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A critical review of analytical methods for quantification of rivaroxaban in biological samples and pharmaceutical formulations

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ABSTRACT

Rivaroxaban (RXN) is a direct oral anticoagulant (direct inhibitor of factor Xa) used in the treatment of atrial fibrillation to reduce the risk of stroke caused by clot, periphery, and coronary heart disease. The foremost goal of the pharmaceutical drug is to exert its therapeutic action and cure the disease. Considering the therapeutic importance of RXN, this review focuses on the various spectrophotometric (UV) and chromatographic methods (thin-layer chromatography, high-performance thin-layer chromatography, high-performance liquid chromatography, ultra-performance liquid chromatography, liquid chromatography-tandem mass spectrometry, ultra-performance liquid chromatography-tandem mass spectrometry) available for the quality control and pharmacokinetic studies of RXN. The analytical methods published provide an overview of the various instrumental techniques that establishes the identity, quality, and purity of RXN. Stability-indicating methods anticipate the degradation products (DPs)/pathways of RXN which would help researchers to elucidate the structure of DP. The proposed review explores the greenness profiles of the reported analytical methods (2010–2024) based on the National Environmental Method Index in view of sustainable development goals 2030. The published bioanalytical methods provide a roadmap for potential researchers in developing more robust, efficient, and eco-friendly methods incorporating sorbent-based microextraction techniques to enhance biomedical research. Also, researchers incorporate analytical quality-based design (AQbD) which augments the quality and shrinks the out-of-trend and out-of-specification to expedite the regulatory flexibility in the analytical method. Overall this review illuminates the future canvassers to develop greener methods using eco-friendly solvents incorporating AQbD for the determination of RXN in biological fluids and drug products.

INTRODUCTION

Direct oral anticoagulants (DOACs) are widely prescribed in general practice to prevent clot formation. Rivaroxaban (RXN) an oxazolidine derivative is a novel DOAC usually referred to as a blood thinner agent which is extensively used to prevent deep vein thrombosis or pulmonary embolism. RXN is chemically 5-Chloro-N-({(5S)-2-oxo-3[4-(3-oxomorpholinyl)phenyl]-1,3oxazolidin-5-yl} methyl)2-thiophene carboxamide with an empirical formula of $C_{19}H_{18}C1N_3O_5S$ and 435.881g/mol as the molecular weight (Fig. 1). It was developed by Bayer and marketed by Janseen Pharmaceuticals, initially approved by FDA on July 1, 2011 [1–3]. Film-coated tablets of RXN are sold under the trade name Xarelto (2.5 mg, 10 mg, 15 mg, and 20 mg). It competitively inhibits free clot bound factor Xa and decreases the clotting ability of blood and prevents the formation of blood clots. In 2019, USFDA has approved RXN as an add-on drug with aspirin for secondary prevention of peripheral arterial disease and acute coronary syndrome [1,2,4]. The development of new analytical methods is flourishing up day by day due to the advancement of hyphenated analytical instruments. The advancement of analytical instruments has led to cost-effective methods with improved precision and

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accuracy. An extensive review of various databases such as Science Direct, Springer, Pubmed, Scopus, Taylor & Francis, and Web of Science was accomplished. The published review lacks the detailed information required for the researchers with respect to the various published methods (Analytical, stability indicating, and bioanalytical methods), their merits and demerits, extraction techniques involved in the bioanalytical method, green assessment of the solvents used in various techniques [5]. Hence the present review surpasses the disadvantages of the existing review and embraces the future investigators with the overview of numerous simple and sophisticated analytical/bioanalytical methods for estimating RXN in different formulations (single and combined dosage forms) and biological matrixes. Also, since most of the methods [high-performance liquid chromatography (HPLC), HPLC/ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)] developed for the estimation of RXN is stability indicating, a special emphasis on the forced degradation conditions and their results are incorporated separately which in turn help to improve the formulation, manufacturing process and determine the storage conditions of RXN. Bioanalytical methods determine the concentration of RXN and its metabolites in biological fluids, i.e., blood, serum, plasma, and urine by HPLC, HPLC/UPLC-MS/MS. Challenges associated during method development include employing suitable extraction techniques such as solid phase extraction (SPE), liquid–liquid extraction (LLE), and protein precipitation to extract RXN from the complex composition of a biological sample (presence of many interfering substances). This review discusses the pros and cons of extraction techniques essential to removing biological matrices and the applicability of the method. In addition, prominence is sited on the sustainability of the reported methods and assessment of greenness profiles as per the National Environmental Method Index (NEMI). Last, a comprehensive view of all the published analytical methods, consisting of chromatographic conditions and the results obtained, merits/demerits, application of the bioanalytical methods along with the greenness profile, is outlined in tabular form for a quick summary. Figure 2 reveals the different analytical methods for the estimation of RXN in single and combined dosage forms from 2012 to 2023. The graphical representation of a number of articles published from 2012 to 2023 is provided in Figure 3.

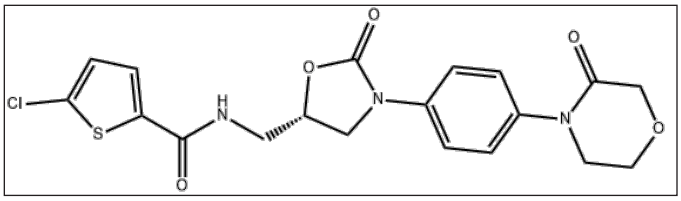


Figure 1. Chemical structure of RXN.

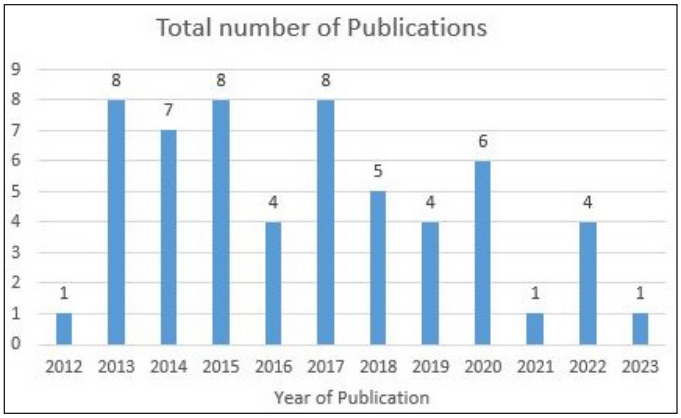


Figure 2. Total number of analytical methods reported during the year 2012–2023. Databases: Science Direct, Springer, Pubmed, Scopus, Taylor & Francis and Web of Science.

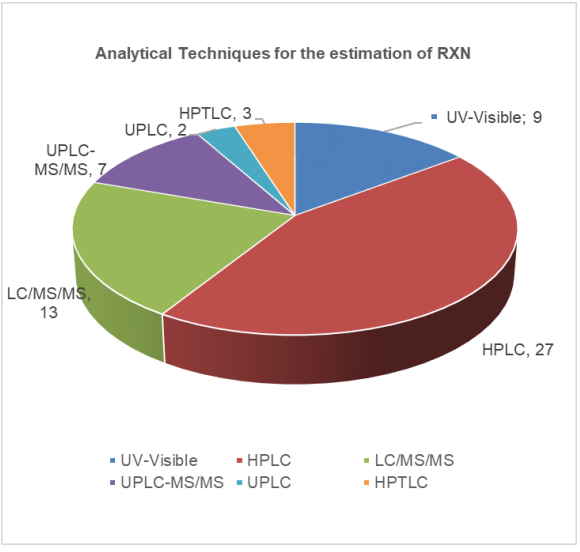


Figure 3. Analytical methods for the estimation of RXN.

ANALYTICAL METHODS FOR DETERMINATION OF RXN

Spectrophotometry

Ultra- violet (UV)-Visible spectrophotometry is a simple, non-destructive, cost-effective versatile technique used to analyse pharmaceutical substances. Because of its simplicity, precise, reliability, and minimum solvent usage, it is widely used for the estimation of RXN. Sekaran *et al.* [6] developed a novel method using dimethyl sulphoxide as the diluent and reported the apparent molar absorptivity (4.825 × 104 l/mol/cm) and Sandell's sensitivity (2.262 × 10^{-3} µgcm^{-2}) which represents the sensitiveness of the method (Fig. 4). Mustafa *et al.* [7] quantified RXN and also estimated the rate of drug release by *in vitro* study (Dissolution—USP Apparatus 2) medium. The drug release was 78% at 15 minutes using pH 4.5 acetate buffer containing 0.4% of sodium dodecyl sulfate as the dissolution medium. Muralikrishna *et al.* [8] reported a simple, sensitive, reproducible, and accurate method with methanol as the diluent. By using methanol as the diluent Kasad Pinaz *et al.* [9] reported a novel Area Under Curve method which would be better than zero-order UV spectrophotometric methods. Seshamamba *et al.* [10] reported derivative spectroscopy

with two different reagents, 4-Chloro-7-nitrobenzo-2-oxa-1, 3-diazole (Method I) and p-Dimethylaminocinnamaldehyde (Method II) for the precise and accurate determination of RXN in bulk and in its tablet dosage forms. El bagary *et al.* [11] reported a thermodynamic simultaneous method for the estimation of cilostazol and RXN based on the oxidation of iron (III) in the presence of 1, 10-phenanthroline to form tris (1, 10-phenanthroline) iron (II) complex (ferroin). The authors evaluated the thermodynamic parameters (free energy, enthalpy, and entropy) of complexation, also estimated the molar absorptivity (53.02×10^3 and 1.28×10^3 l/mol/ cm) and sandell sensitivity (0.0069 and 0.28 µg/cm^{-2}) for cilostazol and RXN, respectively. Simultaneous estimation of RXN and Clopidogrel (CPL) using three UV Spectrophotometric methods (First derivative spectrophotometric method, ratio derivative spectrophotometric method, and absorbance ratio method) with methanol as the diluent was reported by Sharaf *et al.* [12] The reported methods were novel and distinct from each other, whereas methanol is the solvent of choice in most of the methods. The different methods of analysis were Area under the Curve, Derivative spectrophotometry, and thermodynamic method which indicated the applications and importance of the UV spectrophotometric method for the Quality Control of RXN

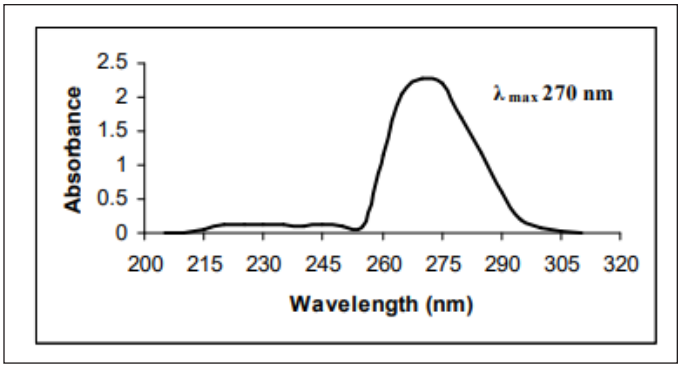


Figure 4. UV spectrum for RXN (adapted from reference 7).

Table 1. Spectrophotometry methods for determination of Rivaroxaban.

S.No	Method/Matrix	Spectrophotometry Method		Results				Ref no
		λ max (nm)	Diluent	Accuracy (%)	Linearity range (µg /ml) / R^2	Precision % RSD	LOD/ LOQ (µg /ml)	
1	UV-visible bulk & tablet	270	DMSO	99.82 – 100.50%	2–20/0.999	0.074%– 1.076%	0.212/0.642	Sekaran *et al*. [6]
2	UV-visible tablet dissolution	248	acetonitrile:Water(50:50 v/v) Acetate buffer with 0.4% SDS (pH 4.5)	102.38	0.5–5/0.9997	0.5– 50/0.9997	0.3/0.5	Celebier *et al*. [7]
3	UV-visible bulk & tablet	248.6	Methanol	100.85 %	2–12 µg/ ml/0.9998	0.08–0.915	0.09842/ 0.2982	Murali krishna *et al*. [8]
4	UV visible	241–260	Methanol	99.31%	2–12/0.999	0.537 % –0.297 %	0.059/0.179	Kasad Pinaz *et al*. [9]
5	UV-visible bulk & tablet	405	4-chloro--nitrobenzo-oxa-1,3-diazole in alkaline medium	99.74 - 100.51	2–20 µg/ml	0.621%– 0.900%	0.110 µg/ml	Seshamba *et al*. [10]
		545	p-Dimethyl aminocinnamaldehyde in acidic medium		25–125 µg/ml		0.483 µg/ml	
6	UV-visible tablet	510 ± 1 nm	1, 10-phenanthroline and formation of tris (1, 10-phenanthroline) iron (II) complex (ferroin)	NA	Cilastazol – 0.5–10 RXN – 30–400	NA	Cilastazol–0.046 RXN–4.719	El bagarey *et al*. [11]
7	Simultaneous estimation RXN and CPL first derivative spectrophotometric method	RXN :289 CPL:249.5	Methanol	NA	RXN :2.0–20.0 CPL:5.0-60	NA	RXN :0.211 CPL:0.361/ RXN:0.641 CPL:1.095	Sharaf *et al*. [12]
	Ratio derivative spectrophotometric method	RXN :256 CPL: 214.5	Methanol	NA	RXN : 2.0–20.0/ CPL: 5.0-60	NA	RXN: 0.137 CPL:0.485/ RXN:0.417 CPL :1.471	
	Absorbance Ratio method	RXN :232 CPL :249	Methanol	NA	RXN : 2.0–20.0 CPL:5.0-60	NA	RXN:0.272 CPL:0.485/ RXN:0.826 CPL:1.471	

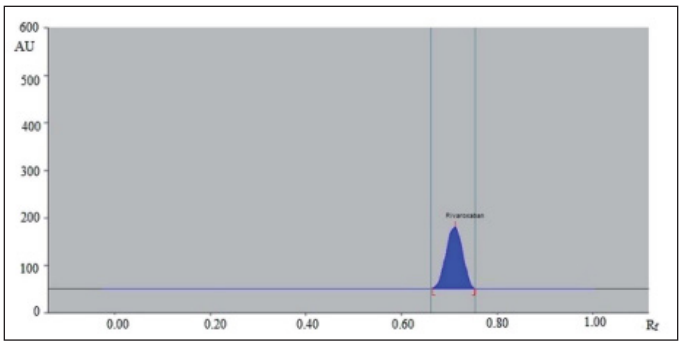


Figure 5. HPTLC densitogram of standard RXN (adapted from reference 15).

in pharmaceutical dosage forms [6–12]. The method and the spectrophotometric results were tabulated in Table 1.

Chromatography methods

High-performance thin-layer chromatography (HPTLC)

HPTLC an effective tool in qualitative analysis, is rapid and economically friendly compared to the hyphenated techniques. Prawez *et al.* [13] developed an eco-friendly method using RP-18 Silica gel 60 F254S as the stationary phase and Ethanol: Water (7:3 v/v) as the mobile phase using a densitometric detection at 253 nm which resulted in an Rf of 0.71 minutes. The method was highly expedient with a shorter run time, minimal sample preparation, and excellent recovery of 99.20 % for the nano formulation (Fig. 5). Darshna *et al.* [14] developed a simple, specific, and cost-effective HPTLC method using Silica Gel 60 F254 under pure nitrogen stream as the stationary phase and Methanol: Toluene: Triethanolamine (7: 2.5:0.5 v/v) as the mobile phase. RXN was detected at 249 nm with an Rf of 0.60 minutes, linearity of 500–3,000 ng/spot, the limit of detection (LOD), and limit of quantification (LOQ) of 127.56 ng/spot and 386.57 ng/spot, respectively. The additives in the pharmaceutical formulations of the assayed analyte did not interfere with the determination of RXN. Both methods used an exclusive novel stationary phase, though the method showed a good recovery without compromising the usage of green solvents [13,14].

HPLC

HPLC, the most prevalent chromatographic method due to its simplicity, versatility, and broad scope. RP-HPLC uses a monolithic stationary phase and mixtures of organic solvents with buffers containing acidic/basic additives as mobile phases to separate moderately hydrophilic/hydrophobic compounds. Amelia *et al.* [15] Sahoo and Mekap *et al.* [16] and Eswarudu *et al.* [17] developed and validated a sensitive and accurate RP-HPLC method for the estimation of RXN. The chromatographic conditions of all three methods were similar, whereas optimization was done by varying the ratio of the mobile phase (Acetonitrile: Water). A sensitive and less time-consuming method with good recovery was achieved by Amelia *et al.* [15] Sunny *et al.* [18] developed an Ion Pair-RP-HPLC method using triethylamine as the ion pair reagent which improved the separation efficiency and peak shape of RXN. The method was cost-effective (Triethyl Amine Buffer (pH: 11.06): Acetonitrile (85:15 v/v)) and less time-consuming (R_t 1.56 minutes) than the reported RP- HPLC methods. A sensitive method using a novel peak additive (Glacial acetic acid) in the mobile phase to improve the peak shape and efficiency of the method was developed by Meenakshi and Nageswara [19] Shivsankar *et al.* [20] developed an accurate, sensitive, precise, linear method but with a longer retention time (Rt) of 7.4 minutes. However, optimization of the mobile phase ratio would have resulted in a rapid method. Chandra shekar *et al.* [21] using the C18 column coupled with the guard column developed an RP-HPLC method that eluted RXN at 3.32 minutes. Awatade *et al.* [22] reported a method with a shorter run time and performed the qualitative analysis by IR Spectroscopy. A cost-effective and sensitive method using Buffer : Acetonitrile (70 : 30) as the mobile phase and C18 column was developed by Khan *et al.* [23] Chromatographic results obtained from Simultaneous estimation by Rupali *et al.* [24] (RXN with CPL) and Sarkis *et al.* [25] (RXN with aspirin) resulted in a rapid, simple, reliable, precise and robust methods. The majority of the methods used C18 as the column with isocratic mode by use of different buffers (Acetate and phosphate buffer), peak modifiers (Triethyl amine, glacial acetic acid), varying the aqueous/organic composition (Water, Buffer/Acetonitrile, methanol), pH (Acidic/Basic) of the mobile phase and detected RXN by UV/PDA detector (220–280 nm). Researchers to develop a method employing eco-friendly solvents that would meet the SDGs. Analytical quality by design (AQbD), an extension of QbD offers a robust approach in the development of analytical methods leading to higher quality products and improved patient safety. An exclusive AQbD method by Santos *et al.* [26] for the enantiometric estimation of S - RXN and its chiral impurity R- RXN by RP-HPLC using a chiral stationary phase. The method was selective, linear, precise, accurate, and sensitive with a LOQ of 0.68 µg/ml for S-RXN and 1.0 µg/ml for the chiral impurity [15–26]. The chromatographic condition of the RP-HPLC methods and their results are presented in Table 2.

STABILITY INDICATING METHOD

An ideal stability-indicating method discriminates the analyte and its degradation products (DPs) efficiently. Forced degradation studies aids in synthesis, formulation development, and packaging where knowledge of chemical behavior may be applied to enhance a therapeutic product [27].

UV Spectrophotometry

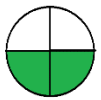
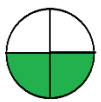
UV spectrophotometry is a cost-effective method that can be used for stress degradation studies of RXN and its degraded products. Girishchandra *et al.* [28] developed an accurate and reliable method in which the assay limit of RXN was as per the WHO limit (NLT 97 % and NMT 101.05 %). RXN degrade much especially on exposure to UV light and acidic medium but do not degrade in basic medium.

Chromatography methods

HPLC

HPLC methods can separate, detect, and quantify the various drug-related degradants of RXN formed on

Table 2. Estimation of Rivaroxaban by RP-HPLC.

S.No	Method	Chromatographic Conditions				Results					Greeness profile	Merits/ Demerits	Ref. no
		Stationary Phase	Mobile Phase	Flow rate mL/ minute	Temperature (°C)	Detector/ detection wavelength (nm)	Rt (minute)	Linearity range (µg/ml)/ R2/ %RSD	% Recovery	LOD/ LOQ			
	RP-HPLC	C18 (250 × 4.6 mm,5 µm)	Acetonitrile: water (80:20 v/v)	1	40	UV/247	2.986	10-70/0.996/<2%	99%–100%	0.009381/0.07862		Sensitve method with shorter run time	Amelia *et al.* [15]
	HPLC	Phenomene× C18 (250 × 4.6 mm, 5 µm)	Acetonitrile : Water (55:45 v/v)	1.2	35	UV/251	3.8	5.0–40	99.0	NA		Simple, linear, accurate method	Suraj and Mekap [16]
	HPLC Method	C18 (250 × 4.6 mm, 5 µm)	Acetonitrile: Water (50:50)	1	NA	PDA/251	4.893	5 –50/0.9992/	98.84	0.054/0.164/0.2		Simple, linear method with PDA detection	Eswarudu *et al.* [17]
	HPLC	Agilent Eclipse C8 LC (150 × 4.6 mm, 5 µm)	Triethyl Amine Buffer (pH:11.06) : Acetonitrile (85:15 v/v)	1.2	25	UV/251	1.56	500–1/0.999 /<2	98–102.4	0.675/0.0786		Use of Peak modifiers, very less time consuming method, One and only method with C8 as the stationary phase	Sunny *et al.* [18]
	RP-HPLC	C18 (250 × 4.6 mm, 5 µm)	0.1% Glacial acetic acid : Acetonitrile (30:70v/v)	1	NA	PDA/250	3.44	2–10/0.9993/<2%	98%–102%	0.008/0.248		Acetic acid improves the peak shape and efficiency of the method	Meenakshi *et al.* [19]
	RP-HPLC	C18 (250 × 4.6 mm, 5 µm)	Potassium di hydrogen phosphate Buffer (PH3): ACN (60:40%)	1	NA	UV/248	7.45	1–5/ 0.9978/<1	100.8	0.093/0.2768		Sensitive method with a longer run time	Shivasankar *et al.* [20]
	RP-HPLC Method	C18 (250 × 4.6 mm,5 µm) coupled with Silica guard column	Acetonitrile: Methanol : Ortho phosphoric acid (90:8:2) pH : 4.06	1.5	NA	UV/234	3.32	50 –200/0.997/0.47 – 0.609	99.708	0.75/2.47		Only method coupled with guard column with a greater flow rate	Shekar *et al.* [21]
	HPLC Method	Sun Q C18	Acetonitrile : Buffer 80 : 20 (Sodium Acetate)	1	ambient	UV/249	3.69	5–30/0.994	100.941	0.439/1.33/0.288		Efficient column at low pH	Awatade *et al.* [22]

Continued

HPLC Method	Hypersil ODS (250 × 4.6 mm, 5 µ)	Buffer : Acetonitrile (70 : 30)	1	30	UV/250	6.177	0.101–24.139/0.9997	99.54	0.004/0.006/0.53		Sensitive cost effective method with good recovery	Khan *et al.* [23]
Simultaneous estimation RXN and CPL	BDS Hypersil C18 (250 mm × 4.6 mm, 5 µ)	Buffer (0.05M KH_2PO_4 pH 4.0) : methanol (30:70)	1	25	UV/220	RXN: 2.39 CPL: 4.40	50–100/0.999/ CPL:0.487–0.760 RXN:0.53–0.596	RXN: 99.73%–101.07% CPL: 100.05%–101.38%	NA		Eco friendly simultaneous method with good recovery	Sajjanwar *et al.* [24]
Simultaneous Estimation of Aspirin and RXN	S.P: C18 (250 × 4.6 mm, 5 µm)	Acetonitrile : Water (65 : 35) pH 5.6 with acetate buffer	1.1	27	UV/245	Aspirin 2.1, RXN 3.2	Aspirin: 10-600 RXN: 10%–100 % R2>0.999 RSD NMT 2	Aspirin 99.61 RXN: 101.28	Aspirin: 18.31/55.47 RXN: 1.69/5.11		Shorter run time with good recovery	Sarkis *et al.* [25]
QbD approach-chiral approach	Chiralpak AD-RH (150 × 4.6 mm, 5 µm)	Acetonitrile: Water: pH-4.5 (92 : 8)	0.35	40	UV/250	NA	NA	99.88 % Chiral cpd – 1.0 µg/ml	LOQ-s-RIV- 0.68 µg/ml		Robust DOE enhanced method for chiral seperation	dos Santos *et al.* [26]

storage/manufacturing [29,30]. Hyphenated techniques (HPLC/UPLC–MS/MS) have become a golden standard in the characterization and quantitative analysis of degradants with improved selectivity, sensitivity, and efficiency. The chromatographic conditions of the stability-indicating methods are represented in Table 2. Kasad Pinaz *et al.* [31] developed and validated a simple, cost effective, less time-consuming, precise and accurate method in which the acidic (3 DP), basic (4 DP), photolytic, and thermal degradation peaks was clearly separated from RXN peak. However, characterization of the degradants would help researchers to understand the degradation pattern of RXN. Seshamamba *et al.* [32] Souri *et al.* [33] Zareen *et al.* [34] Çelebier *et al.* [35] performed a sensitive method with UV detection and isocratic elution. The methods were efficient in detecting the degradants. Seshamamba *et al.* [32] used a cyano column which has led to an efficient and sensitive than the other methods. Souri *et al.* [33] proposed a stability-indicating method that revealed the unstability of RXN in stress degradation conditions, also reported the dissolution profile which indicated >90% release of RXN within 45 minutes (Dissolution medium: 0.6% sodium lauryl sulfate in acetate buffer, Apparatus: paddle at 75 rpm). Walter *et al.* [36] developed a specific and stability-indicating method with an accuracy of 99.77%, hence the method can be applied for the analysis in human plasma and tablet dosage forms. Girase *et al.* [37] Badroon and Sreeramulu *et al.* [38] Sachin *et al.* [39] reported a stability-indicating analytical method using gradient elution and diode array detector (DAD) detection. The peak obtained during the forced degradation condition was homogenous and unaffected by degradation impurities [38]. Monitoring of the commercial route of synthesis and impurity profiling which is very important for the quality of RXN by RP-HPLC was performed by Girase *et al.* [37] and Sachin *et al.* [39] RXN was found to degrade significantly in Acid condition, but impurities (1–10) were well separated with an less RRT and the mass balance of all the peaks was found to be satisfactory. The method developed by Sachin *et al.* [39] eluted all the potential impurities of RXN (Impurity A-H) within 14 minutes [31–41].

UPLC

Ultra-performance liquid chromatography (UPLC) using shorter columns improves analyte resolution and sensitivity, lowers solvent consumption, and shortens run times. Rajan and Anver Basha *et al.* [42] and Rao *et al.* [43] reported a UPLC-DAD method for the estimation of RXN using gradient elution. Both the authors characterized the impurities. Rao *et al.* [43] developed a novel, rapid, stable, sensitive ion pair - UPLC - DAD method using ion pair reagents (octane sulphonic acid sodium) as the mobile phase for estimating RXN in tablet dosage forms. The anionic pair reagent improves the peak shape and retention of RXN and the impurities were characterized. Jabaliya *et al.* [44] transferred the HPLC method to UPLC owing to increased resolution, sensitivity, and reduction in run time [42–44]. The results of UPLC indicated an accurate, precise, linear method than the developed HPLC method. The method would be a great advantage if the impurities were characterized by UPLC-DAD.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS is an ideal technique for detecting nanomolar/picomolar quantities of various drugs, degradants, and food metabolites. Cholleti *et al.* [45] developed and validated an HPLC method and identified the degradants by LC-MS/MS method which separated enantiomers and all the process-related impurities of RXN. Arous *et al.* [46] investigated and found RXN susceptible to acid and base hydrolytic stress conditions. The authors identified, isolated, and characterized the degradation pathways using LC-MS, thin layer chromatography (TLC)-densitometry, NMR spectroscopy, and FT-IR. Three major *DPs* (DP-1, DP-2, DP-3) were detected, separated, and determined and two (DP-2 and DP-3) of them were further characterized by NMR spectroscopy and FT-IR. Ramisetti and Kuntamukkala *et al.* [47] presented a simple, rapid, and isocratic stability indicating HPLC method for the determination of RXN which detected 3 *DP*s formed under stress conditions. The structures of Process related impurities and the *DPs* (DP-1 and DP-3) were quantified by high-resolution ESI-MS/MS analysis and characterized by NMR spectroscopy (Process-related impurities and DP-3 [45–47].

The stability-indicating methods reported were highly sensitive with shorter Rt indicating a cost-effective green approach (Table 3). Most of the methods were stability indicating and detected the degradants employing a gradient program; however, characterization of the degradants formed in the HPLC method could be a better way ahead in these stress degradation studies. Characterization of impurities of RXN and degradation pathways by LC-MS/MS methods establishes biological safety which reveals the need and scope of impurity profiling of RXN in pharmaceutical research.

COMBINED ANALYTICAL TECHNIQUES

RXN is estimated by combined analytical techniques to indicate the accuracy of a method with other methods. These articles provide us the collective information in a single paper. Lories *et al.* [48] reported a HPLC, TLC, and UV method for the estimation of RXN in single dosage form. Method A : Stability indicating analytical method—HPLC method with phenomenex-C18, (150 × 4.6 mm, 5 µm) column and 1.2% w/v Potassium Dihydrogen Phosphate pH 3.5 ± 0.2 and Acetonitrile (70:30, v/v) as the mobile phase at a flow rate of 1.5 ml/minute with UV Detection at 280 nm to detect the impurities. RXN, Timolol Maleate degradant and Bimatoprost degradant eluted at 8.5, 5.3, and 4.2 minute, respectively. The Correlation Coefficient (R^2), % recovery, LOD, and LOQ of Timolol Maleate degradant and Bimatoprost degradant were 0.999, 100.84, 0.62 µg/ml, 0.65 µg/ml, and 0.999, 99.67, 1.3 µg/ml, 3.15 µg/ml, respectively. Method B: TLC method used precoated Silica Gel GF254, 0.25 mm as the stationary phase and Chloroform: Isobutyl Alcohol (50:50 v/v) as the mobile phase with Densitometric detection at 280 nm. The R_f of RXN was 0.60 ± 0.02 with a % RSD of 0.50–4.80 µg/spot. Method C: First derivative method at a wavelength of 237.4 nm was linear over the range of 1.6–22.4 µg/ml. Method D: First derivative ratio method using acetonitrile as the solvent at a wavelength of 236 nm showed a R^2, %RSD, %Recovery, LOD, LOQ of 0.9999, 1.072 %, 99.99 ± 0.242, 0.52 µg/ml and 1.85 µg/ml [48]. HPLC method emerged to be the sensitive and effective method in detecting the degradants at a short run time. Combinatorial methods help researchers explore the pros and cons of various methods from multiple angles and get more confined results.

BIO ANALYTICAL METHODS

Bio-analytical method development is useful in the identity, purity, potency, and bioavailability of drugs. The data that are obtained from bioequivalence and pharmacokinetic studies are required for the successful evaluation of drugs in animal models and human trials. The development of effective bio-analytical methods is therefore crucial for the successful implementation of drug development. Hyphenated techniques have turned out to be quintessential in the assessment of pharmaceuticals in various biological samples. Bio-analytical methods employing HPLC/UPLC/hyphenated techniques are ephemeral and assist in controlling the quality of pharmaceuticals as well as in obtaining pharmacokinetic and toxicokinetic data of RXN in single and combined dosage forms. RXN was extracted from biological samples (Rat Plasma, human plasma/serum, patient samples) employing SPE, LLE, and protein precipitation. Numerous variations depending on the nature and amount of solvent/matrix in the extraction method will be optimized to achieve a better separation of RXN from the biological matrix. Structurally related internal standards used in the research were chloramphenicol, Prednisolone, carbamazepine, Apixaban (AXN), Diazepam, and Risperidone. Few researchers have used stable isotopically labeled (SIL) internal standards and deuterated internal standards which yield robust and reproducible results compared to structurally related internal standards.

UV Spectrophotometry

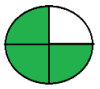
UV Spectrophotometry has emerged as an effective method for estimating the analyte from biological samples. Bhavyasri *et al.* [49] developed a novel UV Spectrophotometric bioanalytical method using human plasma as the matrix and detected RXN at 252 nm with Acetonitrile: Water (60: 40) as the diluent. De proteination of plasma with protein precipitation extraction using Acetonitrile depicted a good recovery (99.04 %–100.74 %), linear (1–20 ppm), LOD/LOQ (0.0121 µg/ml/ 0.0368 µg/ml), and precision (0.02%–0.03 %) which proved to be a simple, cost-effective and sensitive method in the estimation of RXN in spiked human plasma [49].

Chromatography Methods

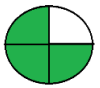
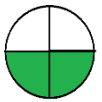
HPTLC

HPTLC is the most flexible, reliable, and cost-efficient separation technique so become one of the most popular methods for bio analysis. Shukla *et al.* [50] reported a bioanalytical method for the estimation of RXN in human plasma. Protein precipitation with acetonitrile as the precipitating reagent gave a compact band of R_f at 0.44 minutes with a recovery of 66.95%–69.03 %. This method was applied to a pharmacokinetic study of RXN and estimated the C_{max} (63.83 ng/ml) and t_{max} (47.3 ng/ml), area under the curve$_{0\text{-}t}$ (290 and 219.0 ng/ml/hour), and

Table 3. Stability indicating UV/HPLC/UPLC/MSMS methods for determination of rivaroxaban.

S.No	Method	Chromatographic Conditions					Results			Greeness profile	Merits/Demerits	Ref. no
		Stationary Phase	Mobile Phase	Flow rate ml/ min	Temperature (°C)	Detector/ detection wavelength (nm)	Rt (min)	Linearity range (µg/ml)/ R2/ %RSD	% Recovery	LOD/ LOQ		
1	Acid Degradation study of Rivaroxaban	Phenomenex C18(250 × 4.6 mm, 5 µm),	Methanol: Acetonitrile (50:50, v/v)	1	NA	UV/250	1.8	20–100/ 0.9999/ 0.143%	99.89 %	0.1277/0.387 µg/ml		Simple HPLC-UV method for acidic degradation/ Characterization would have improved the effectiveness of the method	Pinaz and Muralikrishna [31]
2	Photolytic –thermal degradation study by RP-HPLC	Phenomenex Luna C18 (250 × 4.6 mm,5 µm)	Methanol: acetonitrile (50:50 v/v)	1.2	NA	PDA/250	2.725	20–100 / 09995/0.143	100.5%	0.1277/0.3872		No characterization of the degradants	Kasad [40]
3	Base Degradation Study by RP-HPLC	Phenomenex C18 (250 × 4.6 mm, 5 µm), 100°A particle size	Methanol : Acetonitrile (50:50v/v)	1	NA	PDA/250	1.7	20–100 /0.99995/ 0.143%	100.85%	0.1277/0.3872		No characterization of the degradants	Kasad Pinaz and Muralikrishna [41]
4	HPLC-UV Bulk Tablet	CN (250 × 4.6 mm, 5 µm)	0.1M sodium acetate: Methanol (60.40, v/v)	1	30	UV/247	4.46	1–120/0.9992/ 0.421	99.74%	0.194/0.648		A very sensitive stability indicating cost effective HPLC-DAD method	Seshamamba *et al.* [32]
5	Stability-indicating RP-HPLC	Column:C18 (150 × 4.6 mm, 5 µm)	Acetonitrile : Water (70:30 v/v)	0.7	40	PDA/249	2.9 minutes	0.04–200/ 0.9992	99.77	NA		No characterization of the degradants	Walter *et al.* [36]
6	Stability-indicating RP-HPLC	C18 (250 × 4.6 mm, 3.5 µm)	0.02M po tassium hydrogen phosphate (Acetonitrile: Methanol) Gradient elution	1	45	PDA/240	RRT -1.0	50–250 /0.99/0.72%–2.44%	80%–120 %	0.018/ 0.03		Efficient method to characterize the degradants with good resolution	Girase et al. [37]
7	Stability indicating assay by HPLC	Column: Inertsil C8 (250 × 4.6 mm, 5 µ)	potassium Phosphate Buffer (pH3) : Acetonitrile Gradient elution	1	40	PDA/250	23.05 minutes	300–700/0.9987/ 0.89 %	99.5%–97.5%	NA		No characterization of the degradants	Badroon and Sreeramulu [38]
8	Stability indicating HPLC	Nova-Pak C8 (4 µm, 150 mm × 3.9 mm)	Acetonitrile: Potassium Dihydrogen phosphate 50 mM (pH 3.0) (40:60, v/v)	1	NA	UV/270	2.5	1–50/ 0.999/0.61	NA	0.58/0.19		Degradation with respect to 80°C and room temperature	Souri *et al.* [33]
9	HPLC and Stability Indicating Method	C18 (250 × 4.6 mm, 5 µ)	0.01 M potassium dihydrogen phosphate buffer : Acetonitrile (20 : 80) Gradient Elution	1	35	PDA/250	3.2	50–500/0.999/ 0.3%–0.9 %	98.9 %–110.1 %	NA		Efficient HPLC-PDA method to characterize the impurities.	Garad and Mane [39]

Continued

10	Stability Indicating Method RP-HPLC	C18 (250 × 4.6 mm, 5 µ)	Methanol : Water (50:50)	0.8	30	UV/250	3.37	0.005–40	98.31–101.60 %	NA		Eco-friendly HPLC-UV method	Sayeda and Hangad [34]
11	RP-HPLC Internal standard: Caffeine	Phenomenex Luna C18 LC (250 × 4.6 mm,5 µm)	Acetonitrile :Water (55:45 v/v) mixture	1.2	40	UV/249	Caffeine : 2.21 RXN : 3.37	0.005–40.0/ 0.9997/ 1.17	NA	0.005/0.01		No characterization of the degradants	Çelebier *et al.* [35]
12	Stress study HPLC method: Technology transfer to UPLC	C8 100A (250 × 4.6 mm id, 5 µm)/ UPLC Column : Acquity UPLC (R) BEH C8 (100 × 2.1mm id, 1.7 µm particle size)	0.1% Orthophosphoric acid : Acetonitrile (60: 40 V/V)/0.1% Orthophosphoric acid : acetonitrile (50:45 v/v)	1/0.25	30	PDA/280	NA	20–80/10–40/0.999	99 to 101/99 to 101%	0.08/0.075 0.175/0.150		One and only stability indicating method with successful technology transfer to UPLC	Jebaliya *et al.* [44]
13	Stability – indicating UPLC	Acquity BEH C8 column (100 mm × 2.1 mm, 1.7 µm)	Mobile phase A - 0.05M diammonium hydrogen phosphate (pH 3.0) : acetonitrile (80:20, v/v) and Mobile phase B Acetonitrile : water (90:10, v/v) gradient programme	1	30	PDA/254	15.0	0.999	98.4% and 103.5%	0.0005/ 0.0015		Sensitive UPLC-PDA method with characterization of impurities	Rajan and Anver Basha [42]
14	Stability – indicating UPLC	Acquity UPLC BEH HSS T3 (100 mm × 2.1-mm, 1.8-µm)	1mL Ortho phosphoric acid and 10 mM Octane1-sulphonic acid sodium Buffer and acetonitrile A (90:10) B(20:80) Acetonitrile (60:40%) Gradient elution	0.45	25	PDA/ 248	4.089	50–150 /0.9995/ <6.5%	93.9–107.9	0.05–0.30		UPLC-PDA method with efficient characterization of impurities	Rao *et al*. [43]
15	RXN Impurities –Forced degradation by LC-MS/MS	Chiralpak IC (250 × 4.6 mm, 5 µ)	A:B A : Acetonitrile : Ethanol: n-butylamine B : Water : Methanol : n-butylamine (50 : 50 : 0.5)	0.5	27	254	NA	90–100 %	NA	NA		Development of HPLC method and Characterization of impurities by LC-MS/MS	Cholleti *et al.* [45]
16	Liquid chromatography LC-MS/MS	C18 (250 × 4.6 mm 5 µm)	Acetonitrile and Water	1.5	55	UV/254	25.67 Impurity A : 20.76	300–1,000 /0.9997	NA	0.676/2.230		Elucidation of structure of the degradants	Arous *et al.* [46]
17	Stress Degradation Products of Rivaroxaban LC-MS/MS	Kinetex C18 (150 × 4.6 mm, 5 µm) column	20 mM ammonium acetate and acetonitrile (65 : 35 v/v)	1.0	30	RXN: m/z 436 DP-1: m/z 454 DP-3 : m/z 410 and 408	4.15	50–150/0.9997/ 0.9%	99.29 %	0.05/0.15		Elucidation of structure of the degradants	Ramishetti and Kuntamukkala [47]

$t_{1/2}$ (6.87 and 6.64 hours) for test and marketed formulation of RXN. Also, the study could be extended for the estimation of RXN in urine [50].

HPLC

Bioanalytical method development and validation using HPLC assist in performing quality control of pharmaceuticals as well as in obtaining pharmacokinetic and toxicokinetic data of drugs in less time. Protein precipitation which provides a quick sample clean up and better recovery was utilised by Yadav *et al.* [51] to develop a cost-effective bioanalytical method for the determination of RXN. Human plasma was spiked with RXN and the precipitating agent (acetonitrile) and centrifuged for 10 minutes at 1,000 rpm. The supernatant was injected into the HPLC and the results were monitored. Another author used SPE which effectively extracted RXN from human plasma with high recovery efficiency. Selective extraction of RXN based on their affinity to Phenomenex Strata-X 33-µm polymeric reversed phase cartridge facilitating the removal of interfering substances and enrichment of RXN by washing (methanol: water (50: 50, v/v)). RXN was eluted by methanol: glacial acetic acid (99: 1, v/v), followed by evaporation with a stream of nitrogen and finally reconstituted with the mobile phase before analysis [52]. This method was sensitive and increased the RXN concentration in samples by 7.5 times. Gouveia *et al.* [53] developed a fast and simple HPLC-DAD and applied to quantify the four currently marketed DOAC (AXN, EXN, dabigatran, and RXN). SPE using Oasis PRIME HLB cartridges (1cc/30 mg) for conditioning, the solid residue reconstituted with 0.1 % formic acid, and methanol (50:50, v/v) showed efficient recovery of all the anticoagulants. Also, the developed cost-effective HPLC-DAD bio analytical method can be more widely used in clinical laboratories than expensive LC–MS/MS [51–54].

LC-MS/MS

LC–MS/MS, a selective and sensitive technique used for pharmacokinetic studies, metabolites identification in biosamples. Reddy *et al.* [54] reported a precise, reproducible LC/MS/MS method that can be used for the TDM of RXN. RXN was found to be for about 5 freeze-thaw cycles and reconstituted samples were stable up to 72 hours post to extraction which indicates the effectiveness of the method. Varga *et al.* [55] Derogis *et al.* [56] Rohde [57] Rodina *et al.* [58] developed and validated a rapid, sensitive, high-throughput LC-MS/MS method for the estimation of RXN in human plasma and applied the method successfully in clinical studies. The authors adopted the protein precipitation method using methanol for the extraction of RXN from the biological matrix. The protein precipitation method was effective in extracting the analyte from clinical samples. Also incorporating gradient elution techniques leads to enhanced peak resolution, faster analysis times, and better detectability [56–58]. Simultaneous determination of RXN and dabigatran by LC-MS/MS in human plasma employing protein precipitation with zinc sulphate as the precipitating agent results in effective precipitation through hydrophobic interactions of protein molecules. Also, this method extracted the analyte by precipitating the proteins efficiently even when applied to 65 blood samples obtained from humans [59]. Simultaneous estimation of DOAC (AXN, dabigatran, RXN) in human plasma by LC-MS/MS employing protein precipitation technique was reported by Lagoutte-Renosi *et al.* [60] To compare the pharmacokinetics of RXN and TAK-438 in rats when being administered alone or being co-administered, Wang *et al.* [61] developed a simple, rapid and reliable method for simultaneous determination of the drugs in rat plasma by LC–MS/MS. Lee *et al.* [62] reported the first analytical method employing LLE for determining RXN in rat plasma which can be applied in special clinical situations. LLE is the classical method used for isolation, especially from water and biological fluid samples. Ethyl acetate, dichloromethane, and their mixtures are among the preferred extraction solvents. LLE was performed by mixing aliquot of a rat plasma and RXN with the IS and vortexed for a minute with the addition of Ethyl acetate. After centrifugation, the supernatant was collected, dried, and reconstituted with acetonitrile. The resulting solution was centrifuged and the supernatant solution was injected into the LC-MS/MS system and the results were monitored [55–63].

UPLC-MSMS

In recent years, a UPLC coupled with mass spectroscopy (MS) has been developed to estimate the biological samples, with better accuracy, sensitivity, precision, and high throughput. Iqbal *et al.* [64] developed UPLC/MS/MS method for the estimation of RXN in pharmaceutical dosage forms in the biological matrix (human Plasma) employing protein precipitation. All the other methods were simultaneous estimation of RXN in combined dosage forms by UPLC/MS/MS. Foerster *et al.* [65], Zhang *et al.* [66], Zhao *et al.* [67], Schmitz *et al.* [68], Sheng *et al.*[69], and Kuhn *et al.* [70] developed UPLC/MS/MS method for the estimation of RXN in combined pharmaceutical dosage forms in the biological matrix (human plasma/rat plasma) employing protein precipitation. Foerster *et al.* [65] developed a dried blood spot (DBS) assay that facilitates the sufficient extraction of all DOAC (AXN, dabigatran, EXN, and RXN). Consistent extraction recoveries and matrix effects of all samples were achieved in a dual extraction process of DBS (ultrasonic bath and shaking), which were treated with extraction solvent (methanol/water, 95/5 % v/v). The method was highly advantageous such that the patients can sample DBS at home within the dosing interval. Kuhn *et al.* [70] reported a rapid method with a run time of 2.5 minutes which was found to be one of the fastest methods in the estimation of DOAC. The method reported by Schmitz *et al.* [68] presented the highest precision for the DOAC compared to all the methods [64–70]. Table 4 overviews the LC parameters, validation, and application of the bioanalytical methods. All the UPLC/MS/MS methods were applied for the pharmacokinetics studies in patients, and the results obtained revealed the sensitiveness of the method. Most of the methods used protein precipitation as the major extraction technique followed by the SPE technique, the % recovery obtained with both methods was within the limits. Whereas, micromethods with a limited amount of extraction phase than the sample have

Table 4. Degradation Results of Rivaroxaban by UV/HPLC/LC-MS/

Analytical Technique	Hydrolysis		Oxidation	Photolysis	Thermal	Impurities/ Degradation products	Reference
	Acidic	Alkali					
UV	**68.17 %**	**No degradation**	**-**	**95.27 % Possible by UV light**	**79.04%**	**-**	**Mandake *et al*. [28]**
HPLC	2.21%–32.29 % (0.1 M HCl was used and refluxed for 1,2,4,6 hours and 1 Day and 3 Day at RT, 40 0 C and 60 0 C)	NA	NA	NA	NA	NA	Pinaz and Muralikrishna [31]
	NA	NA	NA	17.47 %–32.25 % (UV light and sunlight)		51.29 %–59.01 % (oven at 60°C and 80°C for 1 hour)	Kasad [40]
	NA	5.04 % to 47.35 % (0.1M NaOH at 1,2,4,6 hours and 1 Day and 3 Day at 40 0 C and 60 0 C)	NA	NA	NA	NA	Kasad and Muralikrishna [41]
	7.705 % (0.1 N HCl for 2 hours at 80ºC)	8.851 %(0.1 N NaOH for 2 hours at 80ºC)	6.55 % (3 % H2O2 for 2 hours at 80ºC)	9.186 % (sunlight for 24 hours)	7.304 % (105 °C for 2 hours)	NA	Seshamamba *et al*. [32]
	NA	NA	NA	NA	NA	NA	Walter *et al*. [36]
	(1 M HCL for 4 hours at 80°C)	1 M NaOH for 4 hours at 80°C	5 %v/v H2O2 for 4 hours at 40°C	1.2 million lux h	80˚C for 12 hours	Impurity 1–10	Girase *et al*. [37]
	93.5% (0.1N HCL 700C/ 2 hours)	85.6 % (0.1N NaOH RT for 1 hour)	99.5 % (3 % H2O2 at 700C/ 48 hours)	99.1 % (1.2 &106 LUX hours)	99.4% (600C/ 7 days)	NA	Badroon and Sreeramulu [38]
	7% (at room temp.) 45% (at 800C)	89 % (at room temp.) 73.8% (at 800C)	39 % (at room temp.) 15% (at 800C)	Solid form- 30% Water form- 84%	Solid form- 21% Water form- 30%	NA	Souri *et al*. [33]
	5.9% (1 N HCI/ 5 ml/60°C/2 hours)	9.9% (1 N NaOH /5 ml/R.T/20 minute)	1.6% (3O% H2O2/ 5 ml/ 60°C/ l2 hours)	0.4% (1.2 million lux hours)	0.3 % (60°C for 7 days)	Impurities A - H	Garad *et al*. [39]
	0.72 % (0.1 N HCL for 4 hours at 70ºC)	18.4 % (0.1 N NaOH for 4 hours at 70ºC)	NA	4.2 % (UV 254 nm for 24 hours)	0.67 % (Oven at 150°C for 4 hours)	NA	Sayeda *et al*. [34]
	83.2 % (0.1 N hydrochloric for 2 hours at 40ºC)	83.0 % (0.1 N sodium hydroxide for 2 hours at 40 ºC)	NA	35.1 % (UV 254 nm for 24 hours)	77.7 % (2 hours at 80 °C)	NA	Çelebier *et al*. [35]
	13% (0.5 N HCl at 80ºC for 45 minutes)	50 % (0.1 N NaOH for 60 minutes at 80ºC)	6 % (30% v/v H2O2 at 60◦C for 2 hour)	1.5 % (72 hours)	3 % (80ºC for 36 hours in hot air oven)	NA	Jebaliya *et al*. [44]

Continued

UPLC	6.4 % (0.1-N HCl at 70 °C for 48 hours, 1 hour, 2 hours, and 48 hours)	11.2% (0.1-N NaOH at RT for 48 hours, 1 hour, 2 hours, and 48 hours)	N.P (3.0 % H2O2 at 70 °C) for 48 hours, 1 hour, 2 hours, and 48 hours	N.P (UV light 254 & 365 nm for 10 days)	N.P (105°C for 10 days)	Impurity 1–*10	Rao *et al.* [43]
Detection of impurities by LC-MS/MS	NA 27.61 % (0.1 N HCl for 72 hours) (1 N HCl for 1 hour)	NA 34.83 % (0.1 N NaOH for 72 hours) 6.1 % (1 N NaOH for 1 hour)	NA 0.92 % (3% H2O2 solution over a period of 72 hours) 18.4 % (10% H2O2 for 24 hours)	NA 0.09* (7 days at 200 W h/m2 of UV light and 1.2 million lux hours of visible light) N.P (UV light for 7 days)	NA 0.32 % (105°C for 7 days) N.P (60 °C for 7 days)	NA Acid-Appearance of DP2 & DP3. Alkali- Appearance of DP1, DP2 & DP3. DP 1 s (S)-2-(2-((4-(5-((5-chlorothiophene-2-carboxamido)methyl)-2-oxooxazolidin-3- yl)phenyl)amino) ethoxy) acetic acid DP 2 No Structural confirmation DP 3 (R)-5-chloro-N-(2-hydroxy-3-((4-(3-oxomorpholino) phenyl)amino) propyl)thiophene-2-carboxamide	Cholleti *et al.* [45] Arous *et al.* [46] Ramisetti *et al.* [47]

emerged as an advantageous method than the usual methods. Syringe-based SPE (Microextraction by packed sorbent), disposable-pipette extraction, liquid-phase microextraction, thin-film microextraction, single-drop microextraction, sorbent-based microextraction techniques, dispersive liquid-liquid microextraction, electromembrane extraction and stirbar sorptive extraction are the numerous micromethods used in clinical investigations. In a nutshell, the incorporation of microsampling techniques and microextraction would be a tremendous spike in the area of bioanalytical research.

GREENER ASSESSMENT OF THE REPORTED LIQUID CHROMATOGRAPHIC METHODS

Sustainable Development Goals 2030 were implemented to fulfill human development goals without exhausting resources for future generations [71,72]. Among the 17 SDGs, SDG Goal 12 (ensure sustainable consumption and production patterns) espouses Green analytical chemistry which deals with the development of efficient and sustainable chemical processes, thus reducing the environmental impacts [73]. NEMI, eco-scale assessments, green analytical procedure index, analytical method volume intensity, and analytical greenness metric are the several tools to evaluate the greenness of analytical methods. NEMI owing to its easiest and rapid greenness assessment, represented by a pictogram provides an adequate inference about the environmental effects of the investigated method. The circular pictogram divided into four quadrants (Fig. 6), where A = persistent, bio-accumulative, and toxic (PBT), B = hazardous, C = corrosive, and D = waste (run time × flow rate) employs the summary of the greenness of the method. The greenness profile of all the reported analytical methods for RXN was assessed and summarised in a tabular form, the quadrants were filled green for the greener methods while left blank for the non-greener ones. All the methods used acetonitrile (PBT and hazardous) and methanol (Not PBT but hazardous) as the mobile phase. Hence, the method with methanol as the mobile phase would show three of the quadrants green (PBT, corrosive, and waste), whereas acetonitrile as the mobile phase would show two quadrants green [74–77]. All the reported methods were found to be non-corrosive (pH 2–12) and generated a low amount of waste (less than 50 g). The greenness assessment using NEMI suggested that among the HPLC methods (12), one method was 75 % and the remaining 11 methods were 50 %. The stability indicating methods (18) was 75 % for 2 methods and 50 % for the

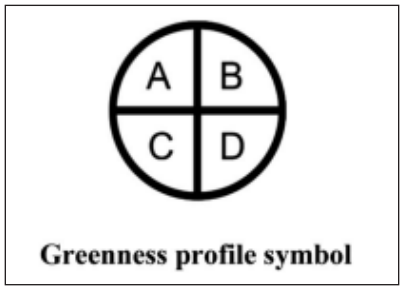


Figure 6. Greeness assessment quadrants by NEMI.

Table 5. Bioanalytical methods for determination of rivaroxaban.

S.no	Method/Matrix	Chromatographic Conditions						Results				Greeness profile	Application	Ref. no
		Stationary phase	Mobile phase	Flow rate ml/ min	Sample extraction Technique	Detector/detection wavelength (nm)	Internal Standard	Rt (minute)	Linearity range (µg/ ml)/ R2/ %RSD	% Recovery	LOD/ LOQ			
1	RP-HPLC/ Human plasma	Phenomenex luna C8 (25 cm × 5 µm 4.6 mm)	methanol: water: dimethyl sulfoxide (50:45:5, v/v/v)	1	Protein Precipitation with acetonitrile	PDA/252	Chloramphenicol	6.2	5–40/0.975 /0.57%–1.15 %	99.38 - 100.44%	1.5/4.5		An greener approach for the quantification of RXN in human plasma	Yadav and Dubey [51]
2	RP-HPLC/ Human plasma	Phenomenex Luna C18 LC (250 × 4.6 mm,5 µm)	Acetonitrile : Water (55:45 v/v) mixture	1.2	Solid Phase Extraction	UV/249	prednisolone	Prednisolone: 2.21 RXN : 3.37	0.005–40.0/ 0.9997/ 1.17 %		0.005/0.01		Can be adapted on liver perfusion and intestinal permeability studies in animal models	Çelebier *et al.* [52]
3	Simultaneous method HPLC/ Human Plasma	Column: C18 (55 mm × 4 mm, 3 µm) Temperature : 30o C	Formic acid (0.1 %,v/v): acetonitrile (55:45v/v) Gradient elution	1.2	Solid Phase Extraction	PDA/ IS-278 nm, dabigatran and AXN – 300 nm , EXN-289 nm, RXN - 249 nm	Chloramphenicol	Dabigatran – 1.6 minutes, EXN- 3.6 minutes, AXN – 4.5 min, RXN - 4.7 min	AXN: 0.017- .28/ 0.017 - 5.28/ 11.89 %; EXN - 0.033-5.28/ 0.066 - 5.28/ 13.58 %; RXN: 0.017- 5.28 / 0.017 - 5.28/ 13.39 % R2: 0.993	AXN -77.75 % dabigatran – 97.41 %, EXN -77.61 %, RX N - 70.70	LLOQ: 12.98%– 5.79 %		Applied successfully to analyse patient samples in TDM	Gouveia *et al.* [53]
4	HPLC-MS/MS/ Human Plasma	Phenomenex Gemini C8 (50 × 4.6 mm × 5 µ)	Acetonitrile: 5 mm Ammonium Formate buffer (pH 3.5 ± 0.3) (85:15 v/v)	0.8 ml/ minute	Solid Phase Extraction	m/z - 436.20>144.80 IS m/z - 440.20>144.70	RXN D4	RIV – 0.82minutes →m/z144.80 RIV D4 – 0.82 min	2.00- 500.93 ng/ ml/0.99/3.8% –0.9 %	Overall recover: >96%	LLOQ :2.01 ng/ mL		Applied for therapeutic drug monitoring of RXN, pharmacokinetic or bioequivalence studies	Reddy *et al.* [54]
5	LC-MS/MS/ Human Plasma	Zorbax SB-C18 (3.0 × 100 mm, 3.5 µm),	0.2 % Formic acid in water : Acetonitrile (65:35v/v)	1	Protein Precipitation with methanol	positive electrospray ionization and MS/ MS mode (sum of m/z 231.1; 289.2 and 318.2 from m/z 436.3)	NA	1.4	24.00-960.00 ng/ ml/0.9927	96.5 %–108.5%	LLOQ : 24.0 ng /ml		Therapeutic drug monitoring of RXN in 29 patients	Varga *et al.* [55]
6	Pharmacokinetic Study Patients Plasma Sample HPLC-MS/MS	Kinetex C18 (100 × 3 mm, 2.6 µm) ESI	Ultra-pure water containing 0.01% Formic acid : 60% Methanol	0.5	Protein Precipitation method	RXN: 435.9 –144.9 IS: 440.1–144.9	RXN D4	1.8	2–500 ng/ml R2: 0.9996	Accuracy : 95.2 – 101.0 %Acceptable Stability : 99.2 -110.3 %	LLOQ :4 pg/ml ULOQ: 500 ng/ml		Applied to 49 patients treated with RXN	Derogis *et al.* [56]
7	LC-MS/MS matrix: Human plasma	C18 (125* 4 mm, 5 µm)	acetonirile: 0.01 mol/l, ammonium acetate (Gradient elution)	1	Protein Precipitation	RXN: m/z 436.1 to 144.9 IS: 464.2→144.9)	([2H5, 15N]) RXN	RXN: 3.30 IS: 3.37	0.50 and 500 g/l/	96.3% and 102.9%	2µg/l		Applied for the accurate determination of RXN and pharmacokinetics in human plasma.	Rohde G [57]

Continued

8	Simultaneous determination of RXN and dabigatran lev by LC-MS/MS in Human plasma	C18 (100* 2.1 mm, 1.7 µm)	0.07 g ammonium acetate with 0.1% formic acid: acetonitrile with 0.1% formic acid (90:20, V/V)	0.3	protein precipitation by ZnSO4 solution	RXN: m/z 436.1→144.9,13 C6 RXN: m/z 442.1→144.9, dabigatran- m/z 472.2→289.0 and m/z 478.2→294.9, 13C6-dabigatran m/z 436.1 to 144.9	13C6-dabigatran and 13C6-RXN	dabigatran and 13C6-dabigatran – 1.45 RXN and 13C6-RXN – 2.15 min	2.5–500 ng/ml/0.995/1.4 – 9.6 %	78.9 – 105.6	2.5 ng/ml		Applied to 65 blood samples obtained in humans.	Korostelev *et al.* [59]
9	LC-MS/MS simultaneous determination of direct oral anticoagulants AXN, dabigatran, RXN	C18 (100* 2 mm, 3 µm)	0.1% Formic acid: Acetonitrile with 0.1% Formic acid (Gradient elution)	0.35	Protein precipitation with methanol	m/z 436.1–144.9	13C6- dabigatran 13C, 2H7 AXN 13C6-RXN	Dabigatran - 2.75 AXN- 3.20 RXN - 3.25	RXN: 5 to 1000 µg/l, AXN and dabigatran: 25 to 1,000 µg/l	NA	RXN: 5µg/l 25 µg/l for apixaban and dabigatran		The method can be used during clinical studies and in daily routine practice for the management of specific clinical situations at reasonable cost	Lagoutte-Renosi *et al.* [60]
10.	LC-MS/MS in Human Blood Serum	C18 (50* 4.6 mm, 3.5 µm)	0.1% formic acid: Acetonitrile with 0.1% Formic acid (Gradient elution)	0.1	Protein Precipitation with Methanol	RXN (436.10 m/z) and promethazine (285.10 m/z)	Promethazine	RXN: 2.42 Promethazine: 2.41	1 – 1000 ng/ml/1.72–7.06/	NA	1.0 ng/ml		Applied at Davydovsky City Hospital, for TDM	Rodina *et al.* [58]
11	RXN and TAK-438 in rat plasma LC-MS/MS	Agilent Eclipse plus C18 column (100 mm × 2.1 mm, 3.5 µm)	0.1% formic acid in acetonitrile (A) and 0.1% aqueous formic acid (B) (A: B = 70: 30% v / v)	0.5	Protein Precipitation with acetonitrile	RXN: m/z 436.1 → 144.9, TAK-438: 346.1 → 173.0, IS: 237.1 → 194.2	carbamazepine	RXN: 2.452, TAK-438; 1.678 and IS: 2.673 min	RXN: 0.5–275.0 ng/ml/0.994 TAK-438: 0.5–100.0 ng/ml/0.997 %RSD: 2.66 -11.87	RXN: 92.26 -109.42 TAK-438:90.68 – 99.55	0.5 ng/ml		Applied to pharmacokinetic interactions study between RXN and TAK-438 for the first time.	Wang, *et al.* [61]
12	LC-MS/MS - RXN in human plasma and its application to a pharmacokinetic study	Gemini C18 (150 mm × 4.6 mm, 5 mm)	Acetonitrile: 5mM ammonium acetate (80:20 v/v)	1.5	Solid phase extraction	RXN: 436/145 (m/z) RXN D4: 440/145 (m/z)	RXN D4	RXN: 1.8 RXN D4: 1.18	5.96–801 ng/ml/ 0.99/ 1.08%–3.75%	88.9% and 89.2%	5.96 ng/ml		Applicable for supporting the pharmacokinetic, bioavailability, and bioequivalence studies of RXN	Shaikh *et al.* [63]
13	LC-MS/MS/ Rat plasma	Luna C18 column (2.0 ×150 mm, 5 mm)	5% (v/v) aqueous acetonitrile solution including 0.1% (v/v) formic acid	0.25	Liquid liquid Extraction using ethyl acetate	436.1m/z and 460.1 m/z	Apixaban	RXN: 2.9 minutes IS: 2.6 minutes	0.5 - 500 ng/ml/0.99/	89.2 - 105.7%	0.5 ng/ml		first analytical method employing LLE for estimation RXN in Rat plasma and can be applied in special clinical situations	Lee *et al.* [62]

Continued

14	UPLC-MS/MS/ plasma	Acquity UPLC BEH C8 column (100 × 2.1 mm, 1.7 m; Waters)	2.5 mM ammonium Formate (pH 3.0): Acetonitrile	800	Protein Precipitation	MS/MS	[13C6]Dabigatran [13C6] RXN, [13C, 2H7] Apixaban	NA	23–750 ng /ml R2 :≥ 0.99	NA	LOD : ≤ 0.025 ng/ mL		UPLC-MS/MS method was compared with coagulation methods by the use of patient samples for dabigatran and RXN	Schmitz *et al.* [68]
15	UPLC-MS/MS/ plasma	Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7 µm)	Formic Acid (0.1 %, v/v) and Acetonitrile (55:45v/v)	0.3	Protein Precipitation with acetonitrile	RXN: 436.1→145.1 m/z Enalapril: 377.3→234.2 m/z Diazepam: 285.2→193.1 m/z	Diazepam	RXN: 2.13, Enalapril: 1.90 Diazepam: 2.56 min	RXN :1.0-200 ng/ml/ 0.9944/2.2 -8.4 Enalapril: 0.5–100 ng/ ml/0.997/2.6–7.9	81.7%–89.4%	RXN: 1.0 ng/ml Enalapril: 0.5 ng/ml		applied to study the pharmacokinetic interaction investigation in rats	Sheng *et al.* [69]
16	UPLC-MRM Mass Spectrometry Method/Plasma Simultaneous method Dabigatran and RXN	Acquity UPLC BEH Phenyl, (50 × 2.1 mm, 1.7 µm)	5%/95% water/ methanol containing also 0.1% formic acid and 2 mmol/l ammonium acetate.	0.35	Protein Precipitation	Dabigatran - 472.2 > 289.2 RXN - 436.1 > 145.0	[13C6]-RXN [13C6]-dabigatran	[13C6]-Dabigatran - 1.47 minutes [13C6]-RXN - 1.97 min	0.8 and 800 µg/l/0.99	Dabigatran - 104.5% RXN - 87.0%	Dabigatran - 0.46 µg/l RXN - 0.54 µg/l		Applied to determine the concentrations of the two drugs independent of inteference factors such as hemolysis or lipaemic plasma	Kuhn *et al.* [70]
17	UHPLC-MS/ MS/ plasma	C18(100*2.1 mm, 1.7 µm)	Acetonitrile: 10mM ammonium acetate (80:20 v/v)	0.3	Protein Precipitation by Acetonitrile	RXN: m/z 436.00 - 144.87 IS: m/z 411.18 - 191.07	Risperidone	RXN:0.81 minutes IS: 0.85 minutes	0.57–625 ng/ ml/0.997/	95.3–102.6	0.57 ng/ml		UPLC–MS/ MS assay was successfully applied to a single dose, oral pharmacokinetic study of RXN (1 mg/kg) suspension in healthy male rats.	Iqbal et al. [64]
18	UPLC-MS/ MS Dried blood spot technique to monitor direct oral anticoagulants	Phenyl(2.1*50 mm, 1.7 µm)	(95%5mM ammonium bicarbonate in water with 5% Acetonitrile gradient)	0.5	Solid Phase Extraction	m/z 436.2–144.9	isotope-labeled internal standard	NA	2.5 (AXN, RXN), 4.4 (dabigatran), and 9.3 ng/ml (edoxaban) to 750 ng/ml	65–81 %	2.5 ng/ml		Correlation between Dried blood spot and plasma concentrations was assessed in 33 patients	Foerster *et al.* [65]
19	UPLC–MS/ MS/ Rat Plasma Determination of RXN, AXN and edoxaban in rat plasma	Acquity UPLC BEH C18 chromato-graphy column (2.1 mm 9 50 mm, 1.7 lm)	Acetonitrile and 0.1% formic acid in water	0.4	Protein precipitation	RXN - m/z 436.1 - 145.1 AXN: m/z 460.0 - 443. EXN: m/z 548.2 - 366.1 IS: m/z 285.2 - 193.1	diazepam	RXN: 1.71 minutes, AXN: 1.69 minutes, edoxaban: 1.58 IS: 1.89	RXN: 1.0–200 ng/ml/ 0.9948 AXN: 1.0–100 ng/ml/0.9971 Edoxaban : 1.0–500 ng/ ml/0.9956	92.8–108.0 %	5 ng/ml		Applied successfully to the pharmacokinetic investigations of RXN, AXN and edoxaban in rats	Zhang *et al.* [66]
20	UHPLC-MS/MS/ Human Plasma - Simultaneous estimation of apixaban, EXN and RXN in human plasma and breast milk	C18(50*2.1 mm, 1.8 µm)	0.1% formic acid in deionized water (A) and acetonitrile (B) Gradient Elution	0.5	Protein Precipitation by methanol	m/z 436.1-144.9	AXN-13C, 2 H8, EXN-2 H6 and RXN-13C6	RXN: 1.84 AXN: 1.81 Edoxaban: 147	5–500 ng/ml in plasma and 5–250 ng/ml in breast milk	106.13%–109.05% in plasma and from 93.40% to 107.91% in breast milk	5 ng/ml		Applied to the analysis of clinical samples in a clinical lactation study involving AXN and RXN	Zhao et al. [67]

remaining. The greenness profile of the bioanalytical methods was 75 % for 3 methods and 50 % for 17 methods. HPTLC methods reported a 100 % greenness profile among all the other analytical techniques. Subsequently, the replacement of toxic solvents like acetonitrile with ethanol or water could be more ecologically to make the existing analytical methods more sustainable meeting the requirement of SDG12. The reported methods pave a roadmap for future researchers that there is a need to use eco-friendly solvents for the chromatographic separation of RXN without compromising the sensitivity of the analytical methods.

CONCLUSION

The review elaborates on the different analytical methods of RXN which will be helpful to the researchers. Validation of all the methods was performed as per ICH guidelines and found to be linear, accurate, precise, specific, and robust. Stability-indicating methods revealed the degradation profile of the drug. Protein precipitation was found to be the most preferred extraction technique adopted for sample pretreatment. Miniaturized novel sample extraction methods can be adopted in the future for the extraction of complex matrices. A substantial emphasis on examining the greenness profile by NEMI, which indicated 50% green for 90% of the methods. Also, concepts of "blueness" and "whiteness" assessment using the recently introduced blue applicability grade index and Red-Green-Blue 12 (RGB 12) algorithms can be incorporated. To put it briefly there is a pressing need for developing eco-friendly methods and applying them in the analytical techniques for the separation of RXN from biological matrices and finished drug products. Future trends incorporating miniaturized techniques would pave a roadmap for the estimation of RXN.

LIST OF ABBREVIATIONS

AQbD: Analytical Quality by Design; AXN: Apixaban; CPL: Clopidogrel; DAD: Diode Array Detector; DMSO: Dimethyl Sulphoxide; DOAC: Direct oral anticoagulants; *DP:Degradation Product;* EXN: Edoxaban; HPLC: High Performance Liquid Chromatography; HPTLC: High Performance Thin Layer Chromatography; LOD: Limit of Detection; LOQ: Limit of Quantification;

LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry; PBT: *Persistent, Bioaccumulative, Toxic;* R^2: Correlation Coefficient; Rt: Retention Time; RXN: Rivaroxaban; TLC: Thin Layer Chromatography; UPLC: Ultra Performance Liquid Chromatography; UPLC-MS/MS: Ultra Performance Liquid Chromatography Tandem Mass Spectrometry; UV: Ultra- Violet.

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All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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