficient and the best results can be achieved using cold reagents. Organic solvents, such as methanol, acetonitrile, acetone and ethanol, although having a relatively low efficiency in removing plasma proteins, have been widely used in bioanalysis because of their compatibility with high-performance liquid chromatography (HPLC) mobile phases. These organic solvents which lower the solubility of proteins and precipitate them from solutions have an effectiveness which is inversely related to their polarity.

Advantages

- Simple.
- Fast sample preparation
- Disadvantages:
- Drugs can precipitate with proteins.
- Sample is diluted, no pre-concentration steps.

2. Liquid–Liquid Extraction

Liquid–liquid extraction (LLE) is the direct extraction of the biological material with a water-immiscible solvent. The analyte is isolated by partitioning between the organic phase and the aqueous phase.

The distribution ratio is affected by a number of factors:

- Choice of extracting solvent
- pH of aqueous phase

The analyte should be preferentially distributed in the organic phase under the chosen conditions. Although a number of factors influence the choice of solvent, the most important factor is the relative lipophilicity or hydrophobicity of the analyte.

There are several disadvantages of LLE

- The technique is not applicable to all compounds. Highly polar molecules can be very difficult, although the use of an ion pairing reagent can extend LLE to molecules of this type.

Another major problem is the formation of emulsions. These can be difficult to break even using centrifugation or ultrasonification and can cause loss of analyte by occlusion within the emulsion. The use of less rigorous mixing or larger volumes of extracting solvent can help reduce the problem with emulsions.

- LLE is also not very readily automatable.

Solid Phase Extraction

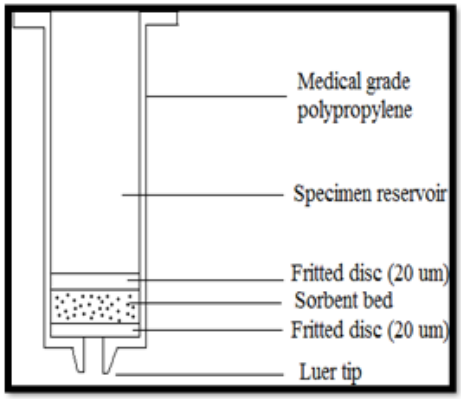

Figure 1.1: Typical SPE Cartridge.

The principal mechanisms of separation and isolation utilised in solid phase separation (SPE), reversed-phase, normal phase and ion exchange are the same as those used in HPLC. Although the mechanisms of separation for the two techniques are the same, the dynamics of each technique is very different. In HPLC the compounds are separated in a continuously flowing system of mobile phase, while SPE is a series of discrete steps. In SPE the analyte is retained on the solid phase while the sample passes through, followed by elution of the analyte with an appropriate solvent. SPE can be considered as a simple on/off type of chromatography. A typical SPE sorbent consists of a 40–60 mm silica particle to which has been bonded a hydrocarbon phase. This bonding is achieved by the reaction of a chlorosilane with the hydroxyl groups of the silica gel to form a silicon– oxygen– silicon link. This mono functional bonded phase was originally the most popular reversed-phase sorbent with 4, 8 or 18 carbon atoms attached (C4, C8 and C18).

As in the development of chromatographic stationary phases the manufacturers have tried to minimise the

effect of these silonal groups. The use of trifunctional derivatives, trichloroalkylsilanes, gives not only greater stability under acid conditions, because the hydrocarbon chain is attached at multiple sites, but reduces the number of free silanols. The number of free silanols is generally further reduced by endcapping, where the derivatised silica gel is reacted with a trimethylchlorosilane reagent. The disposable syringe barrel format of SPE contributed significantly to its success as a sample preparation technique. The syringes are available in 1–25 ml and packing weights from 25 mg to 10 g. The syringe barrel is typically polypropylene with a male Luer tip fitting. The sorbent material is packed between two 20 mm polypropylene frits. A vacuum manifold is used to draw the sample and eluting solvents through the syringe barrel under negative pressure by applying a vacuum to the manifold. Other types of sample processing that may be used include centrifugation and positive pressure.

The SPE is typically carried out using a five-step process:

1. Condition
2. Equilibrate
3. Load
4. Wash
5. Elute

The solid phase sorbent is conditioned by passing a solvent, usually methanol, through the sorbent to wet the packing material and solvate the functional groups of the sorbent. The sorbent is then equilibrated with water or an aqueous buffer. Care must be taken to prevent the phase from drying out before the sample is loaded, otherwise variable recoveries can be obtained. Samples are diluted 1:1 with aqueous prior to loading to reduce viscosity and prevent the sorbent bed from becoming blocked. Aqueous and/ or organic washes are used to remove interferences.

i. Off – line Solid phase extraction:

ii. Advantages

- Selective sample clean up.
- Sample concentration.

Disadvantages

- Labour intensive method development.

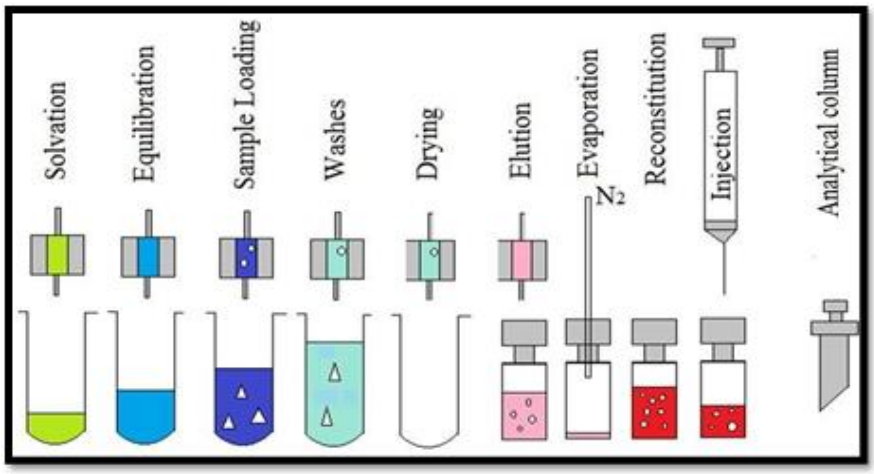

Figure 1.2: Schematic representation of off – line solid phase extraction.

BIONALYTICAL METHOD VALIDATION

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

Analytical methods need to be validated or revalidated

- Before their introduction into routine use;
- Whenever the conditions change for which the method has been validated (e.g., an instrument with different characteristics or samples with a different matrix).
- Whenever the method is changed and the change is outside the original scope of the method.

Bioanalytical method validation (BMV) includes all of the procedures required to demonstrate that a particular bioanalytical method for the quantitative determination of the concentration of an analyte (or series of analytes) in a particular biological matrix is reliable for the intended application. The most widely employed bioanalytical techniques include, but are not limited to, conventional chromatographic based methods (such as GC-ECD and HPLC-UV), mass spectrometry based methods (such as GC-MS and LC- MS), tandem mass spectrometry based methods (such as LC-MS-MS) and ligand- based assays (such as RIA and ELISA).

Different types and levels of validation are defined and characterized as follows

A. Full Validation

- Full validation is important when developing and implementing a bioanalytical method for the first time.
- Full validation is important for a new drug entity.
- A full validation of the revised assay is important if metabolites are added to an existing
- Assay for quantification.

B. Partial Validation

Partial validations are modifications of already validated bioanalytical methods. Partial validation can range from as little as one intra-assay accuracy and precision

- Bioanalytical method transfers between laboratories or analysts
- Change in analytical methodology (e.g., change in detection systems)
- Change in anticoagulant in harvesting biological fluid
- Change in matrix within species (e.g., human plasma to human urine)
- Change in sample processing procedures
- Change in species within matrix (e.g., rat plasma to mouse plasma)
- Change in relevant concentration range
- Changes in instruments and/or software platforms
- Limited sample volume (e.g., pediatric study)
- Rare matrices
- Selectivity demonstration of an analyte in the presence of concomitant medications
- Selectivity demonstration of an analyte in the presence of specific metabolites

C. Cross-Validation

Cross-validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. An example of cross validation would be a situation where an original validated bioanalytical method serves as the reference and the revised bioanalytical method is the comparator. The comparisons should be done both ways. When sample analyses within a single study are conducted at more than one site or more than one laboratory, cross- validation with spiked matrix standards and subject samples should be conducted at each site or laboratory to establish inter- laboratory reliability. Cross-validation should also be considered when data generated using different analytical techniques (e.g., LC-MS-MS vs.ELISA4) in different studies are included in a regulatory submission. All modifications should be assessed to determine the recommended degree of validation.

The process by which a specific bioanalytical method is developed, validated, and used in routine sample analysis can be divided into;

1) Reference standard preparation
2) Bioanalytical method development and establishment of assay procedure
3) Application of validated bioanalytical method to routine drug analysis and acceptance criteria for the analytical run and/or batch.

The following principles of bioanalytical method validation provide steps for the development of a new method or establishing an existing method in a particular laboratory for the first time. The parameters essential to ensure the acceptability of the performance of a bioanalytical method are accuracy, precision, selectivity, sensitivity, reproducibility and stability. All these parameters need to be defined during Full Validation of a bioanalytical method. Full Validation should be performed to support pharmacokinetic, bioavailability, bioequivalence and drug interaction studies in a new drug application (NDA) or an abbreviated new drug application (ANDA).

A. Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ). Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics. If the method is intended to quantify more than one analyte, each analyte should be tested to ensure that there is no interference.

B. Accuracy, Precision, and Recovery

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte.

Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between- run, interbatch precision or repeatability, which measures precision with time, and may involve different analysts, equipment, reagents, and laboratories.

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the

detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unrestricted standards that represent 100% recovery.

C. Calibration/Standard Curve

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the sample. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ.

1. Lower Limit of Quantification (LLOQ)

The lowest standard on the calibration curve should be accepted as the limit of quantification if the following conditions are met:

- The analyte response at the LLOQ should be at least 5 times the response compared to blank response.
- Analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%.

2. Calibration Curve/Standard Curve/Concentration-Response

The simplest model that adequately describes the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation should be justified. The following conditions should be met in developing a calibration curve:

- 20% deviation of the LLOQ from nominal concentration
- 15% deviation of standards other than LLOQ from nominal concentration

At least four out of six non-zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration. Excluding the standards should not change the model used.

D. Stability

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long- term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process.

1. Freeze and Thaw Stability

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions. The freeze–thaw cycle should be repeated two more times, and then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at -70^0C during the three freeze and thaw cycles.

2. Short-Term Temperature Stability

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

3. Long-Term Stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples.

The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back- calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

4. Stock Solution Stability

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

5. Post-Preparative Stability

The stability of processed samples, including the resident time in the autosampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size

in validation samples by determining concentrations on the basis of original calibration standards. Although the traditional approach of comparing analytical results for stored samples with those for freshly prepared samples has been referred to in this guidance, other statistical approaches based on confidence limits for evaluation of analyte stability in a biological matrix can be used. SOPs should clearly describe the statistical method and rules used. Additional validation may include investigation of samples from dosed subjects.

Specific Recommendations for Method Validation:

- The matrix-based standard curve should consist of a minimum of six standard points, excluding blanks, using single or replicate samples. The standard curve should cover the entire range of expected concentrations.
- Standard curve fitting is determined by applying the simplest model that adequately describes the concentration-response relationship using appropriate weighting and statistical tests for goodness of fit.
- LLOQ is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision. The LLOQ should be established using at least five samples independent of standards and determining the coefficient of variation and/or appropriate confidence interval. The LLOQ should serve as the lowest concentration on the standard curve and should not be confused with the limit of detection and/or the low QC sample. The highest standard will define the upper limit of quantification (ULOQ) of an analytical method.
- For validation of the bioanalytical method, accuracy and precision should be determined using a minimum of five determinations per concentration level (excluding blank samples). The mean value should be within ±15% of the theoretical value, except at LLOQ, where it should not deviate by more than ±20%. The precision around the mean value should not exceed 15% of the CV, except for LLOQ, where it should not exceed 20% of the CV. Other methods of assessing accuracy and precision that meet these limits may be equally acceptable.

The accuracy and precision with which known concentrations of analyte in biological matrix can be determined should be demonstrated. This can be accomplished by analysis of replicate sets of analyte samples of known concentrations QC samples from an equivalent biological matrix. At a minimum, three concentrations representing the entire range of the standard curve should be studied: one within 3x the lower limit of quantification (LLOQ) (low QC sample), one near the center (middle QC), and one near the upper boundary of the standard curve (high QC).

- Reported method validation data and the determination of accuracy and precision should include all outliers; however, calculations of accuracy and precision excluding values that are statistically determined as outliers can also be reported.
- The stability of the analyte in biological matrix at intended storage temperatures should be established. The influence of freeze-thaw cycles (a minimum of three cycles at two concentrations in triplicate) should be studied.
- Reinjection reproducibility should be evaluated to determine if an analytical run could be reanalyzed in the case of instrument failure.
- The specificity of the assay methodology should be established using a minimum of six independent sources of the same matrix. Method selectivity should be evaluated during method development and throughout method validation and can continue throughout application of the method to actual study samples.
- Acceptance/rejection criteria for spiked, matrix-based calibration standards and validation QC samples should be based on the nominal (theoretical) concentration of analytes. Specific criteria can be set up in advance and achieved for accuracy and precision over the range of the standards, if so desired.

DRUG PROFILE

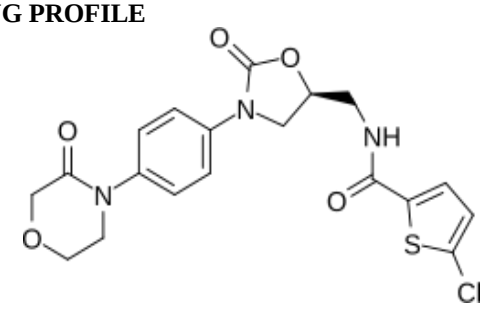

Fig. 1.3: Structure of Rivaroxaban.

- **Molecular Formula** – $C_{19}H_{18}ClN_3O_5S$
- **Molecular Weight** – 435.88 g·mol−1
- **IUPAC name** – (S)-5-chloro-N-{[2-oxo-3-[4-(3-oxomorpholin-4-yl) phenyl]oxazolidin-5-yl]methyl} thiophene-2-carboxamide
- **Description** – White to yellowish powder
- **Solubility** – Slightly soluble in organic solvents (e.g. acetone, polyethylene glycol 400) and is practically insoluble in water and aqueous media.
- **Category** – Antithrombotic agents.
- **Storage** – Store at 25 °C (77 °F) or room temperature; excursions permitted to 15 deg - 30 °C (59 deg - 86 °F).
- **Dose -** 15 to 30 mg daily
- **Plasma protein binding** – 92% to 95%
- **Bioavailability**: For 10 mg dose is >80%
- **Half-life** – 5-9 hours in adults and 11-13 hours in the elderly
- **Metabolism** – Approximately two-thirds of the dose is metabolized. It is metabolized by CYP3A4, CYP3A5, CYP2J2 and CYP-independant mechanism

- **Mechanism of Action**

Rivaroxaban competitively inhibits free and clot bound factor Xa. Factor Xa is needed to activate prothrombin (factor II) to thrombin (factor IIa). Thrombin is a serine protease that is required to activate fibrinogen to fibrin, which is the loose meshwork that completes the clotting process. Since one molecule of factor Xa can generate more than 1000 molecules of thrombin, selective inhibitors of factor Xa are profoundly useful in terminating the amplification of thrombin generation. The action of rivaroxaban is irreversible.

Rivaroxaban, an oral, direct activated factor X (Xa) inhibitor, is an anticoagulant. Factor Xa plays a central role in the blood coagulation cascade by serving as the convergence point for the intrinsic and extrinsic pathways; inhibition of coagulation factor Xa by rivaroxaban prevents conversion of prothrombin to thrombin and subsequent thrombus formation.

- **UV λmax – 271 nm**

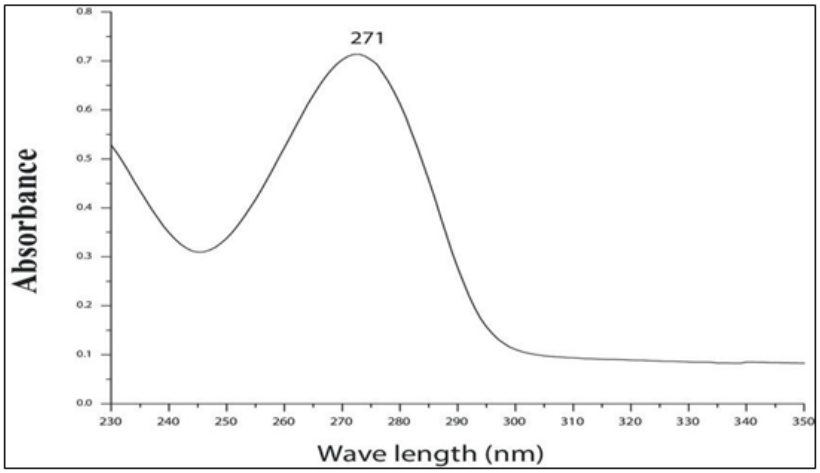

Fig.1.4: λmax of Rivaroxaban in DMSO.

LITERATURE SURVEY

From the literature survey, it was found that there are different methods available for the estimation of Rivaroxaban but nobody has done the bioanalytical method development in human plasma. It was best opportunity to select this method and drug respectively. Some of the reported methods are given below.

Table 1.1: Reported chromatographic methods.

Author & Year	Name of Drug & Study	Mobile Phase	Retention time	Flow Rate	Lambda max
Mustafa Çelebier, 2013	Rivaroxaban & RP- HPLC Simple	ACN:Water (55:45 v/v) mixture	3.37 min	1.2 mL min-1	249 nm
Hemlata Nimje, 2022	Rivaroxaban & RP- HPLC Stability Indicating Method	Water and Acetonitrile (45:55 v/v)	4.191min	1.2 ml/min	249 nm
R. Nageswara Rao, 2017	Rivaroxaban & RP- HPLC Simple	0.1% GAA : ACN in 30:70 %v/v	3.44min	1.1 ml/min	250 nm
Wanbing Rao, 2023	Rivaroxaban & RP- HPLC Stability Indicating Method	RIX Impurity	38 min	--	--
M. M. Eswarudu, 2020	Rivaroxaban & RP- HPLC Simple	Acetonitrile and water in the ratio of 50:50 (v/v)	4.893 min	1.0 ml/min	251 nm

RESULTS AND DISCUSSION

An ANALYTICAL METHOD DEVELOPMENT

1) Preliminary Analysis of Rivaroxaban

Preliminary analysis of Rivaroxaban such as description, solubility, and UV confirms the identification of Rivaroxaban as per available literature.

Solubility study

Slightly soluble in organic solvents (e.g. acetone, polyethylene glycol 400) and is pratically insoluble in water and aqueous media. DMSO showed the maximum absorption at 271 nm wavelengths but in Acetonitrile it shown 251 nm respectively.

1 Selection of analytical wavelength

- Name of drug : Rivaroxaban
- Concentration : 10 µg/ml
- Solvents : DMSO
- Scanned range : 200-400 nm
- Max Absorbance : 271nm

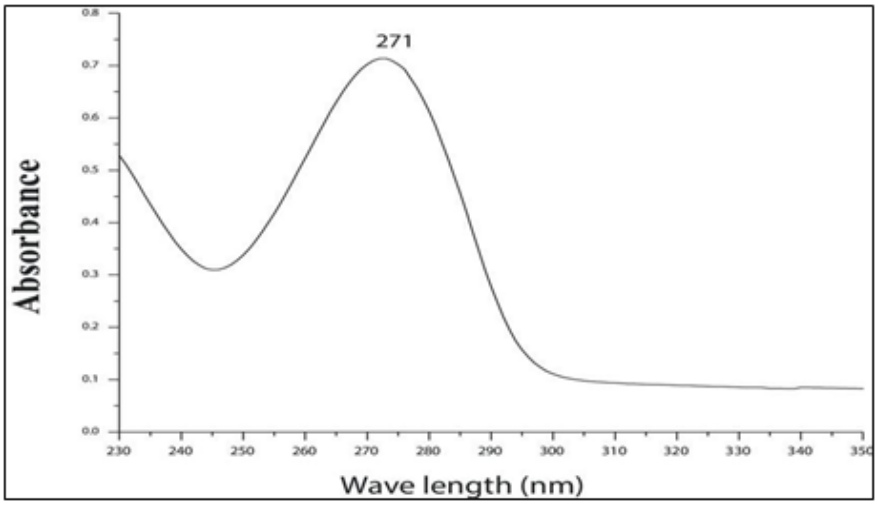

Fig. No 1.5: UV Spectrum of Rivaroxaban.

The maximum UV absorbance of Rivaroxaban was found at 251nm.

Analytical Method Development

The analytical method has been developed after several permutations and combinations of mobile phase with stationary phase. Table 6.1 represents the summary of several mobile phase compositions tried to optimise appropriate mobile phase composition for study. Various combinations showed peak asymmetry, theoretical plate less than 2000 and less retention time. Mobile phase consisting, acetonitrile: ammonium formate buffer pH-4.5 in the ratio of 78:22 v/v was selected as optimized mobile phase as it gave appropriate peak symmetry, theoretical plates and retention time.

Summary of chromatographic parameters selected

a) Column : Symmetry C18, 3.5µm, 250 mm x 4.6 mm
b) Mobile phase : Acetonitrile : Ammonium formate (78:22 v/v)
c) Flow rate : 1 ml/min
d) Detection wavelength : 251nm
e) Sample injector : 20 µl loop
f) Temperature : Ambient
g) Run time : 10 Min

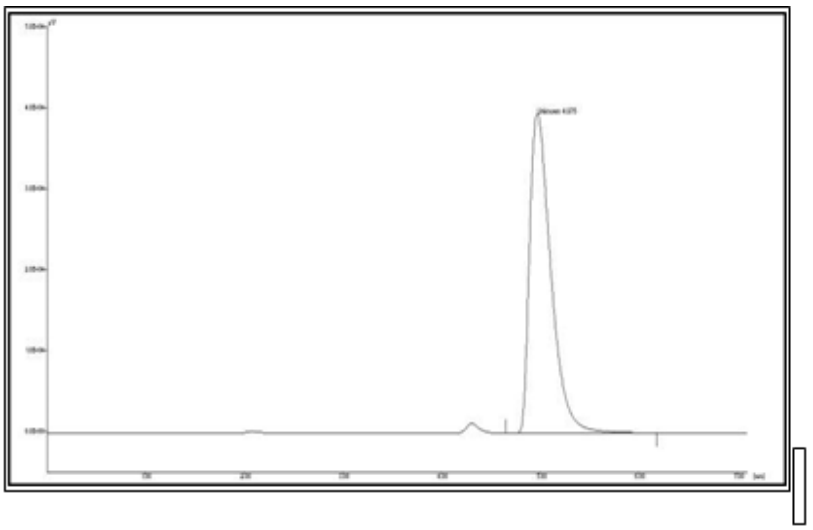

Fig. No. 1.6: HPLC chromatogram of Rivaroxaban (15µg/ml).

A BIOANALYTICAL METHOD DEVELOPMENT

2) Selection of mobile phase

Ammonium formate and acetonitrile was used in various proportions at different pH. After several trials, ammonium formate: acetonitrile was chosen as the mobile phase, which gave good resolution and acceptable peak parameters. The acetonitrile and ammonium formate buffer at 78:22 v/v gives good resolution peaks but retention time was at 3.9 very low. Then by decreasing buffer proportion finally retention time was set at 4.5 min. The mobile phase pH was tested under 3,4 and pH 3.5 presented the best retention. When the ACN content was greater than 10%, the retention time of Rivaroxaban was less than 3.0 which results interference of matrix peaks.

Table No. 1.2: Different Trials For Selection Of The Mobile Phase.

Mobile phase	Composition Ratio	pH	tR
Phosphate buffer: ACN	50:50	3.5	8min
Phosphate buffer: ACN	40:60	3.5	6.5 min
Phosphate buffer: ACN	30:70	3.5	5.8 min
Ammonium formate: ACN	22:78	4.5	4.4 min
Ammonium formate: ACN	30:70	4.5	4.1 min
Ammonium formate: ACN	30:70	3.5	3.9 min
Water: ACN	40:60	3.5	10 min
Water: ACN	30:70	3.5	8 min

3) Optimized chromatographic conditions

Mobile phase: Ammonium formate : Acetonitrile (22:78)
Analytical column: Symmetry C18, 3.5 µm (250x4.6) mm
Injection volume : 20 µl
Flow rate: 1 ml/min
Temperature: ambient
Run time: 10 min Retention time : 4.4 min

B. BIOANLYTICAL METHOD VALIDATION

1. System suitability parameters

Table No 1.3: System suitability parameters.

Sr. No.	Parameters	Rivaroxaban	Limit
1	Resolution	4.4	> 2
2	Column efficiency	6960	> 2000
3	Symmetry factor	1.54	< 2
4	Capacity factor	6.3	> 2

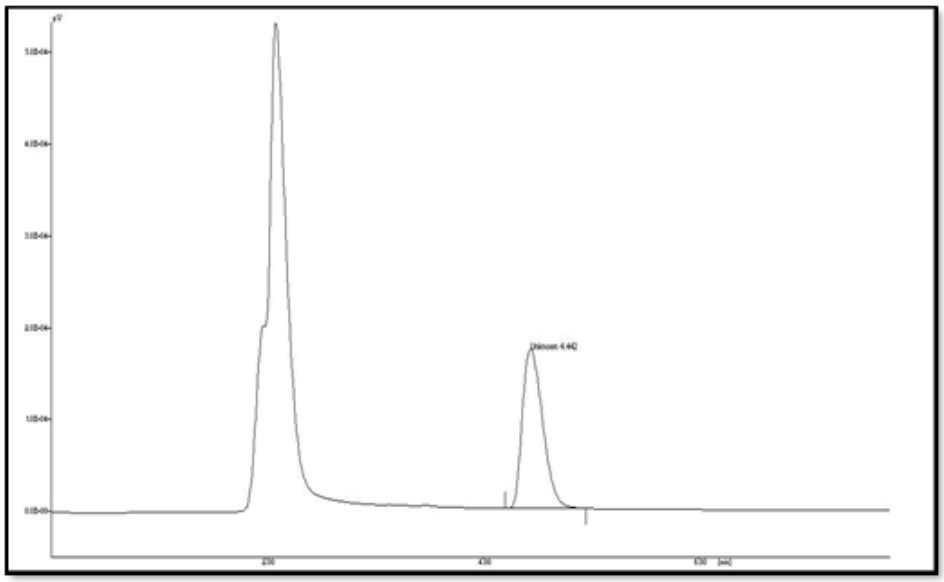

Fig No 1.7 Chromatogram of Rivaroxaban spiked in human plasma (10µg/ml)

Discussion: It was found that the all system suitability parameters are within the acceptance criteria and method is suitable for determination of Rivaroxaban from human plasma sample.

2. Selectivity

For selectivity, the blank samples of the plasma were obtained from six different persons. Each blank sample was tested for interference in Rivaroxaban peak. The plasma and Rivaroxaban peak were well resolved. Typical chromatogram of blank human plasma and spiked in human plasma are shown in Fig. 3.3 and 3.4

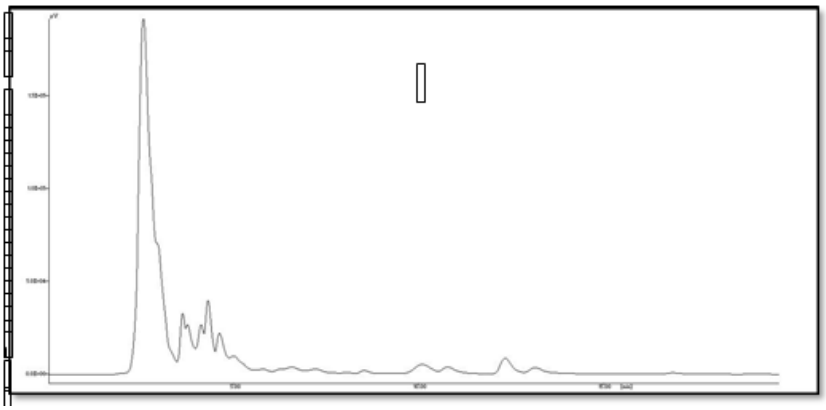

Fig. No. 1.8: A typical chromatogram of blank human plasma.

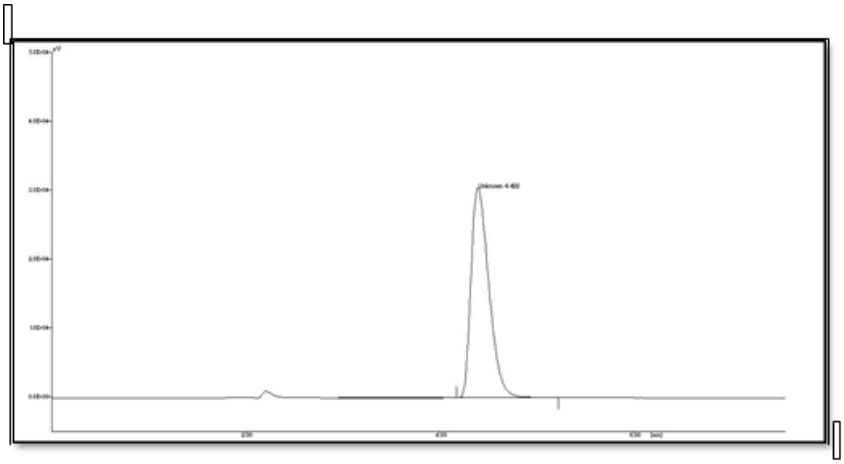

Fig. No. 1.9: A typical chromatogram of Rivaroxaban.

It was found that peak from blank plasma does not interfere with peak of Rivaroxaban. Hence developed method is selective and peak obtained at 4.4 is only because of Rivaroxaban.

3. Linearity and Range

Table No 1.4 Linearity of Rivaroxaban (API)

Sr. No.	Standard concentration (µg/ml)	Peak Area
1	5	183047
2	10	283509
3	15	391946
4	20	500193
5	25	618266

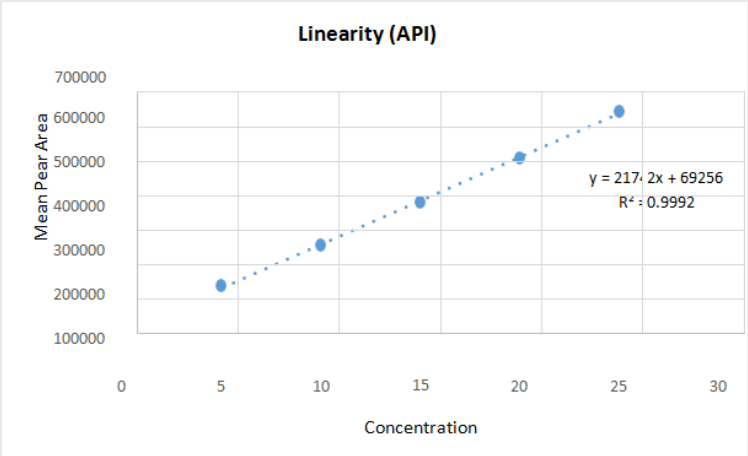

Fig No 1.10: Calibration curve for Rivaroxaban (API).

Table No 1.5 Linearity for Rivaroxaban spiked in human plasma.

Sr. No.	Concentration (µg/ml)	Mean Area
1	5	149500
2	10	214593
3	15	268979
4	20	352025
5	25	420820

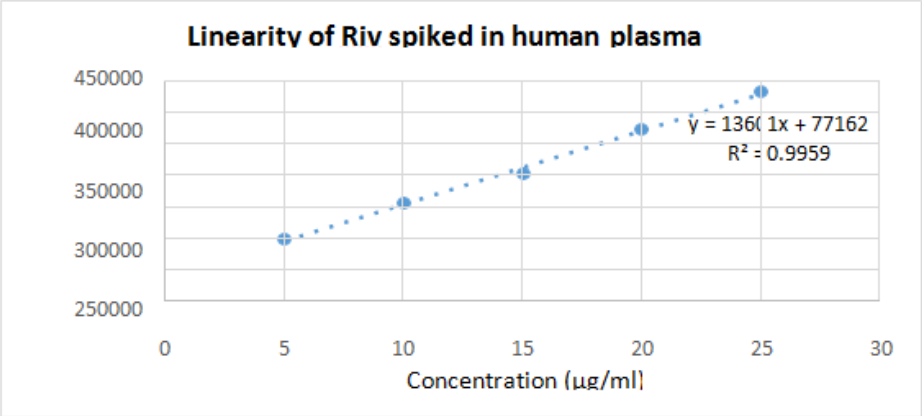

Fig No 1.11: Calibration curve of Rivaroxaban spiked in human plasma.

Discussion: Linearity range of Rivaroxaban was observed at 5, 10, 15, 20 and 25µg/ml. The equation of linearity for Rivaroxaban was y = 21742x + 69256. For all curves the correlation coefficient (r2) is more than 0.999. The linearity range was observed in between 25-150µg/ml.

For spiked human plasma Linearity range of Rivaroxaban was observed at 5, 10, 15, 20 and 25µg/ml. The equation of linearity for Rivaroxaban was y = 13601x + 77162. For all curves the correlation coefficient (r2) was 0.995.

4. Accuracy

Table No 1.6: Accuracy result of Rivaroxaban (8 µg/ml) (LQC).

Sr. No.	Conc. (µg/ml)	Area	% recovery	covered conc. (µg/ml)
1	8	194336	99.14	7.93
2	8	185138	90.5	8.52
3	8	202234	106.53	8.52
Mean				7.89
SD				0.64
% CV				8.10
% Accuracy				98.73

Table No. 1.7: Accuracy result of Rivaroxaban (16 µg/ml) (MQC).

Sr. No.	Conc. (µg/ml)	Area	% recovery	Recovered conc. (µg/ml)
1	16	301600	99.74	15.95
2	16	293857	96.12	15.38
3	16	309416	103.4	16.54
Mean				15.96
SD				0.5822
% CV				3.64
% Accuracy				99.75

Table No 1.8 Accuracy result of Rivaroxaban (24 µg/ml) (HQC)

Sr. No.	Conc. (µg/ml)	Area	% recovery	Recovered conc. (µg/ml)
1	24	401806	97.74	23.45
2	24	413976	101.54	24.37
3	24	401980	94.11	22.58
Mean				23.47
SD				0.8913

% CV	3.79
% Accuracy	97.80
Acceptance Criteria: The % Mean Accuracy for all the samples should be within 85.00 – 115.00 %.	

% Mean Accuracy = (mean of calculated conc./nominal conc.) x 100

Discussion: It can observed that all the obtained results of accuracy were satisfactory. The % mean accuracy lies between 88.00 – 106.00 %. Hence a good accuracy was observed by this method.

5 Precision Interday Precision

Table No 1.9 Interday precision result of Rivaroxaban.

A	DAY 1	DAY 2	DAY 3
	Concentration 8 µg/mL (LQC)		
1	194536	187807	189987
2	189145	190987	184928
3	192245	189234	183025
4	186087	188086	192851
5	179987	179987	190167
6	187895	185453	188042
Mean	**188282**	**186925**	**188166**
SD	**5037.3**	**3853**	**363.65**
% CV	**2.675**	**2.061**	**1.9337**
B	**Concentration 16 µg/mL(MQC)**		
1	301600	301587	298754
2	294876	295776	294536
3	298987	300123	294536
4	293334	298347	294332
5	295112	294434	301165
6	293223	295334	293564
Mean	**296188**	**297600**	**297392**
SD	**3374**	**2874**	**3728**
% CV	**1.139**	**0.965**	**1.25**
C	**Concentration 24 µg/mL (HQC)**		
1	403112	401587	398754
2	405876	410276	404536
3	403223	409123	404332
4	408987	408347	401165
5	402334	404434	405422
6	409112	406516	403564
Mean	**405440**	**405334**	**402962**
SD	**3040**	**3295**	**2581**
% CV	**0.75**	**0.810**	**0.62**

Intraday Precision

Table No 1.10: Intraday precision results of Rivaroxaban.

A	1st reading	2nd reading	3rd reading
	Concentration 8 µg/mL (LQC)		
1	194536	189452	187807
2	189145	183816	190987
3	192245	183259	189234
4	186087	192851	188086
5	179987	190579	179987
6	187895	188042	185453
Mean	**188282**	**187999**	**186925**
SD	**5037.3**	**363.65**	**3853**
% CV	**2.801**	**1.631**	**2.061**
B	**Concentration 16 µg/mL(MQC)**		
1	301600	298025	301587

2	294876	2940391	295776
3	298987	294805	300123
4	293334	294279	298347
5	295112	301851	294434
6	293223	293489	295334
Mean	**296188**	**296801**	**297600**
SD	**3374**	**3728**	**2874**
% CV	**1.537**	**1.35**	**1.28**
C	**Concentration 24 µg/mL (HQC)**		
1	403112	398754	401587
2	405876	404536	410276
3	403223	404332	409123
4	408987	401165	408347
5	402334	405422	404434
6	409112	403564	406516
Mean	**405440**	**402962**	**405334**
SD	**3040**	**2581**	**3295**
% CV	**0.91**	**0.71**	**0.85**

Discussion: It was found that the precision results were found satisfactory with respect to percent coefficient of variation (%CV) for all levels which were within the limit. The % coefficient of variation (%CV) of LQC sample is 1.31 % and t he % CV of MQC and HQC samples are 1.18 and 0.70% respectively. The developed method was précised for estimation of Rivaroxaban from human plasma.

6. Recovery

Table No 1.11 Recovery results of Rivaroxaban (8 µg/ml) (LQC)

Replicate	Peak Area of Standard drug	Peak area of drug Spiked in plasma
1	243509	194336
2	250012	204136
3	249221	195554
Mean	247580	198008
SD	3548	5341
% CV	1.43	2.69
% Mean Recovery	7	9.97

Table No 1.12 Recovery results of Rivaroxaban (16 µg/ml) (MQC)

Replicate	Peak Area of Standard drug	Peak area of drug Spiked in plasma
1	421946	301600
2	419963	298895
3	422015	301945
Mean	421308	300813
SD	1165	1670
% CV	0.27	0.55
% Mean Recovery	7	1.39

Table No 1.13 Recovery results of Rivaroxaban (24 µg/ml) (HQC)

Replicate	Peak Area of Standard drug	Peak Area of spiked plasma drug
1	598222	401806
2	594256	405345
3	589996	399321
Mean	594158	402157
SD	4113	3027
% CV	6.92	0.75
% Mean Recovery	67	68

% mean recovery = (mean area of spiked plasma / mean area of standard) x 100

Discussion: The percent recovery of Rivaroxaban from human plasma was found to be 79.97, 71.39, 67.68 for LQC, MQC and HQC level. The recovery of Rivaroxaban ease consistent, precise and reproducible.

7. Stability

a. Freeze and Thaw stability

Table No 1.14: Freeze and thaw stability result of Rivaroxaban (8 µg/ml) (LQC).

Replicate	Peak Area of standard sample	Peak Area of stability sample
1	7.89	7.01
2	8.06	7.6
3	8.1	7.4
Mean	8.01	7.33
SD	0.11	0.30
% CV	1.35	1.26
% Mean Stability	91.57%	

Table No 1.15: Freeze and thaw stability result of Rivaroxaban (24µg/ml) (HQC).

Replicate	Peak Area of standard sample	Peak Area of stability sample
1	24.18	23.2
2	24.64	23.01
3	23.52	22.5
Mean	24.11	22.90
SD	0.562	0.361
% CV	2.33	1.57
% Mean Stability	94.98%	

% mean stability = (mean of stability sample/mean of comparison sample) x 100

Discussion: Rivaroxaban did not show significant alteration in its concentration even after three cycles of freeze and thaw.

b. Short-term temperature stability

Table No 1.16: Short-term stability results of Rivaroxaban (8 µg/ml) (LQC).

Replicate	Peak Area of standard sample	Peak Area of stability sample
1	8.23	7.8
2	8.06	7.4
3	8.1	7.2
Mean	8.13	7.46
SD	0.08	0.30
% CV	1.08	1.30
% Mean Stability	91.75%	

Table No 1.17: Short-term stability result of Rivaroxaban (24 µg/ml) (HQC).

Replicate	Peak Area of standard sample	Peak Area of stability sample
1	24.01	22.01
2	24.23	22.98
3	23.96	22.45
Mean	24.06	22.48
SD	0.143	0.48
% CV	0.59	2.13
% Mean Stability	93.66%	

% mean stability = (mean of stability sample/mean of comparison sample) x 100

Discussion: According to observed results the concentration of Rivaroxaban in the biological sample did not show significant alteration in its concentration. Samples are stable upto 6 hrs.

c. Long-term stability

Table No 1.18: Long-term stability result of Rivaroxaban (8 µg/ml) (LQC).

Replicate	Peak area of standard sample	Peak area of stability sample
1	7.98	6.85
2	8.1	6.99
3	8.2	7.02
Mean	8.09	6.95
SD	0.11	0.09

% CV	1.35	1.29
% Mean Stability	85.9 %	

Table No 1.19 Long-term stability result of Rivaroxaban (24 µg/ml) (HQC).

Replicate	Peak Area of standard sample	Peak area of stability sample
1	24.02	21.04
2	24.12	21.2
3	24.2	21.5
Mean	24.11	21.14
SD	0.090	0.092
% CV	0.37	0.42
% Mean Stability	88.79%	

% mean stability = (mean of stability sample/mean of comparison sample) x 100

Discussion: The percent mean long-term temperature stability stability of Rivaroxaban in the biological sample did not show significant alteration in its concentration even after 7 days. After 21 days the concentration of Rivaroxaban decreased upto 30% within the time period under the indicated storage conditions, However the days.

d. Stock solution stability

Table No 1.20 Stock solution stability result of Rivaroxaban (8 µg/ml) (LQC).

Replicate	Peak area of standard sample	Peak area of stability sample
1	8.12	7.24
2	8.1	7.12
3	7.86	7.01
Mean	8.02	7.12
SD	0.144	0.11
% CV	1.80	1.61
% Mean Stability	88.74%	

Table No 6.20: Stock solution stability result of Rivaroxaban (24 µg/ml) (HQC).

Replicate	Peak area of standard sample	Peak area of stability sample
1	24.2	21.56
2	23.54	21.01
3	23.98	21.4
Mean	23.76	21.32
SD	0.38	0.282
% CV	1.60	1.326
% Mean Stability	89.74%	

% mean stability = (mean of stability sample/mean of comparison sample) x 100

Discussion: Rivaroxaban when stored at room temperature did not show significant alteration in its concentration; hence the standard stock solution can be stored upto 6 hrs after its preparation. Sample did not show significant alteration in its concentration after 6 hrs.

Table No 6.21: Summary of all validation parameter results.

Validation Parameters	Result
Linearity range (µg/ml)	5-25
Retention time (min)	4.4
Correlation coefficient (r2)	0.995
Intraday Precision (mean %CV)	7.327
Inter day Precision (mean %CV)	6.26
Mean Recovery (%)	73.01
Freeze and Thaw stability % Mean stability	93.27
Short-term temperature stability % Mean stability	92.70
Long-term stability % Mean stability	87.34
Stock solution stability % Mean stability	89.24

All the parameters of validation were found within the acceptance criteria as per USFDA guidelines. Thus, we conclude the developed bioanalytical method was suitable for determination of Rivaroxaban from human plasma and can be further applied in of pharmacokinetic study of Rivaroxaban.

CONCLUSION

Attempts were made to develop RP-HPLC method for Rivaroxaban HCl. RP-HPLC method was developed and validated as per USFDA guideline using Acetonitrile: ammonium formate buffer (78:22 v/v), pH 4.5 as mobile phase. Retention time of Rivaroxaban was found to be 4.4 min at the wavelength, 251 nm and flow rate was 1 ml/min. On the basis of results obtained we concluded that, A simple bioanalytical method was developed to quantify Rivaroxaban in human plasma. The validated method covered the wide range of linearity over 5-25 µg/ml and was therefore suitable for the determination of Rivaroxaban in human plasma at different therapeutic dose levels.

1. In the developed RP-HPLC method drug spiked plasma samples were prepared by protein precipitation technique.
2. The mobile phase used was Acetonitrile: Buffer (78:22 v/v) for the better separation of Rivaroxaban. The % mean recovery was found to be 87.47 %.
3. The developed method was simple, selective, accurate, precise, and cost effective.
4. The stability studies were carried out as per the US-FDA guidelines for bioanalytical method development. Rivaroxaban has been found to be stable when subjected under different stability conditions.
5. The proposed method can be applied to monitor urine concentrations of Rivaroxaban in pharmacokinetic studies. It can also be used for therapeutic drug monitoring in order to optimize drug dosage on an individual basis.

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