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Analytical Method Development and Validation of Antidiabetic Drugs by using RP-HPLC

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Abstract: *This study presents the development and validation of a reverse-phase high-performance liquid chromatography (RP-HPLC) method for the quantitative analysis of the antidiabetic drug saxagliptin monohydrate. Utilizing an HPLC system equipped with a C18 column (4.6 x 250 mm, 5 µm), the method employed a mobile phase of acetonitrile, methanol, and water in a 40:10:50 ratio, adjusted to pH 3.5 with o-phosphoric acid. The detection wavelength was set at 223 nm, with a flow rate of 0.7 ml/min and a sample injection volume of 20 µl. The retention time for saxagliptin monohydrate was observed at 3.98 minutes, and the method demonstrated excellent linearity over a concentration range of 10-50 µg/ml with a regression coefficient (R^2) of 0.999. Validation parameters, including accuracy, precision, linearity, ruggedness, and limits of detection (LOD) and quantitation (LOQ), confirmed the method's reliability and robustness. The LOD and LOQ were determined to be 0.5815 µg/ml and 1.7622 µg/ml, respectively. This RP-HPLC method proved to be effective for the routine analysis of saxagliptin monohydrate in pharmaceutical formulations, providing a specific, sensitive, and reproducible analytical tool.*

Keywords: RP-HPLC, saxagliptin monohydrate, method development, validation, antidiabetic drug, linearity, precision, accuracy

I. INTRODUCTION

The management of diabetes, a chronic disease with increasing global incidence, heavily relies on the availability of effective and reliable antidiabetic medications. Saxagliptin monohydrate, a dipeptidyl peptidase-4 (DPP-4) inhibitor, is a widely prescribed drug for the treatment of type 2 diabetes mellitus. It works by increasing the levels of incretin hormones, which in turn help to regulate blood glucose levels. Given the critical role of saxagliptin in diabetes management, there is a strong need for precise and reliable analytical methods to ensure the quality, efficacy, and safety of pharmaceutical formulations containing this drug.[1,2]

High-performance liquid chromatography (HPLC) is a preferred analytical technique in pharmaceutical analysis due to its high specificity, sensitivity, and capability to analyze complex mixtures. Reverse-phase HPLC (RP-HPLC), in particular, is extensively used for the separation and quantification of pharmaceutical compounds because of its robustness and reproducibility.[3,4]

This study aims to develop and validate an RP-HPLC method for the quantitative analysis of saxagliptin monohydrate. The method development involves optimizing various chromatographic conditions to achieve the best separation and detection of saxagliptin. This includes selecting the appropriate column, mobile phase composition, flow rate, detection wavelength, and pH.[5,6]

Following the development phase, the method is validated according to regulatory guidelines to ensure its reliability and accuracy. Validation parameters include assessments of linearity, precision, accuracy, ruggedness, and the limits of detection (LOD) and quantitation (LOQ). By rigorously validating the method, we ensure that it meets the stringent requirements necessary for routine quality control applications in the pharmaceutical industry.[7,8]

The outcome of this study is expected to provide a robust and validated RP-HPLC method that can be employed for the consistent and reliable analysis of saxagliptin monohydrate in pharmaceutical products, thereby supporting the overall goal of ensuring high-quality antidiabetic medications for patients. [9,10]

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II. MATERIALS AND METHODS

MATERIALS

Water (HPLC Grade) and acetonitrile (HPLC Grade) saxagliptinmonohydrat drug was procured from Glenmark Generics Ltd. Mumbai. As a grft sample tablets were procured from local pharmacy. High performance liquid chromatography of Younglin (S.K) ltd Binary Gradient system Model no HPLC 3000 Series, detector UV-3000-M Column C18 (Grace) 4.6 x 250 mm, digital PH meter EU- Tech,ME-302and analytical balance of acculabWensar High Precision balance Modal PGB 100 were used. All the chromatographs were recorded by using Autochro -3000 softwere.

METHODS

The mobile phase was chosen after several trials with acetonitrile: Methanol: Water in various proportions. The mobile phase consisted of acetonitrile:methanol:water (40:10:50V/V) by adjusting the pH 3.5 with O-phosphoric acid was selected to achieve symmetrical peak. The effects of flow rates in the ranges of 0.5 to 1.1 ml/min were examined. A flow rate of 0.7 ml/min gave good result, system suitability parameter and reasonable retention time. The retention times of saxagliptin monohydrate was observed 3.98min at 223nm wavelengths with 20 µl injection volume. The total run time of analysis was 15min.[11]

Preparation of Standard Stock Solution

Stock Standard Solution was prepared by dissolving 10mg of saxagliptinmonohydrat in 10 ml methanol that gives concentration of 1000 µg/ml[12]

Standard Sample Preparation

To prepare standard samples of Saxagliptin, start by dissolving 10 mg of Saxagliptin in 10 ml of methanol to obtain a stock solution with a concentration of 1000 µg/ml (Stock-I). From this stock solution, prepare a series of dilutions by taking specified aliquots and diluting them to 10 ml with the mobile phase: 0.1 ml to make a 10 µg/ml solution, 0.2 ml for a 20 µg/ml solution, 0.3 ml for a 30 µg/ml solution, 0.4 ml for a 40 µg/ml solution, and 0.5 ml for a 50 µg/ml solution.[12]

Chromatographic Condition

The chromatographic conditions for the analysis of Saxagliptin are specified as follows: use a C18 (Grace) analytical column with dimensions of 4.6 x 250 mm and a particle size of 5 µm. The mobile phase consists of a mixture of acetonitrile, methanol, and water in the ratio of 40:10:50. The detection wavelength is set at 223 nm, with a flow rate of 0.7 ml/min. The column is maintained at ambient temperature. Inject a sample size of 20 µl, and ensure the pH of the mobile phase is adjusted to 3.5. The total run time for the chromatographic analysis is 10 minutes.[13,14]

III. RESULTS AND DISCUSSION

Reverse phase high performance liquid chromatography has gained the valuable position in the field of analysis due to ease of performance, Specificity, Sensitivity and the analysis of sample of complex nature. This technique is commonly used for the quantitative estimation of the drug from their formulation as well as for studying their metabolites of drug and their estimation in their biological fluids. Estimation the constituent's form the multicomponent system is the best advantage of RP-HPLC method of analysis

Determination of λ- max of saxagliptin monohydrate by UV-Visible Seectrophotometer.

Study of Spectra and selection of wavelength:

The aliquot portion of prepared stock standard solution of saxagliptin monohydrate were diluted appropriately with water to obtain concentration of each drug. The solution were scanned in the range 200-400 nm the overlain UVabsorbance spectrum of saxagliptin monohydrate

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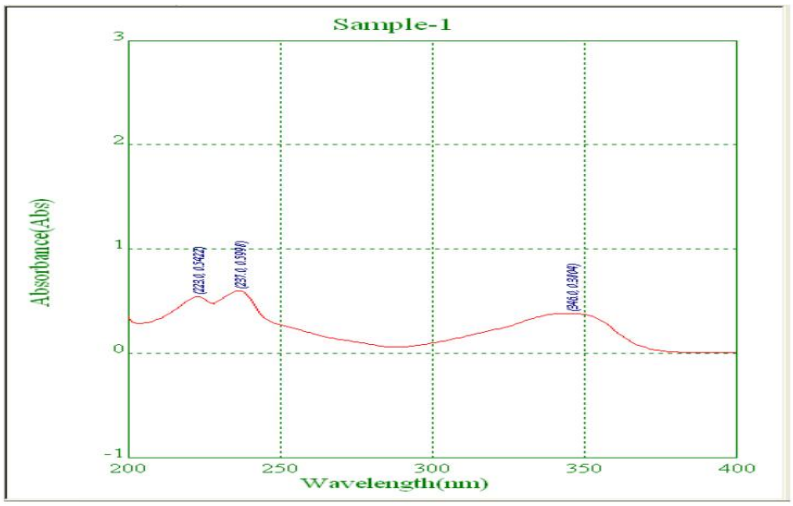

Fig 1: UV Absorbance spectrum of saxagliptin monohydrate

HPLC Method development and optimization: the finally chromatographic condition are,

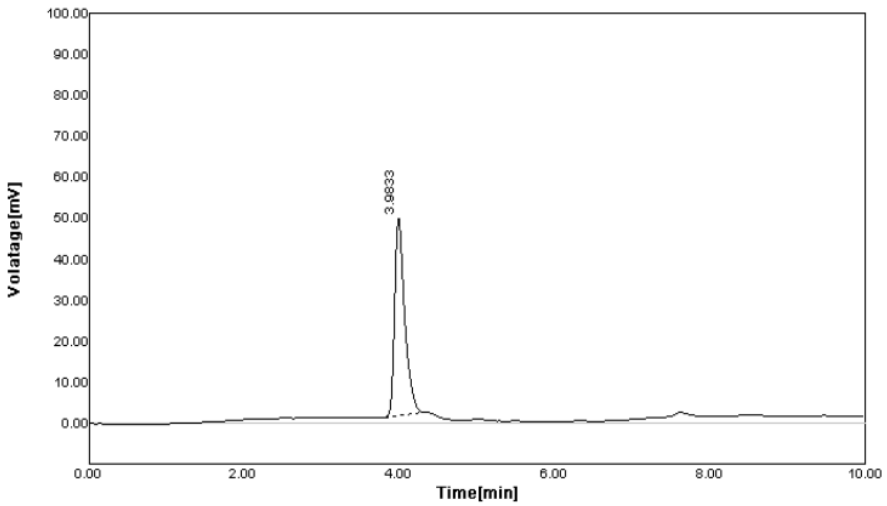

Fig 2: Optimized chromatogram of saxagliptin monohydrate.

Preparation of calibration curve

The absorbance of saxagliptin drug solution. The graph plotted as the concentration of the drug Vs peak area depicted in fig no:10 The standard curve shows linearity and obey Beer,s Lambert law with regression coefficient of (R2) 0.999 For saxagliptin monohydrate respectively over the working concentration range of 0-60 ug/ml.

Table 1: Absorbance's of standard curve of saxagliptin monohydrate.

Sr no.	Conc. (µg/ml)	Absorbance
1	10	267.78
2	20	515.12
3	30	796.75
4	40	1065.67
5	50	1326.2

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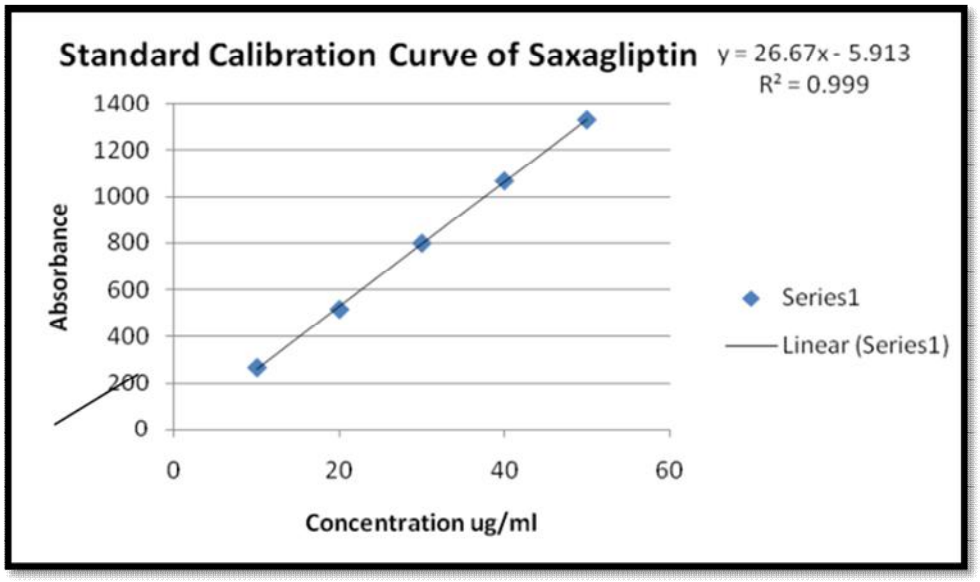

Fig 3: Standard Calibration Curve for Saxagliptin monohydrate

Method Development The saturation of column with the mobile phase, the standard and sample solution were subjected to chromatographic analysis using the above chromatographic condition. Chromatogram so after injecting 20 µ of the standard and test sample

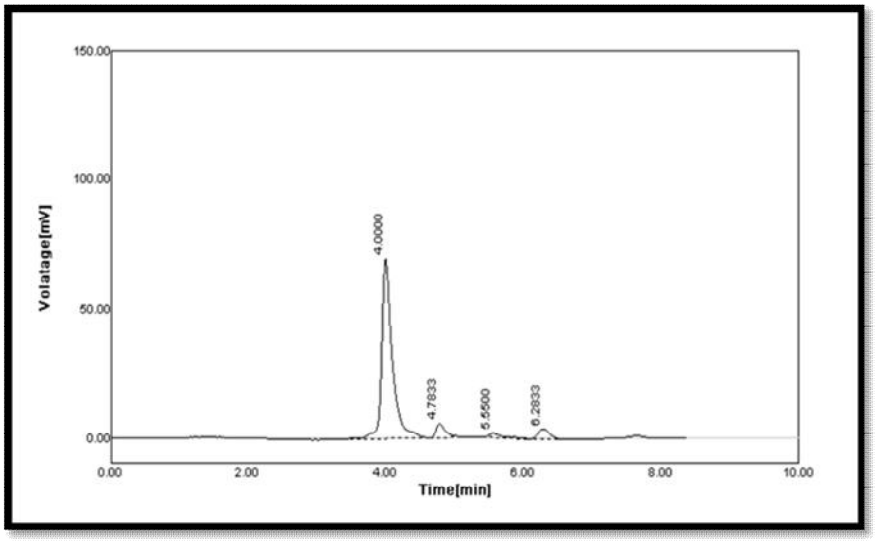

Fig 4: Standard Chromatogram of Saxagliptin monohydrate.

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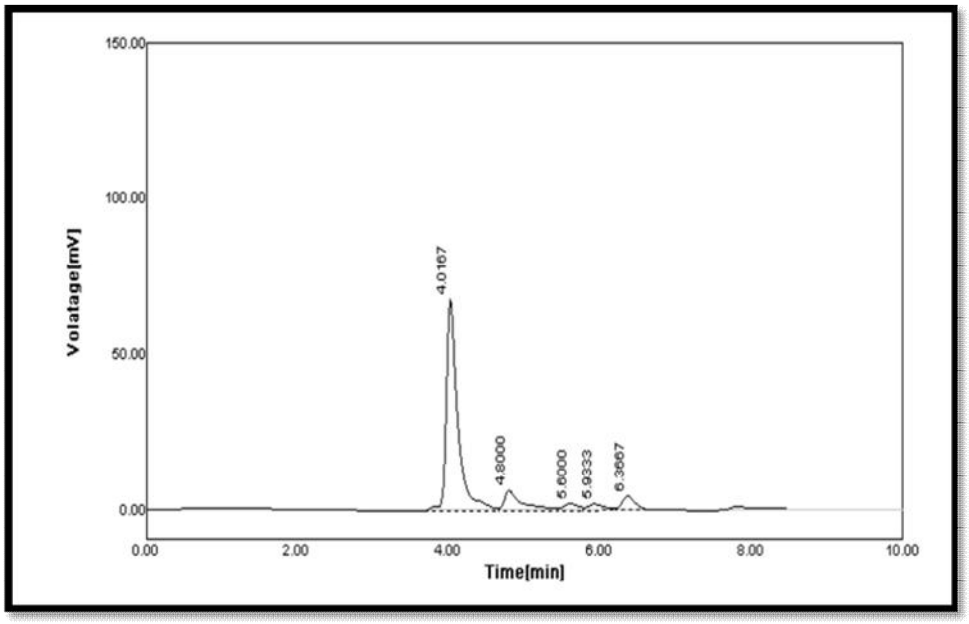

Fig 5: Chromatogram of Saxagliptin monohydrate Tablet.

The chromatogram of standard and sample of saxagliptin are shown in fig no- 5, 6 indicating that the retention time for saxagliptin 4.00 and 4.01 for standard and sample respectively. Which is not so differ. it concludes that, the development RP-HPLC method is accurate and precise without any interference or overlapping. The assay of saxagliptin was calculated and mentioned in table no-3 with relative standard derivation that is not more than 2. This data of drugs complies with the standard specification.

Table 2: Data showing assay of tablet formulation

Component	Label Claim (mg)	% Amount Found	Mean	S.D	% R.S.D
Saxagliptin monohydrate	5	19.77	99.53	0.27	0.27

Parameters of Validation

The final standard solution containing mixture of saxagliptin monohydrate was used for validation study.

Accuracy:

This table shows the accuracy data for saxagliptin monohydrate at different concentration levels, including the retention time (R.T.), mean area, standard deviation (S.D.), and percent relative standard deviation (% R.S.D.). Accuracy is assessed by comparing the measured values with the expected values at each concentration level.

Table 3: Accuracy data for saxagliptin monohydrate at 50 %

Sr no.	Peak		R.T.	Area Mean	S.D.	% R.S.D.
1	Saxagliptin monohydrate	50%	4.01	478.93	0.64	0.62
2		100%	4.0	531.58	0.38	0.38
3		150%	3.9	664.51	1.90	1.87

Precision

Final standard solution of saxagliptin was used. This solution was injected for six times and the area for six injections in HPLC was measured. Precision was performed at two different levels i.e repeatability and intermediate Precision.

Repeatability:

Repeatability of standard solution and measurement of peak area were determined

Final standard solution of saxagliptin was used. This solution was injected for six times and the area for six injections in HPLC was measured.

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Repeatability data for saxagliptin monohydrate.

Table 4: Repeatability data

Repeatability Level	Conc. (µg/ml)	R.T.	Area Mean	S.D.	% R.S.D.
40%	40	4.01	1084.33	1077.62	13.41

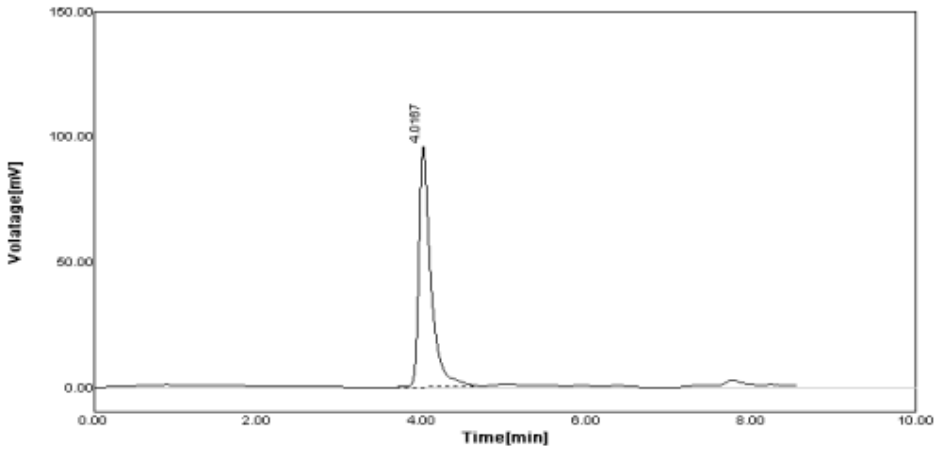

Fig 6: 40% Repeatability chromatogram.

Intermediate Precision

Intra and inter day precision

Intra Method Precision for saxagliptin monohydrate

The assessment of intermediate precision, encompassing both intra and inter-day precision, was conducted to evaluate the reliability and robustness of the analytical method for saxagliptin monohydrate determination. Intra-method precision was examined at different concentration levels (20%, 30%, and 40% µg/ml), revealing consistent retention times and area means across replicates, as indicated by low standard deviations and percent relative standard deviations (% R.S.D.). These findings demonstrate the method's ability to yield reproducible results under varying experimental conditions, essential for ensuring the accuracy and reliability of the analytical procedure in routine use.

Table 5: Intra Method Precision

Precision Level	Conc. (µg/ml)	R.T.	Area Mean	S.D.	% R.S.D.
20%	20	4.03	526.09	527.70	2.27
30%	30	4.03	807.49	800.85	9.93
40%	40	4.01	1067.03	1063.98	4.32

Interday Method Precision for saxagliptin monohydrate

The evaluation of inter-day method precision for saxagliptin monohydrate further confirms the reliability and robustness of the analytical method across different days. At various concentration levels (20%, 30%, and 40% µg/ml), consistent retention times and area means were observed, with standard deviations and percent relative standard deviations (% R.S.D.) indicating acceptable variability within and between days. These results underscore the method's ability to deliver reproducible and accurate measurements over time, highlighting its suitability for routine analysis and ensuring confidence in the reliability of the obtained data

Table 6: Interday Method Precision

Precision Level	Conc. (µg/ml)	R.T.	Area Mean	S.D.	% R.S.D.
20%	20	4.03	520.33	526.28	8.41
30%	30	4.01	810.63	813.50	4.05
40%	40	3.95	1065.32	1068.82	4.95

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Linearity: Five levels in 10%,20%,30%,40%,50%, of both drugs were injected for linearity study. The correlation coefficient for the saxagliptin monohydrate was found less than1, which complies with the standard.

Table 7: Linearity data for saxagliptin monohydrate.

Linearity Level	Conc. (µg/ml)	R.T.	Area Mean	S.D.	% R.S.D.
10%	10	4.08	267.27	264.78	3.51
20%	20	4.06	511.96	515.12	4.48
30%	30	4.06	795.37	796.75	1.96
40%	40	4.05	1060.14	1065.67	7.96
50%	50	4.03	1330.24	1326.20	5.71

Ruggedness

The ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage

Data Showing Ruggedness evaluation of the RP- HPLC method.

A: Flow rate (mL/min)

Table 8: Flow rate

Retention Time	SD	% RSD
0.6	4.6	0.32
4.4	2.81	0.72
0.8	3.4	0.34
3.4	5.59	0.72

B: Wavelength change

Table 9: Wavelength change

Wavelength	Retention Time	SD	% RSD
222	3.4	2.96	0.39
224	3.9	2.75	0.36

C: Mobile phase change

Table 10: Mobile phase change

Mobile Phase	Retention Time	SD	% RSD
9 Methanol + 39 Acetonitrile + 52 Water	4.0	10.49	1.20
11 Methanol + 41 Acetonitrile + 48 Water	3.9	2.50	0.32

The Raggedness study of standard solution indicated that the retention times for drug was not so differ and relative standard derivation of drug is less than 2(Table no 12) this might be possible due to proper system suitability

Limit of Detection and Limit of Quantitation. (LOD& LOQ)

Limit of detection and limit of quantitation of saxagliptin monohydrate was determined successfully with the help of following equation.

LOD& LOQ are calculation using the following equation. LOD = 3.3 X So/b LOQ = 10 X So/b Where, SO & b are the standard derivation and the slope of the calibration line respectively. For, ϖ LOD = 3.3x4.7/26.67= = 0.5815ug/ml ϖ LOQ = 10 x4.7 /26.67 =1.7622 ug/ml.

System suitability test

The table indicates the system suitability data for the drug saxagliptin monohydrate. The Standard theoretical plate number should not be less than 2000. The theoretical plate number for saxagliptin monohydrate peak in standard chromatograph 4910.5 respectively. Tailing factor of saxagliptin monohydrate 1.3 resp., which is the peaks of standard Chromatogram that should be less than 2.

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Retention time (TR): 3.98
Theoretical plate (N): 4910.5
Tailing factor (T): 1.3

IV. DISCUSSION

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) Analysis

The study focused on the development and validation of an RP-HPLC method for the quantitative estimation of saxagliptin monohydrate from its formulation and biological fluids. This technique offers advantages in specificity, sensitivity, and the ability to analyze complex samples.

Spectral Studies and Wavelength Selection

Saxagliptin monohydrate's UV absorbance spectrum was studied by diluting standard solutions and scanning within the 200-400 nm range. This spectrum allowed the determination of the drug's maximum absorbance (λ-max), which is crucial for accurate and consistent HPLC detection.

HPLC Method Development and Optimization

The final chromatographic conditions were optimized to achieve a clear, reproducible chromatogram for saxagliptin monohydrate. The calibration curve demonstrated linearity over a concentration range of 0-60 µg/ml, with a regression coefficient (R^2) of 0.999, indicating compliance with Beer's Lambert Law.

Calibration Curve and Linearity

The absorbance data for saxagliptin monohydrate across different concentrations showed linearity and precision. The standard calibration curve was plotted as concentration versus peak area, depicting excellent linearity and a high correlation coefficient, confirming the method's accuracy and reliability for quantitative analysis.

Chromatographic Analysis

Standard and sample solutions of saxagliptin monohydrate were subjected to chromatographic analysis, yielding retention times of 4.00 and 4.01 minutes, respectively. This close retention time for standard and sample indicates the method's precision and lack of interference or overlap, ensuring accurate quantification.

Validation Parameters

Accuracy

Accuracy was assessed at different concentration levels (50%, 100%, 150%), with retention times and areas recorded for each. The mean area, standard deviation (S.D.), and percent relative standard deviation (% R.S.D.) were calculated, showing high accuracy across all concentrations. For instance, at 50% concentration, the retention time was 4.01 minutes with an area mean of 478.93 and % R.S.D. of 0.62%.

Precision

Precision was evaluated through repeatability and intermediate precision. The repeatability test involved six injections of the standard solution, yielding consistent peak areas with low % R.S.D., confirming the method's repeatability. Intermediate precision, tested over intra-day and inter-day variations, showed consistent retention times and areas, with acceptable % R.S.D., demonstrating the method's robustness and reliability over time.

Linearity

The method's linearity was confirmed by testing five concentration levels (10%, 20%, 30%, 40%, 50%). The correlation coefficient for saxagliptin monohydrate was less than 1, complying with standard requirements. The linearity data indicated a strong linear relationship between concentration and peak area.

Ruggedness

Ruggedness was assessed by varying flow rate, wavelength, and mobile phase composition. The retention times remained consistent with low % R.S.D., indicating the method's robustness against small variations in analytical conditions.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were calculated using the standard deviation and slope of the calibration curve. The LOD was determined to be 0.5815 µg/ml, and the LOQ was 1.7622 µg/ml, indicating the method's sensitivity and ability to detect and quantify low concentrations of saxagliptin monohydrate.

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System Suitability

System suitability tests confirmed the method's effectiveness. The theoretical plate number for saxagliptin monohydrate was 4910.5, and the tailing factor was 1.3, both within acceptable limits, indicating efficient column performance and peak symmetry.

V. CONCLUSION

The developed RP-HPLC method for the quantitative analysis of saxagliptin monohydrate is robust, precise, and accurate. Utilizing a C18 column and a mobile phase of acetonitrile, methanol, and water (40:10:50) at pH 3.5, the method achieved a retention time of 3.98 minutes with detection at 223 nm. Validation parameters, including accuracy, precision, linearity, ruggedness, LOD, and LOQ, demonstrated the method's reliability and suitability for routine analysis of saxagliptin in pharmaceutical formulations. This validated method provides a valuable analytical tool for quality control in the pharmaceutical industry.

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