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Research Article

Stability -Indicating RP-HPLC Method Development and Validation for the Simultaneous Estimation of Alogliptin and Metformin in Bulk and Pharmaceutical Formulations

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A B S T R A C T

A robust and precise reverse-phase high-performance liquid chromatography (RP-HPLC) method has been developed and validated for the simultaneous quantification of Alogliptin and Metformin in combination dosage forms. The analysis was conducted using a Develosil ODS HG-5 RP C18 column (15 cm x 4.6 mm i.d; particle size 5 µm) with a mobile phase comprising acetonitrile and potassium dihydrogen phosphate buffer (0.05M, pH 3.8) in a 70:30 v/v ratio, at a flow rate of 1 ml/min, with ultraviolet detection at 258 nm. The retention times for Alogliptin and Metformin were 1.97 and 5.43 minutes, respectively. The method demonstrated linearity within the concentration range of 0-150 µg/ml for Alogliptin and 0-15 µg/ml for Metformin, with a correlation coefficient (r^2) of 0.998 for both compounds. The limits of detection were determined to be 0.01 and 0.03 g/ml, while the limits of quantification were 0.15 and 0.45 g/ml for Alogliptin and Metformin, respectively. The percentage relative standard deviation (%RSD) values were below 2 for both drugs. The assay results indicated that the content of Alogliptin and Metformin was 99.689% and 100.078%, respectively. The method was validated for parameters including linearity, accuracy, precision, robustness, limit of detection (LOD), and limit of quantification (LOQ) in accordance with International Council for Harmonisation (ICH Q2 (R1)) guidelines. This developed method was successfully applied for the quantitative analysis of commercially available dosage forms.

Keywords: Alogliptin, Metformin, Method development, validation, RP-HPLC

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INTRODUCTION

Alogliptin is an oral antidiabetic agent available in tablet forms of 6.25 mg, 12.5 mg, and 25 mg for oral administration.[5] It is utilized to enhance glycemic control in patients with type II diabetes mellitus, either as monotherapy or in combination with metformin or a peroxisome proliferator-activated receptor gamma (PPARγ) agonist, such as thiazolidinediones, when a single agent does not achieve sufficient glycemic control.[4] Several methods have been reported for the determination of alogliptin using spectrophotometry and high-performance liquid chromatography (HPLC).[6]

Metformin is a potent biguanide antidiabetic agent that has been employed for decades to regulate blood glucose levels in patients with type II diabetes and is regarded as the first-line treatment according to international guidelines.[1,2] The primary molecular mechanisms of metformin involve mitochondrial inhibition and the activation of Adenosine monophosphate activated protein kinase, which collectively suppress hepatic gluconeogenesis. Additionally, metformin can enhance skeletal muscle sensitivity to insulin, both directly and indirectly. [3]

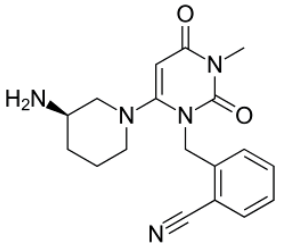

Figure 1: The structure of Alogliptin

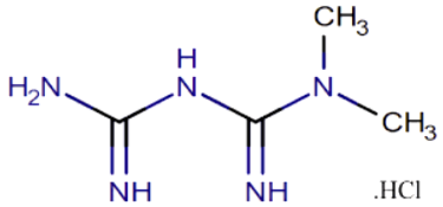

Figure 2: The structure of Metformin

The objective of this study is to develop and validate a precise, accurate, and stability-indicating RP-HPLC method for the simultaneous estimation of Alogliptin and Metformin in bulk and pharmaceutical formulations as per ICH Q2 (R1) guidelines, suitable for routine quality control and stability analysis.

MATERIALS AND METHODS

Chemicals and reagents:

The pure standard drugs Alogliptin and Metformin were procured from Pharmatrain Labs, Hyderabad, India. Sodium dihydrogen orthophosphate and Milli-Q water were supplied by Merck Chemical Laboratories. Kazano tablets, containing a combination of 12.5 mg of Alogliptin and 500 mg of Metformin, were obtained from the local market, manufactured by Takeda Pharmaceuticals North America, Inc.

Instrumentation and Chromatographic Conditions:

High-performance liquid chromatography (HPLC) is an advanced technique within column chromatography. Unlike conventional methods that rely on gravity for solvent movement, HPLC applies high pressure, up to 400 atm, enabling faster and more efficient separations. The fundamental principle remains the same across chromatographic methods: separation of a sample into its components according to their varying affinities (polar or non-polar) for the mobile and stationary phases.[14]

In this study, method development and validation were carried out using a Hitachi LaChrom Binary Gradient System (Model 1575) equipped with a UV detector and pump. The analysis was performed on a Develosil ODS HG-5 RP C18 column (15 cm x 4.6 mm i.d; particle size 5 µm) maintained at 40 °C. The mobile phase consisted of acetonitrile and potassium dihydrogen phosphate buffer (0.05 M, pH 3.8) in a 70:30 (v/v) ratio, delivered at a flow rate of 1.0 mL/min under isocratic conditions. An injection volume of 10 µL was used for all samples, with detection at 258 nm.

Chromatographic method development

To obtain optimal separation and sensitivity, several parameters were systematically evaluated. These included the flow rate of the mobile phase, its composition, the injection volume, and the column temperature. Each factor was carefully varied and assessed to establish the most suitable conditions in terms of resolution and detection limits.

Optimization of chromatographic conditions was carried out with consideration of peak symmetry, tailing factor, theoretical plates, and retention time.[12,13] The mobile phase consisted of acetonitrile and potassium dihydrogen phosphate buffer (0.05 M, pH 3.8)mixed in a70:30 (v/v) ratio. Column temperatures of 35 °C and 40 °C and injection volumes ranging from 10 to 30 µL were tested, while maintaining a constant flow rate of 1.0 mL/min.

Preparation of standard solution

Preparation of standard solution of Alogliptin

Accurately weighed 10 mg of Alogliptin was transferred into a 100 mL volumetric flask. Approximately 10 mL of HPLC-grade methanol was added, and the mixture was sonicated until completely dissolved. The volume was then adjusted to the mark with the same solvent, yielding a final concentration of 100 µg/mL.

Preparation of Standard Solution of Metformin

Similarly, 10 mg of Metformin was accurately weighed and placed into a 100 mL volumetric flask. About 10 mL of HPLC-grade methanol was added and sonicated to achieve dissolution, after which the volume was made up with the same solvent. The resulting solution contained 100µg/mL of Metformin.

Preparation of Mixed Standard Solution of Alogliptin and Metformin

For the preparation of a combined standard solution, 10 mg of Alogliptinand10 mg of Metformin were weighed separately and transferred into two different 10 mL volumetric flasks. To each flask, about3 mL of mobile phase was added, followed by sonication for complete dissolution. The volumes were then made up to the mark with the

same solvent. From these, 1 mL of Alogliptin solution and 0.1 mL of Metformin solution was diluted to 10 mL with the mobile phase. The resulting solution was filtered through a 0.45 µm membrane filter and degassed in an ultrasonic bath prior to use. Several working standard solutions were subsequently prepared by serial dilution**.**

Selection of Wavelength

The absorption spectra of 25 µg/mL solutions of both Alogliptin and Metformin were recorded in the 200–600 nm range using water as a blank on a single-beam UV–Vis spectrophotometer (Thermo Scientific, Model 1530-8000081C, Singapore). The optimal wavelengths for simultaneous detection were determined from the intersection points observed in the overlay of the UV absorption spectra of the two drugs.

Assay of Alogliptin and Metformin

Twenty tablets were weighed, and the I.P. method was employed to calculate the average tablet weight. The tablets were then powdered and triturated using a mortar and pestle. A portion of the powder equivalent to 100 mg of drug was transferred into a 100 mL volumetric flask containing 70 mL of mobile phase. The mixture was sonicated for 15 minutes in an ultrasonicator, and the final volume was adjusted to the mark with the mobile phase. The solution was filtered through a 0.45 µm membrane filter and degassed. From the resulting stock solution, 1 mL was transferred into five separate 10 mL volumetric flasks, and the volume was made up with the mobile phase to prepare working solutions.

The prepared solutions were injected in five replicates into the HPLC system and the observations were recorded.

Table 1: Assay of ALOGLIPTIN & METFORMIN Tablets

Brand name of tablets	Labeled amount of Drug (mg) Alogliptin/Metformin	Mean (±SD) amount (mg) found by the proposed method (n=6)	Mean (± SD) Assay (n = 6)
Kazano Tab from Takeda Pharmaceuticals North Americ	12.5/500	12.41 (±0.08) /499.58 (±0.04)	99.689(±0.59) /100.078(±0.67)

The assay of Kazano Tablets containing alogliptin and metformin was 99.689% and 100.078%, respectively.

Method Validation

HPLC methodologies are established to determine the identity, purity, physicochemical properties, and potency of pharmaceutical products.[6] These methods play a vital role in drug testing against set specifications during manufacturing, quality release processes, and long-term stability assessments.[6] They are also applied in safety evaluations, characterization studies, and investigations of drug performance.[9] As outlined by the International Conference on Harmonization (ICH), the most common analytical procedures include: (i) identification tests, (ii) quantitative assays of the active pharmaceutical ingredient (API) or selected components in the drug product, (iii) quantitative analysis of impurities, and (iv)limit tests for impurity control. Method validation involves the assessment of critical parameters such as accuracy, precision, specificity, limit of detection (LOD), limit of quantitation (LOQ), linearity and range, robustness, and ruggedness, ensuring the method is suitable for its intended analytical purpose

Linearity

The linear response was measured by preparing various linearity concentrations ranging from 0 to 150µg/mL. By plotting the peak area against analyte concentrations, a calibration plot was constructed, and an equation was generated for the quantification of alogliptin and metformin based on the peak areas obtained during analysis. The correlation coefficients (r^2), slope and intercept were determined from their respective calibration plots. The r^2 should 0.999.

Precision System

Precision was estimated from the 06 replicates of the standard solution on the same day and under the same chromatographic conditions. For method precision, the six replicates of the sample solution (50 ppm) were assessed within a day, and the RSD was determined. Six replicates of 50 ppm sample solution were analyzed on different days, varied HPLC, and different columns for intermediate precision. The RSD (≤2%) of the peak areas for alogliptin and metformin were measured.

Accuracy

The sample solution was prepared and analyzed in triplicate at each level by spiking the alogliptin and metformin standard in diluent at about 80%, 100% and 120% of the sample concentration.The percentage recovery was calculated to demonstrate the accuracy of the analyte .Individual and average percentage recovery had to be between 98-102%, and the % RSD had to be below 2.0% at each level.

LOD and LOQ

LOD is the minimum detectable sample concentration but not quantifiable under specified conditions, while LOQ is the lowest determinable concentration. A statistical approach was used to establish the limits of detection and quantification. This involved the use of a calibration curve containing analyte concentrations in close proximity to the respective limits. The limits were computed using the equations for detection and quantitation limit by applying 3.3σ/s and 10σ/s, respectively.

System suitability

The HPLC technique was evaluated to determine its suitability for the intended analytical application and to verify its reproducibility. A solution containing alogliptin and metformin at a known concentration of 50 ppm was analyzed in six replicates for this evaluation. The suitability of the method was determined by assessing the tailing factor and column efficiency in terms of theoretical plates, i.e., >2000, and % RSD should not exceed 2.0%.

Robustness

The robustness evaluation of the method for determining alogliptin and metformin involved minor alterations in chromatographic conditions. This evaluation was conducted to confirm whether the method can maintain the reliability and integrity of the analytical process. System suitability parameters including tailing factor (≤2) and theoretical plates (>2000) must meet the requirements under various robustness conditions. %RSD should not exceed 2%.

Specificity and forced degradation

Specificity is confirmed when the analyte peak is distinctly separated from other peaks. To evaluate the ability of the HPLC method to distinguish the standard, sample, and blank injections were performed. FD is a method used to induce the degradation of alogliptin and metformin under settings exhibiting greater severity than the accelerated conditions and undergoes decomposition to produce degradation components that serve as an indicator of the drug molecule stability and the chemical behavior of the drug, which further support the pharmaceutical product development and their packaging. As per the ICH guideline, the FD study aims to disclose potential degradation products to assist in evaluating the stability and reveal degradation pathways.[8,10]It also validates the stability-indicating methods used. Stress conditions recommended by the ICH for degradation studies include acid, base, photolytic, thermal, and humidity induced degradation. The primary goal is to detect any losses of analytes and the subsequent formation of degradation products.

RESULTS AND DISCUSSION

Analytical method validation

Accurately weighed and transferred 12.5 mg of alogliptin and 500mg of metformin working standards into a 10mL clean dry volumetric flask added approximately 7mL of diluent, sonicated to completely dissolve the mixture, and adjusted the volume to the mark with the same solvent (stock solution).The linearity of the method was determined by pipetting appropriate aliquots of the above stock solution into a series of 10 ml volumetric flasks, and the volume was made up to the mark to obtain concentrations ranging from 0-150µg/ml for Alogliptin and 0-15µg/ml for Metformin.

The λmax values of the two ingredients, that is Alogliptin & Metformin, were found to be 222 nm and 258 nm respectively in mobile phase as solvent system. The isobestic point for the drugs was found to be 258 nm.

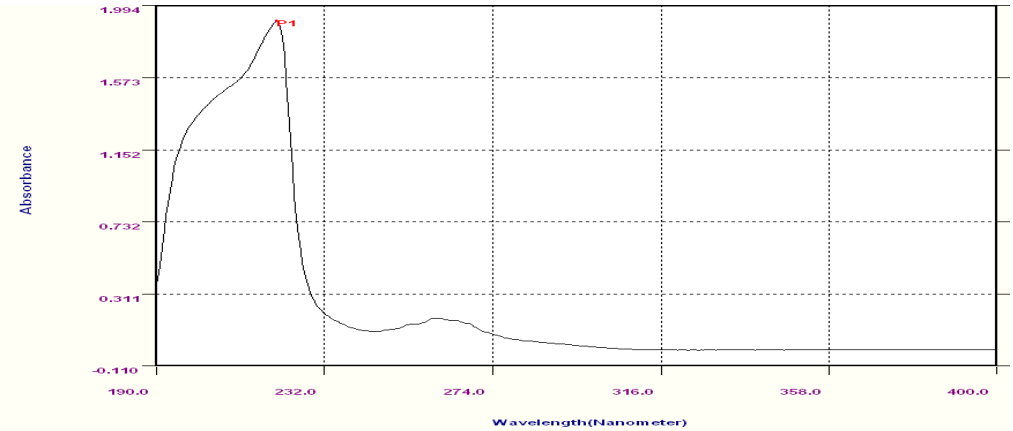

Figure 3: UV-Spectrum for Alogliptin

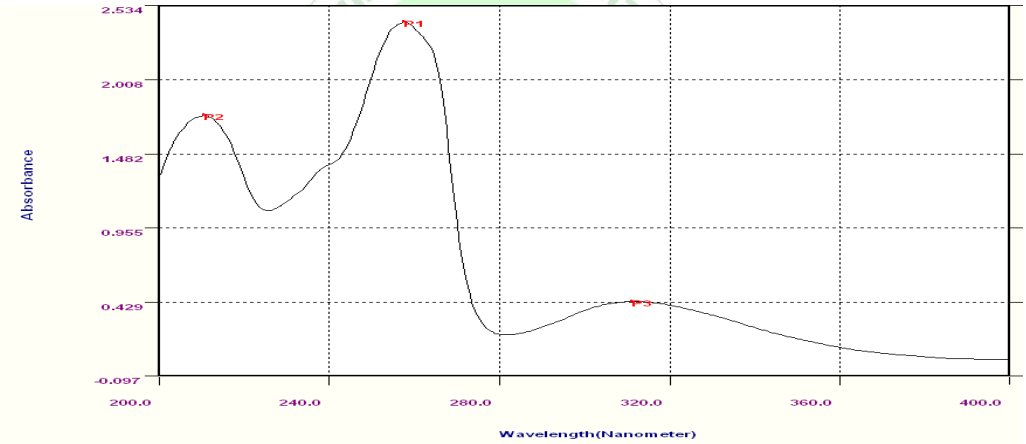

Figure 4: UV-Spectrum for Metformin

Calibration curves were plotted with observed peak areas versus concentration, followed by regression equations. The calibration curves for Alogliptin and Metformin are shown in **Fig. No .5.** and **Fig. No.6** and their respective linearity parameters are listed in **Table 2.**

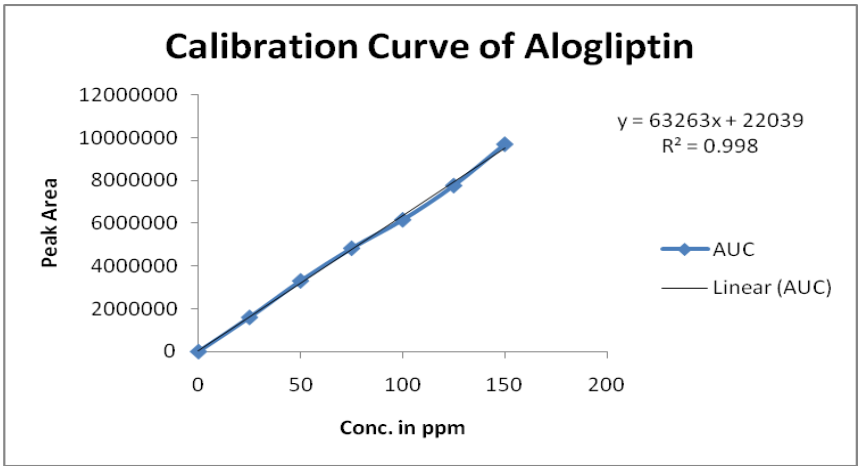

Figure 5: Linearity graph of Alogliptin

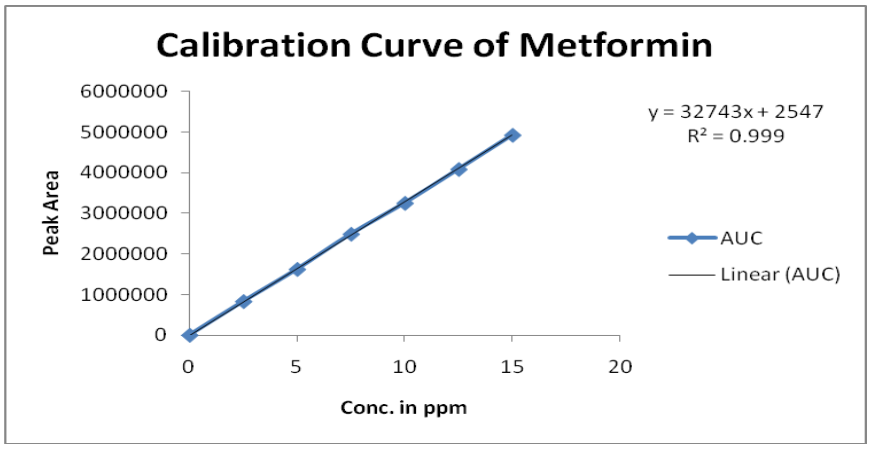

Figure 6: Linearity graph of Metformin

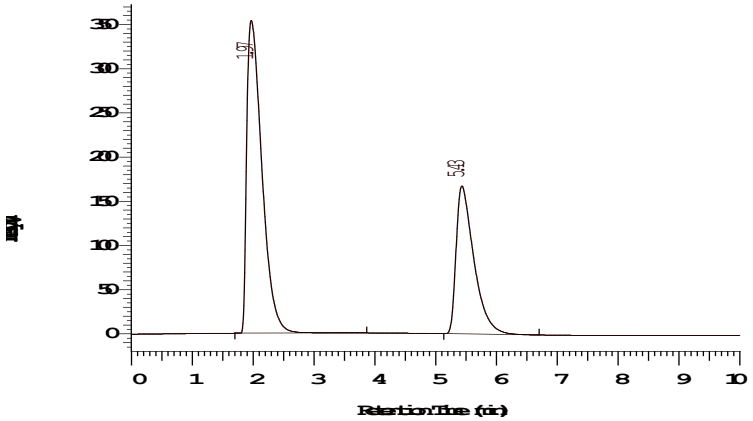

Figure 7: Typical chromatogram of ALOGLIPTIN (RT=1.97 min) and METFORMIN (RT= 5.43 min).

Linearity:

The method was linear in the range of 0-150 µg/ml for Alogliptin and 0-15 µg/ml for Metformin standards. The linear regression data are presented in Table 2.

parameters	Alogliptin	Metformin
Linearity range	0-150 µg/mL	0-15 µg/mL
Correlation coefficient (r^2)	0.998	0.998
Regression line equation	y=63263x +22039	y=32743x +2547

Precision

For intraday studies, the drug having concentration value 80%, 100 % and 120% of the target concentration (n 3), were injected in triplicate into the HPLC system, and for interday studies, the drug at above three concentrations was injected in triplicate into the HPLC system for three days. The data were subjected to statistical treatment to calculate the RSD for Alogliptin and Metformin. The data are presented in Tables **3 and 4**.

Table-3: Data for Alogliptin analysis

Conc. of Alogliptin (API) (µg/ml)	Observed Conc. of Alogliptin (µg/mL) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
80	79.95	1.05	80.01	0.24
100	100.98	0.55	100.051	0.41
120	119.84	0.18	119.95	0.18

Table 4: Data for Metformin analysis

Conc. of Metformin (API) (µg/ml)	Observed Conc. of Metformin (µg/mL) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
8	7.96	0.89	8.09	0.89
10	10.06	0.37	10.08	0.98
12	12.08	0.16	11.98	0.86

Intraday and interday studies showed that the mean RSD (%) was within the acceptance limit (≤2%); therefore, it was concluded that there was no significant difference in the assay, which was tested within and between days. Hence, the method at the selected wavelength was precise.

Repeatability

The repeatability study, which was conducted on a solution with a concentration of approximately 100 µg/ml for alogliptin and 10 µg/ml for metformin (n = 5), showed an RSD of 0.456289% for alogliptin and 0.763828% for metformin. It was concluded that the analytical technique exhibited good repeatability.

Table 5: Results of Repeatability

S. No.	Drug Name	Rt	Peak Area	Theoretical Pla	Tailing Facto
1	Alogliptin	1.97	6130775	3957	1.38
2	Metformin	5.43	3389698	4254	1.46

Accuracy (% Recovery)

The accuracy of an analytical method is the closeness of the test results obtained by that method to the true value. The accuracy of the method was determined by recovery studies at three different concentration levels, 80%, 100%, and120% by spiking known quantities of the drug analyte, and the percentage of recovery was calculated. The results are presented in **Table 6.**The % recovery at each level, mean% recovery, and% RSD met the established acceptance criteria within the given limit of acceptance criteria. Therefore, the method under the selected chromatographic conditions is accurate.

Table 6: % Recovery studies

80%				
Conc.	**Rt**	**Peak area**	**Conc. Found**	**% Recovery**
8	5.39	268759	8.130	101.625
8	5.39	265214	8.022	100.275
8	5.39	264576	8.000	100.000
			Avg.	100.6333
			SD	0.869746
			%RSD	0.864272
100%				
Conc.	**Rt**	**Peak area**	**Conc. Found**	**% Recovery**
10	5.43	329491	9.985	99.85
10	5.42	335642	10.173	101.73
10	5.43	328987	9.969	99.69
			Avg.	100.4233
			SD	1.134431
			%RSD	1.129649
120%				
Conc.	**Rt**	**Peak area**	**Conc. Found**	**% Recovery**
12	5.37	400551	12.155	101.291
12	5.37	396857	12.042	100.35
12	5.35	395861	12.012	100.10
			Avg.	100.5803
			SD	0.628021
			%RSD	0.624397

LOD and LOQ

The LOD is the lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value under the stated experimental conclusions. The quantification limit of an analytical procedure is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ were calculated from the slope(s) and standard deviation (SD) of the peak areas using the formulae LOD = 3.3 σ/s and LOQ = 10 σ/s. The results are presented in **Table 7.**

Table 7: LOD &LOQ for Alogliptin & Metformin

S.NO	DRUGS	LOD	LOQ
1	Alogliptin	0.01 µg/ml	0.15 µg/ml
2	Metformin	0.03 µg/ml	0.45 µg/ml

The LOD was found to be 0.01 and 0.03 µg/ml and the LOQ were found to be 0.15 and 0.45 µg/ml for Alogliptin & Metformin respectively, which indicates that the sensitivity of the method is high.

System Suitability Parameter

System suitability testing is an integral part of many analytical procedures, including HPLC. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. The following system suitability test parameters were established. The data are presented in **Table 18.**

Table 8: Data of System Suitability Parameter

S. No.	Parameter	Limit	Result
1	Resolution	Rs > 2	3.86
2	Asymmetry	T ≤ 2	Alogliptin =0.19 Metformin =0.24
3	Theoretical plate	N > 2000	Alogliptin =4068 Metformin= 5397

Forced Degradation studies:

The following protocol was strictly adhered to for the forced degradation of alogliptin and metformin active pharmaceutical ingredients (API).

The API (Alogliptin and Metformin) was subjected to stress conditions in various ways to observe the rate and extent of degradation that is likely to occur during storage and/or after administration to the body.This is one type of accelerated stability study that helps us determine the fate of the drug that is likely to happen after long-term storage, within a very short time compared to real-time or long-term stability testing.[7]The various degradation pathways studied are acid hydrolysis, basic hydrolysis, thermal degradation, photolytic degradation and oxidative degradation .[11]

Acid degradation studies: Accurately weighed 10 mg of Alogliptin and Metformin were transferred to a clean and dry round-bottom flask. 30 ml of 0.1 N HCl was added and refluxed in a water bath at 60°*C* for 4 hours allowed cooling to room temperature. The sample was then neutralized using *a* dilute NaOH solution and the final volume of the sample was made up to 100ml with water to prepare a100 µg/ml solution.10µl solutions was injected into the system, and the chromatograms were recorded to assess the stability of the sample.

Alkali Degradation studies: Accurately weighed 10mg of pure drug Alogliptin and Metforminwas transferred to a clean anddry round-bottom flask. 30 ml of 0.1 N NaOH was added and refluxed in a water bath at 60°*C* for 4 hours allowed cooling to room temperature. The sample was then neutralized using *a* dilute Hcl solution and the final volume of the sample was made up to 100ml with water to prepare *a* 100 µg/mL solution.10µL solutions were injected into the system, and the chromatograms were recorded to assess the stability of the sample.

Thermal degradation studies: Accurately weighed 10 mg of pure drug was transferred to a clean and dryround-bottomflask.30 ml of HPLC water was added. The mixture was then refluxed in a water bath at 60 °*C* for 6 hr. After the reflux was complete, the drug became soluble, and the drug-water mixture was allowed to cool to room temperature. The final volume was made up to 100 mL with HPLC water to prepare a100 µg/mL solution and 10µL were injected into the system, and the chromatograms were recorded to assess the stability of the sample.

Photostability studies: 10milligrams of the pure drug was placed in a clean and dry Petri dish. It was kept in a UV cabinet at 254 nm for 24 h without interruption. Accurately weighed 1 mg of the UV-exposed drug was transferred to a clean and dry 10 mL volumetric flask. First the UV exposed drug was dissolved in methanol & made up to the mark with mobile phase to get 100 µg/mL solutions and 10 µL were injected into the system and the chromatograms were recorded to assess the stability of sample.

Oxidative degradation: 10 mg of pure drug was taken in a clean & dry 100 mL volumetric flask. 30 mL of 3% H_2O_2 and a little methanol was added to it to make it soluble & then kept as such in dark for 24 hours. Final volume was made up to 100 mL. Using water to prepare 100 µg/mL solutions. Recorded to assess the stability of sample.

The results of the degradation studies indicated the specificity of the method that has been developed. Alogliptin and Metformin were stable in oxidation and thermal stress (wet heat) conditions. The result of forced degradation studies are given in the following **Table-9**.

Table 9: Results of forced degradation studies of Alogliptin and Metformin

Stress condition	Time	Assay of active substanc	Assay of degraded produc	Mass Balance (%)
Acid Hydrolysis (0.1 M Hcl)	4Hrs.	87.63	12.37	100.0
Basic Hydrolysis (0.I M NaOH	4Hrs.	60.32	39.68	100.0
Wet heat	6Hrs.	73.46	26.54	100.0
UV (254nm)	24Hrs.	90.13	9.87	100.0
3 % Hydrogen peroxide	24Hrs.	86.05	13.95	100.0

CONCLUSION

An RP-HPLC method for quantitative detection of Metformin and Alogliptin in both bulk and dosage forms was successfully developed and validated during the investigation. Utilizing a C18 column, an acetonitrile: potassium dihydrogen phosphate buffer (0.05M, pH-3.8) (70:30v/v)mobile phase, and UV detection at 258 nm, the method demonstrated precise retention times of 1.97 minutes for metformin Hcl and 5.43 minutes for alogliptin. Adhering to ICH Q2 (R1) guidelines, the method showed excellent linearity, accuracy, range, and robustness, making it reliable and reproducible. The approach's simplicity, cost-effectiveness, and improved peak resolution, coupled with the shorter retention period and isobestic mode, underscore its superiority over previous methods.

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Conflicts of interest: The authors declare no conflicts of interest in publication of this research.

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