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Development and Validation of RP-HPLC Method for the Determination of Pyridostigmine Drug

Khushboo Vyas[1], Pramod Kumar Goyal[2], Piush Sharma[3]

[1,2,3]Department of Pharmaceutical Chemistry, Maharshi Arvind College of Pharmacy, Ambabari Jaipur, Rajasthan, India

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Corresponding author: Khushboo Vyas

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Abstract:

A reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the quantitative determination of Pyridostigmine, a drug commonly used in the treatment of myasthenia gravis, both in bulk drug and tablet formulations. The method offers high precision, accuracy, and sensitivity, making it suitable for routine quality control analysis. This study outlines the optimization of chromatographic conditions, method validation, and results that comply with ICH guidelines, including specificity, linearity, precision, accuracy, and robustness. This research article focuses on the RP-HPLC method development and validation for Pyridostigmine, ensuring it is fit for use in pharmaceutical applications such as routine[1-4] quality control and analysis. RP-HPLC method development and validation are crucial in the pharmaceutical industry for ensuring the quality, safety, and efficacy of drug products. Pyridostigmine, a drug commonly used to treat myasthenia gravis, requires robust and reliable analytical methods for its quantification and quality control. RP-HPLC (Reversed-Phase High-Performance Liquid Chromatography) is a widely used technique for this purpose due to its versatility, sensitivity, and ability to separate complex mixtures.[4-5]

Keywords: Pyridostigmine, RP-HPLC, Method Development, Validation, Bulk Drug, Tablets, Myasthenia Gravis, Pharmaceutical Analysis

Introduction

Pyridostigmine is an anticholinesterase medication used for the management of myasthenia gravis, a neuromuscular disorder characterized by muscle weakness. It works by inhibiting the breakdown of acetylcholine, improving communication between nerves and muscles. Pyridostigmine is commercially available in various pharmaceutical forms, including tablets, which require rigorous quality control and consistency in terms of drug content.

High-performance liquid chromatography (HPLC), specifically reverse-phase HPLC (RP-HPLC), is widely used in the pharmaceutical industry to analyze the quality and stability of drug substances and formulations. RP-HPLC is ideal for Pyridostigmine due to its relatively low polarity, making it amenable to separation using non-polar stationary phases. This study focuses on the development of an RP-HPLC method for the analysis of

Pyridostigmine in bulk drug and tablet forms, followed by method validation.[6-8]

ble. RP-HPLC method development and validation are crucial in the pharmaceutical industry for ensuring the quality, safety, and efficacy of drug products. Pyridostigmine, a drug commonly used to treat myasthenia gravis, requires robust and reliable analytical methods for its quantification and quality control. RP-HPLC (Reversed-Phase High-Performance Liquid Chromatography) is a widely used technique for this purpose due to its versatility, sensitivity, and ability to separate complex mixtures. This response will delve into the critical aspects of RP-HPLC method development and validation for pyridostigmine bulk drug, drawing upon various research articles and established guidelines.[8-9]

Materials and Methods

Chemicals and Reagent

Pyridostigmine bromide reference standard was procured from a certified supplier. Acetonitrile (HPLC grade), water (HPLC grade), and orthophosphoric acid were used for preparing the mobile phase. All solvents and reagents used were of analytical grade.

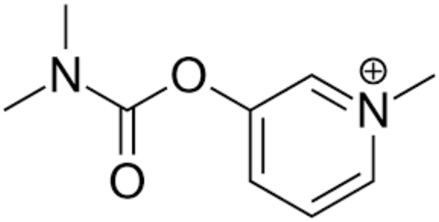

fig: Chemical structure of Pyridostigmine

Instrumentation

The RP-HPLC system used for the analysis consisted of a UV-Visible detector, a binary pump, and a column compartment. The separation was achieved on a C18 column (4.6 mm × 250 mm, 5 µm particle size). Chromatographic data were acquired and processed using the XYZ HPLC software8-9.

Chromatographic Conditions

To develop an optimized RP-HPLC method for Pyridostigmine, several mobile phase compositions, flow rates, and detection wavelengths were tested. The final optimized conditions were as follows:

- **Column:** C18 (4.6 mm × 250 mm, 5 µm)
- **Mobile Phase:** A mixture of acetonitrile and 0.1% orthophosphoric acid in water (50:50, v/v)
- **Flow Rate:** 1.0 mL/min
- **Detection Wavelength:** 280 nm
- **Column Temperature:** 30°C
- **Injection Volume:** 20 µL

Preparation of Stock and Working Solutions

The stock solution of Pyridostigmine was prepared by accurately weighing 10 mg of the Pyridostigmine bromide standard and dissolving it in 10 mL of acetonitrile, giving a final concentration of 1 mg/mL. Working solutions were prepared by diluting the stock solution with the mobile phase to obtain a concentration range of 0.1–20 µg/mL9-10.

Calibration Curve

A series of standard Pyridostigmine solutions were prepared with concentrations ranging from 0.1 to 20 µg/mL. A calibration curve was generated by plotting the peak area against the concentration of Pyridostigmine.

Method Development

Preparation of Stock and Working Solutions

The stock solution of Pyridostigmine was prepared by accurately weighing 10 mg of the Pyridostigmine bromide standard and dissolving it in 10 mL of acetonitrile, giving a final concentration of 1 mg/mL. Working solutions were prepared by diluting the stock solution with the mobile phase to obtain a concentration range of 0.1–20 µg/mL5-7.

Calibration Curve

A series of standard Pyridostigmine solutions were prepared with concentrations ranging from 0.1 to 20 µg/mL. A calibration curve was generated by plotting the peak area against the concentration of Pyridostigmine.

Selection of detection wavelength

In the present study, the drug solutions of 10 µg/ml of SOR were prepared and scanned over a range of 200 - 400 nm. It was observed that the drug showed maximum absorbance at 265.5 nm which was chosen as the detection wavelength for the determination of SOR

Optimization of Mobile Phase

The mobile phase typically consists of a mixture of water and an organic solvent such as acetonitrile or methanol. The ratio of these solvents is carefully optimized to achieve the desired separation. Acetonitrile isoften preferred for its lower viscosity and UV absorbance. , but methanol can provide different selectivity and is sometimes used as an alternative. Buffers are also added to the mobile phase to control pH, which can significantly affect the ionization state of the analyte and, consequently, its retention. Common buffers include phosphate, acetate, and formate buffers. The pH should be chosen based on the pKa of pyridostigmine to ensure it is in a suitable ionization state for optimal separation.

Experiment with different organic solvents (e.g., acetonitrile, methanol) and their ratios to optimize resolution and selectivity. Acetonitrile and methanol are commonly used organic modifiers in RP-HPLC due to their different elution strengths and selectivities for various compounds. Acetonitrile is generally considered to be a stronger eluent than methanol, meaning that it can elute compounds more quickly. Acetonitrile also tends to provide better peak shape and resolution for some compounds, while methanol may be more compatible with certain detectors or sample matrices. The ratio of organic solvent to water in the mobile phase can be adjusted to optimize the retention and selectivity of the separation.

Adjust pH to control ionization of Pyridostigmine and improve peak shape, particularly if Pyridostigmine contains ionizable functional groups9-10. The pH of the mobile phase can significantly affect the ionization state of Pyridostigmine, which can influence its interaction with the stationary phase and its retention time. If Pyridostigmine contains acidic or basic functional groups, adjusting the pH of the mobile phase can control its ionization state and improve peak shape.

Optimization of Flow rate and temperature

Flow rate affects retention time and peak resolution; optimize for efficient separation without compromising resolution. The flow rate of the mobile phase affects the retention time and peak resolution in RP-HPLC. Higher flow rates generally lead to shorter retention times and faster analysis times, but may also compromise resolution. Lower flow rates typically provide better resolution but also increase analysis times. The optimal flow rate should be determined experimentally, balancing the need for efficient separation with the desired resolution and analysis time. Flow rate affects both the resolution and the analysis time. Higher flow rates decrease analysis time but can compromise resolution, while lower flow rates improve resolution but increase analysis time. The optimal flow rate is typically determined empirically. Column temperature can also influence separation by affecting the analyte's and mobile phase's viscosity and vapor pressure

Column temperature can influence separation selectivity and peak shape, particularly for complex mixtures. The column temperature can affect the separation selectivity and peak shape in RP-HPLC. Higher column temperatures generally lead to faster mass transfer and improved peak shape, but may also decrease retention time and selectivity. Lower column temperatures typically provide greater retention and selectivity but may also compromise peak shape. The optimal column temperature should be determined experimentally, considering the chemical properties of Pyridostigmine and the desired separation characteristics. Optimize flow rate and temperature to balance resolution and analysis time, ensuring efficient and effective separation of Pyridostigmine. The flow rate and column temperature are interdependent parameters that should be optimized together to achieve the best possible separation of Pyridostigmine. By carefully adjusting these parameters, it is possible to balance the need for efficient separation with the desired resolution and analysis time. The optimal flow rate and temperature will depend on the specific characteristics of the HPLC system, the stationary phase, the mobile phase, and the sample matrix10-11.

Table 1.a: Optimized chromatographic conditions for the proposed HPLC method

Parameter	**Chromatographic conditions**
Column	Thermo scientific model C18 Column (4.6 mm i.d., X 250 mm; 5 µm particle size) (based on 99.999 % ultra-high purity silica)
Mobile phase	Acetonitrile: buffer (63.35% v/v)
Flow rate	1 mL/min
Run time	7 minutes
Detector	1254 Diode Array Detector.
Detection wavelength	280 nm
The volume of the injection loop	20 µL
Temperature	30 ^{o}C

Method Validation

Table 1.b: System suitability parameters

System suitability parameters	**Limits**	**SOR**
Tailing factor (T)	≤ 1.8	0.721
Number of theoretical plates	NLT 2000	7870
Theoretical plates per meter (N)*	-	1,46,567
Retention time*	-	3 .25 minutes
SD for peak area and RT		0.0128
% RSD	NMT 2.0	0.525

- **Specificity:** The method was specific for Pyridostigmine, as there was no interference from excipients or impurities in the tablet matrix. Specificity is the ability of the method to accurately measure the analyte of interest in the presence of other[8-9], such as impurities, degradation products, and excipients. This is typically assessed by analyzing samples spiked with known impurities and degradation products to ensure they are well-separated from the pyridostigmine peak. Forced degradation studies, where the drug substance is subjected to stress conditions like heat, light, acid, base, and oxidation, are often performed to generate degradation products for specificity testing .

The method should be able to distinguish Pyridostigmine from these other components and provide accurate quantification without significant interference. Use blank samples, placebo, and spiked samples to evaluate specificity and identify potential interference. To evaluate specificity, several types of samples should be analyzed, including blank samples (samples containing only the mobile phase or sample diluent), placebo samples (samples containing all the excipients in the pharmaceutical Formulation but without Dimercaprol), and spiked samples (samples containing Dimercaprol at known concentrations). By comparing the chromatograms of these samples, it is possible to identify any peaks that may interfere with the quantification of Pyridostigmine. Ensure no significant interference at the retention time of Pyridostigmine to confirm the method's selectivity for the target analyte . The absence of significant interference at the retention time of Pyridostigmine is a key indicator of the method's specificity. If there are no other peaks eluting at or near the retention time of Pyridostigmine in the blank or placebo samples, it can be concluded that the method is selective for the target analyte. However, if there are interfering peaks, further method optimization may be necessary to improve the separation and resolution of Pyridostigmine from these interferences.

Table 1.c: Peak Purity

Sample	**Analyte**	**Purity angle**	**Purity threshold**
Standard preparation	SOR	0.128	0.17
Placebo + analyte preparation	SOR	0.129	0.254
Test preparation	SOR	0.128	0.129
Spiked test preparation	SOR	0.129	0.220

Name of the solution	**Retention time (Rt) minutes**
Mobile phase (blank)	No interference at RT of analyte peak
Placebo	No interference at RT of analyte peak
SOR 10 µg/mL (sample)	3.020 minutes

Linearity and range: A linear relationship was observed between the concentration of Pyridostigmine and the peak area over the range of 0.1–20 µg/mL, with a correlation coefficient (R^2) of 0.999, indicating excellent linearity. Linearity refers to the method's ability to produce results that are directly proportional to the concentration of the analyte in the sample. It is typically evaluated by analyzing a series of standard solutions covering a range of concentrations[36]. . A calibration curve is then constructed by plotting the peak area against the concentration, and the correlation coefficient (R^2) is calculated. An R^2 value of ≥0.999 is generally considered acceptable for linearity.

The correlation coefficient (R2) is a statistical measure of the linearity of the calibration curve. It indicates the degree to which the data points on the calibration curve fit a straight line. R2 values close to 1 indicate good linearity, meaning that there is a strong correlation between the concentration of Dimercaprol and the detector response. A minimum R2 value of 0.999 is typically required for RP-HPLC methods used for Quantitative analysis.

Table 3: Linearity data of Pyridostigmine by HPLC

S. No.	**Concentration (µg/mL)**	**Area**
1.	0	0
2.	2	122112
3.	4	298571
4.	6	438632
5.	8	666704
6.	10	689130
7.	Intercept	7665.047619
8.	Slope	5488.15714
9.	CC	0.989504
10.	Squared CC	0.999011

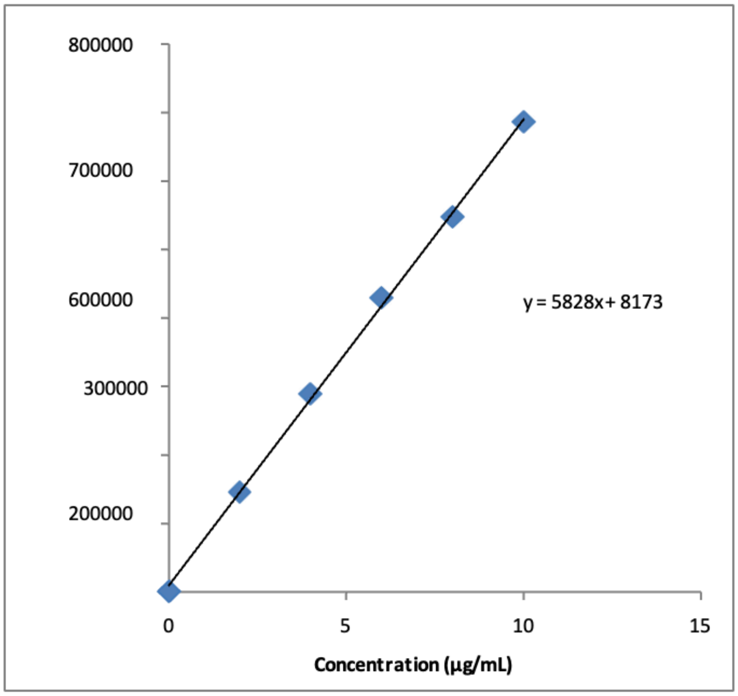

Figure 2.a: Calibration graph of Pyridostigmine by RP- HP

- **Accuracy and Precision:** The accuracy of the method was evaluated by performing recovery studies. Known amounts of Pyridostigmine were added to placebo tablet powder, and the recovery was found to be between 98% and 102%, confirming the accuracy of the method. Accuracy is the closeness of the measured value to the true value. It is typically assessed by analyzing samples spiked with known amounts of pyridostigmine and calculating the percentage recovery. Accuracy should be evaluated at multiple concentration levels to ensure it is consistent across the range of the method.

Table 3.a: Results of Accuracy study

% Recovery Level		Amount Added (mg)	Amount recovered (mg)	% Recovery
	1	10.12	10.09	99.4
50 %	2	10.23	10.11	99.2
	3	9.19	8.56	99.4
	1	20.53	20.21	98.1
100 %	2	25.64	25.59	99.2
	3	23.71	23.61	99.4
	1	35.89	35.78	99.5
150 %	2	38.32	37.88	99.7
	3	36.76	36.72	99.5

The precision of the method was demonstrated by intra-day and inter-day studies. The relative standard deviation (RSD) for both intra-day and inter-day precision was less than 2%, indicating good reproducibility. . Precision is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogeneous sample. It is usually expressed as the relative standard deviation (RSD) of a series of measurements. Precision is evaluated at two levels: repeatability (intra-day precision) and intermediate precision (inter-day precision). Repeatability is assessed by analyzing multiple replicates of a sample on the same day, while intermediate precision is assessed by analyzing multiple replicates of a sample on different days, by different analysts, and on different instruments.

Table 2.b: Complied data of Method Precision & Intermediate Precision

Injection No.	Method Precision	Intermediate Precision
1	97.3	99.3
2	98.6	98.4
3	98.4	98.2
4	99.4	98.5
5	98.1	98.4
6	98.7	99.6
Mean	98.1654	99.1000
% RSD	0.540332	0.605449
Cumulative	**RSD**	1.14565

Table 2.c: Precision at 50 %, 100 %, 150 % (Precision at different levels)

S. No.	50%	100%	150%
1	145328	288941	427689
2	145742	287465	428864
3	146230	288129	428513
4	145896	288752	428567
5	145246	289148	427614
6	145847	288465	428954
Mean	14571.8	288483.333	428366.833
% RSD	0.25417	0.21291251	0.13530080

Table 3.b: Results of Precision at Accuracy study

S. No.	Concentration % of spiked level	% recovery	% RSD
1	50 %	99.4	0.2661
2	100 %	99.0	0.7070
3	150 %	99.6	0.5798

Robustness: The method was found to be robust, as small variations in flow rate (±0.1 mL/min), mobile phase composition (±2%), and column temperature (±2°C) did not significantly affect the chromatographic results, demonstrating the method's reliability under different operating conditions. . Robustness is a measure of the method's capacity to remain unaffected by small, but deliberate variations in method parameters, such as flow rate, temperature, mobile phase composition, and pH. . Robustness is typically evaluated by making small changes to these parameters and assessing the impact on the resolution, retention time, and peak area of pyridostigmine.

Table 3.c: Results of Robustness study

Parameters	Optimized	Used	Retention time (min)	Plate count $	Peak asymmetry #	Remarks
Flow rate (± 0.2 mL/min)	1.0 mL/min	0.8 mL/min	3.298	8823	1.10	*Robust
		1.0 mL/min	3.223	8765	1.11	*Robust
		1.2 mL/min	3.19	8723	1.12	*Robust
Detection wavelength (± 5 nm)		211 nm	3.223	8761	1.13	Robust
	216 nm	216 nm	3.223	8767	1.21	Robust
		218nm	3.223	8762	1.14	Robust
Mobile phase composition Methanol: Acetonitrile (± 5 %)		59.70% v/v	3.327	8776	1.13	*Robust
	63.35%v/v	60.56% v/v	3.223	8789	1.11	*Robust
		64.56% v/v	3.209	8790	1.13	*Robust

LOD and LOQ

Limit of Detection is the lowest concentration in a sample that can be detected but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. The LOD and LOQ are 0.523 µg/mL and 1.565 µg/mL respectively.

Stability studies

Forced Degradation Studies

Stability testing is performed to ensure that the analyte is stable in the sample matrix and under the storage conditions used during analysis: Stability testing is performed to ensure that the analyte is stable in the sample matrix and under the storage conditions used during analysis. This includes evaluating the stability of pyridostigmine in the mobile phase, standard solutions, and sample solutions, as well as its stability under different storage temperatures and durations.

Subject Pyridostigmine to stress conditions (acidic, basic, oxidative, thermal, and photolytic) to generate degradation products and assess the method's stability-indicating properties**[41]**. Forced degradation studies, also known as stress testing, are used to evaluate the stability of Pyridostigmine under various stress conditions. These studies involve subjecting Pyridostigmine to extreme conditions, such as acidic, basic, oxidative, thermal, and photolytic stress, to accelerate its degradation and generate degradation products. The purpose of forced degradation studies is to assess the method's ability to separate and quantify Pyridostigmine in the presence of its degradation products, and to determine the degradation pathways of the drug.

Sample Solution Stability

Assess the stability of Pyridostigmine in solution over time under different storage conditions (e.g., room temperature, refrigerated) to determine its degradation rate and storage requirements **[41]**. Sample solution stability studies are used to assess the stability of Pyridostigmine in solution over time under different storage conditions. These studies involve preparing solutions of Pyridostigmine in the mobile phase or sample diluent and storing them at different temperatures, such as room temperature and refrigerated temperature. The solutions are then analyzed at regular intervals to determine the concentration of Pyridostigmine and to identify any degradation products that may be formed. Determine the time frame within which the sample solution remains stable and can be accurately analyzed, ensuring reliable results during routine analysis The time frame within which the sample solution remains stable is a critical parameter for routine analysis. If the sample solution is not stable, the concentration of Pyridostigmine may change over time, leading to inaccurate results. The sample solution stability study should be used to determine the maximum time that the sample solution can be stored before analysis without significant degradation. This ensures that the method provides reliable results during routine analysis, minimizing the impact of degradation on the accuracy of Pyridostigmine quantification **[41]**. By determining the stability of Pyridostigmine in solution, the method can be used to provide reliable results during routine analysis. This helps to minimize the impact of degradation on the accuracy of Pyridostigmine quantification and ensures that the results are representative of the true concentration of the drug in the sample[16-18].

Application to Pyridostigmine Tablets

The developed RP-HPLC method was applied to the analysis of Pyridostigmine tablets. The results showed that the drug content in the tablets was consistent with the labeled amount. The method was able to detect and quantify Pyridostigmine in the presence of tablet excipients, ensuring its suitability for routine quality control analysis of the tablet formulation.

Result and Discussion

Present the optimized mobile phase composition, flow rate, column temperature, and detection wavelength. The optimized chromatographic conditions for the RP-HPLC method should be clearly presented, including the mobile phase composition (e.g., ratio of buffer to organic solvent), pH, flow rate, column temperature, and detection wavelength. These conditions represent the culmination of the method development process and should provide the best separation and detection of Pyridostigmine.

Provide representative chromatograms showing separation of Pyridostigmine from potential interferences. Representative chromatograms should be included to illustrate the separation of Pyridostigmine from potential interferences, such as excipients, degradation products, and matrix components. The chromatograms should demonstrate that Pyridostigmine is well-resolved from these interferences and that the peak shape is symmetrical and sharp. Discuss the rationale behind the chosen chromatographic conditions and their impact on separation efficiency **[3]**. The rationale behind the chosen chromatographic conditions should be discussed, explaining why these conditions were selected and how they impact the separation efficiency. This discussion should include the effects of the mobile phase composition, pH, flow rate, and column temperature on the retention, selectivity, and peak shape of Pyridostigmine.

Lab- Sea coin Research and laboratory PVT. Ltd

Date- 2025-3-28   8:30:21

Analyzer: Mukesh

Column-C18

Spl. Name: Pyridostigmine

Temp-30**°C**

Wave length- 280nm

Batch no. - 1

Flow- 1ml

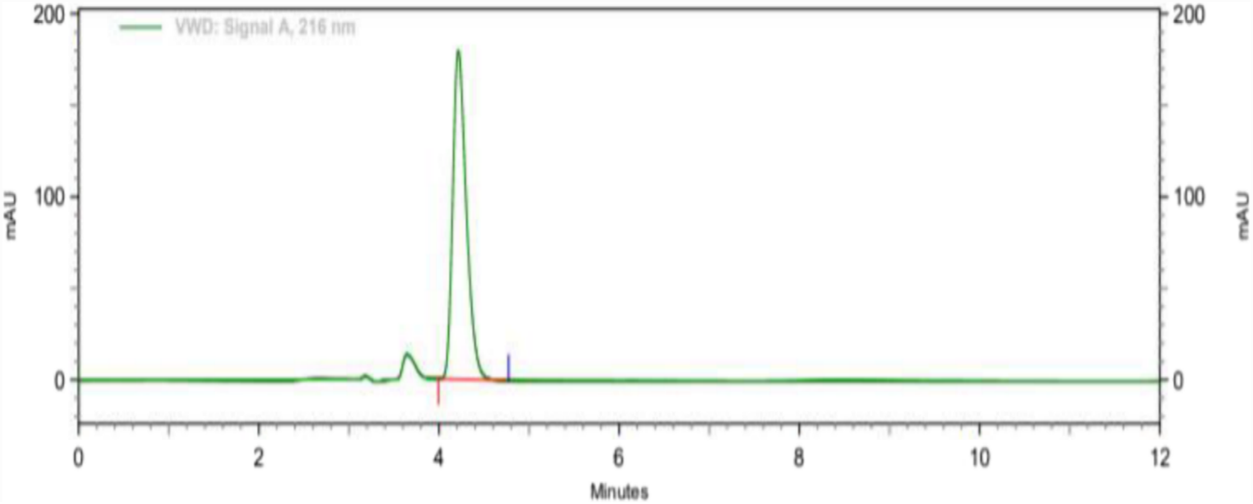

Figure 3: Standard chromatogram of Pyridostigmine (2 µg/mL)

Lab- Sea coin Research and laboratory PVT. Ltd

Date- 2025-3-28 8:30:21

Analyzer: Mukesh

Column-C18

Spl. Name: Pyridostigmine

Temp- 30**°C**

Wave length- 206 nm

Batch no. - 1

Flow- 1ml

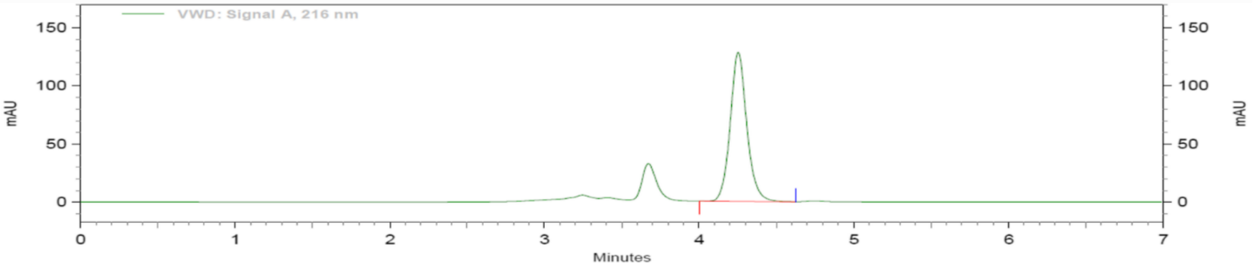

Figure 3.a: Standard chromatogram of Pyridostigmine (4 µg/mL)

Lab- Sea coin Research and laboratory PVT. Ltd

Date- 2025-3-28 8:30:21

Analyzer: Mukesh

Column-C18

Spl. Name: Pyridostigmine

Temp-30**°C**

Wave length- 280 nm

Batch no. - 1

Flow- 1ml

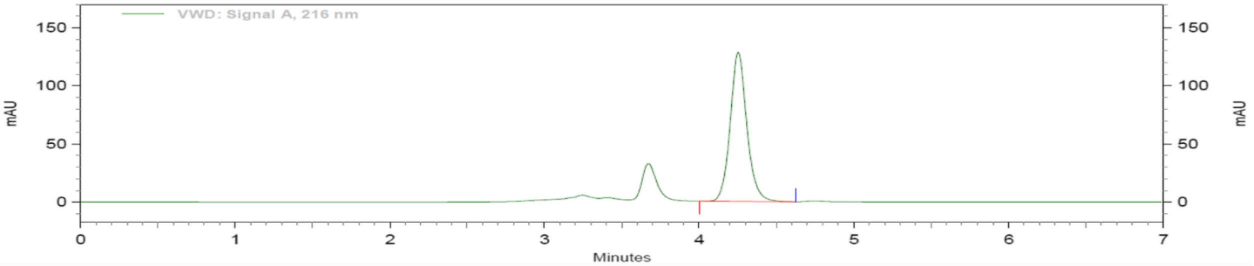

Figure 3.c: Standard chromatogram of Pyridostigmine (8 µg/mL)

Lab- Sea coin Research and laboratory PVT. Ltd

Date- 2025-3-28 8:30:21

Analyzer: Mukesh

Column-C18

Spl. Name: Pyridostigmine

Temp-30**°C**

Wave length- 280 nm

Batch no. - 1

Flow- 1ml

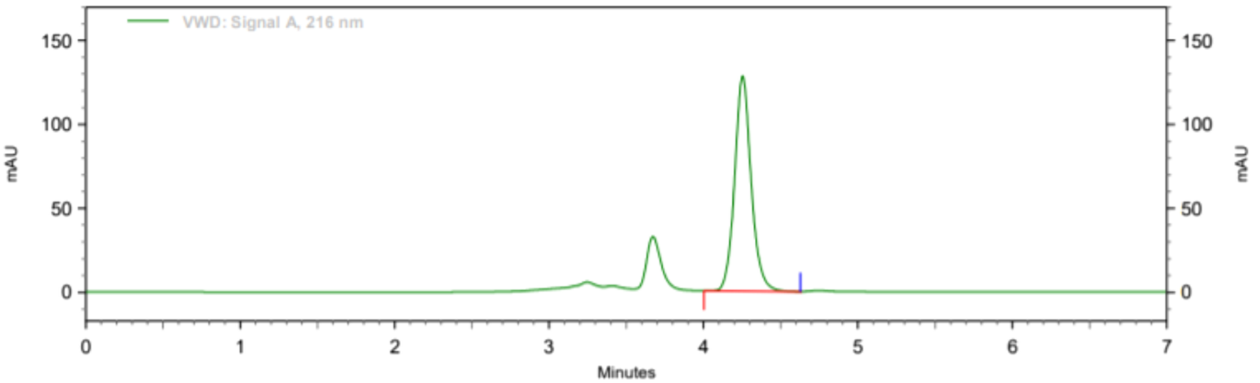

Figure 3.d: Standard chromatogram of Pyridostigmine (10 µg/mL)

Present the validation data for specificity, linearity, accuracy, precision, and robustness in a clear and organized manner. The validation data for specificity, linearity, accuracy, precision, and robustness should be presented in a clear and organized manner, typically in the form of tables or graphs. The data should include the results of the specificity studies, the calibration curve and correlation coefficient for linearity, the recovery data for accuracy, the %RSD values for precision, and the results of the robustness studies. Include calibration curves, recovery data, and %RSD values to support the validation claims. Calibration curves should be included to demonstrate the linearity of the method. Recovery data should be included to demonstrate the accuracy of the method.

Validation Results

Present the validation data for specificity, linearity, accuracy, precision, and robustness in a clear and organized manner. The validation data for specificity, linearity, accuracy, precision, and robustness should be presented in a clear and organized manner, typically in the form of tables or graphs. The data should include the results of the specificity studies, the calibration curve and correlation coefficient for linearity, the recovery data for accuracy, the %RSD values for precision, and the results of the robustness studies. Include calibration curves, recovery data, and %RSD values to support the validation claims. Calibration curves should be included to demonstrate the linearity of the method. Recovery data should be included to demonstrate the accuracy of the method. %RSD values should be included to demonstrate the precision of the method. The validation results should be compared with established acceptance criteria to demonstrate that the method is suitable for its intended purpose. The acceptance criteria should be based on regulatory guidelines and industry standards.

Comparison with existing Methods

Compare the developed method with existing HPLC or other analytical techniques for Pyridostigmine analysis. The developed RP-HPLC method should be compared with existing HPLC or other analytical techniques for Dimercaprol analysis, such as spectrophotometry or titrimetric. This comparison should include the advantages and disadvantages of each method in terms of sensitivity, selectivity, analysis time, cost-effectiveness, and ease of use. Highlight the advantages of the new method in terms of sensitivity, selectivity, analysis time, or cost-effectiveness. . The advantages of the new RP-HPLC method should be highlighted, such as its improved sensitivity, selectivity, , reduced analysis time, or cost-effectiveness.

These advantages should be supported by data from the method development and validation studies. Discuss any limitations of the developed method and potential areas for improvement. The limitations of the developed RP-HPLC method should be discussed, such as its limited linear range or susceptibility to matrix effects. Potential areas for improvement should also be identified, such as the use of a more sensitive detector or the optimization of the sample preparation procedure.

Applications of the method

Apply the validated method to quantify Pyridostigmine in pharmaceutical formulations (e.g., injections) and ensure product quality . The validated RP-HPLC method can be applied to quantify Pyridostigmine in pharmaceutical formulations, such as injections, to ensure that the drug product meets the required specifications for potency and purity. This involves preparing the pharmaceutical formulation for analysis, typically by dissolving or diluting the sample in a suitable solvent, and then injecting the sample into the RP-HPLC system. The concentration of Pyridostigmine is then determined by comparing the peak area of the sample to that of known standards.

Assess the content uniformity and assay of the drug product to meet regulatory requirements. Content uniformity refers to the consistency of the Pyridostigmine content in individual dosage units within a batch of the pharmaceutical Formulations. Assay refers to the determination of the amount of Pyridostigmine in a representative sample of the drug products. Both content uniformity and assay are important quality control parameters that must be assessed to meet regulatory requirements and ensure that the drug product is safe and effective. Compare the results with the labeled claim to ensure product quality and accuracy of the reported dosage the results of the pharmaceutical formulation analysis should be compared with the labeled claim, which is the amount of Dimercaprol that is stated on the product label. The measured concentration of Dimercaprol should be within a specified range of the labeled claim, typically 90-110%, to ensure product quality and accuracy of the reported dosage.[16-19]

Table 4.a: Assay results of marketed formulation

S. No.	**Formulation**	**Labeled claim**	**Amount found**	**Mean % recovery± SD***	**% RSD**
1	Pyridostigmine drug	4 gm	2.8gm	99.95 ± 12	**1.21**

Summary:

To summarize the methods employed and the results obtained in the study of SOR, it is mentioned below in Table 4.b.

Table 4.b: Summary of the RP-HPLC of Pyridostigmine

S. No.	**Validation Parameter**	**Acceptance Criteria**	**Results**

1	System Suitability	1. The ICH regulation between degradants peak and SOR peak in the system suitability should not be less than 2.0. 2. The ICH tailing factor for SOR peak in standard preparation should not be less than 2.0. 3. The ICH theoretical plate count for the SOR peak in standard preparation should not be less than 3000. 4. The % Relative standard deviation in six replicate injections of standard preparation should not be more than 2.0 %.	Complies
2.	Specificity Interference from blank, placebo, and impurities	1. There should not be any significant interference (NMT 0.2 % of the target concentration) from blank, placebo, and impurities with the analyte. 2. Peak purity of the analyte peak should pass (Purity angle should be less than Purity threshold).	No interference observed, and peak purity complies
3.	Precision		
	1. System precision	% RSD for six replicate injections should not be more than 2.0.	SOR: % RSD - 0.02967
	2. Method precision	% RSD for assay of six test preparations should not be more than 2.0.	SOR: % RSD - 0.54033
	3. Intermediate precision	1) % RSD for the assay of six test preparations should not be more than 2.0. 2) Cumulative % RSD of 12 determinations (method and intermediate precision) should not be more than 2.0.	SOR: %RSD - 0.605449 SOR: Cumulative %RSD - 1.14578
	4. Precision at different levels	% RSD for six replicate injections at 50 %, 100 %, 150 % levels should not be more than 2.0	50% - 0.25417 100% - 0.21291 150 % - 0.13530
4.	Linearity	The squared correlation coefficient should not be less than 0.997	0.99901
5.	Accuracy Study	The mean % recovery should be between 98.0 to 102.0	50 % - 99.4 100 % - 99.0 150 % - 99.6
6.	Robustness	1) System suitability should pass. 2) Assay value should not be more than ± 2 % from the mean value of method precision, or the result should be within the range ofmethod precision results.	Complies

Applications of Design of Experiment

Design of Experiments (DoE) is a systematic approach to method development that allows for the efficient optimization of multiple method parameters simultaneously. By using DoE, the effects of different parameters and their interactions can be studied in a structured manner, leading to a more robust and reliable method.

Green Analytical Chemistry Consideration

There has been increasing emphasis on developing analytical methods that are environmentally friendly. Green Analytical Chemistry (GAC) principles aim to reduce or eliminate the use and generation of hazardous substances in analytical processes For RP-HPLC method development, GAC considerations include minimizing solvent consumption, using less toxic solvents, and reducing waste generation. This can be achieved by optimizing the mobile phase. composition, using smaller column dimensions, and employing techniques such as solvent recycling.

Examples of Some Case Studies

Several research articles have reported on the development and validation of RP-HPLC methods for pyridostigmine and related compounds. These studies provide valuable insights into the practical aspects of method development and validation.

Example 1 Developing of Stability Indicating RP-HPLC Method -: A stability-indicating method is one that can accurately measure the analyte of interest in the presence of its degradation products. Such methods are crucial for assessing the stability of drug products and ensuring their quality throughout their shelf life. To develop a stability-indicating RP-HPLC method for pyridostigmine, forced degradation studies would be conducted under various stress conditions (heat, light, acid, base, oxidation) to generate degradation Products. The chromatographic conditions would then be optimized to ensure adequate separation of pyridostigmine from its degradation Products.

Example 2 Application of DOE for Method Optimisation-:

DoE can be used to optimize the mobile phase composition and flow rate for an RP-HPLC method for pyridostigmine. A factorial design or a response surface methodology (RSM) can be employed to study the effects of these parameters on the resolution, retention time, and peak area of pyridostigmine. The results of the DoE study can then be used to identify the optimal chromatographic conditions that provide the best separation and sensitivity.

Example 3 Green HPLC Method Development-:

To develop a green RP-HPLC method for pyridostigmine, efforts would be made to minimize solvent consumption and use less toxic solvents. This could involve using a smaller column dimension optimizing the mobile phase composition to reduce the amount of organic solvent, and employing techniques such as solvent recycling. The greenness of the method can be evaluated using metrics metrics such as the Analytical Eco-Scale or the Green Analytical Procedure Index (GAPI).

Regulatory Guideline

The validation of analytical methods is a regulatory requirement in the pharmaceutical industry. Several regulatory agencies, such as the International Council for Harmonization (ICH), the United States Food and Drug.

Conclusion

The RP-HPLC method developed for the analysis of Pyridostigmine bulk drug and tablet formulations is reliable, precise, and accurate. The method was validated according to ICH guidelines, confirming its robustness, specificity, and suitability for routine pharmaceutical analysis This method can be utilized for the quality control of

Pyridostigmine tablets, ensuring their compliance with pharmacopeial standards. RP-HPLC method development and validation are essential for ensuring the quality, safety, and efficacy of pyridostigmine bulk drug. A systematic approach to method development, including careful selection of stationary phase, mobile phase optimization, and optimization of chromatographic conditions, is crucial for achieving adequate separation, resolution, and sensitivity. Method validation should be performed according to established guidelines to demonstrate demonstrate that the method is suitable for its intended purpose. The application of DoE can facilitate method optimization, while GAC principles can help to develop environmentally friendly methods. By following these principles and guidelines, robust and reliable RP-HPLC methods can be developed and validated for the analysis of pyridostigmine bulk drug.[12-15]

.The method's suitability for routine analysis and quality control applications should be emphasized, highlighting its potential for improving the quality and safety of Dimercaprol-containing products.The RP-HPLC method developed for the analysis of Dimercaprol is efficient, reliable, and suitable for quality control applications. The method was optimized and validated according to ICH guidelines, ensuring its specificity, accuracy, precision, and robustness. This RP-HPLC method can be employed for routine analysis of Dimercaprol in pharmaceutical formulations and in pharmacokinetic studies.

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