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Original Article

Comprehensive analysis of Aspirin and Apixaban: thedevelopment, validation, and forced degradation studies of bulk drugs and *in-house* capsule formulations using the RP-HPLC method

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Abstract: Objectives: This study aimed to develop a robust Reverse Phase-High-Performance Liquid Chromatography (RP-HPLC) method for simultaneous determination of Aspirin (ASP) and Apixaban (API) in bulk and in-house capsule formulations. Methods: The separation was conducted on a Phenomenex Luna C_{18} column using a Shimadzu LC20AT High-performance liquid chromatography (HPLC) system. The mobile phase consisted of 40:60 Acetonitrile (ACN): phosphate buffer (pH 4) modified by O-Phosphoric Acid (OPA). The parameters included a flow rate of 1 ml/min, a column temperature of 30°C, and Ultra-Violet (UV) detection at 227 nm. Method validation encompassed linearity, precision (Intraday and Interday), accuracy (% Recovery), and sensitivity (Limit of Detection (LOD) and Limit of Quantification (LOQ)). Stability testing followed The International Council for Harmonization (ICH) guidelines. Results: The developed method demonstrated reliable separation of Aspirin and Apixaban with retention times of 5.37 min and 7.10 min, respectively. It exhibited linearity over the concentration ranges of 50-300 µg/mL for Aspirin and 5-15 µg/mL for Apixaban. The recovery percentage ranged from 90.02% to 101% for Aspirin and 98.18% to 101.18% for Apixaban. LOD and LOQ were determined as 0.84 µg/mL and 2.55 µg/mL for Aspirin, and 0.41 µg/mL and 1.24 µg/mL for Apixaban, respectively. Stability testing confirmed the method's robustness under various stress conditions. Conclusions: The validated RP-HPLC method offers a reliable tool for routine analysis of Aspirin and Apixaban in pharmaceutical formulations, highlighting its potential for combined dosage applications and routine quality control.

Keywords: Aspirin, Apixaban, forced degradation studies, RP-HPLC, method development and validation, ICH

Introduction

Peripheral artery disease (PAD) is becoming increasingly common worldwide and results in critical limb ischemia (CLI) in a significant number of people. Death, amputation, and a worse quality of life are associated with the last stages of PAD and CLI. CLI treatments differ enormously, resulting in a wide range of therapeutic and therapeutic outcomes. Endovascular therapies for CLI are increasingly becoming more common due to their lower morbidity and mortality rates compared to open revascularization. Despite the enormous number of PAD procedures performed around the world, few studies have been completed to assess the use of antiplatelet and antithrombotic medications after surgery [1]. Because these procedures are primarily based on coronary methodological approaches to postintervention medications, the current guidelines for PAD treatment provide insufficient evidence to support any decision. Antithrombotic therapies are administered to patients with PAD to prevent microem-

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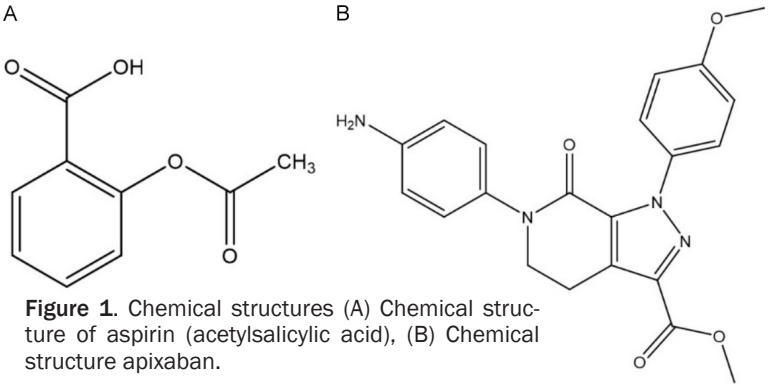

Figure 1. Chemical structures (A) Chemical structure of aspirin (acetylsalicylic acid), (B) Chemical structure apixaban.

bolization and thrombus formation in the treated artery, resulting in early thrombosis inhibition. Most patients with PAD have long occlusions and a significant thrombus load due to the blocked length of the arteries, particularly in the infrapopliteal arteries [2].

In a recent study of infrapopliteal arteries after limb amputation due to CLI, researchers discovered that 72.7% of infrapopliteal arteries with less than 70% stenosis developed acute or chronic thrombi. Notably, atherosclerotic plaques did not always correlate with this phenomenon. The majority of the arteries involved in luminal thrombus formation had few atherosclerotic plaques (67.5%) [3]. This raises the possibility of a link between atherosclerosis and thrombotic events. Following surgery, the infrapopliteal vessels are frequently narrower and more prone to obstruction. In patients with tissue loss, occlusions in such arteries are more common than in those with stenosis. According to research, thrombus components of the arterial wall are more common in below-the-knee arteries. Repeat surgeries and infrapopliteal arterial restenosis are more common and begin sooner than in the femoropopliteal arteries. In comparison to more proximal angioplasties, the proposed anticoagulant approach using the infrapopliteal segment would most likely require fewer patients to demonstrate its safety and efficacy. Inferolateral angioplasty is a surgery in which narrowed or obstructed blood vessels that feed the heart with oxygen and nutrients are opened. The coronary arteries are the names of these blood vessels. A coronary artery stent is a tiny metal mesh tube that expands inside the artery. Angioplasty is frequently followed by stent placement. This approach helps to prevent the artery from closing again. A drug-eluting stent contains medication that keeps the artery open over time

The AGRIPPA study revealed that dual-route treatment with 100 mg aspirin and 2.5 mg aspirin twice daily was effective [4]. In chronic diseases, the AGRIPPA trial was the most recent experimental test of this dual pathway methodology. In patients with PAD who underwent infrapopliteal angioplasty, 2.5 mg of apixaban twice daily plus aspirin was compared to the standard of care. Apixaban is a reversible small-molecule factor Xa inhibitor with a plasma half-life of 8-15 hours. Direct oral anticoagulants are safer and more effective than vitamin K antagonists at preventing strokes and treating venous thromboembolism (VTE) in people with atrial fibrillation. In these cases, apixaban is a treatment option. Researchers have thoroughly investigated the safety of this medication in a variety of contexts, and confirmed that combining it with aspirin results in a low risk of bleeding. The chemical structures of aspirin and apixaban are shown in **Figure 1**. **Figure 1A** shows the chemical structure of aspirin (acetylsalicylic acid), a widely used antiplatelet agent known for its role in cardiovascular disease prevention. **Figure 1B** shows the chemical structure of apixaban, an oral anticoagulant belonging to the direct factor Xa inhibitor class, which is used primarily for stroke prevention in atrial fibrillation and venous thromboembolism treatment. Recently, the Augustus experiment demonstrated the safety and effectiveness of apixaban in combination with antiplatelet therapy in patients with acute myocardial infarction (AF) undergoing percutaneous coronary intervention [5].

For the simultaneous determination of ASP and API, several analytical techniques have been published for single drugs but not for this combination. Several UV methods [6-10], HPLC methods [11-31] and HPTLC methods [32-38] are among the methods used for single-drug combinations and combinations with other drugs. Analytical method validation assures that various HPLC analytical techniques produce consistent and reproducible results; this is an important stage in the development of new dosage forms since it offers information

on accuracy, linearity, precision, detection, and quantitation limits. According to the ICH regulations, "the goal of validation of an analytical process is to demonstrate that it is suitable for its intended purpose". The provision of validation data for the responsible authorities is now required during the medication development process. The ICH has standards for validating analytical methods. A new sensitive and quick RP-HPLC method for determining the concentration of aspirin and apixaban in bulk and pharmaceutical formulations was developed in this research article, and the method was validated as per ICH regulations. We conducted forced degradation studies following the ICH Q1A (R2) Guidelines [39, 40].

Material and methods

Instrumentation

The experiment was carried out on a Shimadzu-LC 20AT HPLC system equipped with a photodiode array detector, prominence pump, column oven, LC solution software (version 1.25), and manual sample injector. The system was operated by software (version 1.25), and a Phenomenex C18 column (250 mm × 4.6 mm, 5 µm) was used for separation.

Materials and reagents

The aspirin standard and API were purchased from Himedia Laboratories Pvt. Ltd., India. The apixaban standard and API were gifted by Bio Organics & Applied Materials Pvt. Ltd., Bangalore, India. Merck Life Science Pvt. Ltd., Mumbai, India, delivered HPLC-grade solventssuch as acetonitrile, OPA, methanol, IPA, TEA, and HCL. The Slab Reagents (Vadodara, India) supplied potassium dihydrogen orthophosphate, sodium hydroxide, and ethyl cellulose. Loba Chemie Pvt. Ltd., Mumbai, India, delivered hydrogen peroxide lactose, magnesium stearate, microcrystalline cellulose, talc, and starch. The rest of the reagents were of analytical grade. For separation, the mobile phase consisted of a 60:40 v/v mixture of potassium dihydrogen phosphate buffer (pH 4) and ACN. The flow rate was held constant at 1.0 mL/min, the column temperature was held at 30°C, the eluents were detected with a UV detector at 227 nm, and the injection volume was 20 µL.

Methodology

Buffer solution preparation (20 ppm): A total of 2.72 g of potassium dihydrogen phosphate (KH_2PO_4) was weighed and dissolved in a 1000 ml volumetric flask with Milli-Q water. Then, 1 ml of triethylamine (TEA) was added to the solution. The pH was adjusted to 4 using o-phosphoric acid (OPA).

Mobile-phase preparation: Forty volumes of acetonitrile (ACN) were blended with sixty volumes of buffer, with the pH subsequently adjusted to 4 utilizing o-phosphoric acid (OPA). Following this, the solution underwent filtration through a 0.45 µm membrane filter, and the mobile phase was subjected to sonication for a duration of 25 minutes in a sonicator.

Stock solution preparation: A total of 25 mg of aspirin standard was meticulously weighed and transferred into a 25 ml volumetric flask. Subsequently, acetonitrile (ACN) was added to dissolve the aspirin, following which the volume was adjusted to match that of ACN. Consequently, the final stock solution prepared was 1000 µg/ml of aspirin (ASP).

Preparation of working stock solutions: Ten milligrams of the apixaban standard were accurately weighed and transferred into a 10 ml volumetric flask. The solution was then dissolved and diluted to volume with acetonitrile (resulting in a concentration of 1000 µg/ml). Subsequently, 1 ml of the standard solution was pipetted into a 10 ml volumetric flask and further diluted with acetonitrile to obtain a concentration of 100 µg/ml. From this 100 µg/ml solution, 5 ml of the apixaban solution was withdrawn and added to the previously prepared stock solution. The final working standard solutions thus obtained contained 1000 µg/ml of aspirin and 50 µg/ml of apixaban. From this combined solution, volumes ranging from 0.5 to 3 ml were pipetted into 10 ml volumetric flasks. Consequently, the final concentrations of the solutions ranged from 50-300 µg/ml for aspirin and 2.5-15 µg/ml for apixaban.

Preparation of an in-house capsule dosage form: The aspirin powder and apixaban tablets were prepared at a volumetric ratio of 100:5. To coat the apixaban tablets, a 3% ethyl cellulose solution was applied using a coating pan. Inside the capsules, aspirin powder was inserted,

while apixaban tablets were placed above the capsule. Various common excipients, including starch, talc, microcrystalline cellulose, ethyl cellulose, and magnesium stearate, were utilized in the formulation.

Method validation

The method was validated according to ICH regulations ICH Q2 (R1), with validation parameters such as specificity, linearity, range, accuracy, precision, LOQ, LOD, and robustness.

For aspirin and apixaban, the specificity of the established analytical approach was tested. To ensure that no contaminants influenced the results, a placebo solution was placed onto the C_{18} column.

System suitability

The theoretical plates, tailing factor, resolution, peak asymmetry, and % RSD for the peak area were considered for system suitability. The values were calculated for aspirin and apixaban using a developed chromatogram and adhering to acceptance criteria. The chromatograms were recorded after injecting a stock solution containing a mixture of 100 µg/mL aspirin and 5 µg/mL apixaban three times.

Linearity and range

Linearity refers to an analytical procedure's ability (within a given range) to produce test results that are directly proportional to the concentration (amount) of analyte in the sample. The calibration curve method was used to determine the LOD and LOQ of ASP and API. Aspirin and apixaban solutions containing 50-300 µg/mL and 2.5-15 µg/mL, respectively, were prepared and injected five times.

Limit of detection (LOD) and limit of quantitation (LOQ)

The calibration curve method was used to determine the LOD and LOQ of aspirin and apixaban. Aspirin and apixaban solutions were prepared at concentrations of 50-300 µg/mL and 2.5-15 µg/mL, respectively. The samples were injected in triplicate.

Precision

Repeatability: Six injections of the same concentration of aspirin (50-300 µg/ml) or apixaban (2.5-15 µg/ml) were used to test the repeatability of the results. The repeatability parameter shows the peak area of both peaks, as well as the % RSD calculated.

Intraday precision: The intraday precision was assessed by calculating the % RSD for three distinct concentrations of aspirin (100, 200, and 300 µg/mL) and apixaban (5, 10, and 15 µg/mL) over three different days.

Interday precision: Interday accuracy was established by calculating the % RSD for three different concentrations of aspirin (100, 200, and 300 µg/mL) and apixaban (5, 10, and 15 µg/mL) every day for three days.

Accuracy

The method's accuracy was determined through recovery studies at three levels using the standard addition method. The % recovery was calculated for each level and to establish analytical methods for assessing the recoveries of aspirin and apixaban. The % recovery should not be less than 98.0% and not more than 102.0%.

Robustness

Various parameters, such as flowrate changes, wavelength changes, mobile phase ratio changes, pH changes, and other changes, were changed for the developed RP-HPLC method for simultaneous estimation of ASP and API.

Assays of in-house formulations

Twenty capsules were weighed and ground into fine pieces with a dry mortar and pestle. The equivalent powder was weighed to an average weight, and a single capsule (565 mg powder) was transferred to a 100 ml volumetric flask. The volume was adjusted using acetonitrile after 25 minutes of sonication, after which a stock solution was obtained. To eliminate excipients, the solution was filtered through a membrane (0.45 µm). This solution was also diluted to prepare test solutions for analysis.

Forced degradation studies

In the ICH Q1A (R2) section 2.1.2, parameters for performing forced degradation tests on drug ingredients and drug products are recommended (stress testing), where temperature, humidity, oxidation, and photolysis are all ad-

vised. Aspirin and Apixaban are subjected to the above-mentioned stress conditions. For acidic and alkali degradation, samples were refluxed with 0.1 N HCl and 0.1 N NaOH at 40°C for 60 min. For oxidative degradation, 30% v/v H_2O was used and the same was refluxed at 60°C for 30 min. For thermal degradation, a sample was placed in an oven at 105°C for 6 h; for photostability degradation, the drug was exposed to UV light by keeping the sample in a UV chamber for 7 days or 200 W h/m^2 in a photostability chamber; for neutral degradation, the drugs were refluxed in water for 6 h at 60°C. Sample were subjected to refrigerated temperature for 5 days. All the samples were diluted to obtain a final concentration of 100 µg/mL of Aspirin and 5 µg/mL of Apixaban. Twenty microliters of the samples were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Solution stability

The drug solution's stability underwent assessment for short-term and autosampler stability. Short-term stability was evaluated by storing samples at room temperature (25°C) for 24 hours, while autosampler stability was assessed by storing samples in the autosampler for the same duration. Each sample underwent six injections into the HPLC, and the results were compared with the nominal values of QC samples.

Statistical parameters

It is necessary to do statistical analysis on the data collected during a method validation in order to prove the accuracy and reliability of the analytical method. The key factors utilized for analyzing the outcomes of analytical technique validation include the computation of the mean (or average), standard deviation, relative standard deviation, and regression analysis.

Mean: The mean or average of a data collection is a fundamental and widely used statistical measure. The mean is determined by summing all data points and dividing the total by the number of samples.

Standard deviation: The standard deviation of a data set is a statistical metric that quantifies the extent to which the values in the sample set deviate from the mean. It is calculated by determining the difference between the mean and each individual value in the set.

Relative standard deviation: The relative standard deviation is calculated by multiplying the standard deviation of the sample set by 100% and dividing it by the average of the sample set. The relative standard deviation is shown as a percentage. Typically, the criteria for determining the accuracy, precision, and repeatability of data is expressed as the percentage relative standard deviation (% RSD).

Regression analysis: Regression analysis is employed to assess the linear correlation between test findings. A linear relationship is typically assessed across the entire range of the analytical method. The data acquired from analyzing the solutions prepared at various concentration levels is often examined by graphing it. Linear regression is a statistical method that examines the connection between two variables by creating a linear equation based on observed data. Other significant computations typically given include the coefficient of determination (R2) and linear correlation coefficient (r). The coefficient of determination (R2) quantifies the amount of variation that is accounted for by the model. Optimally, R2 should have a value of one, indicating a complete absence of error.

Results

Optimization of chromatographic conditions

Different chromatographic conditions were explored to develop a suitable RP-HPLC method for the simultaneous detection of aspirin and apixaban. The optimized chromatographic conditions, detailed in **Table 1**, were established as follows: utilizing a Shimadzu HPLC-LC20AT instrument, a Phenomenex Luna C_{18} stationary phase (250*4.6 mm id., 5 µm), and a mobile phase consisting of phosphate buffer pH 4 and acetonitrile in a ratio of 60:40% v/v. The injection volume was set at 20 µl, using a sample mixture of aspirin (50 µg/ml) and apixaban (2.5 µg/ml). The column oven temperature was maintained at 30°C, with detection performed at a wavelength of 227 nm using a PDA Shimadzu detector. The flow rate was set at 1 ml/min, with a column saturation time of 90 minutes prior to analysis. The chromatographic separation achieved by the above condition was represented in **Figure 2** where a guardian peak is observed on the chromatogram with proper retention time, tailing factor, resolution, and other parameters observed.

Table 1. Optimized chromatographic conditions

Parameter	Condition
Column	Phenomenex Luna C_{18} (250*4.6 mm id., 5 µm)
Mobile phase	Phosphate Buffer pH 4: Acetonitrile (60:40% v/v)
Diluent	Acetonitrile
Column temperature	30°C
Wavelength	227 nm
Flow rate	1 ml/min
Run Time	12 Min
Injection volume	20 µl

Specificity

For aspirin and apixaban, the specificity of the established analytical approach was tested. To ensure that no contaminants influenced the results, a placebo solution was placed onto the C_{18} column, as mentioned in **Figure 2**, shows the method is specific. Chromatogram depicting (**Figure 2A**) blank chromatogram, (**Figure 2B**) optimized chromatogram of Aspirin and Apixaban in mixture, (**Figure 2C**) peak purity curve of aspirin, and (**Figure 2D**) peak purity curve of apixaban. This analysis highlights the distinct chromatographic profiles and purity assessments critical for the quality control of these pharmaceutical compounds.

System suitability

The theoretical plates, tailing factor, resolution, peak asymmetry, and % RSD for the peak area were considered for system suitability. The values were calculated for aspirin and apixaban using a developed chromatogram and adhering to acceptance criteria. The chromatograms were recorded after injecting a stock solution containing a mixture of 100 µg/mL aspirin and 5 µg/mL apixaban three times. The system suitability parameters included the number of theoretical plates, resolution, and peak asymmetry, which were calculated to determine whether the results met the recommended limits described in **Table 2**.

Linearity and range

Linearity refers to an analytical procedure's ability (within a given range) to produce test results that are directly proportional to the concentration (amount) of analyte in the sample. The calibration curve method was used to determine the LOD and LOQ of aspirin and apixaban. Aspirin and apixaban solutions containing 50-300 µg/mL and 2.5-15 µg/mL, respectively, were prepared and injected five times.

Limit of detection (LOD) and limit of quantitation (LOQ)

The calibration curve method was used to determine the LOD and LOQ of aspirin and apixaban. Aspirin and apixaban solutions were prepared at concentrations of 50-300 µg/mL and 2.5-15 µg/mL, respectively. The samples were injected in triplicate. The LOD and LOQ for aspirin were 0.84 µg/ml and 2.55 µg/ml, respectively, while those for apixaban were 0.41 µg/ml and 1.24 µg/ml, respectively, which demonstrated that the method is sensitive.

Precision

Repeatability: Six injections of the same concentration of aspirin (50-300 µg/ml) or apixaban (2.5-15 µg/ml) were used to test the repeatability of the results. The repeatability parameter shows the peak area of both peaks, as well as the % RSD calculated, % RSD was less than 1.5%.

Intraday precision: The intraday precision was assessed by calculating the % RSD for three distinct concentrations of aspirin (100, 200, and 300 µg/mL) and apixaban (5, 10, and 15 µg/mL) over three different days. The intraday precision shows various factors, such as the mean area, standard deviation, and % RSD.

Interday precision: Interday accuracy was established by calculating the % RSD for three different concentrations of aspirin (100, 200, and 300 µg/mL) and apixaban (5, 10, and 15 µg/mL) every day for three days. Interday precision describes the mean area, standard deviation, and % RSD for peak areas within limits.

Accuracy

The method's accuracy was determined through recovery studies at three levels using the standard addition method. Aspirin and apixaban had mean percentage recoveries of 99.02-101% and 98.18-100.18%, respectively.

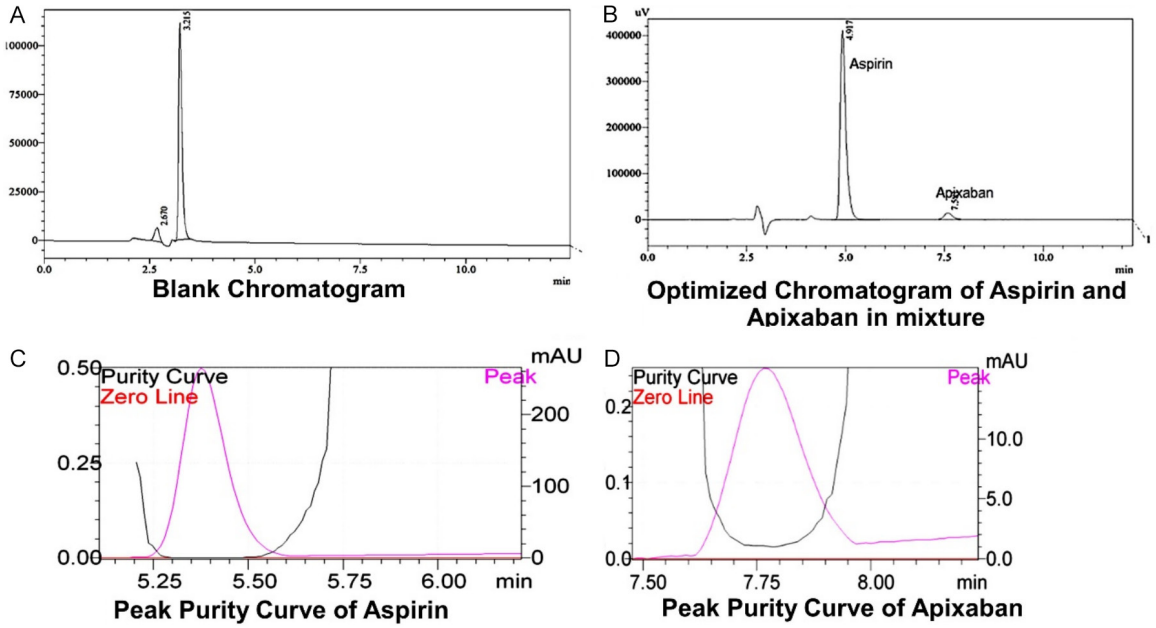

Figure 2. Chromatogram analysis under various conditions. A. Blank chromatogram. B. Optimized chromatogram of Aspirin and Apixaban in mixture. C. Peak purity curve of aspirin. D. Peak purity curve of apixaban.

Table 2. Summary of system suitability parameters

Parameter	Aspirin	Apixaban
Retention time (min)	5.37	7.10
Tailing factor	1.366	1.256
Theoretical plates	8588.88	9794.39
Resolution	-	8.57
Purity	0.999995	0.999983
Repeatability [RSD, % (n=6)]	1.48	1.22

RSD: Relative Standard Deviation.

The % recovery was calculated for each level and was found to be under the limit, indicating the accuracy of the established analytical methods for assessing the recoveries of aspirin and apixaban. The % recovery should not be less than 98.0% and not more than 102.0%.

Robustness

Various parameters, such as flowrate changes, wavelength changes, mobile phase ratio changes, pH changes, and other changes, were changed, and the developed RP-HPLC method for simultaneous estimation of aspirin and apixaban was found to be robust with a % RSD of less than 1.5.

A summary of all validation parameters is mentioned in **Table 3**.

Results of the in-house formulation

Assay % of *in-house* formulations should range between 98% and 103%. Assays for *in-house* formulations are within the acceptance criteria, and analysis of the formulation is performed with the developed method shown in **Table 4** and chromatogram in **Figure 3**, illustrating (**Figure 3A**) placebo, (**Figure 3B**) chromatogram of in-house capsule formulation, (**Figure 3C**) peak purity curve of aspirin (In-house formulation), and (**Figure 3D**) peak purity curve of apixaban (In-house formulation). These results underscore the robustness of the chromatographic method in differentiating between active pharmaceutical ingredients and excipients in formulations.

Forced degradation

The standard solutions underwent a series of stress tests as outlined in the experimental procedure. **Figures 4-9** present chromatograms depicting degradation profiles under various stress conditions. Under acidic conditions, aspirin and apixaban underwent degradation rates of around 2.34% and 3.04%,

Table 3. Summary of validation parameters

Sr. No	Parameters	Results	
		Aspirin	Apixaban
1	Linearity range (µg/mL)	20-300	2.5-15
2	Regression equation	Y = 43529 × + 117489	Y = 50526 × - 2376.2
3	Regression coefficient	0.9984	0.9994
4	Intraday precision [RSD, % (n=3)]	0.65-1.42	0.77-1.31
5	Interday precision [RSD, % (n=3)]	0.95-1.50	0.68-1.12
6	Repeatability [RSD, % (n=6)]	0.95	0.81
7	Accuracy (% recovery, n=3)	99.02-101.0	98.18-100.18
8	LOD (µg/mL)	3.69	0.07
9	LOQ (µg/mL)	11.19	0.23
10	% Assay (n=6)	100.3	99.6
11	Robustness	Robust	Robust
12	Solvent Stability	Stable	Stable

Table 4. Assay of an *In house* Capsule formulation

Aspirin			Apixaban		
Conc. (µg/ml)	Mean Area (n=6)	% Assay (n=6)	Conc. (µg/ml)	Mean Area (n=6)	% Assay (n=6)
100	4524214	99.5	5	252989	99.9
	4545688	99.9		257839	101.9
	4588920	100.9		253856	100.3
	4580561	100.7		250699	99.1
	4561363	100.3		256370	101.3
	4560568	100.3		250857	101.5
Mean area	4560219	100.31	Mean area	254756.5	100.68
± SD (n=6)	23437.1		± SD (n=6)	2706.22	
% RSD (n=6)	0.51		% RSD (n=6)	1.06	

respectively, in their bulk forms, as illustrated in **Figure 4A**. In formulation, these degradation rates were 1.63% for aspirin and 8.0% for apixaban, as depicted in **Figure 4B**. These results emphasize the compounds' susceptibility to acid-induced degradation, which is critical for assessing their stability in acidic environments relevant to pharmaceutical processing and storage.

Under alkaline conditions, aspirin and apixaban underwent degradation of about 2.59% and 3.5%, respectively, in bulk as shown in **Figure 5A**. In formulation, these percentages were 1.63% and 9.0% for aspirin and apixaban, respectively, as depicted in **Figure 5B**. The chromatograms in **Figure 5** illustrate the degradation of peaks in both aspirin and apixaban under basic stress conditions, offering insights into their behavior in alkaline environments.

Under oxidative stress conditions, aspirin and apixaban exhibited degradation rates of approximately 9.7% and 1.07%, respectively, in their bulk forms as shown in **Figure 6A**. In formulation, these degradation rates were 9.5% for aspirin and 1.05% for apixaban, as indicated in **Figure 6B**. This highlights the vulnerability of these compounds to oxidation-induced degradation, crucial for understanding their stability in environments pertinent to pharmaceutical production and storage.

Under photolytic stress conditions, aspirin and apixaban experienced degradation rates of approximately 10.1% and 2.2%, respectively, in their bulk forms as mentioned in **Figure 7A**. In formulation, these degradation rates were 9.1% for aspirin and 2.0% for apixaban, as shown in **Figure 7B**. This depicts chromatograms illustrating degradation induced by light exposure, emphasizing the compounds' sus-

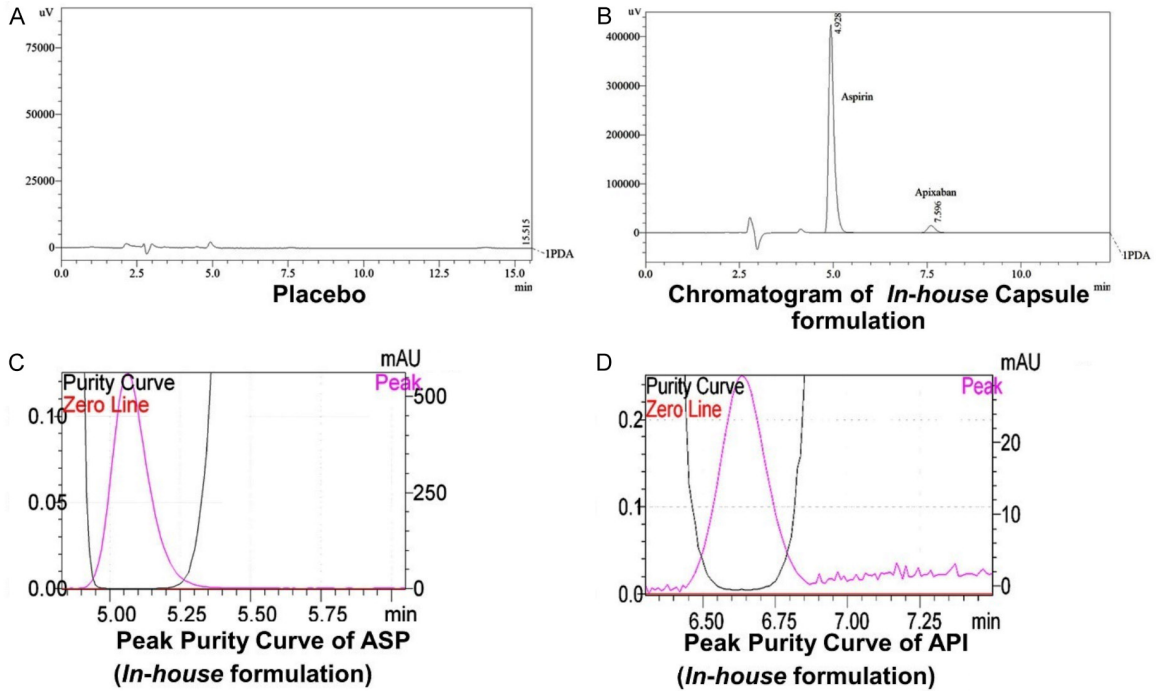

Figure 3. Chromatogram analysis of placebo and formulation samples under optimized conditions. A. Placebo. B. Chromatogram of In-house capsule formulation. C. Peak purity curve of aspirin (In-house formulation). D. Peak purity curve of apixaban (In-house formulation).

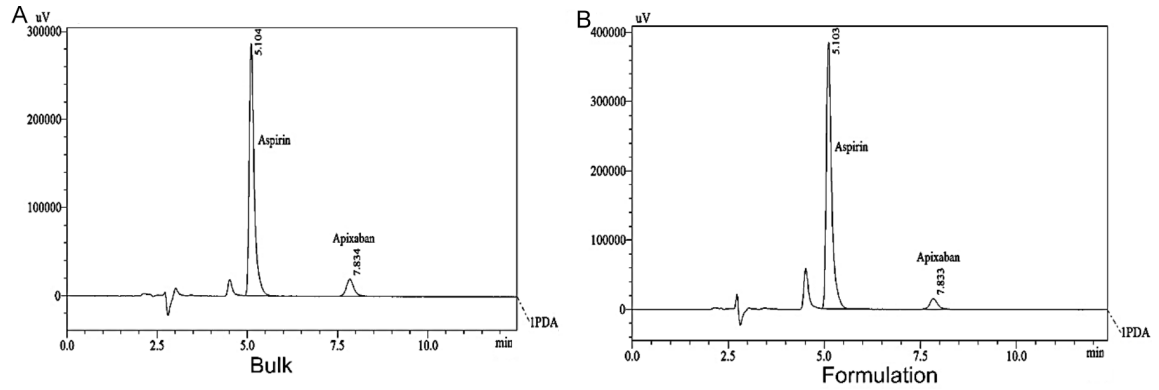

Figure 4. Chromatographic profiles illustrating peak degradation under acidic conditions in (A) bulk and (B) formulation samples.

ceptibility to photolytic degradation and its implications for formulation stability.

Under thermal stress conditions, aspirin and apixaban exhibited degradation rates of approximately 8.2% and 1.4%, respectively, in their bulk forms as shown in the chromatogram in **Figure 8A**. In formulation, these degradation rates were 8.9% for aspirin and 1.3% for apixaban, as depicted in **Figure 8B**. The chromatograms in **Figure 8** demonstrate degradation under temperature-induced stress, critical for assessing compound stability during manufacturing, transportation, and storage.

Under refrigerated conditions (2-5°C), both aspirin and apixaban exhibit minimal degradation (<1%) in both bulk (**Figure 9A**) and formulation (**Figure 9B**), as indicated by the absence of significant degradation peaks. These chromatograms highlight the compounds' stability under low-temperature stress, providing

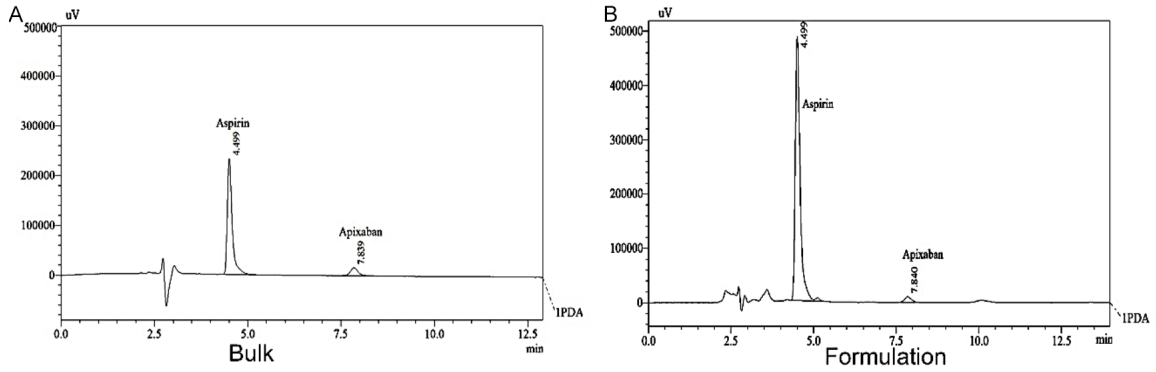

Figure 5. Chromatographic analysis of peak degradation under alkaline conditions in (A) bulk and (B) formulation samples.

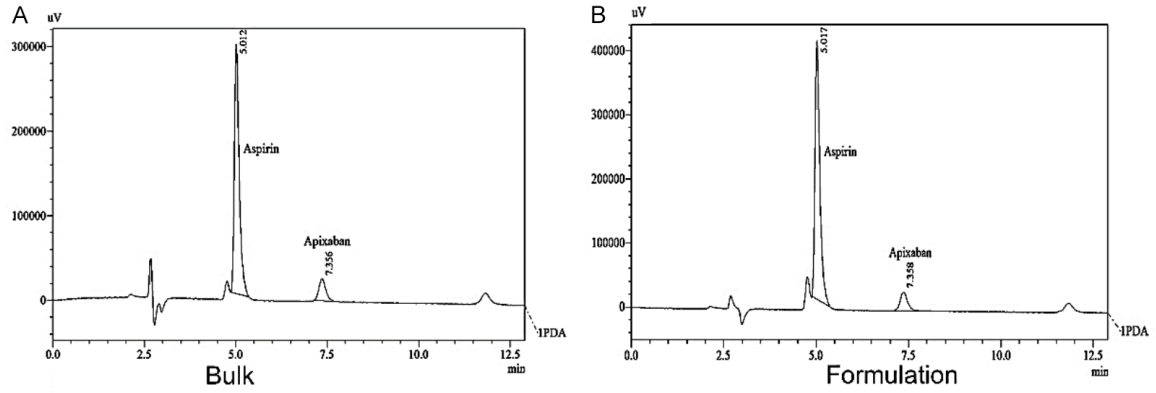

Figure 6. Chromatographic profiles illustrating peak degradation under oxidative conditions in (A) bulk and (B) formulation samples.

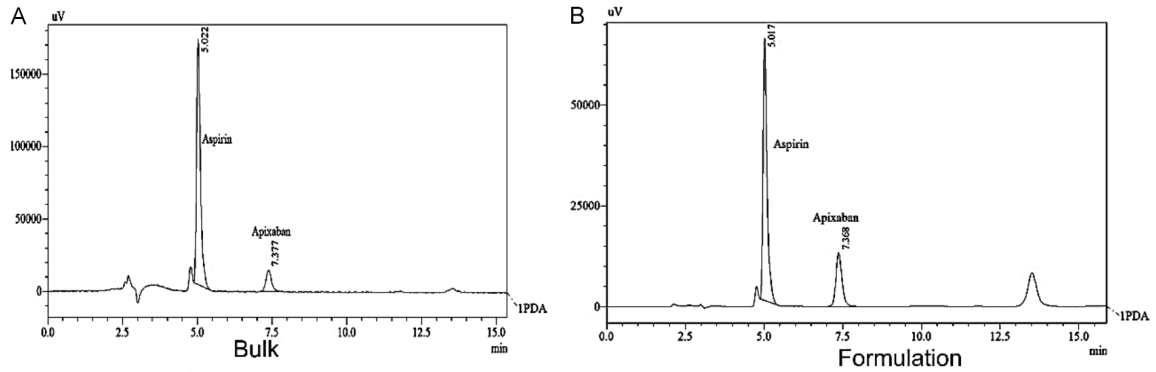

Figure 7. Chromatographic profiles illustrating peak degradation under photolytic stress conditions in (A) bulk and (B) formulation samples.

insights into their behavior during prolonged storage and distribution in pharmaceutical settings. **Table 5** presents a detailed summary of the degradation conditions and recovery of aspirin and apixaban for comprehensive analysis and comparison.

Discussion

The entire experiment was conducted in accordance with ICH Q2 (R1) Guidelines, wherein the Peak Purity Index and Single Point Threshold of both drugs were determined to be nearly 0.99,

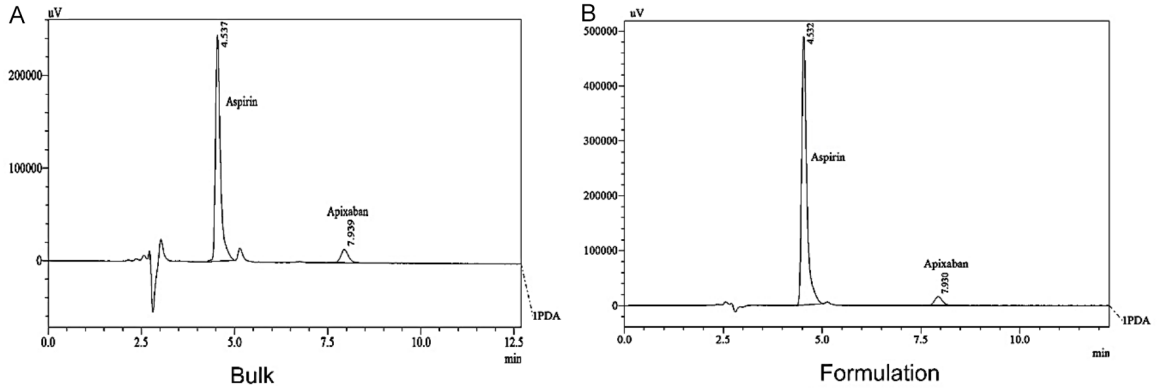

Figure 8. Chromatographic profiles illustrating peak degradation under thermal stress conditions in (A) bulk and (B) formulation samples.

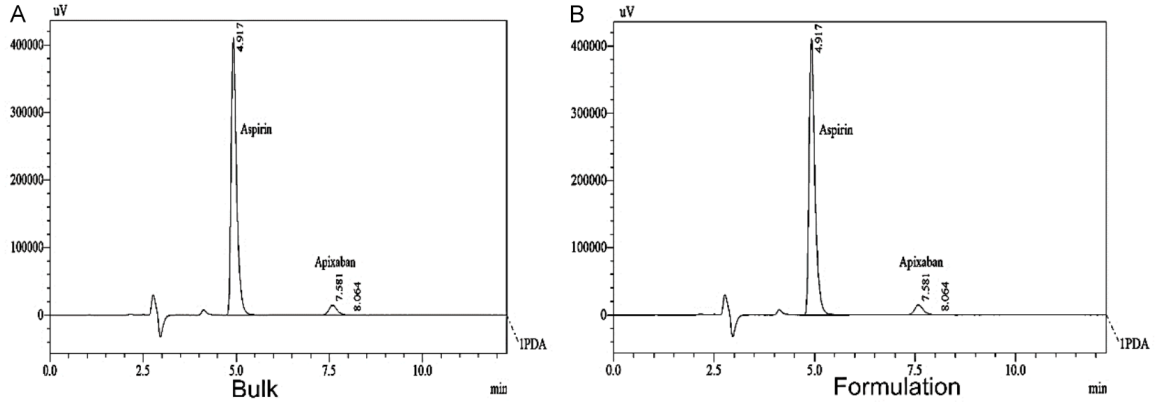

Figure 9. Chromatographic profiles illustrating peak degradation under refrigerated temperature conditions in (A) bulk and (B) formulation samples.

surpassing the required minimum of 0.95. System suitability parameters including theoretical plates, tailing factor, and % RSD for peak area were rigorously evaluated. The values obtained for aspirin and apixaban from the chromatogram met the acceptance criteria, which specify that the method must achieve theoretical plates, a tailing factor of not more than 1.5, and % RSD of less than 2.0%. Additionally, the resolution between peaks exceeded 2, confirming that all system appropriateness characteristics were within defined limits.

Linearity and range parameters such as slope, intercept, % RSD, LOD, LOQ, and the coefficient of determination (R2) for the calibration curves were found to be within acceptable limits. The R2 value for the linearity curve exceeded 0.99, indicating excellent correlation between concentration and chromatographic response for both apixaban and aspirin across their working ranges. The method exhibited sensitivity with low LOD and LOQ values: 0.84 µg/ml and 2.55 µg/ml for aspirin, and 0.41 µg/ml and 1.24 µg/ml for apixaban, respectively.

Precision was assessed using repeatability, with six replicate injections at the same concentration, yielding mean, standard deviation (SD), and % RSD values within acceptable limits. Intraday and Interday precision studies across different days and instruments showed % RSD values consistently below 1.5%, demonstrating the method's precision and reproducibility over time and across different analytical setups.

Accuracy was evaluated through recovery studies where concentrations of 80%, 100%, and 120% were spiked, resulting in recovery percentages between 98.0% and 102.0% for both aspirin and apixaban at each level. These

Table 5. Summary of forced degradation studies of Aspirin and Apixaban from Bulk drug and *in-house* capsule formulation

Sr No	Degradation Type	Degradation Condition	Sample Type	Amount of ASP degraded (%)	Amount of ASP Recovered (%)	Amount of API degraded (%)	Amount of API Recovered (%)
1	Acid Hydrolysis	0.1 N HCl, 1 hour, 40°C	Bulk drug	2.34	97.66	3.04	96.96
		0.1 N HCl, 1 hour, 40°C	*In-house* formulation	1.63	98.37	8.0	92.0
2	Alkaline Hydrolysis	0.1 N NaOH, 1 hour, 40°C	Bulk drug	2.59	97.41	3.5	96.5
		0.1 N NaOH, 1 hour, 40°C	*In-house* formulation	1.63	98.37	9	91
3	Oxidative Stress	3% H_2O_2, 1 hour	Bulk drug	9.7	90.3	1.07	98.93
		3% H_2O_2, 1 hour	*In-house* formulation	9.5	90.5	1.05	98.95
4	Photolytic Stress	Kept in UV light, 1 hour	Bulk drug	10.1	89.9	2.2	97.8
		Kept in UV light, 1 hour	*In-house* formulation	9.1	90.9	2.0	98
5	Thermal Stress	80°C, 1 hour	Bulk drug	8.2	91.8	1.4	98.6
		80°C, 1 hour	*In-house* formulation	8.9	91.1	1.3	98.7
6	Refrigerated Temperature	2-5°C, 5 days	Bulk drug	No Degradation Observed	100	No Degradation Observed	100
		2-5°C, 5 days	*In-house* formulation	No Degradation Observed	100	No Degradation Observed	100

results fell within the specified recovery range, indicating the accuracy and reliability of the developed analytical procedure.

Robustness testing confirmed the method's resilience to small variations in temperature, flow rate, wavelength, pH, and mobile phase ratio, with % RSD values remaining within acceptable limits under different conditions.

Analysis of an in-house capsule formulation demonstrated assay results of approximately 100.31% for aspirin and 100.68% for apixaban, meeting the acceptance criteria of 98-103%. Forced degradation studies under acid hydrolysis, alkaline hydrolysis, oxidative, photolytic, thermal, and refrigerated conditions showed degradation percentages between 5% and 20%, indicating the stability of both drugs under various stress conditions, particularly at refrigerated temperatures.

Conclusion

The development of a precise, accurate, and robust RP-HPLC method for the simultaneous quantification of aspirin and apixaban in both bulk and in-house capsule formulations represents a significant advancement in pharmaceutical analytical chemistry. The developed methodology showcases notable attributes including simplicity, sensitivity, robustness, precision, and efficiency, rendering it a valuable tool for routine laboratory analyses and combined dosage applications within the pharmaceutical industry. Key optimizations, such as the utilization of a Phenomenex Luna C_{18} column, a mobile phase comprising a 40:60 ratio of acetonitrile to phosphate buffer at pH 4, and UV detection at 227 nm, facilitated successful chromatographic separation and quantification of both analytes. Method validation, in accordance with ICH Q2 (R1) guidelines, revealed excellent linearity, recovery, and precision, with all parameters meeting predefined acceptance criteria. Moreover, forced degradation studies, conducted per ICH Q1A (R2) guidelines, confirmed the stability of aspirin and apixaban under various stress conditions, underscoring the reliability and robustness of the proposed RP-HPLC method in which the refrigerated condition is found to be best storage condition for both the drugs. With a rapid run time of less than 10 minutes, this method offers practical utility in pharmaceutical analysis, enabling efficient quantification of aspirin and apixaban combinations across diverse dosage forms, thus addressing a critical need in drug development and quality control.

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Disclosure of conflict of interest

None.

Abbreviations

ACN, Acetonitrile; OPA, Orthophosphoric acid; API, Apixaban; IPA, Isopropyl Alcohol; ASP, Aspirin; TEA, Triethylamine; PAD, Peripheral artery disease; HCL, Hydrochloric acid; VTE, venous thromboembolism; Con, Concentration; VKA, Vitamin K antagonists; NaOH, Sodium hydroxide; CLI, Critical limb ischemia; H_2O_2, Hydrogen Peroxide; AF, Atrial fibrillation; DOAC, Direct oral anticoagulant; ICH, International Council for Harmonization; mins, Minutes; mg, Milligram; UV, Ultra-Violet; RP-HPLC, reverse-phase high-performance liquid chromatography.

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