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Method Development and Validation for The Simultaneous Estimation of Alogliptin and Metformin Using RP-HPLC Method in Both Bulk and Marketed Pharmaceutical Dosage Form

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Abstract

Analytical Method Development and Validation for Alogliptin and Metformin in bulk and Combine Dosage Form by RP-HPLC, New method was established for simultaneous estimation of Alogliptin and Metformin by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Alogliptin and Metformin by using Symmetry C18 5µm (4.6 x 150mm), flow rate was 1.0 ml/min, mobile phase ratio was Phosphate buffer (0.02M) pH-3.8: Methanol: Acetonitrile (60:20:20%v/v), detection wavelength was 260nm. The retention times of Alogliptin and Metformin were found to be 2.324mins and 4.314mins respectively. The % purity of Alogliptin and Metformin was found to be 99.865% and 99.658% respectively. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study n Alogliptin and Metformin was found in concentration range of 0µg-36µg and 0µg-39µg and correlation coefficient (r2) was found to be 0.9995 and 0.9998, % recovery was found to be 100.280, %RSD for repeatability was 0.174 and 0.709, % RSD for intermediate precision was 0.093 and 0.937 respectively. The precision study was precise, robust, and repeatable. LOD value was 1.377 and 1.079, and LOQ value was 4.174 and 3.272 respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Alogliptin and Metformin in API and Pharmaceutical dosage form.

Keywords

Alogliptin and Metformin, Method Development, Validation, Accuracy.

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INTRODUCTION

Alogliptin[1] is a dipeptidyl peptidase-4 (DPP-4) inhibitor which is used in combination with diet and exercise in the therapy of type 2 diabetes, either alone or in combination with other oral hypoglycemic agents. Alogliptin has been reported to cause liver injury, but the characteristics and details of the injury have not been defined in the published literature. Alogliptin[2] is a selective, orally bioavailable, pyrimidinedione-based inhibitor of dipeptidyl peptidase 4 (DPP-4), with hypoglycemic activity. In addition to its effect on glucose levels, Alogliptin may inhibit inflammatory responses by preventing the toll-like receptor 4 (TLR-4)-mediated

formation of proinflammatory cytokines. Alogliptin[3] inhibits dipeptidyl peptidase 4 (DPP-4), which normally degrades the incretins glucose-dependent insulinotropic polypeptide (GIP) and glucagon like peptide 1 (GLP-1). The inhibition of DPP-4 increases the amount of active plasma incretins which helps with glycemic control. GIP and GLP-1 stimulate glucose dependent secretion of insulin in pancreatic beta cells. GLP-1 has the additional effects of suppressing glucose dependent glucagon secretion, inducing satiety, reducing food intake, and reducing gastric emptying. The IUPAC Name of Alogliptin is 2-[[6-[(3R)-3-aminopiperidin-1-yl]-3-methyl-2, 4-dioxo pyrimidin-1-yl] methyl] benzonitrile. The Chemical Structure of Alogliptin is as follows

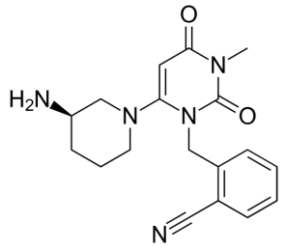

Fig-1: Chemical Structure of Alogliptin

Metformin is an oral antihyperglycemic agent that improves glucose tolerance in patients with NIDDM, lowering both basal and postprandial plasma glucose. Metformin[4] is not chemically or pharmacologically related to any other class of oral antihyperglycemic agents. Unlike sulfonylureas, metformin does not produce hypoglycemia in either patients with NIDDM or healthy subjects and does not cause hyperinsulinemia. Metformin does not affect insulin secretion. Metformin's mechanisms of action differ from other classes of oral antihyperglycemic agents. Metformin[5] decreases blood glucose levels by decreasing hepatic glucose production, decreasing intestinal absorption of glucose, and improving insulin sensitivity by increasing peripheral glucose uptake and utilization. These effects are mediated by the initial activation by metformin of AMP-activated protein kinase (AMPK), a liver enzyme that plays an important role in insulin signaling, whole body energy balance, and the metabolism of glucose and fats. Activation of AMPK is required for metformin's inhibitory effect on the production of glucose by liver cells. Increased peripheral utilization of glucose may be due to improved insulin binding to insulin receptors. Metformin[6] administration also increases AMPK activity in skeletal muscle. AMPK is known to cause GLUT4 deployment to the plasma membrane, resulting in insulin-independent glucose uptake. The rare side effect, lactic acidosis, is thought to be caused by decreased liver uptake of serum lactate, one of the substrates of gluconeogenesis. In those with healthy renal function, the slight excess is simply cleared. However, those with severe renal impairment may accumulate clinically significant serum lactic acid levels. Other conditions that may precipitate lactic acidosis include severe hepatic disease and acute/decompensated heart failure. The IUPAC Name of Metformin is 1-carbamimidamido-N, N-dimethylmethanimidamide. The Chemical Structure of Metformin is following

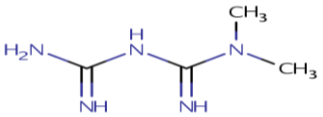

Fig-2: Chemical Structure of Metformin

However, an extensive literature search[36-39] didn't reveal any estimation method for Alogliptin and Metformin in API and Tablet dosage form. Therefore, an attempt has been made to develop and validate simple, precise, accurate HPLC method for estimation of Alogliptin and Metformin in API and Tablet dosage form.

MATERIALS AND METHODS

Table-1: List of Equipments

S.No.	Instrument	Model No.	Software	Manufacturer's Name
1	HPLC Alliance	Waters	Empower	Waters
2	UV Double Beam Spectrophotometer	UV 3000	UV Win 5	Lab India
3	Digital Weighing Balance	BSA224SCW	-	Sartorius
4	pH meter	AD102U	-	Lab India
5	Ultra Sonicator	SE60US	-	-
6	Suction Pump	VE115N	-	-

Table-2: List of Chemicals

S.No.	Chemical	Manufacturer	Grade
1	Water	Merck	HPLC Grade
2	Methanol	Merck	HPLC Grade
3	Acetonitrile	Merck	HPLC Grade
4	Potassium dihydrogen orthophosphate	Merck	A. R

Selection of Wavelength:

The detection wavelength[7] was selected by dissolving the drug in mobile phase to get a concentration of 10µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400nm. The overlay spectrum of Alogliptin and Metformin was obtained and the isobestic point of Alogliptin and Metformin showed absorbance's maxima at 260 nm.

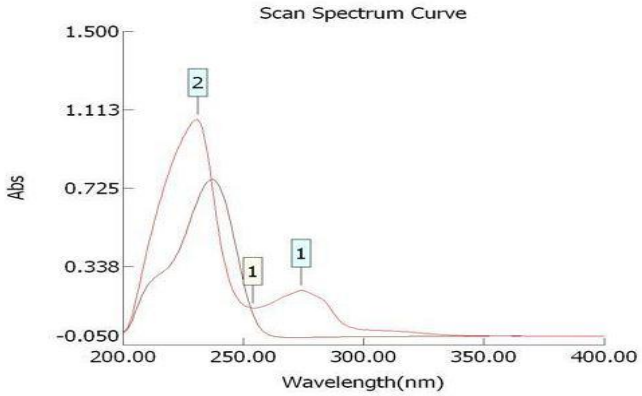

Fig-3: Overlay spectrum for Alogliptin and Metformin

Method Development

Preparations and Procedures:

Preparation of Phosphate buffer: (pH: 3.8): Weighed 0.136086 grams of KH_2PO_4 was taken into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water, adjusted the pH to 3.8 with ortho phosphoric acid.

Preparation of Mobile Phase: A mixture of pH 3.8 Phosphate buffer 600 mL (60%), 200 mL of MEOH (20%) and 200 mL of Acetonitrile are taken and degassed in ultrasonic water bath for 15 minutes. Then this solution is filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation: Mobile phase is used as Diluent**.**

Preparation of the individual Alogliptin standard preparation: 10mg of Alogliptin working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and about 2ml of Diluent[8] is added. Then it is sonicated to dissolve it completely and made volume up to the mark with the diluent. (Stock solution). Further 1.0 ml from the above stock solution is pipette into a 100 ml volumetric flask and was diluted up to the mark with diluent.

Preparation of the individual Metformin standard preparation: 10mg of Metformin working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and about 2ml of Diluent is added. Then it is sonicated to dissolve it completely and made volume up to the mark with the diluent. (Stock solution). Further 1.0ml from the above stock solution is pipette into a 100 ml volumetric flask and was diluted up to the mark with diluent.

Preparation of Sample Solution: (Tablet) Accurately 10 tablets are weighed and crushed in mortar and pestle and weight equivalent to 10 mg of Alogliptin and Metformin (marketed formulation) sample into a 10mL clean dry volumetric flask and about 7mL of Diluents is added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further 3 ml of above stock solution was pipetted into a10ml volumetric flask and diluted up to the mark with diluent.

Procedure: 20µL of the standard, sample are injected into the chromatographic system and the areas for Alogliptin and Metformin peaks are measured and the %Assay is calculated by using the formulae[9].

Method Validation

The method validation was done according to the ICH guidelines[35] in terms of system suitability, selectivity, specificity, linearity, sensitivity, precision, accuracy and robustness.

System Suitability

Prior to the validation study, system suitability[10-12] tests were performed by measurement of general characteristics such as peak symmetry, number of theoretical plates, retention time, tailing factor etc. The results obtained were satisfactory and in accordance with guidelines.

Specificity

Specificity of an analytical method is its capability to measure the analyte precisely and particularly in presence of parts that may be likely to be present in the sample matrix. Chromatograms of standard and sample prove that the method was specific[13].

Accuracy

The method accuracy for Alogliptin and Metformin in was determined by analyzing standard solution at 50, 100, 150% level. The accuracy[14-16] of the results was demonstrated by calculating the percent recovery. The results showed adequate accuracy performance for the determination of Alogliptin and Metformin in tables 5 & 6.

Linearity

The linearity[17,18] plot (Figure 5 & 6) was constructed with five concentrations at the level of 12-36% and 13-39% (12, 18, 24, 30, 36 µg/ml of Alogliptin & 13, 19.5, 26, 32.5, 39 µg/ml of Metformin) respectively (Table 3 & 4). The response of the drug was found to be linear in the studied concentration range and the linear regression equation[19] was y = 41014x + 15181 & y = 4910.7x + 1112.1 for Alogliptin and Metformin respectively. The correlation coefficient was found to be 0.9995 & 0.9998 for Alogliptin and Metformin respectively.

Precision

Intra and inter-day precision[20-21] of the analytical method was determined by performing method precision for three times in same day and followed by three consequent days. %RSD was calculated and found to be within the specified limits (<2 %). Table 9 & 10 shows the results of precision.

Limits of detection and quantitation:

The limit of detection [22](LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantified. It is expressed as a concentration at a specified signal: noise ratio, usually 3:1. The limit of quantitation[23] (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. The ICH has recommended a signal: noise ratio 10:1. LOD and LOQ may also be calculated based on the standard deviation of the response (SD) and the slope of the calibration[24] curve(s) at levels approximating the LOD according to the formulae:

LOD=3.3(SD/S) and LOQ=10(SD/S).

Robustness

Method robustness[25-27] was established by deliberately varying the experimental conditions such as flow rate (±0.1 ml/min), column oven temperature (±2°C), mobile phase components ratio (±5%), pH of mobile phase (±0.2 units) and detection wavelength (±2 nm).The study was carried out on the same day with for Alogliptin and Metformin standard solution of concentration 24 µg/ml & 26 µg/ml for Alogliptin and Metformin respectively. In each case, plate count and peak tailing were calculated. The calculated values were within the acceptance limits. Therefore, the method is considered as robust and shown in table 11 and 12.

Estimation of Alogliptin & Metformin in TABLET Dosage Form

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Finally the weighed tablets are powdered and triturated well by using mortar and pestle. A quantity of powder which is equivalent to the 100mg of drugs were transferred to a clean and dry 100ml of volumetric flask and add 70 ml of mobile phase and the resulted solution was sonicated for 15 minutes by using ultra sonicator, Then the final volume was make up to the mark with the mobile phase. The final solution was filtered through a selected membrane filter (0.45 µm) and in order to sonicate to degas the mobile phase (Solvent system). From this above stock solution (1 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system (Mobile phase).

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

A duplicate injection (Blank Solution) of the standard solution also injected into the HPLC system[28] and the chromatograms and peak areas were recorded and calculated. The obtained data are shown in Table 40.

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Average weight} = \text{mg/tab}$$

Where:

AT = Test Preparation Peak Area

AS = Standard preparation Peak Area
WS = Working standard weight taken in mg
WT = Sample weight taken in mg
DS = Standard solution dilution
DT = Sample solution dilution
P = Working standard percentage purity

Forced Degradation Studies

Forced Degradation Study[29-31] of Alogliptin and Metformin API and Tablet dosage form was carried out under different stress conditions as mentioned in ICH guideline[32] Q1A (R2). The standard solution containing 24µg/ml of Alogliptin API and 26µg/ml of Metformin API respectively were subjected to acid, alkali hydrolysis, and peroxide, thermal and photolytic degradation. The results of forced degradation studies were shown in table 14 and 15.

RESULTS AND DISCUSSION

Method Development

Optimized Chromatographic Method:

Column: Symmetry C18 5µm (4.6 x 150mm)
Mobile phase ratio: Phosphate buffer (0.02M) pH-3.8: Methanol: Acetonitrile (60:20:20%v/v)
Detection wavelength: 260nm
Flow rate : 1ml/min
Injection volume : 20µl
Column temperature : Ambient
Auto sampler temperature: Ambient

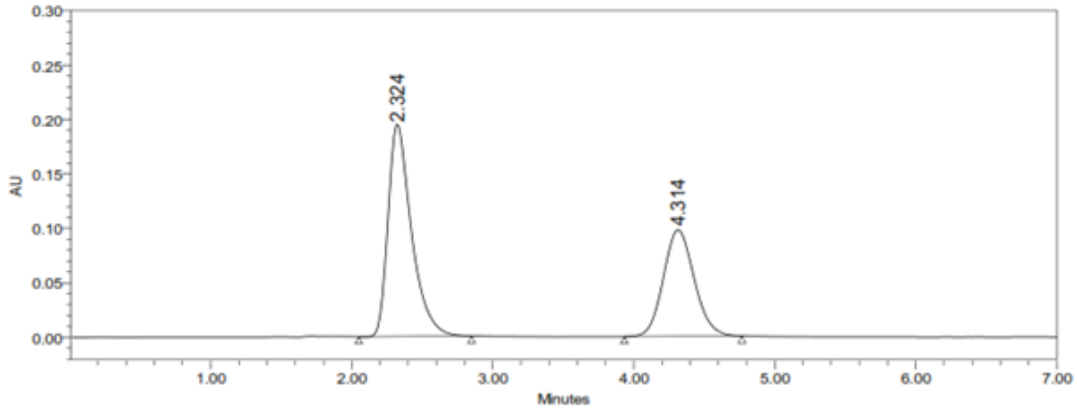

Fig-4: Chromatogram of Optimized Method

Method Validation

System Suitability: System Suitability was the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters[33] such as tailing factor, resolution, plate count and reproducibility are determined and compared against the specification suitable for the method.

Linearity: Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration[34] within a given range. Linearity is generally reported as the variance of the slope of the regression line.

Table-3: Chromatographic Data for Linearity Study of Alogliptin

Concentration (µg/ml)	Average Peak Area
0	0
12	523864
18	764875
24	999874
30	1235658
36	1488542

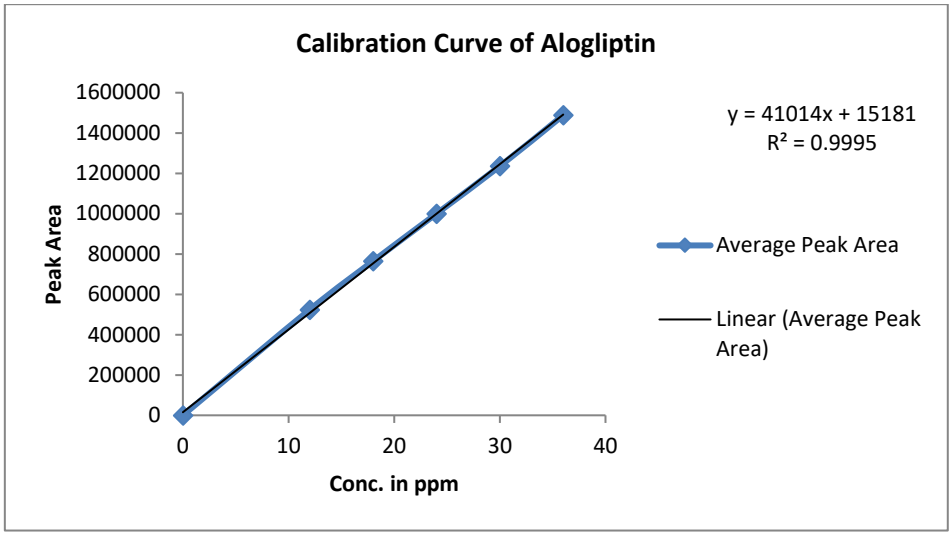

Fig-5: Calibration Curve of Alogliptin

Table-4: Chromatographic Data for Linearity Study of Metformin

Concentration µg/ml	Average Peak Area
0	0
13	65698
19.5	98254
26	128587
32.5	160648
39	191874

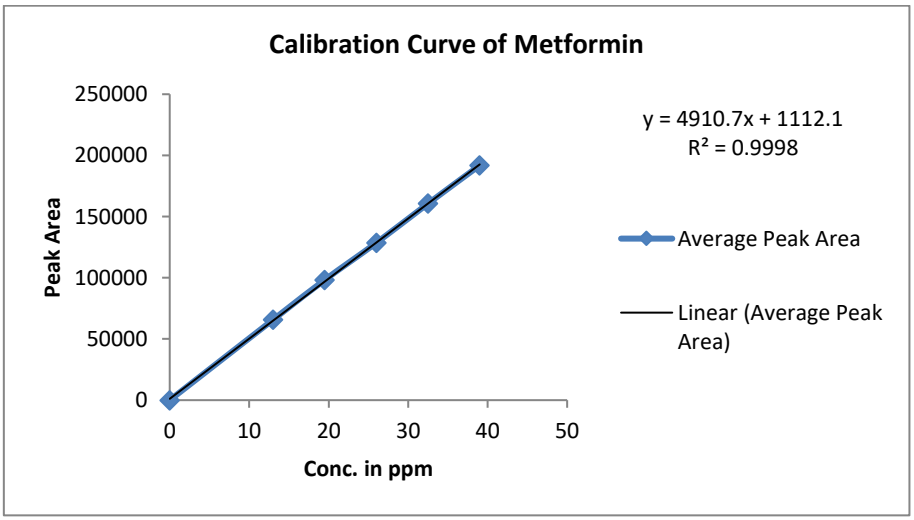

Fig-6: Calibration Curve of Metformin

Accuracy:

The accuracy of the test method was demonstrated by % recovery across its range by making three different concentrations at 50%, 100% and 150 % level using API method.

Table-5: Accuracy results of Alogliptin

%Concentration (At specification	Area	Amount added (mg)	Amount found (mg)	% Recovery	Mean Recovery
50%	508367	12	12.024	100.200%	
100%	999100.3	24	23.989	99.954%	100.150%
150%	1496200.3	36	36.110	100.305%	

Table-6: Accuracy results of Metformin

%Concentration (at specification Level)	Area	Amount Added(mg)	Amount Found(mg)	% Recovery	Mean Recovery
50%	65093.67	13	13.029	100.223%	
100%	129339.3	26	26.111	100.426%	100.280%
150%	178242.7	39	36.070	100.194%	

Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Repeatability

Obtained five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table-7: Results of repeatability for Alogliptin:

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	Resolution
1	Alogliptin	2.321	946253	155465	5326	1.36	8.25
2	Alogliptin	2.317	947845	154578	5246	1.37	8.26
3	Alogliptin	2.323	945867	155845	5478	1.35	8.34
4	Alogliptin	2.322	948572	155698	5425	1.38	8.37
5	Alogliptin	2.324	949857	154857	5326	1.36	8.39
Mean			**947678.8**				
Std. Dev			**1649.66**				
%RSD			**0.174074**				

Table-8: Results of Repeatability for Metformin:

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Metformin	4.304	111563	13254	3869	1.42
2	Metformin	4.300	111254	13425	3852	1.43
3	Metformin	4.308	111672	13254	3896	1.45
4	Metformin	4.310	112654	13265	3962	1.42
5	Metformin	4.314	113123	13154	3874	1.48
Mean			**112053.2**			
Std. Dev			**795.2614**			
%RSD			**0.709718**			

Intermediate Precision/Ruggedness:

Inter-day precision was performed by injecting standard preparations three times into chromatographic system on 2 different days by maintaining the optimized chromatographic conditions and calculate % RSD of retention time and peak areas for Alogliptin and Metformin respectively.

Table-9: Results of Intermediate precision for Alogliptin

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USPPlate Count	USPTailing	Resolution
1	Alogliptin	2.328	956325	156325	5246	1.35	8.24
2	Alogliptin	2.326	958741	157854	5367	1.38	8.26
3	Alogliptin	2.327	957542	156986	5265	1.34	8.47
4	Alogliptin	2.326	956895	158547	5384	1.39	8.29
5	Alogliptin	2.331	957486	156985	5297	1.35	8.34
Mean			**957397.8**				
Std. Dev.			**899.5091**				
% RSD			**0.093954**				

Table-10: Results of Intermediate precision for Metformin

S.No.	Peak Name	Rt	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Metformin	4.335	121231	13458	3896	1.52
2	Metformin	4.336	121457	13674	3785	1.54
3	Metformin	4.334	123142	13485	3969	1.58
4	Metformin	4.337	121325	13958	3859	1.57
5	Metformin	4.340	123654	13875	3789	1.59
Mean			**122161.8**			
Std. Dev.			**1145.733**			
% RSD			**0.937882**			

Method Robustness: Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±5nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-4, % RSD < 2%) the developed RP-HPLC method for the analysis of Alogliptin (API).

Table-11: Result of Method Robustness Test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.96
Flow (0.9 ml/min)	0.84
Temperature (27^{0}C)	0.81
Temperature (23^{0}C)	0.94
Wavelength of Detection (265 nm)	0.56
Wavelength of detection (255 nm)	0.17

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±5nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-4, % RSD < 2%) the developed RP-HPLC method for the analysis of Metformin (API).

Table-12: Result of method robustness test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.58
Flow (0.9 ml/min)	0.64
Temperature (27^{0}C)	0.72
Temperature (23^{0}C)	0.91
Wavelength of Detection (265 nm)	0.86
Wavelength of detection (255 nm)	0.78

Limit of detection (LOD) & Limit of quantification (LOQ):

The detection limit (LOD) and quantitation limit (LOQ) may be expressed as:

L.O.D. = 3.3 (SD/S).

L.O.Q. = 10 (SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Result & Discussion: The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 1.377µg/ml & 4.174µg/ml respectively for Alogliptin. The LOD was found to be 1.079µg/ml and LOQ was found to be 3.272µg/ml for Metformin which represents that sensitivity of the method is high.

Estimation of Alogliptin & Metformin in TABLET Dosage Form

Table-13: Assay of Alogliptin & Metformin Tablets

Brand Name of Tablets	Labelled Amount of Drug (mg) Alogliptin & Metformin	Mean (±SD) amount (mg) found by the proposed method (n=6)	Mean (± SD) Assay (n = 6)
Kazano Tablet (Takeda Pharma)	12.5/1000mg	12.387 (±0.09) /999.685 (±0.08)	99.865 (±0.245) /99.658 (±0.354)

Result and Discussion: The assay of Kazano Tablets containing Alogliptin and Metformin was found to be 99.865% and Metformin was found to be 99.658%.

Forced Degradation Studies

Table-14: Results of Force Degradation Studies of Alogliptin API

Stress Condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	87.316	12.684	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	78.155	21.845	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	86.215	13.785	100.00
UV (254nm)	24Hrs.	76.346	23.654	100.00
3% Hydrogen Peroxide	24Hrs.	75.104	24.896	100.00

Table-15: Results of Force Degradation Studies of Metformin API

Stress Condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	85.155	14.845	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	77.514	22.486	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	84.522	15.478	100.00
UV (254nm)	24Hrs.	74.251	25.749	100.00
3% Hydrogen Peroxide	24Hrs.	73.015	26.985	100.00

CONCLUSION

For the first time, a stability indicating HPLC with UV detector method has been developed and validated for the assay of Alogliptin and Metformin in bulk and marketed tablet dosage form. Analysis of Alogliptin and Metformin in vitro by the proposed method was valid. All parameters satisfied the acceptance criteria of the ICH guidelines. The stability indicating nature of the developed method indicated that the Alogliptin and Metformin could be assayed in the presence of their degradation products. Therefore, the developed and validated stability indicating method can be employed for the routine estimation of Alogliptin and Metformin in quality control laboratories.

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