ORIGINAL ARTICLE

A C G publications

J. Chem. Metrol. 16:2 (2022) 125-134

journal of chemical metrology

Development and application of HPTLC method for estimation of Rivaroxaban and Aspirin in bulk drug and in-house tablet form

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(Received November 14, 2022; Revised December 26, 2022; Accepted December 28, 2022)

Abstract: In the present work, simple, precise, accurate high-performance thin-layer chromatography was developed, optimized, and validated for quantitation of Rivaroxaban (RIV) and Aspirin (ASP). The developed method was applied for quantification of both the drugs simultaneously in bulk drug and in-house tablet formulation. In this study, the Camag Linomat V HPTLC system and win CATS software V1.4.7 were used. Both molecules were separated using a chromatographic method consisting of toluene: ethyl acetate: methanol: glacial acetic acid (6:3:0.5:0.5 v/v/v/v) as mobile phase and an aluminum pre-coated plate with silica gel 60 F254 as the stationary phase. Both drugs were detected at 256nm. With R_f values of 0.23 and 0.72, respectively, Rivaroxaban and Aspirin were satisfactorily resolved. Moreover, as per the ICHQ2 (R1) guideline, Specificity, precision, accuracy, robustness, linearity, the limit of detection, and the limit of quantification were performed. The method was found linear in the range of 100-400 ng/band and 2000-8000 ng/band of Rivaroxaban and Aspirin, respectively. The precision of the method was determined by %RSD and it was found in range. *In-house* tablet formulation was prepared and applied the developed method for assay of RIV and ASP. The % Assay (%v/v) of RIV and ASP was found 100.61 %w/w and 100.29 %w/w. In a conclusion, the accurate, precise, sensitive, and robust HPTLC method was optimized, developed, and validated as per ICH Q2 (R1) guideline, which was applied for *in-house* tablet formulation. The result of the assay suggests that the developed method can be used for simultaneous estimation of RIV and ASP for their dosage form as a part of regulatory submission.

Keywords: Rivaroxaban; high performance thin layer chromatography; validation; ICH guidelines; tablet formulation.

1. Introduction

Rivaroxaban an anti-coagulant and its chemical formula is (5-chloro-N-(5S)-2-oxo-3-[4-(3-oxo-4-morpholinyl) phenyl])-1, 3-oxazolidin-5-yl} methyl-2-thiophene carboxamide, Figure 1) which is taken orally to prevent thromboembolism in patients after surgery. It's a specific and very effective inhibitor of factor Xa. Factor Xa inhibition causes thrombin production to stop and thrombin-mediated coagulation to be reduced. Rivaroxaban is a blood thinner that is used to prevent thromboembolism following hip or knee replacement surgery [1-3].

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The article was published by ACG Publications
http://www.acgpubs.org/journal/journal-of-chemical-metrology July-December 2022 EISSN:1307-6183
DOI:http://doi.org/10.25135/jcm.78.2211.2636
Available online: December 30, 2022

2-acetoxybenzoic acid is common drug and commercially known as aspirin worldwide. (Figure 1). It's regarded as lowering prostaglandin and thromboxane synthesis. The capacity of 2-acetoxybenzoic acid to inhibit prostaglandin and thromboxane synthesis is attributed to the cyclooxygenase (COX) enzyme being inactivated irreversibly. The enzyme cyclooxygenase is essential to produce prostaglandins and thromboxane [4, 5].

Patients with CAD who received dual antiplatelet therapy had significantly better protection against CVS events than patients who received either Aspirin or Clopidogrel alone, according to several studies. Some studies show that Rivaroxaban is effective in reducing atherothrombosis and avoiding atherosclerotic complications. Compared to Clopidogrel and aspirin, the combination of Rivaroxaban and Aspirin was found to be marginally more effective in preventing atherosclerotic events in this study. Combination therapy with Aspirin and Rivaroxaban reduced MACE (stroke, myocardial infarction, and cardiovascular related death) by 28% and MALE (chronic limb-threatening ischemia, arterial bypass, and amputation) by 46% in patients with PAD. Study suggests that even at a modest dose of its equivalent of 2.5 mg, Rivaroxaban was able to dramatically decrease arachidonic acid-induced platelet aggregation in the presence of Aspirin *ex vivo*, and that this effect was seen in 58% of patients who had been previously Aspirin-intolerant. Patients who are not sensitive to Aspirin may benefit from taking 81 mg of Aspirin daily in addition to 2.5 mg of Rivaroxaban twice daily. [6-12]

In terms of both quality and cost, high-performance thin-layer chromatography (HPTLC) is hard to beat as a separation technique for scientific investigation. Plus, it's a flexible analytical methodology, so you may adjust the analysis to fit the specifics of each stage of the process. An HPTLC fingerprint can provide not only the identity of a sample, but also certain semi-quantitative details (band intensity) about it. Quantitative assessments of analytes are possible with the help of hyperspectral data, densitograms, and picture profiles. The flexibility to apply different detection techniques on the same sample and plate is another benefit of HPTLC. These techniques include ultraviolet/visible light, fluorescence, hyperspectral, derivatization, and effect directed. The analytes from the sample are left on the plate, unlike in other chromatographic methods. HPTLC allows for non-invasive, parallel analysis rather than sequential methods. Since only one plate is used for each sample, there is little chance of cross-contamination, and the HPTLC process only takes a brief amount of time to run.

A literature survey reveals that many analytical methods were developed for quantification of Rivaroxaban and Aspirin individually including UV spectrophotometry [13–15] high-performance liquid chromatography [16–20], high-performance thin layer chromatography [21, 22], and LC-MS (liquid chromatography mass spectrometry [23] methods. However, there have been no published methods for estimation of Rivaroxaban and Aspirin simultaneously by HPTLC methods reported yet. The development and validation of the HPTLC method for the simultaneous estimation of Rivaroxaban and Aspirin in bulk drugs and in-house formulations for the first time is presented as per ICH guidelines.

2. Experimental

2.1. Materials and Methods

During the experiment, Camag HPTLC system was used. It includes Hamilton Syringe (100µl) for spotting the sample. Camag TLC Scanner IV for scanning the spot was used. While for the development of TLC, Camag Twin Trough chamber was used. HPTLC system operated by winCATS software V1.4. Gratis samples of Rivaroxaban (99.7%) and 2-acetoxybenzoic (99.8%) were received from the Bio-organics and Applied Materials Pvt Ltd. AR grade Toluene, ethyl acetate, methanol, and glacial acetic acid were procured from Merck Chemicals of Mumbai, India.

2.2. Mobile Phase Optimization for HPTLC

During the development, various mobile phase and their proportion were tried for optimization and separation of both the peaks. The mobile phase comprises Toluene: Ethyl Acetate: Methanol: GAA (6:3:0.5:0.5 %v/v/v/v) shows both peaks better resolved and found reproducible. HPTLC method was adjusted to establish a Rivaroxaban and Aspirin simultaneous test method. The solution (15 µg/mL Rivaroxaban 300 µg/mL Aspirin) was used, and 10 µL TLC plates were used to spot samples, which were then processed in a variety of solvent systems. Because both medicines did not migrate, optimizing the HPTLC approach was extremely challenging in this circumstance. After numerous tests, it was discovered

that the movement of both medications needed the use of a polar mobile phase. As a result, a ratio of 6:3:0.5:0.5%v/v/v/v of toluene, ethyl acetate, methanol, and glacial acetic acid was found to be optimal. The TLC chamber was flooded for 20 minutes to lessen the neckless effect. The mobile phase was carried out up to an 8cm distance, and the TLC plate took around 22 minutes to fully expand.

2.3. Preparation of Solutions

In this experiment, 5mg of Rivaroxaban and 100mg of Aspirin medication powder were precisely weighed put in a 10ml volumetric flask, where Rivaroxaban dissolved in the smallest amount of Acetonitrile possible. Methanol was used to fill up the gaps, and Aspirin was dissolved in the smallest amount of Methanol possible. Methanol was used to make up for the difference in volume. Pipette aliquots the standard stock solution into a 10ml volumetric flask dilute with methanol up to the mark to a concentration of 15 µg/mL Rivaroxaban and 300 µg/mL Aspirin.

2.4. Methods

2.4.1. Specificity

Analyzing tests and standard drug samples determined the method's specificity. The purity of spectra was assessed at three different levels: the beginning, middle, and end. The correlation between the spectra of the standard and the spectra of the drug in the sample track was used to evaluate peak purity. The peak purity of Rivaroxaban and Aspirin was determined on the spot: peak (S), peak (M), and peak (E).

2.4.2. Linearity

A 10 µL solution was spotted on the TLC plate from the mixed standard solution, 15 µg/mL Rivaroxaban 300 µg/mL Aspirin, to get final concentrations of Rivaroxaban of 100-400ng/band and Aspirin of 2000-8000ng/band for Aspirin. On the TLC plate, each concentration was tested six times. The calibration curves were constructed by plotting the peak areas against the relevant concentrations after developing the plate with the previously described mobile phase. The method's linearity was tested by injecting seven different concentrations of the drug produced in the mobile phase into the system in triplicate while keeping the injection volume constant. Peak regions were plotted against concentrations to construct the calibration plots.

2.4.3. Calculation of Limit of Detection and Limit of Quantitation

The calibration curve of three low-concentration samples was used to calculate LOD and LOQ. The formulas 3 σ/S and 10 σ/S of the calibration curve were used for the estimation of LOD and LOQ, respectively.

2.4.4. Precision

The method's precision was confirmed by repeatability and intermediate precision studies. Researchers tested three different concentrations six times on the same day (150ng/band, 250ng/band, and 350 ng/band for Rivaroxaban, and 3000ng/band, 5000ng/band, 7000 ng/band for Aspirin). By repeating tests three times on various days, the method's intermediate precision was tested.

2.4.5. Accuracy

The accuracy of the method was determined by running a chromatogram in an optimized mobile phase on a drug sample (Rivaroxaban and Aspirin combination in-house tablet). According to the standard

addition method, 80, 100, and 120 percent, standard samples of Rivaroxaban and Aspirin had been added and analyzed.

$$\% \, Spiked \; concentration = \frac{Mean \; Concentration}{Spiked \; Concentration} X100$$

2.4.6. Robustness

The effects on the results were investigated after slight modifications in the mobile phase composition (less than 10% for each component). Toluene, for example, is a mobile phase with a unique composition: (1) (6.6: 3: 0.5: 0.5 v/v/v/v) (2) (5.4: 3: 0.5: 0.5 v/v/v/v) (3) (6: 3.3: 0.5: 0.5 v/v/v/v) (4) (6: 2.7: 0.5: 0.5 v/v/v/v)Prior to chromatography, the plates were pre-activated for 2, 5, or 7 minutes at 110°C after being pre-washed with methanol. The duration between spotting and chromatography, as well as the time between chromatography and scanning, varied between 10 and 20 minutes. The method's robustness was tested for Rivaroxaban (150ng/band, 250ng/band, and 350ng/band) Aspirin (3000ng/band, 5000ng/band, 7000ng/band). The formulation sample solution (10 µl) and working standard solution (14 µl) were analyzed on a pre-washed TLC plate.

3. Results and Discussion

3.1. Chromatographic Separation

The separation of RIV and ASP are carried out in the stationary phase consisting of 10x10 and 10x20 cm plates of alumina sheet with G60 F254 while the mobile phase is composed of toluene, ethyl acetate, methanol, and glacial acetic acid (6:3:0.5:0.5 %v/v/v/v/v) and detected and scanned at 256 nm. Figure 1 demonstrates resolved peak of RIV and ASP. The R_f values of Rivaroxaban and Aspirin are 0.23 and 0.72, respectively, in a run distance of 90 mm as shown in Figure 1.

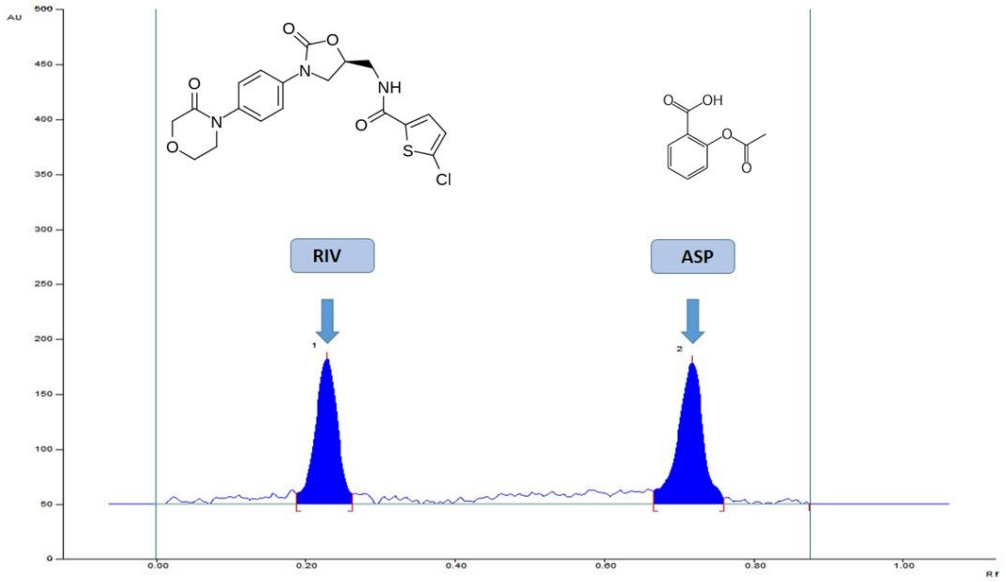

Figure 1. Densitogram of Rivaroxaban R_f (0.23) Aspirin (2-acetoxy benzoic acid) R_f (0.72)

During the development of the method, various parameters and their effect were checked by changing one parameter and holding other parameters constant.

3.2. Mobile Phase Characteristics

To achieve a sharp chromatographic peak without any interference, several trials were carried taken. The flow rate of the mobile phase and the solvents ratio of the mobile phase changed frequently

during this trial. Toluene, ethyl acetate, methanol, and glacial acetic acid (6:3:0.5:0.5 percent v/v/v/v/v) proved to be the best of all combinations for drug separation as both the drug were separated with optimum retention factor and there was no tailing or fronting in the peaks as shown in Figure 2.

3.3. Method Validation

3.3.1. *Linearity*

Linearity was performed to check the correlation in the range of 100-400ng/band for Rivaroxaban 2000-8000ng/band for Aspirin. The overlay linearity chromatogram of RIV and ASP has shown in Figure 2. The regression coefficient was found 0.998 and 0.998 for Rivaroxaban and Aspirin, respectively. The correlation coefficient was R^2= 0.998 for both the drugs, which was displayed in Figure S1 in supporting information. Linearity data is presented in Table 1.

Table 1. Linearity data of RIV and ASP (n=6)

No	RIV			ASP		
	Conc. (ng/band)	**Peak area (Mean±SD)**	**%RSD**	**Conc. (ng/band)**	**Peak area (Mean±SD)**	**%RSD**
1	100	1715.8 ± 14.33	0.83	2000	1914.06 ± 7.29	0.38
2	150	2111.92 ± 10.10	0.47	3000	2676.74 ± 14.62	0.54
3	200	2521.64 ± 19.78	0.78	4000	3129.84 ± 16.36	0.52
4	250	3026.04 ± 15.28	0.50	5000	3841.32 ± 25.74	0.67
5	300	3417.36 ± 13.27	0.38	6000	4542.34 ± 36.95	0.81
6	350	3839.4 ± 31.55	0.82	7000	5145.18 ± 29.75	0.57
7	400	4367.7 ± 26.46	0.60	8000	5739.44 ± 28.09	0.48

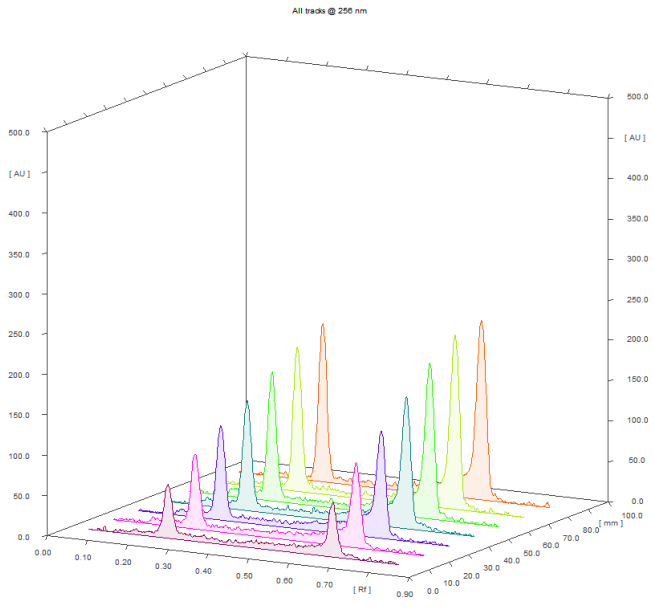

Figure 2. Overlay linearity chromatogram of RIV and ASP 100-400 ng/band and 2000-8000 ng/band

3.3.2. Accuracy

Percentage recovery and relative standard deviation percentage (%RSD) were determined, and results are provided in Tables 2 and 3.

Table 2. Accuracy data of RIV (n=3)

% Level	Sample Concentration (ng/band)	Standard added (ng/band)	Total concentration (ng/band)	Amount Recovered (ng/band)	% Recovery	Mean ± SD	% RSD
80%	150	120	270	268.8	99.5%	3165.233 ± 4.56	0.14
100%	150	150	300	299.65	99.88%	3436.36 ± 31.78	0.92
120%	150	180	330	332.18	100.66%	3722.3 ±2.66	0.85

Table 3. Accuracy data of ASP (n=3)

% Level	Sample Concentration (ng/band)	Standard added (ng/band)	Total concentration (ng/band)	Amount found (ng/band)	% Amount found	Mean area ± SD	% RSD
80%	3000	2400	5400	5393.2	99.80%	4102.7 ± 3.20	0.85
100%	3000	3000	6000	6025.9	100.43%	4504.93 ± 4.66	0.1
120%	3000	3600	6600	6655.3	100.83%	4905.26 ± 4.05	0.45

3.3.3. Precision

Intra-day precision -The percent RSD was calculated and found to be 0.43- 0.68 and 0.31-0.85 for RIV and ASP, respectively, which are less than 2.0 and under the acceptable limit. Table 4 shows the outcome.

Table 4. Intraday precision data of RIV and ASP for HPTLC method (n=6)

Conc. (ng/band)		Peak area (Mean ± SD)		%RSD	
RIV	**ASP**	**RIV**	**ASP**	**RIV**	**ASP**
150	3000	2120.4 ± 11.23	2714.6 ± 12.50	0.53	0.46
250	5000	2941.46 ± 20.70	3814.33 ± 11.95	0.68	0.31
350	7000	3819.2 ± 16.72	5053.26 ± 43.28	0.43	0.85

Inter-day precision-The percent RSD was calculated and found to be 0.34 – 1.02 for RIV and 0.44 – 0.70 for ASP, respectively, which are not more than 2.0 indicating the method is precise. Table 5 displays the outcome.

Table 5. Interday precision data of RIV and ASP for HPTLC method (n=6)

Conc. (ng/band)		Peak area (Mean ± SD)		%RSD	
RIV	**ASP**	**RIV**	**ASP**	**RIV**	**ASP**
150	3000	2164.5 ± 23.55	2756.7 ± 17.5	1.08	0.63
250	5000	3038.16 ± 31.21	3852.36 ± 27.25	1.02	0.70
350	7000	3866.83 ± 13.42	5075.5 ± 22.49	0.34	0.44

3.3.4. Specificity

When the chromatograms of the formulation and standard solution were compared, it found that the R_f values were found to be identical, i.e 0.23 for RIV and 0.72 for ASP. The tablet's excipient and other ingredients did not affect the separation and resolution of RIV and ASP. A high degree of correlation was found when the spectra scanned at the peak start (S), middle (M), and end (E) were compared (above 0.990) mentioned in Table 6. The purity of the matching locations was thus established. In addition, the individual drug's spectrum was compared to that of normal RIV and ASP as shown in Figure S2 in supporting information.

Table 6. Peak purity of RIV and ASP

Sr. No.	Drug component	Type	r (S, M)
1	RIV	Standard	0.996
2	ASP	Standard	0.999

3.3.5. Calculation of Limit of Detection (LOD) and Limit of Quantification (LOQ)

LoD and LoQ values for Rivaroxaban were found to be 0.59µg/mL and 1.80µg/mL and for Aspirin were found to be 6.93µg/mL and 21.01µg/mL respectively which indicate that the method is sensitive.

3.3.6. Robustness Using Five Variables

The developed HPTLC method was tested for robustness using selected variables included. (1) Alteration in Toluene. (2) Alteration in Ethyl Acetate. (3) Alteration in Methanol. (4) Alternation in GAA. (5) Alternation in Detection Wavelength. (6) Alternation in Saturation time. (7) Alternation in Run Distance. The results of the robustness study are mentioned in Table 7.

Table 7. Robustness studies with five variables

Parameters	Factor	Drug	Peak Area Mean ± SD	%RSD
Wavelength (256nm)	251nm	RIV	2161.467 ± 27.77	1.02
	(-5nm)	ASP	3860.23 ± 25.08	0.64
	261nm	RIV	3052 ± 34.47	1.12
	(+5nm)	ASP	3825.067 ± 35.94	0.93
Run Distance (90mm)	85mm	RIV	3032.367 ± 40.31	1.32
	(-5mm)	ASP	3838.267 ± 45.85	1.19
	95mm	RIV	3032.367 ± 40.31	1.32
	(+5mm)	ASP	3838.267 ± 45.85	1.19
Saturation Time (15min)	10min	RIV	3250.033 ± 40.20	1.23
	(-5min)	ASP	3951.233 ± 33.25	0.84
	20min	RIV	2976.067 ± 15.36	0.51
	(+5min)	ASP	3772.46 ± 4.27	0.11
Mobile phase ratio (6:3:0.5:0.5)	5.4	RIV	3132.967 ± 25.83	0.82
	(-10% Toluene)	ASP	3938.367 ± 27.17	0.69
	6.6	RIV	3046.06 ± 26.71	0.86
	(+10% Toluene)	ASP	3830.83 ± 33.99	0.62
	2.7	RIV	2973.26 ± 17.89	0.60
	(-10% Ethyl Acetate)	ASP	3756.53 ± 36.00	0.95
	3.3	RIV	3043.9 ± 42.42	1.39
	(+10% Ethyl Acetate)	ASP	3949.46 ± 45.30	1.14
	0.45	RIV	3040.23 ± 39.99	1.31
	(-10% Methanol)	ASP	3849.23 ± 31.40	0.81
	0.55	RIV	3032.36 ± 40.31	0.44
	(+ 10% Methanol)	ASP	3835.56 ± 26.34	0.50
	0.45	RIV	3065.2 ± 24.75	0.47
	(-10% GAA)	ASP	3878.7 ± 17.68	0.41
	0.55	RIV	3032.967 ± 25.83	0.85
	(+10% GAA)	ASP	3838.367 ± 27.17	0.70

3.4. Assay of In-house Tablet Formulation by Developed HPTLC Method

The amount of Rivaroxaban and Aspirin in a tablet was found to be in good agreement with the experimental results, implying that there is no influence from any of the excipients commonly included in tablets. The proposed approach was used for simultaneous estimation of Rivaroxaban and Aspirin in powder as well as tablet dosage forms. The chromatograph peaks at R_f values 0.23 and 0.72 were observed in the densitogram for RIV and ASP respectively. The equation y = 8.790x + 802.4 for RIV and y = 0.636x + 672.4 for ASP was used to determine its concentration. The % assay result is mentioned in Table 8.

Table 8. Assay of *in-house* tablet formulation by HPTLC method (Where, n=5)

Labeled claim (%W/W)		**Peak area**		**%Assay**	
RIV	**ASP**	**RIV**	**ASP**	**RIV**	**ASP**
400	8000	4398.8	5799.3	100.11	100.86
400	8000	4386.6	5687.8	100.20	98.98
400	8000	4399.7	5791.6	101.21	101.75
400	8000	4397.3	5701.7	100.22	98.88
400	8000	4391.8	5799.8	101.35	100.99
		MEAN		100.61	100.29
		% RSD		0.12	0.97

Table 9. Summary of results of HPTLC method

No.	**Parameters**	**RIV**	**ASP**
1	Linearity (ng/band)	100-400	2000-8000
2	Linearity equation	y = 8.7903x + 802.41	y = 0.636x + 672.4
3	Correlation co-efficient	0.998	0.998
4	Recovery study (Mean %RSD)	0.12	0.97
5	Intraday Precision (%RSD)	0.43- 0.68	0.31-0.85
6	Interday Precision (%RSD)	0.34 - 1.02	0.44 – 0.70
7	Repeatability Precision (%RSD)	1.16	1.05
8	LOD (µg/mL)	0.59	6.93
9	LOQ (µg/mL)	1.80	21.01
10	% Assay	100.61	100.29

4. Conclusions

In a conclusion, for separation of RIV and ASP in powder or tablet form the developed method shows better resolution of peaks as well as optimized R_f value for both the drugs. The HPTLC method was validated as per the ICH (Q2) R1 Guidelines. Table 9 shows the summary of validation parameters results that are within the approval requirements.

This research involves the development of a new tablet formulation containing Rivaroxaban and Aspirin. Moreover, the development and validation of a simple, accurate, sensitive, and cost-effective HPTLC method for simultaneous quantitation of RIV and ASP were carried out. The developed method was applied to in-house formulation and an assay was performed. The data of % recoveries showed the accuracy and efficiency of the proposed HPTLC method. The percentage assay values for formulations research were found to be between 99-101%. The R_f between RIV and ASP with optimized resolution indicates a better separation of both the drugs. All system suitability parameters are observed to be in optimal condition, and the range for RIV and ASP estimation was found. Considering the numerous validation factors and results, the developed technique appears to be selective, specific, accurate, linear, exact, and robust. As a result, the accurate, precise, sensitive, and robust HPTLC method was optimized, developed, and validated as per ICH Q2 (R1) guideline, which was applied for *in-house* tablet

formulation. The result of the assay suggests that the developed method can be used for simultaneous estimation of RIV and ASP for their dosage form as a part of regulatory submission.

Acknowledgements

The authors are thankful to Ramanbhai Patel College of Pharmacy, Changa, Gujarat, India for supporting this work.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/ journal-of-chemical-metrology

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