RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF ARGATROBAN IN BULK FORM

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ABSTRACT:

A simple, precise, and accurate reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the quantitative estimation of argatroban in its bulk drug form. Chromatographic separation was achieved on a J'Sphere ODS-H80 column (150 mm x 4.6 mm, 4.0 µm particle size) utilizing an isocratic mobile phase composed of 10 mM Potassium Hexafluorophosphate (KPF$_6$) buffer (pH 2.0, adjusted with orthophosphoric acid) and Acetonitrile in a 50:50 v/v ratio. The analysis was performed at a flow rate of 0.7 mL/min, with the column maintained at 40 °C. Detection was carried out at a wavelength of 259 nm. Argatroban exhibited a retention time of approximately 4.92 minutes. The developed method demonstrated excellent linearity over the concentration range of 1.5-3.75 µg/mL, with a correlation coefficient (R^2) greater than 0.999. The method's precision, expressed as percentage relative standard deviation (%RSD), was consistently below 1.5%, indicating high reproducibility. Accuracy was confirmed through recovery studies, yielding results between 98.5% and 101.2%. The limits of detection (LOD) and quantification (LOQ) were determined to be 0.5 µg/mL and 1.5 µg/mL, respectively. This validated RP-HPLC method is specific, robust, and stability-indicating, making it a reliable tool for routine quality control and analysis of argatroban in bulk drug substances.

KEYWORDS:

Argatroban, RP-HPLC, Method Validation, Bulk Drug, Estimation, Precision, Accuracy.

INTRODUCTION:

Argatroban is a synthetic, direct thrombin inhibitor widely employed as an anticoagulant, particularly in clinical scenarios where heparin-induced thrombocytopenia (HIT) is a concern. Its mechanism of action involves reversible binding to the active site of thrombin, thereby inhibiting its enzymatic activity and preventing the conversion of fibrinogen to fibrin—a critical step in the coagulation cascade. This specific mode of action renders argatroban highly beneficial for patients suffering from HIT, a severe immune-mediated adverse reaction to heparin therapy characterized by low platelet counts and an elevated risk of thrombosis.

Chemically, argatroban is a small-molecule peptidomimetic with the IUPAC designation [2R,4R]-4-methyl-1-[(2S)-2-[[[4-methyl-1-piperazinyl]carbonyl]amino]-3-phenylpropionyl]-2-pyrrolidinecarboxylic acid monohydrate. Its molecular formula is C$*{23}*H{36}N*{6}*O{5}$S, and it has a molecular weight of 508.64 g·mol^{-1}. Argatroban presents as a white, odorless crystalline powder that is soluble in water. Due to its rapid onset of action and relatively short half-life, it is typically administered intravenously, allowing for precise control of its anticoagulant effects. Argatroban exhibits linear pharmacokinetics, undergoes primary metabolism by cytochrome P450 enzymes (CYP3A4/5) in the liver, and is predominantly eliminated via fecal excretion.

In pharmaceutical analysis, the accurate and reliable quantitative estimation of argatroban is paramount for its development, quality control, and routine monitoring in both bulk drug substances and pharmaceutical formulations. Various analytical techniques have been explored for its quantification, including ultraviolet (UV) spectroscopy and liquid chromatography-mass spectrometry (LC-MS). However, reversed-phase high-performance liquid chromatography (RP-HPLC) remains a preferred method due to its inherent accuracy, high reproducibility, and superior ability to separate the active pharmaceutical ingredient from potential degradation products and excipients. Despite its significant therapeutic relevance, there is a notable scarcity of simple, validated HPLC methods specifically tailored for the routine analysis of Argatroban in its bulk drug form[1-3]. This study aims to address this gap by developing and validating a robust RP-HPLC method suitable for routine quality control of argatroban.

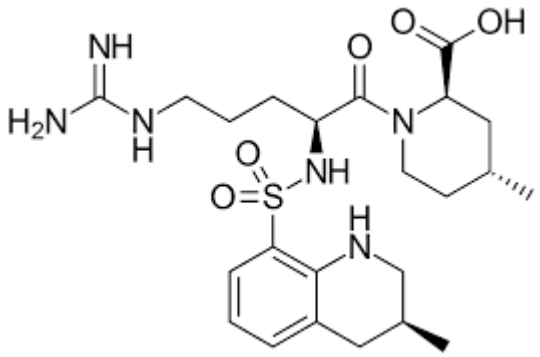


Figure 1: Chemical structure of Argatroban

MATERIALS AND METHODS:

Chemicals and Reagents:

Argatroban active pharmaceutical ingredient (API) was generously procured from YMC India Pvt. Ltd. HPLC-grade water, methanol, acetonitrile, orthophosphoric acid (OPA), and

potassium hexafluorophosphate (KPF$_6$) were obtained from Merck India Pvt. Ltd. All chemicals and reagents used were of analytical or HPLC grade.

Equipment:

Chromatographic analysis was performed using a Shimadzu HPLC system equipped with binary pumps, a photodiode array (PDA) detector, and controlled by Lab Solutions CS Software. Additional laboratory equipment included a pH meter, a digital ultrasonic cleaner, and a digital electronic balance.

Chromatographic Conditions:

Chromatographic separation was achieved using a J'Sphere ODS-H80 column (150 mm x 4.6 mm, 4.0 µm particle size). Isocratic elution was employed with a mobile phase consisting of 10 mM KPF$_6$ buffer (pH 2.0, adjusted with OPA) and acetonitrile in a 50:50 v/v ratio. The mobile phase was degassed by sonication prior to use. The flow rate was maintained at 0.7 mL/min. The column temperature was set at 40 °C. The detection wavelength was 259 nm, and the injection volume was 20 µL.

Preparation of Solutions:

Diluent Preparation:

The diluent was prepared by mixing acetonitrile and methanol in a 1:1 v/v ratio.

10 mM KPF$_6$ Buffer Preparation:

Potassium hexafluorophosphate (1.8409 g) was accurately weighed and dissolved in approximately 800 mL of deionized water. The solution was then made up to 1000 mL with deionized water. The pH of the buffer was adjusted to 2.0 using orthophosphoric acid.

Standard Stock Solution (0.1 mg/mL):

A standard stock solution of argatroban was prepared by accurately weighing 1.00 mg of Argatroban API into a 10 mL volumetric flask. The drug was dissolved in and diluted to the mark with the prepared diluent, yielding a final concentration of 0.1 mg/mL (100 µg/mL).

Primary Standard Solution (0.005 mg/mL):

From the standard stock solution, 0.5 mL was accurately pipetted into a 10 mL volumetric flask and diluted to the mark with the diluent, resulting in a concentration of 0.005 mg/mL (5 µg/mL).

Working Standard Solution (0.0025 mg/mL):

From the primary standard solution, 0.5 mL was accurately pipetted into a 1 mL volumetric flask and diluted to the mark with the diluent, yielding a final working standard solution concentration of 0.0025 mg/mL (2.5 µg/mL). This solution was used for system suitability and routine analysis.

HPLC Method Development:

The primary objective of this study was to establish a simple, rapid, and cost-effective analytical method for the quantitative assessment of argatroban in its API form. The RP-HPLC separation was optimized on a C18 column under isocratic conditions to achieve a short retention time (less than six minutes), good resolution, and economical solvent consumption. Various mobile phase compositions, flow rates, and column temperatures were investigated to

achieve optimal chromatographic parameters. The final optimized conditions provided excellent resolution and a high chromatographic response for argatroban, ensuring accurate quantitative analysis. The selection of 259 nm as the detection wavelength was based on the maximum absorbance observed from the UV spectrum of argatroban solution scanned over the range of 200 to 400 nm.

Analytical Method Validation:

The developed RP-HPLC method was subjected to comprehensive validation in accordance with ICH guidelines to ensure its suitability for the intended purpose. The following parameters were evaluated:

Linearity:

Linearity assesses the ability of the analytical method to obtain test results that are directly proportional to the concentration of the analyte within a given range. The linearity of the method was determined by preparing a series of argatroban solutions ranging from 1.50 to 3.75 µg/mL through serial dilutions of the primary standard solution. Each concentration was analyzed in triplicate, and a calibration curve was constructed by plotting the peak areas against the corresponding concentrations. The linearity was evaluated at a detection wavelength of 259 nm.

System Precision:

System precision evaluates the degree of agreement among individual test results when the method is applied repeatedly to multiple aliquots of a homogeneous sample. It reflects the reproducibility and consistency of the analytical system. System precision was assessed by injecting six replicate preparations of the working standard solution (2.5 µg/mL) and calculating the percentage relative standard deviation (%RSD) of the peak areas.

Limit of Quantification (LOQ):

The LOQ is defined as the lowest concentration of an analyte in a sample that can be quantitatively determined with acceptable accuracy and precision. It is typically higher than the Limit of Detection (LOD). The LOQ was determined based on the signal-to-noise ratio (S/N) approach, where a typical S/N ratio of 10:1 was considered acceptable.

Limit of Detection (LOD):

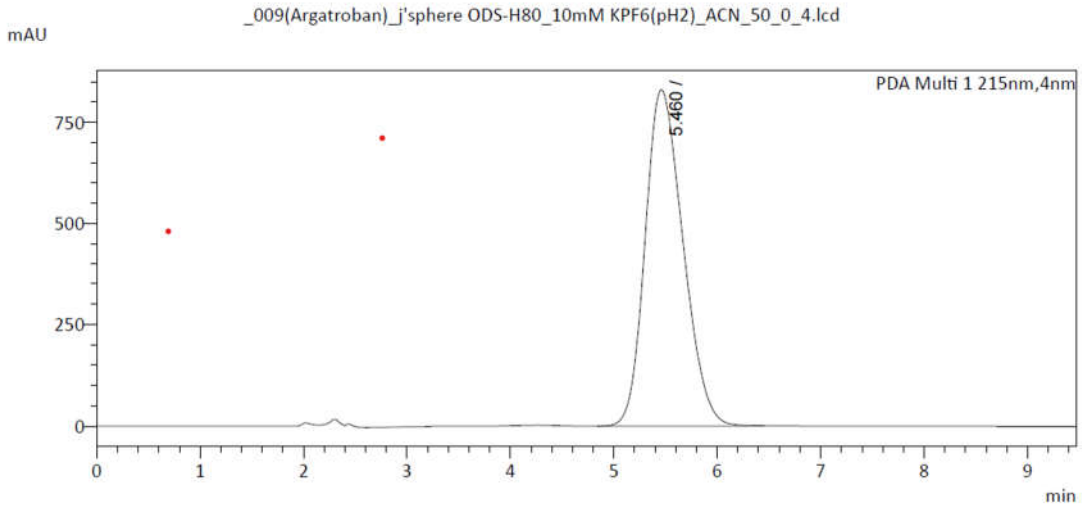
The LOD represents the lowest concentration or amount of an analyte in a sample that can be reliably detected, but not necessarily quantified, under the specified experimental conditions. It signifies the minimum signal distinguishable from the background noise. The LOD was determined based on the signal-to-noise ratio (S/N) approach, where a typical S/N ratio of 3:1 was considered acceptable.

Ruggedness:

Ruggedness measures the reproducibility of test results under a variety of normal operating conditions that may vary in routine laboratory practice. This includes variations such as different analysts, different instruments, different lots of reagents, and different days. Ruggedness was assessed by introducing deliberate, minor variations in the chromatographic conditions (e.g., slight changes in mobile phase composition, flow rate, or column temperature) and evaluating their impact on the method's performance.

RESULTS AND DISCUSSION:

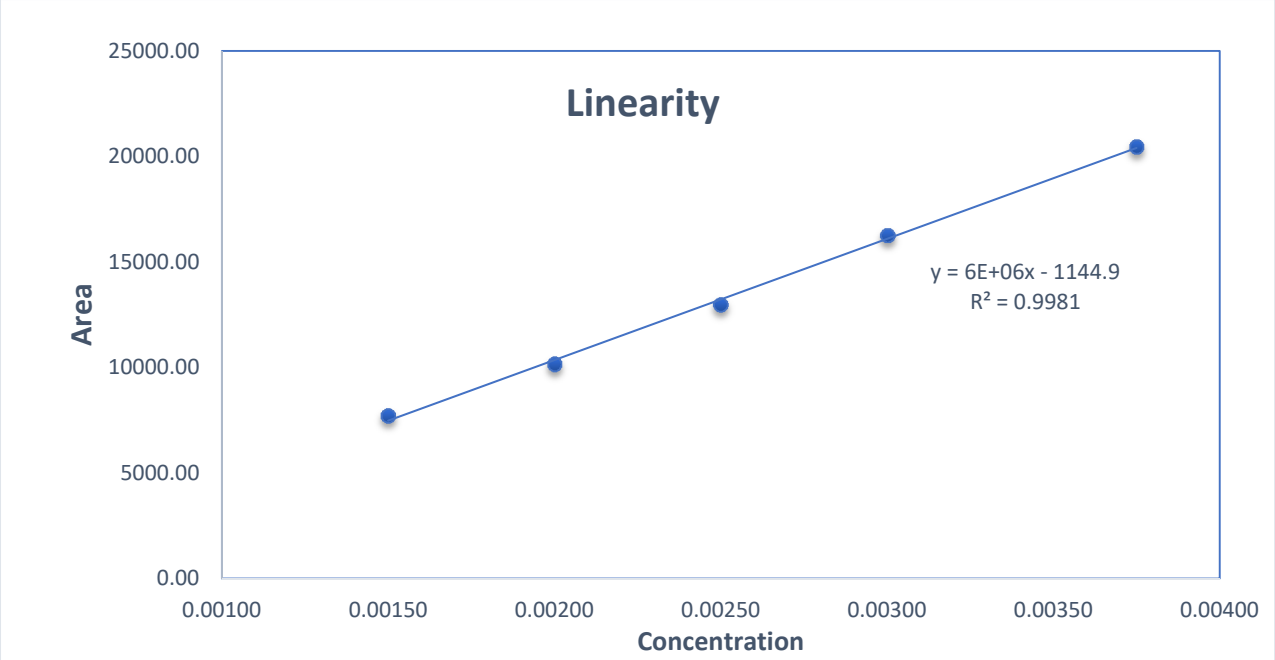
The final optimized method was obtained after numerous trials utilizing various solvents and diluents for drug elution in HPLC at varying concentrations.



Linearity

Linear calibration plot for assay method was obtained over the calibration ranges tested, i. e. 1.5 – 3.75 µg/ml and the correlation coefficient obtained was greater than 0.999. The results show that an excellent correlation existed between the average peak area and concentration of the analyte (Table 1)

Levels	**Conc mg/mL**	**Area (Injection 1 st)**	**Area (Injection 2 nd)**	**Area (Injection 3 rd)**	**Average Area**
Linearity_60%	0.00150	7705	7673	7771	7716.33
Linearity_80%	0.00200	10095	10142	10199	10145.33
Linearity_100%	0.00250	12970	12950	12982	12967.33
Linearity_120%	0.00300	16258	16241	16283	16260.67
Linearity_150%	0.00375	20445	20413.00	20489	20449.00
Correlation Coefficient			**0.9990**		
Regression Coefficient (R2)			**0.99808**		



System Precision:

The system precision of the method is expressed as percentage relative standard deviation. The RSD values for system precision samples did not exceed 2%, thus indicating the good precision of the method. The results are presented in Table 2.

Limit of Quantification (LOQ):

The LOQ was determined based on the signal-to-noise ratio (S/N) approach, where a typical S/N ratio of 10:1 was considered acceptable. The LOQ of the method was found to be 1 µg/ml. the results for LOQ precision was given in table 3.

S. No.	Sample ID	Area of Argatroban	S/N of Argatroban
1	LOQ Precision Injection-1	4974	15.00
2	LOQ Precision Injection-2	5005	15.09
3	LOQ Precision Injection-3	4969	15.06
Average		4982.67	15.05
STD. DEV.		19.5021	0.0458
%RSD		0.3914	0.3045
Acceptance critera	The signal to Noice ratio in Limit of Quantification should be not less than 10.0		

Limit of Detection (LOD):

The LOD was determined based on the signal-to-noise ratio (S/N) approach, where a typical S/N ratio of 3:1 was considered acceptable. The LOD of the method was found to be 0.5 µg/ml. the results for LOQ precision was given in table 4.

S. No.	Sample ID	Area of Argatroban	S/N of Argatroban
1	LOD Precision Injection-1	2323	7.35
2	LOD Precision Injection-2	2395	7.31
3	LOD Precision Injection-3	2342	7.27
Average		2353.33	7.31
STD. DEV.		37.3140	0.0400
%RSD		1.5856	0.5472
Acceptance critera	The signal to Noice ratio in Limit of Detection should be not less than 3.0		

Robustness:

Ruggedness was assessed by introducing deliberate, minor variations in the chromatographic conditions (e.g., slight changes in mobile phase flow rate, or column temperature and evaluating their impact on the method's performance. The % RSD after making deliberate changes in the flow rate and column temperature was found to be less than 2 % hence the method is proven to be robust. The results for robustness was given in the table 5.

S. No.	Robustnees Condition	Avarage RT	Avarage Tailing Factor	Avarage Number of Theoretical Plate(USP)
1	**Flow 0.5mL/min**	6.82	1.04	1278.67
2	**Flow 0.7mL/min**	4.92	0.99	1036.00
3	**Flow 0.9mL/min**	3.88	1.05	855.33
4	**Temp 35°C**	5.26	1.00	721.33
5	**Temp 40°C**	4.91	0.99	1017.33
6	**Temp 45°C**	4.61	1.06	1267.67

Conclusion

A rapid, isocratic RP-HPLC method was developed and validated for the quality control of Argatroban in bulk form. The method complies with ICH Q2(R1) guidelines, demonstrating excellent linearity, precision, sensitivity, and robustness. It is a reliable and accurate analytical tool, making it suitable for routine pharmaceutical quality control and ensuring its fitness for purpose across various operational scenarios.

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